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Effects of Pb in *Tagetes minuta* L. (Asteraceae) leaves and its relationship with volatile compounds

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ABSTRACT

Soil has traditionally been the disposal site for most heavy metal waste. Since metals can not be degraded, soil remediation requires their removal with phytoextraction, being a cost effective alternative in which metal accumulator plants are used to remove toxic metals from soil. Aromatic crops, used for the production of essential oils, may be a suitable alternative to be used in agricultural soils contaminated with heavy metals, since after removal, a marketable product is left. The present study investigated the effects that high concentrations of lead (Pb) have on the volatile compounds in leaves of *Tagetes minuta* L., growing near a battery recycling plant and also determined whether Pb was present in its essential oil. In this way, it could be evaluated whether the use of this species for Pb phytoremediation supports safe production. To carry this out, it was determined whether the essential oil obtained by hydrodistillation contained Pb, which was also checked for each individual of *T. minuta*, by measuring the amount of this metal in leaves using the technique of X-ray fluorescence and calculating the percentages of volatiles by the HS-SPME technique.

The essential oils extracted by hydrodistillation did not have any detectable Pb, there by demonstrating that it is not transferred to the essential oil. Regarding the volatiles, the highest concentrations encountered were for *cis*-tagetone, dihydrotagetone and verberone. Furthermore, the compounds β-ocimene and α-thujone correlated significantly with the Pb concentration in leaves, indicating that the increase of Pb in *T. minuta* may favor synthesis of these compounds. In conclusion, *T. minuta* is a resistant plant that can grow in Pb polluted soils and accumulate the contaminant in aerial tissues, while providing an economic return through the production of essential oil free of Pb.

1. Introduction

The contamination of agricultural soil with non-biodegradable heavy metals affects the growth of plants due to their toxicity and leads to bioaccumulation in different crops (Angelova et al., 2004). Thus, heavy metals become more concentrated in the tissues than in the surrounding environment and attain high and hazardous levels at the top of the food chain, creating a health risk for consumers (Lasat, 2002). Among the most common and toxic heavy metals found are lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As), with exposure leading to adverse effects on human health, such as neurotoxicity and carcinogenicity (ATSDR, 2007, 2008; García-Lestón et al., 2012).

Pb is a heavy metal that has been used historically in industry for various purposes, and is widely distributed in the environment. This, coupled with its high toxicity, makes Pb one of the major environmental pollutants and pathologically a potential danger to the human population, either through consumption or inhalation (Kabata-Pendias and Pendias, 2001). The main sources of Pb are mining, metallurgical industries, the development and manufacture of paints, and recycling of acid batteries (Anttila et al., 1996; García-Lestón et al., 2012; Morais et al., 2012).

Once in the human body, Pb causes disruptions in cellular physiology and replaces cations such as calcium, zinc, magnesium via their transport mechanisms. In the bloodstream, most of the Pb binds to plasma proteins or erythrocytes, causing anemia. In addition, a small portion remains free in the blood and can enter bone tissue. Currently, the replacement of calcium ions at the protein binding sites is considered to be the major pathogenic mechanism of Pb, affecting the correct folding of the protein (Garza et al., 2005). For these reasons, the high concentrations of Pb in edible vegetables grown near urban and industrial areas imply a risk for human health (Kabata-Pendias and Pendias, 2001).

