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Tagetone Induces Changes in Lipid Composition of *Panicum Miliaceum* L. Roots

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Abstract: Terpenes are thought to be important in plant-plant interactions, because of their phytotoxic action on seed germination and growth. Despite of some efforts in studying the mode of action of terpenes at molecular level, little is known about their effect on plant lipid composition. Here, we demonstrate the ability of tagetone to inhibit the root growth of *Panicum miliaceum* L. seedlings, which was related with changes in sterols, alkanes and fatty acids composition. Some sterol and fatty acid ratios are discussed in relation to their function. The results suggest that tagetone exert their effect through impairing the structural and functional properties of root membrane.

Key Word: alkanes; allelopathy; fatty acids; sterols; tagetone.

Introduction: Plants have evolved several strategies to interact with other organisms (Rice 1984; Van der Putten *et al.* 2001). The production and accumulation of secondary metabolites, which inhibit and/or stimulate germination and development of other plants, is an important mechanism in the interaction between plants (allelopathy). Aromatic plants, known to be rich in active principles such as monoterpenes, can play an important role in plant-plant interactions and constitute a primary source of potential allelochemicals (Fischer 1986; Mizutani 1989). Although no single structural feature of the monoterpenes appeared to be a critical factor on germination inhibition, several of the most phytotoxic compounds are ketones (Asplund 1968; Bradow 1993; Vaughn and Spencer 1993; Scrivanti *et al.* 2003). *Tagetes minuta* L., is known to produce copious amounts of ketones, such as tagetone, dihydrotagetone and ocimenone (Zygadlo *et al.* 1990; Garg and Mehta 1998). Moreover, *Tagetes*' essential oil also inhibited the growth of some mushroom and bacteria (Hethelyi *et al.* 1986) and inhibited root growth of maize seedlings (Scrivanti *et al.* 2003). In previous works, we

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showed that two monoterpene ketones, ocimenone and camphor, produced lipid oxidation in maize roots (Scrivanti *et al.* 2003; Zunino and Zygadlo 2004). However, the mode and mechanisms by which these ketones affect the growth of other plant species have not been examined at the molecular level.

Sterols, although a minor class of the complex lipid domain, are important in the plant cell division. It is known that they participate in the formation of membranes. Moreover, their role is not only structural, but also functional (Kemp *et al.* 1967; Nes 1973; Izzo and Navari-Izzo 1993; Moreau *et al.* 1998). Alkanes are constituents of epicuticular waxes of higher plants (Shepherd *et al.* 1995) and are synthesized in the epidermal layer of cells, they probably increase disease resistance from the attack of insects, chemicals and germinating spores of rusts (Tulloch 1976; Lemieux 1996; Post-Beittenmiller 1996; Rhee 1998). We consider that the knowledge of changes in lipid compounds is necessary to get a better understanding of the obviously complex process of inhibition on seed germination by phytotoxic terpenes. Thus, the aim of this work was to investigate possible correlations between the effects of different tagetone concentrations on chemical and developmental parameters.

Materials and Methods

Essential oil extraction and analysis: Essential oil from *Tagetes minuta* L. was extracted by steam distillation. Ketones were the major components of the essential oils (>90%). The oil obtained was dehydrated over anhydrous Na_2SO_4. The compounds were characterized for GC/MS as previously described (Zygadlo *et al.* 1993). Tagetone (2,6-dimethyl-5,7-octadien-4-one,) was extracted from the essential oil by TLC using different solvent systems: n-C_6H_{14} (100%), $C_4H_{10}O$:n- C_6H_{14} (1:9 v/v), (3:7 v/v), (5:5 v/v), (7:3 v/v) and (9:1 v/v). Then, the cleannes of tagetone was checked by GC and GC/MS.

Seed germination bioassay: Seeds of *Panicum miliaceum* L. were used to compare the bioactivity of tagetone. Groups of 200 seeds were germinated during 96 h between two layers of filter paper, Whatman n°1. Then, 20 seedlings of 96 h were placed in rolled towels. The rolls were imbibed with aqueous solutions of tagetone at different concentrations (0.3 ppm; 3.0 ppm; 7.5 ppm; 15.0 ppm and 30.0 ppm) and placed in upright position, for 96 h in darkness at 25°C ± 2°C. Tween-20 was added to the system in order to enhance tagetone dispersion, at a final concentration of 0.1 %. A control sample was prepared, under the same conditions, but without tagetone application.

