Biochemical Systematics and Ecology 36 (2009) 882–890

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Allelopathic potential of *Tagetes minuta* terpenes by a chemical, anatomical and phytotoxic approach

M. Liza López [a,b,*], Norma E. Bonzani [a,c], Julio A. Zygadlo [a,b]

[a] *Instituto Multidisciplinario de Biología Vegetal, IMBIV-CONICET, Av. Vélez Sarsfield 299, X5000CCL, Córdoba, Argentina*
[b] *Cátedra de Química Orgánica, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 1611, X5000CCL, Córdoba, Argentina*
[c] *Cátedra de Botánica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Av. Vélez Sarsfield 299, X5000CCL, Córdoba, Argentina*

ARTICLE INFO

Article history:
Received 4 July 2007
Accepted 2 November 2008

Keywords:
Allelopathy
Germination
Ocimenones
Secretory cavities
Spathulenol
Spatio-temporal variation
Tagetones
Terpenes

ABSTRACT

The dominant terpenes in the essential oils of the leaves and reproductive structures of *Tagetes minuta* L. (Asteraceae) were studied throughout its life cycle in a natural population. The anatomy of the secretory cavities was described in order to correlate the changes in terpene content with structural changes. Finally, the phytotoxic effect of ocimenones on germination was also evaluated. Monoterpenes increased in both green leaves and reproductive structures throughout the plant's life cycle, whereas the opposite occurred in senescent leaves. Spathulenol was the main component in senescing leaves. The highest content of ocimenones, the bioactive components of the essential oil, was found in the reproductive structures. Bioassays showed that both pure ocimenones and fruit material from *T. minuta* delayed and inhibited the germination of cohabitant species. A relationship between allelopathy, biosynthesis, catabolism and terpene release is proposed for the chemical ecological effect of *T. minuta*.

1. Introduction

It has been suggested that the pool of many terpenoids, alkaloids and other types of secondary products is maintained via a fast metabolic turnover (Gershenzon et al., 1993). Plants accumulating terpenes have both short- and long-term turnover rates. Long-term variations occur during late development and involve a net decrease of monoterpenes (Croteau, 1986). Metabolic turnover of secondary metabolites is an aspect of curiosity for ecologists because many compounds act as a defence mechanism against herbivores and pathogens (Paré and Tumilnson, 1997; Kessler and Baldwing, 2001; Wittstock and Gershenzon, 2002). Thus, metabolic turnover significantly modifies the susceptibility of plants to herbivory (Gershenzon et al., 1993). However, changes in the chemical composition of plants not only have ecological connotations on herbivory but also on the production, permanency and inactivation rates of allelochemicals along the life cycle of an allelopathic species. Various examples of both these effects are provided by the literature (Nilsson et al., 1998; Dudai et al., 2001; Batish et al., 2006).

Temporal variations on chemical composition have ecological connotations related to alterations in the allelopathic potential; at the spatial scale the production of allelochemicals depends on different plant parts such as leaves, stems or flowers (Werker, 1993; Sangwan et al., 2001). Furthermore, the production and storage potential of secretory structures is in direct bearing with the developmental dynamics of the producer system (Sharma et al., 2003). For example, as glandular trichomes over-mature, subcellular integrity deteriorates and cellular constituents slowly disappear leaving the secretory cell

* Corresponding author. Present address: Instituto de Biotecnología, Facultad de Ingeniería, Universidad Nacional de San Juan, Av. Libertador Gral, San Martín 1109 (O), 5400 San Juan, Argentina. Tel./fax: +54 264 4211700.
E-mail address: mllopez@unsj.edu.ar (M.L. López).

0305-1978/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bse.2008.11.003

vacant and empty (Shanker et al., 1999). Therefore, the spatio-temporal variations of allelochemicals in a putative allelopathic species could depend on the phenological stage and producer structures, tissues and organs.

Tagetes minuta L. (Asteraceae) is an annual aromatic species native to the grasslands and mountain regions of South America, although it has become widespread throughout the world. Since the Spanish Conquest it has been introduced to Europe, Asia, Africa, Madagascar, India, Australia and Hawaii (Soule, 1993 and references therein). It is a colonizing species of the early stages of plant succession (Gil et al., 2000). In the Altas Cumbres mountain range in Córdoba, Argentina, we have observed that the natural temperate grasslands of *Stipa eriostachya* HBK are recurrently invaded by *T. minuta* after suffering disturbances. Attendant species of *T. minuta* are usually ruderal broad-leaved species such as *Bidens pilosa* L. and *Bidens subalternans* DC. Although *T. minuta* and *B. subalternans* share similar habitat and biotic characteristics, *T. minuta* populations are denser (Campos, 1997). The establishment of almost pure or mono-specific stands requires unusually potent mechanisms (Hierro and Callaway, 2003) such as allelopathy (Ahmed and Wardle, 1994; Ridenour and Callaway, 2001; Hierro and Callaway, 2003 and references therein). Previous studies have shown that ocimenones, one of the terpenes in *T. minuta* essential oil, increase the level of malondialdehyde in the roots of maize seedlings (Scrivanti et al., 2003). Also, advances on the phytotoxic effects of *T. minuta* terpenes indicate that this plant can be presumed an allelopathic species (Zunino et al., 2005).