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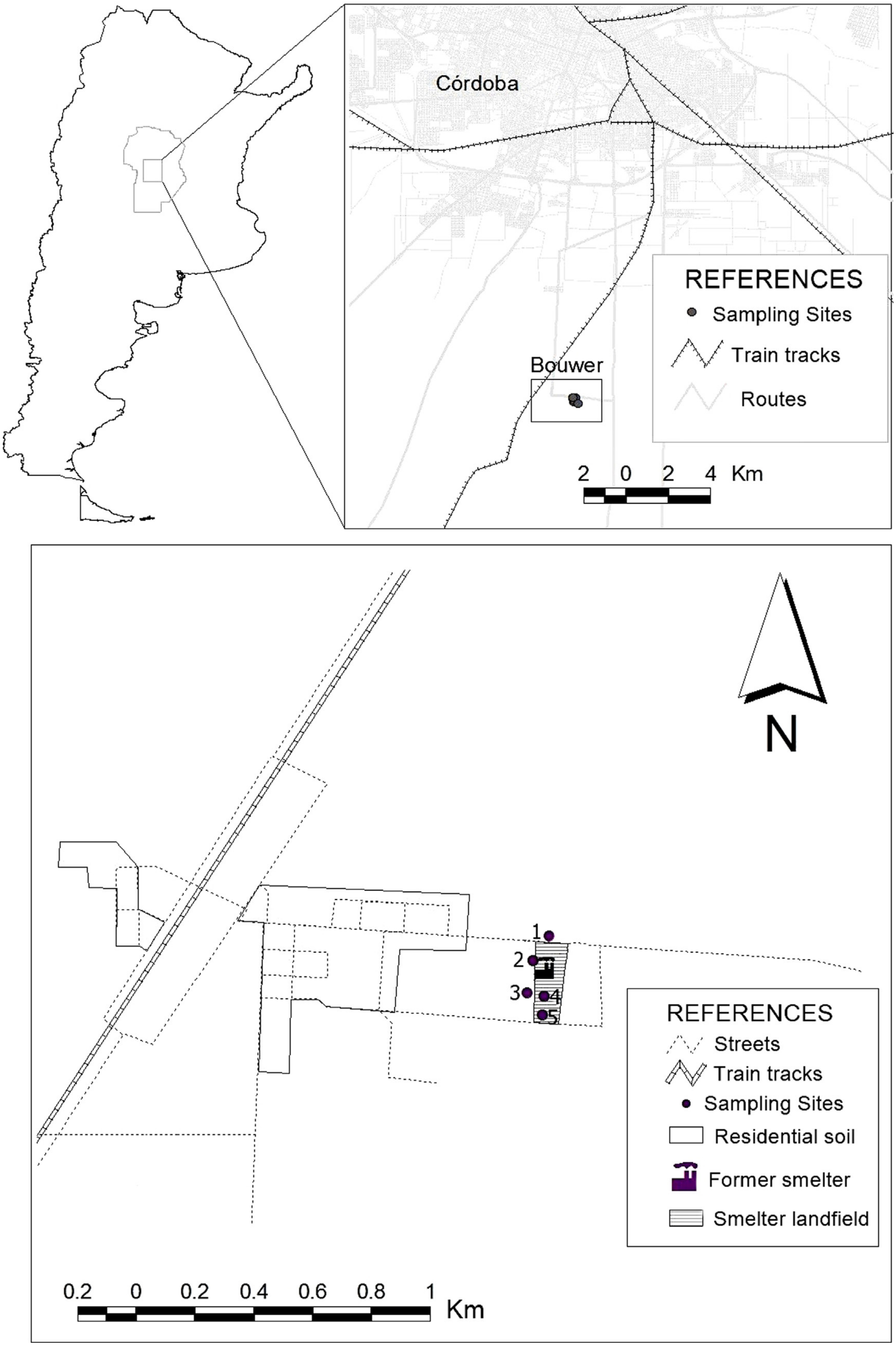

Fig. 1. Location of the study area and sampling sites.