Sterols, alkanes and fatty acid analysis: Roots from seedlings of *P. miliaceum* were milled and extracted with 200 ml of n- C_6H_{14} in a Soxhlet apparatus. The extracted lipids were dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure in a rotatory film evaporator. The lipids were subjected to alkaline saponifications (1N-MeOH-KOH). Unsaponifiable matter was extracted with n-C_6H_{14}. The unsaponifiable material was chromatographied by preparative thin-layer chromatography (TLC). Plates were coated with 0.5 cm layer of silica gel and developed with Cl_3CH/ $C_4H_{10}O$ (9:1 v/v) as eluent. After developing, the plates were splayed with a solution of rhodamine in EtOH 90.5 g litre^{-1} and observed under UV light. The sterol and alkanes zone were carefully scraped

from the plates and extracted with MeOH and Cl_3CH respectively for subsequent GC and GC/MS analysis (Zygadlo 1994). Fatty acids were made methyl esters derivated from saponifiable material (Zygadlo *et al.* 1994). Fragmentation patterns obtained agreed with those of NIST 3.0 database. Sterols, alkanes and fatty acids concentrations were determined using sitosterol, untriacontane and stearic acid respectively as external standars.

Statistical analysis: Each treatment was performed in triplicate and parallel controls were also run. Kruskal-Wallis analysis were performed on the data, and Mann-Whitney-Wilcoxon test was used to compare the medians. Pearson product-moment correlation coefficients between the length of roots and one another lipids analyzed was used for correlation analysis (Sokal and Rohlf 1981).

Results

Effect of tagetone on *P. miliaceum* root growth: We examined the effects of various concentrations of tagetone on the *P. miliaceum* root growth. When *P. miliaceum* seedlings were grown for 96 h in the presence of various concentrations of tagetone, the roots were found to be shorter as the concentration of tagetone increased. Only at the lowest concentration (0.3 ppm) there was not significantly differences with control (Fig. 1).

The value of correlation coefficient (R=-0.84; p=0.04) evidence a strong negative, direct correlation between increasing concentration of tagetone applied and the root length. When tagetone source was removed, the inhibitory effects do not disappear (data not shown) suggesting that they were irreversible.

Changes in sterol content and composition: We analysed the principal plant sterols, campesterol, sitosterol, stigmasterol and avenasterol. The total sterol content declined for concentrations equal or higher than 15 ppm. However, each individual sterol studied changed since the lowest tagetone concentration (0.3 ppm) (Table 1). The principal sterol in P. miliaceum root was stigmasterol (59.1 %). This sterol decreased with 0.3 ppm treatment, while at 3 and 7.5 ppm treatment did not show significant differences with control. Finally at the highest concentrations of tagetone, 15 and 30 ppm, the content of stigmasterol decresed at lower amounts than those founded at 0.3 ppm treatment. However, it can be emphasized that the percentage of stigmasterol remain near the control (55.7 and 56.4 % respectively). On the other hand, campesterol increased since 3 ppm of tagetone, while sitosterol decresed. Only with 0.3 ppm treatment sitosterol presented an increase. Avenasterol, was the lowest sterol affected . It only decreased with the higher tagetone concentrations (Table 1).

In Figure 2 we can see the sterol ratio of *P. miliaceum* roots. Campesterol to sitosterol ratio in root control was nearly 1. The 0.3 ppm of tagetone treatment decrease this ratio, while higher concentrations increase it to about three fold compared to control. On the contrary, sitosterol to stigmasterol ratio presented an opposing behaviour. Concentration of 0.3 ppm increase this ratio, while the other concentrations decrease it. Stigmasterol to the other sterols ratio showed values of 1 or higher.

Changes in fatty acid content and composition: The effect of tagetone triggered

off a continuous decrease of total fatty acids of *P. miliaceum* roots with rising concentrations. The correlation coefficient was -0.94 (p=0.01) (Table 2). At the first two tagetone concentrations not significantly differences were detected with control and this was observed too with the proportions of four individual fatty acids (16:0; 16:1; 18:0 and 18:2) (Table 2). Fatty acid 18:1 increased their percentage at 3 ppm treatment. Linoleic and palmitic acid were predominant in control and in roots treated with 0.3 and 3 ppm of tagetone, while since 7.5 ppm concentration the unsaturated fatty acids 18:1 and 18:2 were predominant. This results are shown in Fig. 3, through the unsaturated to saturated ratio, that increase for the three highest concentrations (7.5; 15 and 30 ppm). Moreover, we found a strong value of correlation coefficient (R) between unsaturated to saturated ratio and tagetone concentration (R= 0.99; p=5.4 x 10^{-5}).

Changes in alkanes content and composition: The total alkanes content decrease with the arise of tagetone concentration since 3 ppm of tagetone (Table 3). The correlation coefficient was high and negative (R=-0.85; p=0.03).