Determining spatial and temporal variations of *T. minuta* terpenes and their effect on attendant species will contribute to a further understanding of the ecological relations and management of this species. Consequently, in this study we present chemical, anatomical and phytotoxic advances that address the allelopathic potential of *T. minuta.* First, we determined the spatio-temporal variations of the main essential oil terpenes during the life cycle of *T. minuta*. Then, we related these variations with the anatomy of secretory cavities in leaves and reproductive structures and studied the phytotoxic potential of ocimenones on the seed germination of attendant species.

2. Materials and methods

2.1. Sampling

Samples were taken during the autumn of 2002 from a natural population of *T. minuta* located near the town of La Cumbre in the Córdoba hills, Argentina (31° 00′11″ S – 64° 26′38″ W, 1434 masl; hereafter La Cumbre-2002). Sampling was carried out from March to June, which is the main period of *T. minuta* vegetative and reproductive activity. Six to 10 samples of green leaves, brown leaves, flowers and fruits were collected. Green leaves were denominated YL (young leaves), brown decaying leaves SL (senescing leaves) and the reproductive structures FF (inflorescences and infructescences). Samples were air dried and kept at room temperature until analysed. A voucher specimen (Fernando Biurrun 8756 – IZAC) was added to the herbarium of La Rioja University, Argentina.

2.2. Extraction of the essential oil

One gram of dry plant material (YL, SL, and FF) was hydro-distilled during 45 min in a micro-Clevenger-type apparatus. The resulting product was then partitioned with Cl_2CH_2 and dried over anhydrous Na_2SO_4. Finally, the solvent was evaporated at reduced pressure. Essential oils were stored frozen at −18 °C until used.

2.3. Quantitative determination of the essential oil

Essential oil components were quantified using a Shimadzu GG-R1A chromatograph fitted with a DB-5 capillary column (30 m × 0.25 mm, 0.25 micron m film thickness) using nitrogen as carrier gas at a flow of 0.9 ml min^{-1}, the injection port and flame ionisation detector at 250 °C and a temperature programme from 60 °C (1 min) to 240 °C at 4 °C/min. Quantification was performed using an external standard calibration curve obtained with standard solutions of pure ocimenones.

2.4. Qualitative determination of the essential oil

To identify the components of the essential oil, GC–MS studies were performed using a Perkin Elmer Q-700 instrument fitted with an SE-30 capillary column (30 m × 0.25 mm; 0.25 micron m film thickness). Helium was used as carrier gas at a constant flow of 0.9 ml min^{-1}, the injection port and mass selective detector were set at 250 °C and the ion source filament was held at a voltage of 70 eV. Component identification was based on matching mass spectral fragmentation and retention with commercial MS libraries such as Wiley 275, those compiled by Adams (1995) and other literature (El-Deeb et al., 2004; Zygadlo et al., 1990).

2.5. Light microscopy

Small fresh sections of YL, SL and FF were fixed in formalin–acetic acid–ethanol (FAA). The material was then dehydrated, embedded in paraffin, sliced with a rotary microtome (10–14 μm) and stained with safranin fast green and haematoxylin. Reproductive structures were stained with an aqueous solution of brilliant cresyl blue (0.05%) (Pérez and Tomasi, 2002).

Preparations were mounted in Canada balsam (Conn et al., 1960; ĎAmbrogio de Argüeso, 1986; Johansen, 1940). Photomicrographs were taken with an Axiophot microscope (Zeiss Göttingen, Germany). Stereomicroscope observations of the plant material were used to locate and record the number of glands.

2.6. TLC-Agar plate bioassay

Bioactive components in the essential oil were detected as proposed by Inoue et al. (1992). Briefly, the essential oil was analysed by preparative TLC using *n*-hexane as running solvent. Preparative TLC was carried out on 20 cm × 20 cm silica gel 60G glass plates (Merck, 0.50 mm thickness). Following solvent evaporation, the chromatogram was carefully covered with a layer of agar (0.5% w/v, 0.50 mm thick) and radish seeds (*Raphanus sativus* L.) were sown perpendicularly to the chromatogram bands. This species was selected due to its fast germination and high sensitivity, both convenient conditions in bio-guided type assays. The seeds were incubated in a growth chamber at 24–26 °C under saturated humidity to prevent agar dehydration. Seed germination and seedling growth were examined after 48 h by visual inspection. Once the bioactive fraction was localized, the components were eluted with MeOH from a newly prepared TLC and identified by GC–MS analysis.

2.7. Extraction and purification of essential oils for the bioassays on attendant species

Essential oils were obtained from fresh stems, leaves and flowers by steam-distillation for 2 h. The extracts were partitioned with Cl_2CH_2 and dried over dry Na_2SO_4. The bioactive fractions were obtained by preparative TLC as described in the previous bioassay (TLC-Agar bioassay). Finally, the purity of the bioactive extracts was verified by GC.

2.8. Plant species

Tagetes minuta is a ruderal species from perennial grasses and annual broad-leaved plant communities. Hence, the test species were selected based on this type of plant life history and on real and potential cohabitants. The following species were used: *B. subalternans* DC., *Taraxacum officinale* Hall., *Mikania cordifolia* (L.F.) Willdenow as broad-leaved species and *S. eriostachya* HBK and *Cynodon dactylon* (L.) Pers. as graminoid species. Seeds were collected from wild sites from September to March 2002. All seeds were preserved at 4 °C until bioassayed.