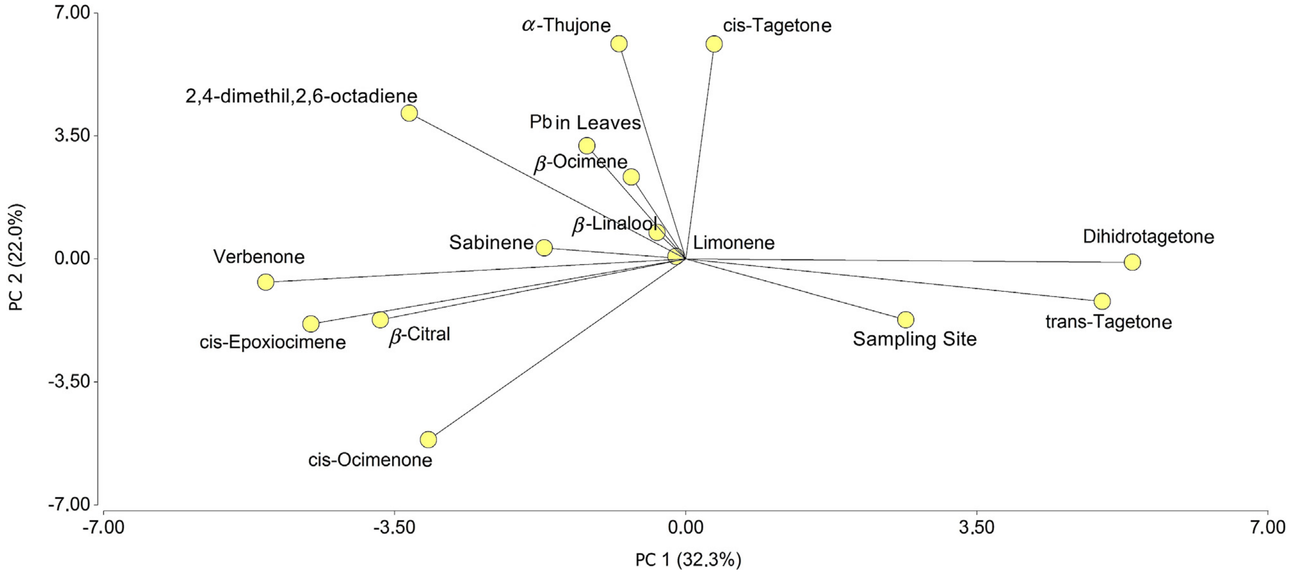

Fig. 2. Principal component analysis (PCA) for the volatiles compounds of *Tagetes minuta* L. and concentration of Pb in their leaves.

Soil behaves as a main heavy metal sink, and since metals can not be degraded, soil remediation requires their removal (Jadia and Fulekar, 2009; Lasat, 2002; Raskin et al., 1997). However, remediation of contaminated soil by conventional methods presents high costs and also harms the environment in different ways (Aboulroos et al., 2006; Alkorta et al., 2004; Cunningham and Ow, 1996; Salt et al., 1995). One possible alternative, is the use of plants for contaminated soil remediation, referred to as phytoremediation (Cunningham and Ow, 1996). This is a low-cost option with no destructive impact on soil fertility or structure compared to traditional rehabilitation technologies, such as soil excavation, extraction of metals with acid or washing floors (Blaylock and Huang, 2000; Jadia and Fulekar, 2009; Salt et al., 1998; Schmidt, 2003). There are different available phytoremediation options, such as phytoextraction, in which metal accumulator plants are used to remove toxic metals from soil (Salt et al., 1995). This procedure is considered to be an attractive low environmental impact technology that remediates pollution while simultaneously providing plant cover for the soil to reduce erosion and leaching. Using this technique, the pollution levels are reduced by successive crops and harvests (Jadia and Fulekar, 2009; Padmavathiamma and Li, 2007), and the method is suitable for large areas of land (Blaylock and Huang, 2000).

Plant characteristics considered to be optimal for the phytoextraction process are not only the ability to tolerate and accumulate high levels of heavy metals in their harvestable parts, but also to show a high rate of growth and the potential to produce wealth biomass in field conditions (Salt et al., 1995). The aromatic crops associated with essential oil production may be a suitable alternative to be used in heavy metal contaminated agricultural soils. Some herbs also seem to be able to accumulate these metals (Zheljazkov et al., 2006; Zheljazkov and Nielsen, 1996; Zheljazkov and Warman, 2003), suggesting the possibility of their use in phytoremediation. However, studies concerning the effects of heavy metals on both plant development and essential oil production and composition are scarce. New phytoremediation projects that can combine the extraction of metals with the ability to provide a return on investment are more likely to be accepted and implemented by affected landowners (Danh et al., 2010), with obtaining essential oils implying a good monetary return.