We described 11 n-alkanes (Table 3). Among the predominant compounds we could mentioned C_{31}-alkanes for the control and all the treatments, while C_{33}-alkanes were predominant in control roots; C_{29}-alkanes in roots treated with 0.3; 3.0; 7.5 and 15 ppm of tagetone and C_{35}-alkanes were present with high percentage value in roots treated with the highest tagetone concentration (30 ppm). For all tagetone treatments, C_{16}-, C_{17}-, C_{21}-, C_{23}-C_{25}- and C_{33}-alkanes concentrations were lower than control. Only C_{22}-alkanes did not present significantly differences with control. C_{27}-alkanes decreased their values with tagetone concentrations since 7.5 ppm, while C_{29}-, C_{31}- and C_{35}-alkanes increased for all tagetone concentration. With 30 ppm of tagetone, the percentage of the later alkane was almost 13-fold higher than the control (Table 3).

Discussion: In summary, application of enhanced concentrations of tagetone to P. miliaceum seedlings resulted in a progressive diminution of root length as well as in decreased contents of the fatty acids, alkanes and to a lesser extent sterols. The lowest concentration of tagetone, 0.3 ppm, presented a different behaviour compared to the highest concentrations tested. It did not affect root growth and showed a different pattern of changes in lipid fractions composition.

The decreased campesterol to sitosterol ratio observed by the lowest treatment could be due to an increased activity of the enzyme sterol methyltransferase2 (SMT2). According to this mechanism, the increased campesterol to sitosterol ratio observed with highest concentrations of tagetone may be due to a re-orientation of the sterol biosynthetic flux towards the 24-methyl sterol biosynthetic segment because of a decrease of the SMT2 activity. Changes in the campesterol to sitosterol ratio is associated with growth and developmental modifications. The pivotal role of campesterol as a precursor of the plant-growth regulators called brassinosteroids has been established through the study of mutants blocked in the steroid biosynthesis pathway (Schaeffer *et al.* 2001). Brassinosteroids have various physiological and morphological effects on plants, and are involved in the elongation of stems and responses to environmental stress (Bishop and Yokota 2001). Although the biosynthesis of

24-ethyl sterols (sitosterol, stigmasterol and avenasterol) is kinetically favoured over the 24-methyl sterols (campesterol) (Guo *et al.* 1995), the increased campesterol proportion in treated seedlings suggest that specific signalling events might be triggered through the brassinosteroid biosynthesis.

It has been suggested that stigmasterol has metabolic roles mainly. However, due to its dominating role in some tissues and species as well as its involvement in senescence and stress responses it is likely that stigmasterol plays a yet unknown role in the regulation of structure and/or function of plasma membranes (Hellgren and Sandelius 2001). In this study, we observed that the percentage of stigmasterol remains high even at the highest tagetone treatments, at expense of a parallel diminution of sitosterol content, indicating probably an increased activity of the enzime C22-desaturase responsible for stigmasterol synthesis. Moreover, at 15 and 30 ppm treatments, where the total sterol content was affected, the percentage of stigamesterol was maintained trough a significative consumption of the precursors sitosterol and avenasterol.

The increase of unsaturated fatty acids has been observed in several plant species subjected to different environmental stresses such as oxygen-, salt- and water-stresses, extreme temperatures and xenobiotics (Cooke *et al.* 1991; Pastori and Trippi 1995; Zunino and Zygadlo 2004). So, tagetone treatment also showed an arise of unsaturated to saturated fatty acid ratio. This arise has been related with a larger membrane fluidity (Karp 1987, Siegenthaler and Tremolieres 1998).

The surface wax of most higher plants contains mixtures of saturated straight-chain hydrocarbons (n-alkanes) having 21-35 carbon atoms. Alkanes with odd-numbered carbon chains predominate (Kolatukudy *et al.* 1976). Epicuticular waxes of higher plants provide a physiochemical barrier that aid plant in their resistance to drought and disease (Shepherd *et al.* 1995) due to its position at the interface between the plant and its environment (Jetter and Schäffer 2001). Under the application of different concentration of tagetone, lesser amounts of n-alkanes than control as well as changes in n-alkanes composition were observed. Treated plants showed lesser proportions of C16- to C27-alkanes, while the proportion of C29- C31- and C35-alkanes were higher. Higher concentrations of longer carbon chains is consistent with a model for the adaptation to reduced cuticular permeability (Dodd *et al.* 1998). Changes in lipidic fractions studied suggest that tagetone treatments might affect the structural and functional properties of membrane, resulting in a stress factor for the normal development of *P. miliaceum* seedlings.