2.9. Phytotoxicity on the germination of attendant species

Aliquots of 0–10 μl purified compound were dissolved in *n*-hexane and placed in 10 cm Petri dishes lined with filter paper and cotton. The solvent was allowed to evaporate and 10 ml of distilled water were added to meet concentrations of 0 (control), 100, 300, 500, 700 and 1000 ppm. Fifteen seeds of each test species were placed in the Petri plates. Treatments were kept at 25 ± 1 °C under a 16 h photoperiod. Germination was recorded after an interval of 2–3 days. The experiments were completely randomised and replicated three times. A germination index (GI) was calculated based on Maguire's formula (1962),

$$GI = \Sigma(Gt/Tt)$$

where Gt = germination percentage at *t*th day and Tt = day of germination test.

2.10. Phytotoxicity assay with T. minuta fruits

When ripe, *T. minuta* fruits drop to the soil for seed dispersal. Consequently, the terpenes from the fruits are probably released straight into the soil. Therefore, a germination test using *T. minuta* fruits was carried out on *T. minuta*, *B. subalternans*, *T. officinale* and *S. eriostachya* seeds. For this, 250 mg of FF were placed on a layer of cotton and covered with filter paper in Petri dishes with 10 ml of distilled water. Then, 15 seeds of each test species were placed on the filter paper in separate Petri dishes. Experiments were performed by triplicate. Treatments were maintained for 8 days in a growth chamber under controlled conditions of light (16 h light/8 h darkness) and temperature (27 ± 1°C). The number of germinations per day was recorded.

2.11. Statistical analysis

Quantitative variations of the main terpenes studied were analysed by ANOVA. Differences among means were detected with Duncan's multiple range test. The significance of the germination percentages were analysed with Tukey's test ($p < 0.05$). GI values were subjected to non-linear regression analysis. Inhibitory concentration values (IC_{50}) of GI inhibition were calculated based on these regression functions.

3. Results

3.1. Spatio-temporal variation of the main essential oil terpenes

GC and GC–MS analysis of the oil samples identified 22 compounds that represented between 81.4 and 91.3% of the oil (Table 1). The oils of both YL and FF samples were characterised by large amounts of oxygenated monoterpenes (67.8 and 91% respectively) while the oil of SL contained a lower content of oxygenated monoterpenes (19.5%) and a larger amount of the oxygenated sesquiterpene spathulenol. The dominant components of the essential oil in the three types of plant material were tagetones (5,7-octadien-4-one, 2,6-dimethyl, ⟨Z–E⟩), ocimenones (2,5,7-octatrien-4-one, 2,6-dimethyl, ⟨Z–E⟩), and the sesquiterpene spathulenol (1H-cyclopren[*e*]azulen-7-ol,decahydro-1,1,7-trimethyl-4-methylene-(1aR-1a.alpha,4a.alpha,7.-beta,7b.alpha)). Accordingly, these components were studied during the life cycle of *T. minuta*.

YL were found from March to May, whereas FF appeared in April. SL were found throughout entire sampling period. The content of tagetones (Fig. 1) varied significantly between the months sampled and the type of plant material ($p = 0.0090$), with maximum concentrations in May and June. Although the content of ocimenones in FF increased mainly in June ($p = 0.0068$, Fig. 2), the highest ocimenone contents in YL and SL samples were found in May. The content of spathulenol varied significantly in SL ($p = 0.0282$, Fig. 3), with greater concentrations in March and June. Spathulenol was the only terpene with no statistical interaction between the months sampled and the type of plant material.

3.2. Secretory structures of T. minuta in YL, SL and FF

The secretory cavities are pellucid, large and rounded-elliptic to linear in shape. With one cavity located in each leaf blade half, they are larger (150–200 μm) than the secretory cavities found close to the margin of the midvein (ca. 70 μm). The secretory cavity is surrounded by a conspicuous parenchyma sheath (Fig. 4A). The parenchyma sheath is comprised by 1–3 colourless epithelial cells. The epithelium is a multilayer of narrowly cylindrical cells with cutinized walls and conspicuous nuclei (Fig. 4A). In the adaxial face, the secretory cavity is limited by chlorenchyma, whereas in the abaxial face the sheath is adjacent to the epidermis. A striated cuticle covers the epidermis of both the adaxial and the abaxial faces of the secretory cavity, although the cuticle is smooth in the rest of the mesophyll (Fig. 4C). There are two vascular bundles close to the sheath, one at each extreme of the secretory cavity. In the senescent stage, the secretory epithelium of the secretory glands in the leaves is collapsed (Fig. 4B). In SL, the epidermis of the secretory cavity is collapsed, strongly stained and presents a fragile aspect (Fig. 4B), and the chlorenchyma is reduced in the adaxial face. In the reproductive structures, the secretory cavities are inserted in the involucral phyllaries (Fig. 4D). In the external face, the sclerenchyma is interrupted in front of the gland. As in the leaves, the secretory epithelium is multilayered. The secretory cavities are uniform along the entire phyllaries, both in structure and anatomy. Secretory ducts (20–50 μm) were observed in the corolla, stamen and the style.

Table 1
Composition of young and senescent leaves (YL, SL) and flowers and fruits (FF) oils as revealed by GC and GC–MS analysis.