Tagetes minuta L. is an annual and aromatic plant that belongs to the Asteraceae family, originally from temperate regions of South America and more recently introduced in several regions of the world for medicinal purposes. The plant has a rich chemistry for natural products and yields volatile essential oils, obtained by hydrodistillation, that are widely used in cosmetics, perfumery (with its aroma being, classified as sweet, similar to citrus fruits), as a food flavoring and in beverages and medicine (Vasudevan et al., 1997). Its properties include antioxidant and anti-inflammatory effects (Ali et al., 2014; Karimian et al., 2014) and anxiolytic (Rocabado et al., 2011) and antibacterial activities (Shirazi et al., 2014). Not only does it have excellent medicinal properties, but it also has been found to have nematicidal and antimicrobial (Tereschuk et al., 1997; Tomova et al., 2005), effects, and can be used in repellents for mosquitoes, ticks and mites (Green et al., 1991; López et al., 2011; Okoth, 1973; Perich et al., 1994; Wanzala et al., 2014; Wanzala and Ogoma, 2013), as well as in insecticides against family Psychodidae and vectors of diseases such as leishmaniasis (Dinesh et al., 2014). It was even reported that their aqueous extracts can inhibit the germination of some species, such as *Lotus corniculata* and *Lactuca sativa* (Kil et al., 2002; Meissner et al., 1986).

Many studies of *T. minuta* L. have been carried out on the composition of the volatile compounds extracted from the plant, which may vary according to the location of collection (Vázquez et al., 2011; Zygadlo et al., 1990), the growth stage, plant part (Chalchat et al., 1995; Weaver et al., 1994) and environmental parameters such as soil composition, the amount of exposure to sunlight and temperature fluctuations (Chamorro et al., 2008; El-Deeb et al., 2004; Meshkatalsadat et al., 2010). In additions, parallel it is known that this species can accumulate Pb under certain conditions (Salazar and Pignata, 2014). However, there is a lack of knowledge about the effect of heavy metals on the composition of the volatiles. Therefore, in this study, we investigated the effect of high concentrations of Pb in leaves of *T. minuta*, growing near a former Pb smelter, on the composition of the volatile compounds, to determine whether its use for phytoremediation supports a safe production of good quality essential oil.

2. Materials and methods

2.1. Study area

The study area was comprised of a plot of approximately 1 ha in the town of Bouwer (Argentina), which is located 18 km South of

Table 1
Percentage of volatile compounds found in leaves of *T. minuta* at different sampling sites.

Compounds	Sites 1	2	3	4	5
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.
Sabinene	0.150 ± 0.157	0.238 ± 0.270	0.085 ± 0.055	0.101 ± 0.086	0.063 ± 0.024
Limonene	0.514 ± 0.235	1.789 ± 2.177	0.908 ± 0.223	1.141 ± 0.398	0.610 ± 0.173
β-Ocimene	2.181 ± 0.973	3.406 ± 2.979	1.269 ± 0.509	3.990 ± 2.145	1.513 ± 0.510
Dihydrotagetone	25.63 ± 13.39	32.59 ± 20.65	26.54 ± 16.26	29.74 ± 5.99	36.64 ± 14.79
β-Citral	1.059 ± 0.098	0.783 ± 0.320	1.010 ± 0.115	0.876 ± 0.154	0.874 ± 0.145
α-Thujone	0.586 ± 0.029	0.449 ± 0.130	0.501 ± 0.155	0.664 ± 0.114	0.575 ± 0.068
β-Linalool	2.040 ± 0.237	2.167 ± 0.280	2.254 ± 0.395	1.945 ± 0.732	4.877 ± 9.390
2,4-Dimethyl, 2,6-octadiene	0.473 ± 0.133	0.287 ± 0.166	0.365 ± 0.085	0.399 ± 0.022	0.411 ± 0.127
cis-Epoxiocimene	2.656 ± 0.143	2.036 ± 0.673	2.734 ± 0.517	2.539 ± 0.067	2.352 ± 0.299
cis-Tagetone	44.39 ± 2.67	35.12 ± 12.83	33.18 ± 17.71	43.89 ± 3.32	42.56 ± 6.91
trans-Tagetone	0.254 ± 0.144	0.476 ± 0.308	0.266 ± 0.159	0.208 ± 0.025	0.029 ± 0.182
cis-Ocimenone	1.648 ± 0.844	1.701 ± 1.813	12.945 ± 21.95	1.228 ±n0.395	1.010 ± 0.683
Verbenone	18.42 ± 10.27	19.10 ± 20.45	17.94 ± 11.46	13.28 ± 3.52	11.18 ± 8.10

