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ORIGINAL ARTICLE

Tagetes minuta L. Variability in Terms of Lead Phytoextraction from Polluted Soils: Is Historical Exposure a Determining Factor?

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Abstract *Tagetes minuta* L. is a plant which accumulates Pb under certain conditions, making it a candidate for phytoextraction projects because it also produces marketable essential oils without detectable Pb levels. Although extraction efficiency has been shown to significantly vary between individuals, these results have been obtained using only historically exposed populations, which leads to the questions: Is the ability to tolerate and accumulate Pb a property of the species? Or is it a characteristic of some individuals from a historically exposed population? In this context, a greenhouse experiment was performed to analyse the intrapopulation and interpopulation variability in response to Pb among individuals from historically unexposed and exposed populations. In addition, we also attempted to identify relationships between certain capabilities (toleration and accumulation of Pb) and the physiological parameters related to oxidative stress or the volatile compounds of the essential oils. The Pb concentration was determined by total reflection X-ray fluorescence, physiological parameters were obtained by spectrophotometry, and essential oils were analysed by gas chromatography. The results demonstrated that adequate tolerance and accumulation capabilities are present in *T. minuta*, irrespective of the exposure history. These findings may be associated to a hormesis response, which includes enhancement of pigments, biomass production and the uptake of other heavy metals such as micronutrients. Nevertheless, the historically exposed population had a better tolerance to Pb, since it presented defence characteristics reflected in the essential oil composition and in the avoidance of damage at the lipid peroxidation level after Pb uptake.

Keywords Pb pollution · Phytoremediation · Tolerance · Essential oils

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1 Introduction

Agricultural soil pollution with heavy metals produces a risk for agricultural product consumers and for the environment (Kelepertzis 2014). Lead (Pb) is among the most toxic heavy metals, having a wide distribution. Its effects on health are well known (Karri et al. 2016), with high concentrations of Pb in soils and crops grown in or near industrial areas representing a human health risk (Kabata-Pendias and Pendias 2001). For this reason, agricultural polluted soils require remediation, with this being a current global challenge (Jadia and Fulekar 2009). However, conventional methods of remediation are expensive, harm the environment and destroy the soil's ability to be used as a productive resource (Dixit et al. 2015). In this context, phytoremediation is an inexpensive alternative which does not cause degradation of soil fertility or structure (Blaylock and Huang 2000). Phytoremediation contemplates soil conservation using plants, with there being different options available; for example, phytoextraction (in which toxic metals are removed by plants), with the pollutant concentration in soil being reduced every harvest (Jadia and Fulekar 2009). Tolerance, high accumulation levels in plant harvestable parts, and high biomass production have been reported to be the most important requirements for species selection (Dixit et al. 2015).

Certain plant species produce essential oils and accumulate heavy metals (Zheljazkov et al. 2006; Gautam and Agrawal 2017), which often makes these species more profitable because they extract metals while they also provide economic benefits (Danh et al. 2010). One of these, is *Tagetes minuta* L., an annual and aromatic plant from the Asteraceae family that produces volatile essential oils, which are extracted by hydrodistillation and used in several industries, such as cosmetics, perfumery, as a food flavouring and in beverages, and in pesticides as well as medicine (Vasudevan et al. 1997; Rocabado et al. 2011; Dinesh et al. 2014; Karimian et al. 2014; Shirazi et al. 2014; Wanzala et al. 2014; Chaaban et al. 2017). The volatile compound compositions of *Tagetes minuta* L. have been widely studied (Zygadlo et al. 1990; Chalchat et al. 1995; Vázquez et al. 2011). In addition, *T. minuta* accumulates Pb with the essential oil produced having no detectable Pb levels, even when its concentration in leaves is high (Salazar and Pignata 2014; Sosa et al. 2016). This indicates that *T. minuta* is useful for phytoextraction and simultaneously provides a marketable and safe product.