	Compound	Retention time DB-5	YL	FF	SL
1	*p*-Cymene	**471**	0.4	Tr	1.1
2	Limonene	**481**	–	–	Tr
3	⟨Z⟩ Ocimene	**498**	–	Tr	Tr
4	⟨E⟩ Ocimene	**519**	–	–	Tr
5	Dihydro-tagetone	**529**	Tr	Tr	Tr
6	Artemesia ketone	**545**	–	–	1.0
7	Chrysantenone	**686**	0.4	–	–
8	Nopinone	**718**	–	2.1	–
9	**⟨E⟩ Tagetone**	**741**	**5.1**	**2.5**	**1.8**
10	Myrcenone	**746**	**2.5**	**0.9**	–
11	**⟨Z⟩ Tagetone**	**758**	**2.4**	**4.3**	**1.1**
12	Borneol	**789**	1.6	0.7	–
13	*p*-Mentha-1.5-dien-8-ol	**792**	0.3	0.8	Tr
14	Santalone	**833**	2.0	4.7	–
15	**⟨Z⟩Ocimenone**	**957**	**19.5**	**14.4**	**7.5**
16	**⟨E⟩Ocimenone**	**978**	**22.4**	**48.1**	**8.1**
17	Carvone	**984**	1.01	9.3	–
18	Thymol	**1113**	1.2	–	–
19	Isopiperitenone		7.4	–	–
20	Piperitenone	**1233**	2.0	3.2	–
21	⟨E–E⟩ α- Farnesene	**1664**	2.1	–	–
22	**Spathulenol**	**1825**	**13.9**	**0.3**	**60.8**
	Total		**84.2**	**91.3**	**81.4**

tr: trace at <0.1%.

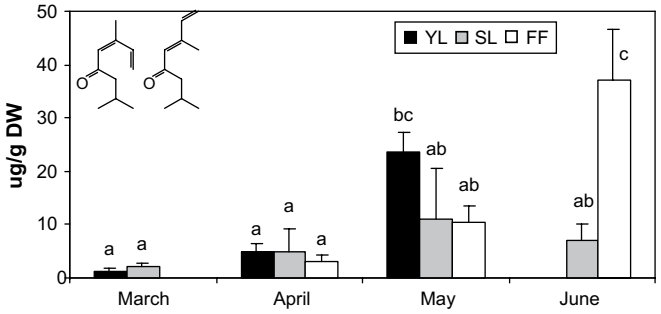

Fig. 1. Monthly variation in tagetone content (mean ± SE) obtained from different *T. minuta* plant parts. Different letters represent significant differences ($p < 0.05$) according to Duncan's multiple range test.

3.3. TLC-Agar bioassay

Bioactivity was observed on the uppermost chromatographic band immediately next to the origin. This band presented a yellowish colour under visible light and an opaque purple colour under UV_{365}. The main component, ca. 85–90%, was identified as 2,5,7-octatrien-4-one, 2,6-dimethyl, Z–E isomers (ocimenones) by GC–MS. Two oxygenated monoterpenes, tagetone (Z–E) and dihydrotagetone, were also detected but in a non-significant proportion. Given the high content of the isomeric mixture of ocimenone (Z–E) in the isolated fraction, it was considered analytically pure in the following bioassays.

3.4. Phytotoxicity on the germination of attendant species

The exponential model was the best fitted regression model for the GI, with the best *P* values and lowest mean square error. The function obtained was:

$$\mathrm{GI} = \alpha\, \mathrm{e}^{(\beta\ \mathrm{concentration})}$$

The values of alfa (α) and beta (β) for each test species are shown in Fig. 5. All species presented a significant reduction in GI as the concentration of ocimenones increased (Fig. 5). The GI of broad-leaved species was similar, but *B. subalternans* presented the highest rate of germination. The most sensitive species was *B. subalternans* that abruptly decreased its GI with a lower concentration of ocimenones (Fig. 5A). On the other hand, the germination rates of the grasses were the lowest as they require longer to germinate (Fig. 5B).

Comparing the broad-leaved species to the grass species, the former showed the lowest values of IC_{50}. Within this first group, *B. subalternans* was the most sensitive species with an IC_{50} of 154 ppm, whereas *T. minuta* had an IC_{50} of 315 ppm, and *M. cordifolia* and *T. officinale* presented intermediate values (248 ppm and 277 ppm respectively). Among the species tested, the highest IC_{50} was recorded for *C. dactylon* (495 ppm), indicating that it was the most tolerant species. The IC_{50} for *S. eriostachya* was 385 ppm.

3.5. Phytotoxicity assay with T. minuta fruits

Since the fruits of *T. minuta* drop to the soil, these organs have a great potential for directly inhibiting the germination of cohabitant species, especially considering that they present the highest the content of ocimenones. The potential of fruits in

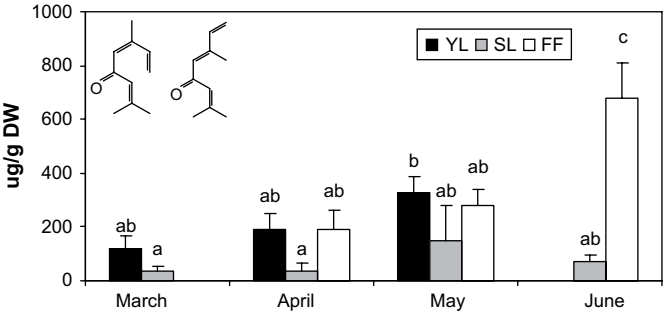

Fig. 2. Monthly variation in ocimenone content (mean ± SE) obtained from different *T. minuta* plant parts. Different letters represent significant differences ($p < 0.05$) according to Duncan's multiple range test.