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Table 1. Effect of tagetone on sterol composition (μg of sterols/ g dry weigth) of *P. miliaceum* roots. Those data having the same superscript symbol are not significantly different at 1% level.

Sterols	Treatments					
	Control	0.3 ppm	3.0 ppm	7.5 ppm	15.0 ppm	30.0 ppm
Campesterol	356.6 ± 28.4 [a]	381.5 ± 7.7 [a]	595.0 ± 15.7 [b]	617.4 ± 9.1 [b]	407.4 ± 5.8 [c]	509.1 ± 16.2 [d]
Sitosterol	316.4 ± 50.0 [a]	466.1 ± 16.2 [b]	176.1 ± 29.2 [cd]	218.4 ± 14.8 [c]	162.0 ± 15.6 [d]	148.0 ± 18.0 [d]
Stigmasterol	1228.7 ± 93.9 [a]	1019.2 ± 26.4 [b]	1241.4 ± 38.5 [a]	1122.1 ± 19.5 [a]	777.9 ± 22.1 [c]	887.1 ± 44.9 [c]
Avenasterol	177.1 ± 21.2 [abc]	206.7 ± 12.1 [ac]	149.7 ± 17.6 [bc]	211.1 ± 51.6 [c]	49.8 ± 6.3 [d]	27.5 ± 9.9 [d]
Total	2078.8 ± 8.6 [a]	2079.6 ± 21.1 [a]	2161.9 ±102.3 [a]	2169.0 ±111.5 [a]	1397.2 ± 84.5 [b]	1571.7 ± 40.0 [b]

Table 2. Effect of tagetone on fatty acids composition of *P. miliaceum* roots. Those data having the same superscript symbol are not significantly different at 1% level.

Fatty acids (% of total)	Treatments					
	Control	0.3 ppm	3.0 ppm	7.5 ppm	15.0 ppm	30.0 ppm
16:0	24.3 ± 1.7 a	25.1 ± 2.1 a	23.8 ± 2.6 a	17.4 ± 1.5 b	15.3 ± 0.3 b	10.7 ± 1.5 c
16:1	1.5 ± 0.3 a	1.5 ± 0.2 a	1.0 ± 0.2 ab	0.9 ± 0.1 b	0.3 ± 0.1 c	0.5 ± 0.1 c
18:0	3.2 ± 0.2 a	4.0 ± 0.3 a	3.3 ± 0.8 ab	2.4 ± 0.2 b	2.2 ± 0.4 c	1.7 ± 0.5 c
18:1	19.0 ± 0.7 a	19.3 ± 0.5 a	21.7 ± 0.8 b	20.4 ± 0.9 ab	27.0 ± 1.0 c	25.1 ± 0.5 c
18:2	48.1 ± 1.6 a	45.0 ± 2.6 a	46.4 ± 3.1 a	51.9 ± 1.0 b	54.1 ± 1.4 bc	58.6 ± 1.6 c
Total (µg/g dry weigth)	956.0 ± 87.1 a	896.4 ± 96.1 a	792.6 ± 94.7 a	548.7 ± 63.4 b	508.9 ± 57.8 b	289.7 ± 30.4 c

Table 3. Effect of tagetone on alkanes composition (percentage) of *P. miliaceum* roots. Those data having the same superscript symbol are not significantly different at 1% level.