Córdoba city (Fig. 1). The soil is an Entic Haplustoll and the climate is mild, with an annual mean temperature of 15 °C and an average annual rainfall of 500–900 mm. This area is characterized by the presence of a former battery recycling plant that operated from 1984 to 2005, but was subsequently closed down after an inspection conducted by supervisory authorities found Pb levels 35 times greater than allowable emission levels. More recent studies carried out in the soils of the study area, have revealed that they are still contaminated with Pb (Rodriguez et al., 2014; Salazar and Pignata, 2014; Salazar et al., 2012).

Table 2
Lead concentration in leaves of *T. minuta*, mean and standard deviation for each sampling site and results of ANOVA for comparison among sites.

Sites	Pb (μg/g PS) Mean ± SD	ANOVA $p = 0.0210$
Basal	6.22 ± 2.35	B
1	15.41 ± 9.55	B
2	9.00 ± 4.49	B
3	9.80 ± 4.07	B
4	41.21 ± 4.76	A
5	16.41 ± 19.54	B

2.2. *Sample collection of* Tagetes minuta *L.*

Within the study area, five sites where selected, with *T. minuta* plants being collected at the same stage of growth (vegetative growth stage). To select these sampling sites, we took into account the heterogeneity of the area according to previous studies where concentrations of Pb in soils were found to be between 20 μg g^{-1} and >1000 μg g^{-1} (Salazar and Pignata, 2014). At each sampling site (plot of 9 m^2), 5 or more plants of *T. minuta* were collected and arranged in individual bags; additionally, a composite sample (pool of other 5 individuals) was collected. One sampling site was selected 3 km from the contaminated area to have a reference value of Pb concentration (Basal). Individual samples were used to quantify the concentration of Pb in each plant, and also to determine the percentage of volatiles, with the pools being used for extraction of essential oil by hydrodistillation.

Once in the laboratory, plant samples were fractionated to obtain leave samples, all the further analysis were conducted only for leaves. The pools were kept in a freezer until hydrodistillation. Regarding individual samples, leaves of each plant were divided, with one part being kept in a freezer and to other part oven dry at 45 °C until dry weight, after washed with Milli Q water. The frozen aliquot was used for volatile compound analysis and the dried one for Pb content analysis.

2.3. *Essential oil by hydrodistillation*

Leaves from pool samples of *T. minuta* L. were used to obtain the essential oils by hydrodistillation for 4 h in a Clevenger-type apparatus, until achieving complete exhaustion. Then, the oils obtained were dried over anhydrous sodium sulphate.

2.4. *Determination of Pb in leaves of* minuta *L.*

The Pb analysis in *T. minuta* leaves was conducted by taking two subsamples of 50 mg dry weight (DW) of leaves per plant, which were then digested with 100 μL of concentrated nitric acid (37% V/V Merk) for 72 h and added to an internal Ga standard (10 ppm). Then, the supernatant was filtered with filter paper of 2 μm (Munktell, Germany) and stored in an Ependorf tube.