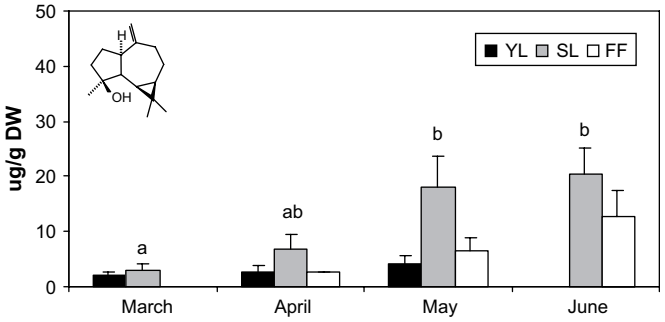

Fig. 3. Monthly variation in spathulenol content (mean ± SE) obtained from different *T. minuta* plant parts. Different letters represent significant differences ($p < 0.05$) according to Duncan's multiple range test.

inhibiting the germination of attendant species is presented in Table 2. In all cases, the controls germinated between day 1 and 5. The treatment reduced the germination of *T. minuta*, *B. subalternans* and *T. officinale* ($P < 0.05$). *Tagetes minuta* was the only species that presented a statistical interaction between the treatment and time of germination. *Bidens subalternans* showed the highest speed of germination, with a 96% of germination after 24 h in the controls. This rapid germination of *B. subalternans* was also registered in the preceding bioassay with pure ocimenones. Nevertheless, this species did not show statistical differences along the time of treatment, indicating that the effect of the treatment could not be reverted. On the contrary, important differences were observed for *T. minuta* between the control and treatment at the beginning of the test: the percentage of germination increased progressively, finally reaching the control levels of germination. *Taraxacum officinale* showed a pattern of response similar to *B. subalternans* as the germination percentages were statistically different from the control levels at the end of the assay. The germination of *S. eriostachya* showed a clear tendency to decrease, especially during the first days of treatment, although no statistical differences were detected.

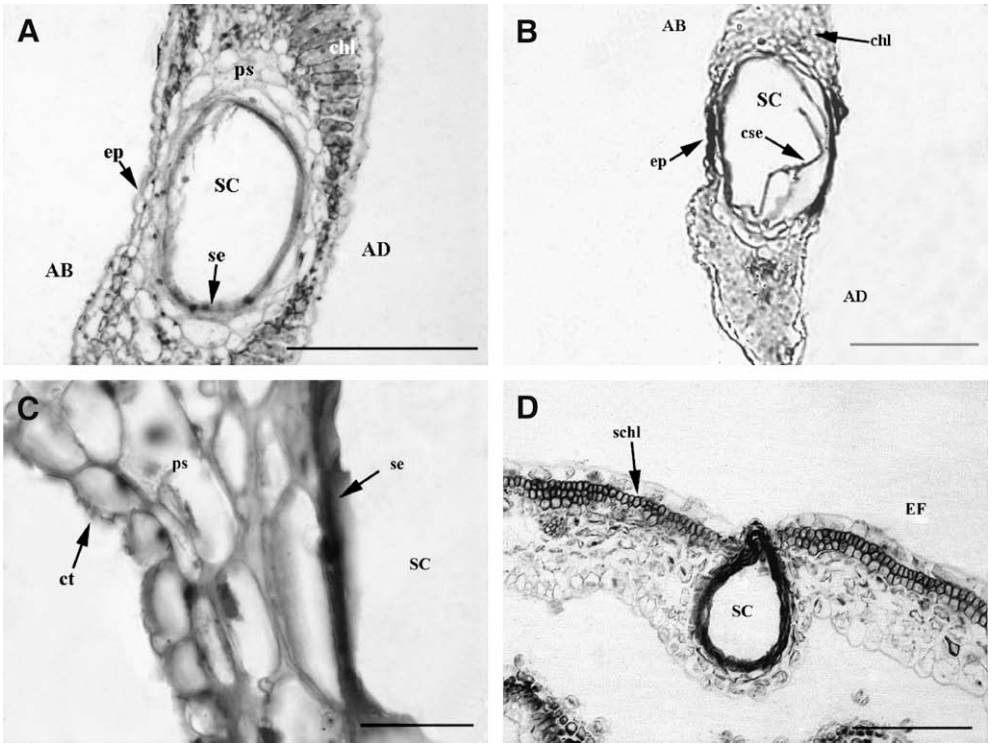

Fig. 4. A: Light photomicrograph of the cross section of a young leaf from *Tagetes minuta* L. The secretory cavity shows the multilayered epithelium with cutinised cells and conspicuous nuclei. The epithelium is surrounded by a parenchyma sheath with colourless and conspicuous cells. One layer of chlorenchyma lies between the epidermis and the parenchyma sheath in the adaxial face. B: Light photomicrograph of the cross section of a senescent leaf showing the deterioration of the secretory cavity. The chlorenchyma is reduced, and the secretory epithelium is broken and collapsed. C: Enlarged view of the secretory cavity showing the striated cuticle on the abaxial face of the young leaf. D: secretory cavity in the phyllaries of the involucre. Scale bars 200 μm on A and B; 75 μm on C; 50 μm on D. AB: abaxial face; AD: adaxial face; SC: secretory cavity; EF: external face; ep: epidermis; chl: chlorenchyma; ct: cuticle; se: secretory epithelium; ps: parenchyma sheath; schl: sclerenchyma; cse: collapsed secretory epithelium.