Research on Pb accumulation by *T. minuta* has revealed a high variability in the extraction efficiency between individuals. In this regard, Posthuma et al. (1993), established that heavy metal pollution is a selection pressure, which operates in a stable, permanent and intense way, with this being a crucial factor in the phytoremediation field (Küpper et al. 2007). Chronic exposure may result in the appearance of tolerant ecotypes that prevent, decrease or repair the harm produced by the metal. These capabilities can be developed by acclimation, during individual exposure, by adaptation, through selection acting upon genetic variation in tolerance, or by a combination of both (Posthuma et al. 1993; Meyer et al. 2010). Lead exposure results in oxidative stress in most plants, leading to physiological consequences (Sharma et al. 2012). In order to be tolerant and accumulator individuals, populations or species must have a specific profile with respect to the physiological parameters related to oxidative stresses.

The present study analysed the intrapopulation and interpopulation variability of *T. minuta* behaviour in response to Pb among individuals from a population historically exposed to this contaminant and from an unexposed population. The aim was to elucidate if tolerance and accumulation capability are specific to the species or exposed population, and whether any relationship exists between these capabilities and the physiological parameters related to oxidative stress or volatile compounds of the essential oils.

2 Experimental

2.1 Seed and Soil Collection

Tagetes minuta L. seeds were collected from two natural populations during the mature stage (March–April). One population, the Bouwer population (BP) has been growing in Pb-polluted soils for the last 30 years due to a battery recycling smelter and is located 18 km south of Córdoba City. In contrast, the Intiyaco population (IP), has never been exposed to Pb and is located 80 km southwest of Córdoba City.

Seeds were sterilised and then germinated for four days at 25 °C in sand. The resulting seedlings were grown in pots for the different treatments under greenhouse conditions (temperature from 18 to 35 °C and summer photoperiod).

Top soil (15 cm) was collected around a former battery recycling plant in Bouwer using a stainless-steel shovel. Soil collection was carried out in two previously studied areas which present different soil Pb concentrations (Salazar and Pignata 2014). These areas were defined as "polluted soil" and "control soil", with Table 1 displaying their physicochemical properties. Soil samples were placed in plastic bags, transported to the laboratory, sieved to ≤2 mm and homogenised.

2.2 Experimental Design

The experimental design was established to investigate two factors (population origin and Pb concentration in soil), with two levels studied for each factor (BP or IP and polluted or control soil, respectively), implying four treatments. Individuals from each population were numerically identified and assigned to each of the two soil treatments in a completely randomised experimental design. Random groups of 25 plants were grown together in 15-L pots, thereby sharing the rhizosphere as occurs in the field. Four pots with polluted soil were prepared for each population, while one pot per population was filled with control soil. The unbalanced design was considered during the statistical analysis.

Table 1 Mean values ± SE and results of analysis of variance (ANOVA) for physicochemical properties of soils

Variable	Control soil	Polluted soil	ANOVA
Distance to the smelter (m)	1011	31	
pH	6.5 ± 0.1	6.7 ± 0.1	0.09
Electrical conductivity (μs cm^2)	64 ± 3	72 ± 5	0.06
Organic matter (%)	9 ± 1	8.5 ± 0.8	0.41
Carbon:Nitrogen ratio (%)	9.5 ± 0.1	8.9 ± 0.1	0.09
Particle size distribution (%)			
Sand (2–0.05 mm)	16.0 ± 0.4	15.9 ± 0.9	0.77
Silt (0.05–0.002 mm)	82 ± 1 a	78.0 ± 0.1 b	0.0001
Clay (<0.002 mm)	2.0 ± 0.8 b	6.1 ± .08 a	0.0001
Pb I (mg kg^{-1})	21 ± 2 b	168 ± 6 a	0.0001
Pb II (mg kg^{-1})	0.6 ± 0.1 b	311 ± 17 a	0.0001
Pb III (mg kg^{-1})	5 ± 2 b	734 ± 5 a	0.0001
Pb Pseudototal (mg kg^{-1})	27 ± 1 b	1212 ± 13 a	0.0001