To determine whether the essential oils contained Pb, 50 μL of the essential oil obtained from each pool were removed, digested with 400 μL of concentrated nitric acid (37% V/V) for 72 h, added to internal standard of Ga (10 ppm) and stored in an Ependorf tube, and subsequently, 7 μL was pipetted in the center of an acrylic support. Standard solutions with known concentrations of Pb were prepared to calibrate the system. The samples were measured for 200 s, using the total reflection set up at the X-ray fluorescence beamline, with a white beam (approximately 0.3 mm wide and 2 mm high) being used for excitation. For X-ray detection, a Ge detector was used with an energy resolution of 148 eV, at 5.9 keV, with a 0.8 mm collimator in the detector (Wannaz et al., 2011).

2.5. *Quality control*

As a quality control, blanks and samples of the standard reference material "CTA-OTL-1" (oriental tobacco leaves, Institute of Nuclear Chemistry and Technology, Poland) were prepared in the same way as described above for plants, which were run after ten determinations to calibrate the instrument and to check potential sample contamination during analysis. These results were found to be between 87% and 93% of the certified value, with the data errors being low and typically less than 15%. The coefficients of variation of the replicate analyses were calculated for different determinations, and found to be less than 10%.

2.6. *Volatile compounds by solid phase micro extraction (SPME)*

To perform this technique, 100 mg of fresh material were placed in a 10 mL vial, which was sealed and placed in a water bath at 40 °C for 10 min. Subsequently, was introduced into the vial a fiber polydimethylsiloxane (PDMS, 100 μm) obtained from Supelco Inc., which was exposed for 30 min whit the temperature kept constant at 40 °C (Vázquez et al., 2011). Once this exposure time elapsed, substances extracted by the fiber were thermally desorbed at 250 °C for 10 min in the injection port of a gas chromatograph.

The identification of volatile components was performed using a gas chromatograph PerkinElmer Q-700 apparatus coupled with an ion trap mass detector (PerkinElmer, Shelton, CT, USA). A DB-5 column (30 m 9 0.25 mm i.d. and 0.25 m coating thickness) was used, and the PerkinElmer Q-700 was equipped with a manual injection port operating in a splitless mode. The working conditions were: injector: 225 °C, initial temperature: 40 °C (5 min), final temperature: 200 °C (5 min), heating rate: 5 °C/min; interface: 230 °C, gas carrier: He 99.99%; head pressure: 5 psi. The mass spectrometer was operated at 70 eV and the spectra were recorded in the range of m/z 25–550 amu in the acquisition mode "scan-full". The identification of the volatile components was performed by comparing the spectra obtained with those provided by the NIST 2.0 and WILEY 7n libraries.

2.7. Statistical analysis

Statistical analyses were performed using InfoStat/E coupled to R. An analysis of variance was performed to compare the mean concentrations of Pb found at each sampling site, using Tukey as a posteriori test. Furthermore, a Pearson correlation analysis between the concentration of Pb in the leaves of *T. minuta* and the percentage of the volatiles was performed. Finally, a principal component analysis (PCA) was carried out using all the quantified variables.

3. Results and discussion

3.1. Analysis of the volatiles by solid phase micro extraction

The volatile compounds analyzed by the SPME technique are presented in Table 1, with a total of thirteen of these compounds being quantified and the highest percentages corresponding to *cis*-tagetone (39.54%), dihydrotagetone (30.67%) and verberona (16.03%). These results are agreement with those found in a previous study in populations of *T. minuta* in the province of Chaco, Argentina, where the compounds that constituted the essential oil extracted by hydrodistillation were analyzed that study, there was a preponderance of dihydrotagetone (42.9%) and *cis*-tagetone (16.8%), whose proportions remained relatively constant in all samples for different localities of this province (Chamorro et al., 2008).

Regarding the composition of *T. minuta* oil in other regions of the world, studies from India, Rwanda, France and Saudi Arabia have also shown that the compounds found most were dihydrotagetone and *cis*-tagetone (El-Deeb et al., 2004; Prakasa Rao et al., 1999), with the latter being the major component in the essences of Saudi Arabia, concurring with our results.