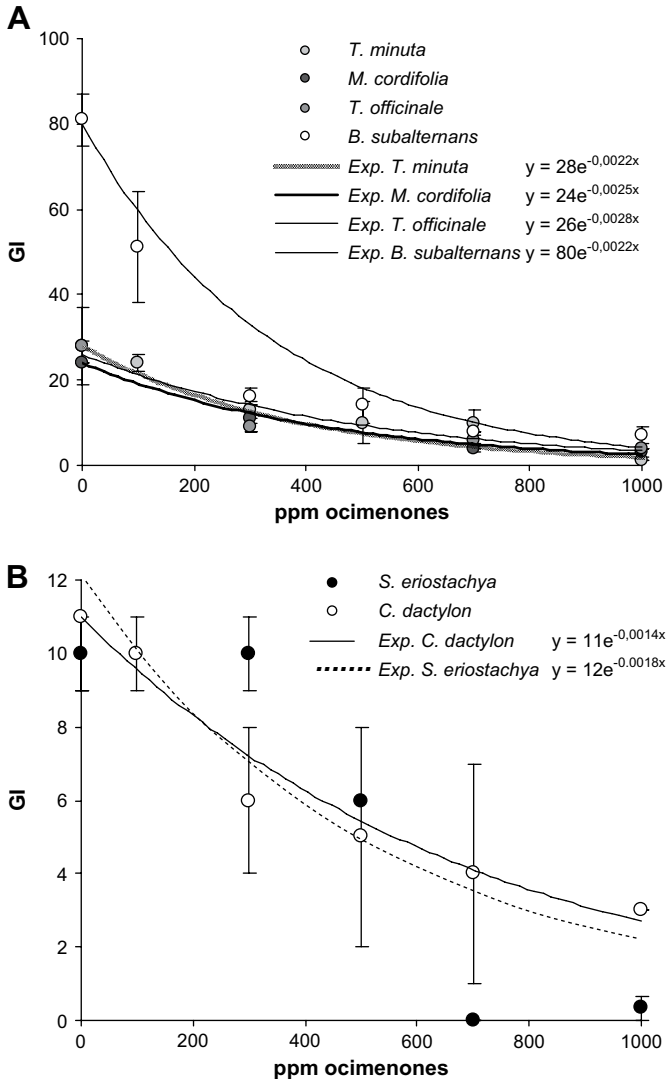

Fig. 5. Germination Index (GI) (mean ± SE) and exponential curves (Exp) fitted to the GI of the tested species. a) Broad-leaved species; b) Grass species. The estimated parameters from the exponential regression models are shown in the references.

4. Discussion

4.1. Spatio-temporal variation of the main terpenes compared to the anatomical changes

Tagetones, ocimenones and spathulenol are the main components reported in previous studies carried out at the studied site (Zygadlo et al., 1990, 1993). Roots were not evaluated because they produce polyacetylenes and thiophenes (Gil et al., 2002). A preliminary extraction carried out by steam distillation and GC–MS analysis confirmed this absence of essential oils in roots (data not shown). A close coordination between plant ontogeny and essential oil accumulation and biogenesis has been demonstrated in aromatic plants (Gershenzon et al., 2000; Sangwan et al., 2001). However, the presence of terpenes in the essential oil from *T. minuta* senescing leaves has not been previously reported.

Simon et al. (2002) studied the occurrence, structure and distribution of secretory structures in the different organs of *T. minuta*. Our purpose in describing the secretory cavities of *T. minuta* was to associate structural changes in the secretory cavities with changes in terpene content, which in turn reflect the biosynthetic and catabolic conditions of the plant tissue/organ. Comparing the compositional profiles of the essential oils of SL, YL and FF allowed making some considerations on the metabolism and physiology of the plant.

According to Croteau (1986), essential oil yield and composition must be viewed in a regulatory context involving a dynamic balance between biosynthetic and catabolic processes. On one hand, the process of monoterpenes biosynthesis is strictly dependant on photosynthesis. The reduced chlorenchyma next to the secretory cavities found in SL indicates

Table 2
Percentage of germination of cohabitant species treated with the addition of fruits of *T. minuta* on cotton and filter paper germination beds.