Abbreviations: Pb I: mobile or exchangeable Pb (extracted with 1 M $CaCl_2$ pH 7); Pb II: Mobilisable Pb (extracted with 0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M TEA pH 7.3); Pb III: total Pb concentration (pure HNO_3 extraction). Values in each row (ANOVA) followed by the same letter do not differ significantly at $p <$ 0.05

Plants were collected at the beginning of the flowering stage. In the first step, six leaves from each plant were collected and conserved at −80 °C for further analysis of the physiological parameters and volatile composition. In a second step, each plant was harvested for root exudate collection following a Niu et al. (2011) and Salazar et al. (2016b) methodology to carry out further analyses of the heavy metal extraction power. For the final step, individual plants were washed and sonicated with ultrapure water (Milli-Q), separated by organs (root, stem and leaves) and dried at 60 °C. The dried biomass per plant was determined, and the samples were ground, homogenised and conserved for further analyses of the heavy metal concentrations.

2.3 Determinations

2.3.1 Exudate Extraction Power

The collected root exudate samples were used as the extraction solution for Pb-polluted soil (properties in Table 1). A volume of 15 mL of root exudates was mixed with 1 g of soil and stirred for 6 h (Salazar et al. 2016b). Once centrifuged, the filtered supernatant was stored for metal quantification by total reflection X-ray fluorescence (TXRF), following the methodology described for plants below. The same procedure was performed using tap water instead of root exudate solution. The difference between the metals extracted by tap water and those extracted by the exudate solution was registered as the exudate extraction power relative to water (EEP_w), expressed as either negative or positive values.

2.3.2 Determination of Pb in Plant Organs

Lead concentrations in root, stem and leaves were determined according to Wannaz et al. (2011) by (TXRF), using synchrotron radiation at the XRF beamline at the National Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil.

Quality control and calibration was conducted using blanks, standard solutions with known concentrations of several heavy metals, and samples of the standard reference material "CTA-OTL-1" (oriental tobacco leaves, Institute of Nuclear Chemistry and Technology, Warsaw, Poland), which were prepared following the same methodology used for plant samples. The results were above 85% of the certified value, with the data errors below 10% and the standard deviation between replicates below 8%.

2.3.3 Determination of Physiological Parameters

For the determination of physiological parameters, 100 mg (fresh weight) were ground in liquid N_2 and homogenised with 1500 μL of ethylic alcohol 80% *v*/v analytical grade. Subsequently, the samples were centrifuged at 14,000 RPM for 10 min at 4 °C. A supernatant aliquot was used for determining the ferric-reducing ability of plasma (FRAP) according to Robert et al. (2014). The pellet was resuspended in the remaining supernatant and incubated at 80 °C for 20 min. Once cooled to room temperature, it was centrifuged at 14,000 rpm for 10 min. The supernatant was used for quantification of malondialdehyde (MDA) (Heath and Packer 1968), a, b and total chlorophyll, carotenoids (Lichtenthaler 1987; Palta 1990) and soluble sugars (SS) (Leyva et al. 2008), while the pellet was dried at 60 °C for dry weight determination. All physiological determinations were read in 96-well microplates in a spectrophotometer (Thermo Scientific Multiskan Spectrum). For MDA, chlorophyll and carotenoid calculations, the following equations were used, while for FRAP and SS, the calibration curves of Trolox and glucose, respectively, were determined.

$[MDA] = (Abs_{532} - Abs_{600})/(\varepsilon \cdot l)$, where *Abs* is absorbance at the wave longitude indicated by the subscript, ε is the molar absorptivity coefficient for MDA (155 mM^{-1} cm^{-1}) and l is the optical path.

$$Chl_{Total} = (1000 \cdot Abs_{654})/(39.8 \cdot l)$$
$$Chl_a = (13.36 \cdot Abs_{663}/l)/(5.19 \cdot Abs_{645}/l)$$
$$Chl_b = (27.43 \cdot Abs_{645}/l)/(8.12 \cdot Abs_{663}/l)$$
$$Carotenoid = \left(1000 \cdot \frac{Abs_{470}}{l} - 2.13 \cdot Chl_a - 97.64 \cdot Chl_b\right)/209$$

where Chl is the chlorophyll concentration, with the subscript denoting a, b or total; *Abs* is the absorbance at the wave longitude indicated by the subscript and l is the optical path.