Zygadlo et al. (1990) also found a great variation in the composition of essential oils among the different populations of *T. minuta*, with soil composition and genetic constitution possibly being responsible for these results. Composition variability may also depend on harvest site, growth stage, deficiencies in some nutrients (nitrogen, phosphorus and sulfur) and the light/shadow proportion (Chalchat et al., 1995; Graven et al., 1991; Kumar et al., 2014; Moghaddam et al., 2007; Omidbaigi et al., 2008; Zygadlo et al., 1990). Thus, there are many factors that can influence the chemical composition of essential oils in different populations on a regional or global scale.

3.2. Determination of Pb minuta L. essential oil

In the elemental analysis conducted for essential oil samples extracted by hydrodistillation, Pb was not present in any of the tested pools. This demonstrates that Pb was not transferred to the essential oil. This is consistent with a previous study where several species, such as cilantro (*Coriandrum sativum*), sage (*Salvia officinalis*), basil (*Ocimum basilicum*), dill (*Anethum graveolens*), chamomile (*Chamaemelum nobile*), hyssop (*Hyssopus officinalis*) and lemon balm (*Melissa officinalis*) growing near a Zn–Cu smelter and under high concentrations of heavy metals (Zn, Pb, Mn, Cu, Cd) in soil or culture medium, were tested for these contaminants and it was found that they were not transferred to the essential oil (Zheljazkov et al., 2008). In addition, similar results have been reported in studies with dill (*A. graveolens*), peppermint (*Mentha piperita*) and basil (*O. basilicum*) growing in areas exposed to different concentrations and combinations of Cd, Pb and Cu, and for three species of *Ocimum* spp. exposed to arsenic (Scora and Chang, 1997; Siddiqui et al., 2013; Zheljazkov et al., 2006). Thus, these finding indicate that the cultivation of aromatic plants is a good strategy for phytoremediation and may have an advantage over other crops harvested since the foliage is a source of heavy metal-free essential oil, which can generate income (Scora and Chang, 1997; Siddiqui et al., 2013; Zheljazkov et al., 2006). Additionally, this practice does not represent a risk from introducing heavy metals into the food chain, as is the case with most food crops such as soybeans (Salazar et al., 2012), lettuce, parsley, carrots, potatoes (Lăcătuşu and Lăcătuşu, 2008) or medicinal crops such as *Ocimum tenuiflorum* L. (Rai et al., 2004). This phenomenon may be related to the essential oil extraction process, with heavy metals being retained in the aromatic plant residues, thereby avoiding their presence in the commercial product and facilitating the disposal of waste (Zheljazkov et al., 2008).

Table 3
Pearson correlation analysis between the Pb concentration in the leaves of *T. minuta* and volatile compounds (%).

Compounds	Pearson coeficient	Probability
Sabinene	−0.08	0.620
Limonene	0.05	0.770
β-Ocimene	0.32	0.050
Dihydrotagetone	−0.26	0.110
β-Citral	−0.14	0.390
α-Thujone	0.50	0.001
β-Linalool	−0.13	0.440
2,4-Dimethyl, 2,6-octadiene	0.20	0.230
cis-Epoxiocimene	0.06	0.720
cis-Tagetone	0.26	0.110
trans-Tagetone	−0.24	0.140
cis-Ocimenone	−0.09	0.590
Verbenone	0.20	0.230

3.3. Determination of Pb in leaves of minuta

Table 2 shows the concentrations of Pb quantified in *T. minuta* leaves collected at different sampling sites. The analysis of variance conducted between sites showed significant differences between site 4 and the others. Moreover, the variance for concentrations within sites was high, in agreement with those obtained by Salazar and Pignata (2014), who found a wide variation in the Pb concentration in leaves of *T. minuta* growing in the same Pb enriched soil, which was attributed to genetic differences at the individual level. In addition, these researchers found Pb concentrations of 380.5 $\mu g\, g^{-1}$ in leaves of *T. minuta* in the same study area, demonstrating the great capacity of this plant to accumulate Pb when growing in contaminated soil. In our study the maximum concentration of 41.42 $\mu g\, g^{-1}$, was obtained at site 4 which was 6 times higher than that obtained in the plant leaves growing at the basal site.