Days	*S. eriostachya*		*T. officinalle*		*T. minuta*		*B. subalternans*	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
1	0^{A}	0^{A}	0^{A1}	0^{B1}	0^{a}	0^{a}	96^{A}	67^{B}
4	72^{A}	33^{A}	0^{A1}	0^{B1}	83^{c}	12^{ab}	96^{A}	78^{B}
5	78^{A}	45^{A}	86^{A2}	50^{B2}	83^{c}	25^{ab}	98^{A}	81^{B}
6	79^{A}	52^{A}	86^{A2}	53^{B2}	95^{c}	46^{abc}	98^{A}	81^{B}
7	79^{A}	56^{A}	92^{A2}	53^{B2}	95^{c}	61^{abc}	98^{A}	82^{B}
8	79^{A}	61^{A}	92^{A2}	53^{B2}	97^{c}	77^{bc}	98^{A}	83^{B}

Values having different capital letter in the same row indicate differences between the control and the treatment, values having different numbers in the same column are significantly different from each other and small letters indicate differences for the interaction time × treatment at $P \leq 0.05$ according to the Tukey test.

a reduction in photosynthesis. Subsequently, the content of monoterpenes in SL was low. These results, along with the high content of spathulenol in SL suggest that leaf senescence primarily affects the production of monoterpenes while the production of sesquiterpenes remains active for longer. On the other hand, the low content of monoterpenes in SL could be due to a faster rate of monoterpenes catabolism compared to that of sesquiterpenes. For example, the catabolism of monoterpenes in *Mentha* is not exhaustive and essential oils are evident in leaves after senescence (Croteau and Sood, 1985; Mihaliak et al., 1991).

Additionally, the secretory structures of essential oils collapse during leaf senescence (Croteau, 1986; Sharma et al., 2003). The broken and collapsed secretory epithelium and the fragile cuticle and epidermis of the secretory cavities in SL constitute less anatomical restricting conditions against leaching and volatilisation of essential oils components. A low hydrophilicity and consequently a high log *P* value (a well-established measure of a compound's hydrophilicity) produce very little permeation. Predicted log *P* values for the main terpenes studied here are 3.23 for tagetone cis–trans, 3.01 for ocimenone cis–trans and 3.49 for spathulenol (Osiris Property Explorer et al., 2001). Therefore, greater amounts of ocimenones and tagetones may be extracted from SL by water leaching.

Together, these results support the idea that biosynthesis, catabolism, leaching and volatilisation are simultaneously happening but at dissimilar rates according to the plant part and phenological stage.

4.2. *Allelopathic potential of* T. minuta *terpenes on seed germination of cohabiting species*

To our knowledge, this is the first report of the bioactivity of ocimenones on seed germination. Since *T. minuta* seeds preferentially germinate under conditions of recent disturbance that also promote the germination of other species contributing to the seed bank (Márquez et al., 2002), an intense competition between *T. minuta* seedlings and other competitor species is likely to occur. For this reason, the use of non-standardized test species in the quantitative bioassays has a great ecological significance. Under field conditions, the presence of ocimenones in the soil may modify the chemical environment of the seed bank. Low concentrations of ocimenones could convert the aggressiveness of *B. subalternans* into a more vulnerable situation as suggested by the results from the assays with pure ocimenones and fruits. However, the opposite was observed with *T. minuta,* indicating a possible mechanism for overcoming autotoxicity.

In summary, although there is no proof of allelopathy under field conditions, there is evidence supporting the concept that *T. minuta* tissues could potentially interfere with the germination of attendant species by producing and releasing ocimenones. The spatio-temporal variations in the production of terpenes causing periodic emissions throughout the life cycle of *T. minuta* individuals may confer an advantage against ruderal competing species.

References

Adams, R.P., 1995. Identification of Essential Oils Components by Gas Chromatography and Mass Spectrometry. Allured Publishers Corp., Carol Stream, Illinois.

Ahmed, M., Wardle, D.A., 1994. Allelopathic potential of vegetative and flowering ragwort (*Senecio jacobaea* L.) plants against associated pasture species. Plant Soil 164, 61–68.

Batish, D.R., Singh, H.P., Setia, N., Kaur, S., Kohlia, R.K., 2006. Chemical composition and phytotoxicity of volatile essential oil from intact and fallen leaves of *Eucalyptus citriodora*. Z. Naturforsch. 61c, 465–471.

Campos, P.E., 1997. Chinchilla (*Tagetes minuta* L.). Amor seco (*Bidens subalternans* DC). Malezas de creciente importancia en los cultivos de verano del sudoeste bonaerense. Bol. Téc. EEA INTA Bordenave 13, 3–16. Technical inform.

Conn, A.P., Darrow, M.A., Emmel, V.M., 1960. Staining Procedures. Williams and Wilkins Co., Baltimore.

Croteau, R., 1986. Catabolism of monoterpenes in essential oil plants. In: Lawrence, B.M., Mookherjee, B.D., Willis, B.J. (Eds.), Flavors and Fragrances: a World Perspective. Elsevier Science Publishers B.V., Amsterdam, pp. 65–84.

Croteau, R., Sood, V.K., 1985. Metabolism of monoterpenes. Evidence for the function of monoterpene catabolism in peppermint (*Mentha piperita*) rhizomes. Plant Physiol. 77, 801–806.

ÐAmbrogio de Argüeso, A., 1986. Manual de técnicas de histología vegetal. Hemisferio Sur S.A, Buenos Aires.

Dudai, N., Larkov, O., Putiesvsky, E., Ravid, U., Lewinsohn, E., 2001. Developmental control of monoterpene content and composition in *Micromeria fruticosa* (L.) Druce. Ann. Bot. 88, 349–354.

El-Deeb, K.S., Abbas, F.A., El Fishawy, A., Mossa, J.S., 2004. Chemical composition of the essential oil of *Tagetes minuta* growing in Saudi Arabia. Saudi Pharm. J. 12 (1), 51–53.