2.3.4 Volatile Compounds by Solid Phase Micro Extraction (SPME)

For determination of volatile compounds, 100 mg of the material conserved at −80 °C were placed in a sealed vial in a water bath at 40 °C for 10 min, after which, a fibre of polydimethylsiloxane (PDMS, 100 μm) was introduced into the vial and exposed for 30 min at 40 °C (Vázquez et al. 2011). Then, substances extracted by the fibre were desorbed at 250 °C for 10 min in the injection port of a gas chromatograph Q-700 coupled with an ion trap mass detector and equipped with a manual injection port operating in a splitless mode (PerkinElmer, Shelton, CT, USA) for volatile component identification utilising a DB-5 column (30 m, 90.25 mm i.d. and 0.25 m coating thickness). The working conditions used were those reported by Vázquez et al. (2011). NIST 2.0 and the WILEY 7n library were consulted for identification of the volatile components.

2.4 Data Analysis

2.4.1 Translocation and Total Transference Factors and Total Extraction

The quotient between Pb concentration in the aerial part of the plants and the root is known as the translocation factor (TF), and was calculated according to Bu-Olayan and Thomas (2009). As Pb concentration was determined in leaves and stem separately, Pb concentration in the aerial parts was calculated as follows:

$$[Pb]_{aerial} = \frac{[Pb]_{leaves} \cdot BM_{leaves} + [Pb]_{stem} \cdot BM_{stem}}{BM_{leaves} + BM_{stem}}$$

The total transfer factor (TTF) considers the migration of Pb from root to aerial organs in terms of mass of Pb instead of concentration, which was calculated according to Salazar et al. (2016a). Then, the total extraction (TE) of Pb per plant in each organ was obtained as follows:

$$TE_{leaves} = [Pb]_{leaves} \cdot BM_{leaves}$$
$$TE_{stem} = [Pb]_{stem} \cdot BM_{stem}$$
$$TE_{root} = [Pb]_{root} \cdot BM_{root}$$
$$TE_{plant} = TE_{leaves} + TE_{stem} + TE_{root}$$

where [Pb] is the concentration of Pb in the organ indicated in the subscript, and BM is the total dry biomass produced by each plant in the organ indicated in the subscript.

2.4.2 Statistical Analysis

Analysis of variance was conducted to compare mean values of the response variables among the four treatments, considering the unbalanced design. The distribution of the variables was tested for normality by the Shapiro-Wilks test. Heteroscedasticity was incorporated into the model using Infostat/E coupled to R. Whenever the ANOVA indicated significant effects ($p < 0.05$), a comparison of mean pairs was carried out by using the Tukey test. Principal components analysis was performed to evaluate the differences among treatments.

Pearson's correlation coefficients were calculated to relate the Pb concentrations in plant organs with physiological parameters and volatile compound compositions.

3 Results

3.1 Soil Pb Pollution Effect on *T. minuta* Populations

In a first approach, the BP and IP response to Pb presence in soil was compared for variables related to the Pb extraction efficiency, physiological parameters and volatile compounds (Table 2). Survival was determined as the percentage of plants that remained alive in each pot; therefore, for this variable only, $n = 4$ for polluted soil treatments, with no standard error for the control soil treatment because there was only one pot. The IP presented a significantly higher survival than BP when growing in polluted soils. Aerial biomass production was enhanced in BP in response to Pb-polluted soils. The Pb concentration in leaves was highly variable for both populations, with values ranging from 10 to 70 $\mu g\ g^{-1}$ when they grew in polluted soils, with only one individual in BP which presented 195 $\mu g\ g^{-1}$ of Pb in leaves. The high variability of Pb concentration in leaves impeded the detection of differences between plants grown in polluted and in control soils. The Pb concentrations in stems and roots were significantly higher for polluted soils, with no differences between populations. The Pb total extraction in each organ and per plant, the Pb extraction power of the exudates, and TF and TTF did not differ significantly between populations.