n-alkanes (% of total)	Treatments					
	Control	0.3 ppm	3.0 ppm	7.5 ppm	15.0 ppm	30.0 ppm
C 16	3.5 ± 0.3 a	0.9 ± 0.1 b	1.2 ± 0.3 bc	1.6 ± 0.4 bc	1.7 ± 0.4 bc	2.0 ± 0.3 c
C 17	3.7 ± 0.1 a	2.4 ± 0.5 b	2.4 ± 0.2 b	1.6 ± 0.4 b	1.2 ± 0.5 b	1.2 ± 0.4 b
C 21	8.2 ± 0.7 a	2.4 ± 0.3 b	4.1 ± 0.2 c	4.9 ± 0.7 c	3.3 ± 0.5 c	3.3 ± 0.8 c
C 22	2.2 ± 0.4 a	3.9 ± 0.4 a	3.0 ± 0.4 a	2.1 ± 0.4 a	2.8 ± 0.1 a	2.9 ± 0.4 a
C 23	10.4 ± 0.4 a	7.6 ± 0.3 b	7.9 ± 0.4 b	8.7 ± 0.3 b	7.3 ± 0.5 b	6.5 ± 0.4 b
C 25	10.8 ± 0.4 a	6.7 ± 0.2 b	8.7 ± 0.4 c	7.9 ± 0.9 b	8.1 ± 0.5 c	9.1 ± 0.5 c
C 27	12.1 ± 0.7 a	15.6 ± 1.3 a	13.4 ± 0.4 a	9.1 ± 0.5 b	9.7 ± 0.1 b	9.0 ± 0.3 b
C 29	9.9 ± 0.4 a	21.6 ± 1.1 b	20.4 ± 1.2 b	17.2 ± 0.6 c	16.0 ± 0.4 c	15.1 ± 0.4 c
C 31	14.7 ± 0.5 a	19.3 ± 0.6 b	20.8 ± 0.7 b	19.0 ± 0.9 bc	17.5 ± 0.8 bc	17.1 ± 0.5 c
C 33	16.7 ± 0.3 a	8.5 ± 0.4 b	11.2 ± 0.3 c	13.8 ± 0.6 de	11.7 ± 0.4 c	12.1 ± 0.6 ce
C 35	1.5 ± 0.1 a	4.0 ± 0.1 b	3.5 ± 0.3 b	2.6 ± 0.3 b	11.6 ± 1.4 c	19.0 ± 1.3 d
Total (mg/ g dry weigth)	187.8 ± 22.1 a	195.3 ± 17.4 a	93.7 ± 7.8 b	88.6 ± 9.0 b	30.1 ± 2.5 c	16.8 ± 3.1 c

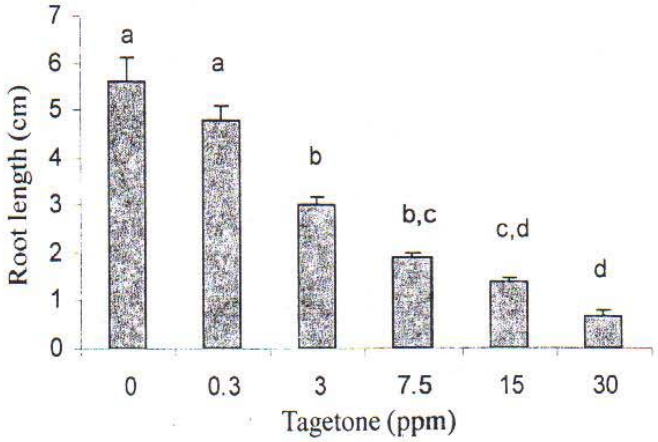

Fig. 1. Effect of different tagetone concentration on *Panicum miliaceum* roots growth. Values are means ± DS. Those bars having the same letter are not significantly different at 1% level.

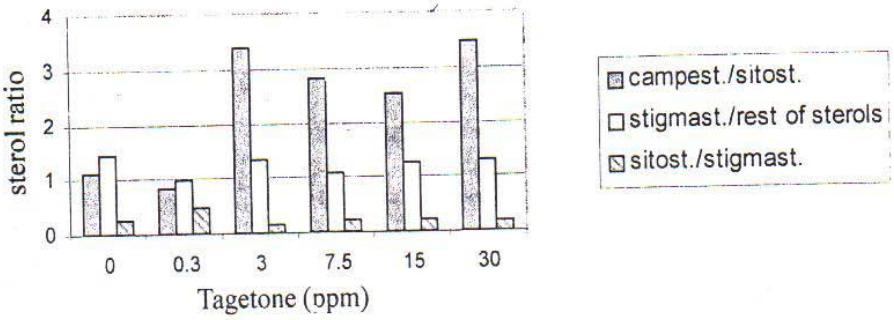

Fig. 2. Effect of tagetone on different sterols ratio of *P. miliaceum* roots.

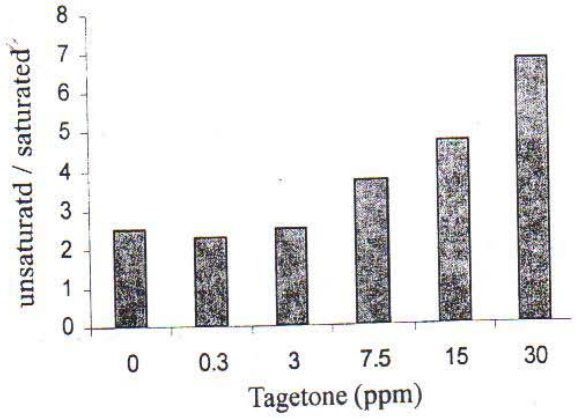

Fig. 3. Effect of tagetone on unsaturated to saturated fatty acids ratio of *P. miliaceum* root.