3.4. Principal component analysis (PCA)

In order to observe which compounds in the essential oil of *T. minuta* leaves were related to the Pb concentration, a PCA analysis was performed. Fig. 2 shows the biplot graph obtained from the analysis, where it can be seen that the compounds most closely related to the Pb concentration in leaves were β-ocimene, β-linalool, α-thujone and 2,4-dimethyl 2,6-octadiene. It is noteworthy that both β-linalool and β-ocimene shared the same metabolic pathway, which may indicate that the presence of Pb can affect these pathways, although α-thujone has another biosynthetic pathway (Dewick, 2002).

3.5. Correlation analysis

A correlation analysis between the Pb concentration in the leaves of *T. minuta* and the percentages of volatile compounds obtained by the SPME technique was performed. Table 3 presents the correlation coefficients, showing that the β-ocimene and α-thujone compounds were significantly correlated with the concentration of Pb in leaves, suggesting that the increase of Pb in *T. minuta* might favor the synthesis of these two compounds. In fact, the emission rates of volatile compounds by plants are influenced by many different factors that can affect their synthesis. These factors can be genetic, biochemical, biotic or abiotic. Among the latter, the most important are temperature, light, water availability, humidity (Céspedes and Loaiza, 2007), and as found in the present study, contaminants. Abiotic stress caused by these factors may lead to morphological, physiological, biochemical and molecular changes (Bajguz and Hayat, 2009).

Contamination with metals such as Cd, Cr, Cu, Hg, Ni, Pb, Sn, Sb, Ti, Zn, and As, increases the activity of various enzymes, most of which are involved in plant defense against oxidative stress, with specific differences depending on the applied element (Clijsters et al., 1999). The most common response to the presence of these toxic elements is the excessive production of reactive oxygen species (ROS), which leads to direct cell damage in plants through the oxidation of biological compounds such as nucleic acids, proteins and lipids. Eventually, this can cause cell death as a defense mechanism against oxidative stress, and in some cases, the plant reacts forming secondary metabolites such as terpenes (Taiz and Zeiger, 2006), which may be part of a defense strategy adopted by the plants to a foreign and negative stimulus such as the presence of heavy metals (Rai et al., 2004).

Previous studies have demonstrated that the compound β-ocimene participates in the activation of defense genes. Arimura et al. (2002) found that when a mite infestation (*Tetranychus urticae*) or artificial damage occurred in lima bean (*Phaseolus lunatus*) leaves, the plant produced a mixture of volatile compounds including β-ocimene. Moreover, if healthy leaves were exposed to this compound, then transcription genes for the biosynthesis of molecules involved in the plant response against stressful stimuli were activated.

It has been shown that α-thujone is produced by *Salvia officinalis* L. plants under hydric stress (Nowak et al., 2012) or salinity stress (Taarit et al., 2010). Also, *Tanacetum vulgare* (Asteraceae) growing in contaminated soils produced 9 times more α-thujone than in uncontaminated areas (Stević et al., 2011). In our investigation, the percentages of α-thujone and β-ocimene increased with the presence of Pb in leaves, indicating a tolerance mechanism. However, as these are minority compounds, the main composition of volatiles did not change substantially. Consequently this plant species can be used in Pb contaminated areas in order to obtain a good quality and pollutant-free essential oil.

4. Conclusions

In this study, it was shown that *T. minuta* plants growing in soil contaminated with Pb accumulated the contaminant in their leaves, while it was not present in their essential oil. It was also demonstrated that the Pb concentration in leaves affected the production of some components, increasing the content of α-thujone and β-ocimene. Then as these compounds are involved in the plant response against stress a high Pb tolerance resulted.

Finally, we can conclude that *T. minuta* can be used for phytoremediation of Pb contaminated soils, as this metal was removed from the ground, with simultaneously, a marketable essential oil being obtained. However, Pb removal rate from soil was not determined, being necessary further studies.

Conflicts of interest

The authors have declared no conflicts of interest.

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