Gershenzon, J., Murtagh, G.J., Croteau, R., 1993. Absence of rapid terpene turnover in several diverse species of terpene-accumulating plants. Oecologia 96, 583–592.
Gershenzon, J., Mc Conkey, M.E., Croteau, R., 2000. Regulation of monoterpene accumulation in leaves of Peppermint. Plant Physiol. 122, 205–213.
Gil, A., Ghersa, C.M., Leicach, S., 2000. Essential oil yield and composition of *Tagetes minuta* accessions from Argentina. Biochem. Syst. Ecol. 28, 261–274.
Gil, A., Ghersa, C.M., Perelman, S., 2002. Root thiophenes in *Tagetes minuta* L. accessions from Argentina: genetic and environmental contribution to changes in concentration and composition. Biochem. Syst. Ecol. 30, 1–13.
Hierro, J.L., Callaway, R.M., 2003. Allelopathy and exotic plant invasion. Plant Soil 256, 29–39.
Inoue, M., Nishimura, H., Li, H.H., Mizutani, J., 1992. Allelochemicals from *Polygonum sachalinenes* Fr. Schm. (Polygonaceae). J. Chem. Ecol. 18, 1833–1840.
Johansen, D.A., 1940. Plant Microtechnique. McGraw-Hill Book Co. Inc., New York, 523 pp.
Kessler, A., Baldwing, J.A., 2001. Defensive function of herbivore-induced plant volatile emissions in nature. Science 291, 2141–2144.
Maguire, J.D., 1962. Speed of germination aid in selection and evaluation for seedling emergence and vigour. Crop Sci. 2, 176–177.
Márquez, S., Funes, G., Cabido, M., Pucheta, E., 2002. Efectos del pastoreo sobre el banco de semillas germinable y la vegetación establecida en pastizales de montaña del centro de Argentina. Rev. Chil. Hist. Nat. 75, 327–337.
Mihaliak, Ch., Gershenzon, J., Croteau, R., 1991. Lack of rapid monoterpene turnover in rooted plants: implications for theories of plant chemical defense. Oecologia 87, 373–376.
Nilsson, M-Ch., Gallet, Ch., Wallstedt, A., 1998. Temporal variability of phenolics and Batatasin-III in *Empetrum hermaphroditum* leaves over an eight-year period: interpretations of ecological function. Oikos 81 (1), 6–16.
Osiris Property Explorer, Thomas Sander, Actelion Pharmaceuticals Ltd., 2001. Available from: http://www.organic-chemistry.org/prog/peo/.
Paré, P.W., Tumilnson, J.H., 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol. 114, 1161–1167.
Pérez, A.N., Tomasi, V.H., 2002. Tinción con Azul brillante de Cresilo en secciones vegetales con parafina. Bol. Soc. Argent. Bot. 37 (3–4), 211–215.
Ridenour, W.M., Callaway, R.M., 2001. The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. Oecologia 126, 444–450.
Sangwan, N.S., Farooqi, A.H.A., Shabih, F., Sangwan, R.S., 2001. Regulation of essential oil production in plants. Plant Growth Regul. 24, 3–21.
Shanker, S., Ajaykumar, P.V., Sangwan, N.S., Kumar, S., Sangran, R.S., 1999. Developmental analysis of essential oil gland count and ultrastructure in *M. arvensis* L. leaf. Biologia. Plant. 42, 379–387.
Sharma, S., Sangwan, N.S., Sangwan, R.S., 2003. Developmental process of essential oil glandular trichome collapsing in menthol mint. Curr. Sci. 84 (4), 544–550.
Scrivanti, R.L., Zunino, M.P., Zygadlo, J.A., 2003. *Tagetes minuta* and *Schinus areira* essential oils as allelopathic agents. Biochem. Syst. Ecol. 31, 563–572.
Simon, P.M., Katinas, L., Arambarri, A.M., 2002. Secretory structures in *Tagetes minuta* (Asteraceae, Heleniae). Bol. Soc. Argent. Bot. 37 (3–4), 181–191.
Soule, J.A., 1993. *Tagetes minuta*: a potential new herb from South America. In: Janick, J., Simon, J.E. (Eds.), New Crops. Wiley, New York, pp. 649–654.
Werker, E., 1993. Function of essential oil-secreting glandular hairs in aromatic plants of the Lamiaceae – a review. Flav. Frag. J. 8, 249–255.
Wittstock, U., Gershenzon, J., 2002. Constitutive plant toxins and their role in defense against herbivore and pathogens. Curr. Opin. Plant Biol. 5, 1–8.
Zunino, M.P., López, M.L., Zygadlo, J.A., 2005. Tagetone induces changes in lipid composition of *Panicum miliacum* roots. J. Essent. Oil Bearing Plants 8 (3), 239–249.
Zygadlo, J.A., Grosso, N.R., Aburra, R.E., Guzmán, C.A., 1990. Essential oil variation in *Tagetes minuta* populations. Biochem. Syst. Ecol. 18 (6), 405–407.
Zygadlo, J.A., Lamarque, A.L., Maestri, D.M., Guzmán, C.A., 1993. Composition of the inflorescences oils of some *Tagetes* species from Argentina. J. Essent. Oil. Res. 5, 679–681.