With regard to the physiological parameters, the results for FRAP indicated that the antioxidant capacity was reduced when plants from both populations grew in polluted soil compared to control soil, remaining higher for IP. Plants from the BP experiment revealed a reduction in the content of soluble sugars when Pb was present in soil, while IP plants showed an enhancement. Plants from both populations presented no significant differences in MDA due to Pb exposure, indicating that this factor did not induce lipid peroxidation. The chlorophyll and carotenoid contents were enhanced in both populations in response to Pb pollution of the soil.

Volatile compounds are the principal characteristic of *T. minuta* essential oils. The studied populations presented many of these compounds, with the most dominant volatile compound for BP being dihydrotagetone, while IP showed an even greater diversity of compounds, mostly *cis*-tagetone, verbenone, limonene and β-ocimene. In the Bouwer population, the composition of volatile compounds was not modified in response to Pb pollution, while IP showed increased percentages of sabinene, limonene, α-thujone and *cis*-, and a reduced percentage of β-Linalool. Figure 1 shows the volatile composition profiles for each treatment.

Table 2 Mean values ± SE and results of analysis of variance (ANOVA) for variables related to Pb extraction efficiency, physiological parameters in leaves and volatile compound composition

	Bouwer Population		Intiyaco Population		ANOVA (p)
	Polluted Soil	Control Soil	Polluted Soil	Control Soil	
Variables related to Pb extraction eficiency					
Survival (%)	67 ± 12b	88	90 ± 4 a	86	0.03
BM_{Leaves} (g $plant^{-1}$)	1.6 ± 0.3 a	0.8 ± 0.3 b	1.9 ± 0.2 a	1.7 ± 0.4 a	0.04
BM_{Stem} (g $plant^{-1}$)	3.7 ± 0.9 a	1.3 ± 0.8 b	5.0 ± 0.7 a	2.9 ± 1.4 a	0.05
BM_{Root} (g $plant^{-1}$)	0.3 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0.2	0.09
$[Pb]_{Leaves}$ (μg g^{-1})	36 ± 8	24 ± 3	28 ± 2	22 ± 4	0.06
$[Pb]_{Stem}$ (μg g^{-1})	50 ± 7 a	6 ± 1 b	56 ± 5 a	7 ± 1 b	0.0001
$[Pb]_{Root}$ (μg g^{-1})	342 ± 41 a	15 ± 2 b	409 ± 22 a	10 ± 3 b	0.0001
TE_{Leaves} (μg $plant^{-1}$)	51 ± 10 a	20 ± 6 b	45 ± 96 a	39 ± 7 a	0.02
TE_{Stem} (μg $plant^{-1}$)	109 ± 36 b	9 ± 3 c	296 ± 88 a	17 ± 4 c	0.0001
TE_{Root} (μg $plant^{-1}$)	104 ± 29 a	6 ± 3 b	170 ± 24 a	4 ± 3 b	0.0001
TE_{Plant} (μg $plant^{-1}$)	264 ± 130 a	35 ± 8 b	510 ± 105 a	45 ± 9 b	0.0001
Pb- EEP_w (μg g^{-1})	−0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.3	1.5 ± 0.7	0.06
TF_{Pb}	0.16 ± 0.02 b	1.1 ± 0.2 a	0.12 ± 0.01 b	2.2 ± 0.5 a	0.0001
TTF_{Pb}	2.5 ± 0.4 c	11 ± 2 b	2.0 ± 0.3 c	32 ± 7 a	0.0001
Physiological parameters					
FRAP (umol Trolox g^{-1})	306 ± 56 c	905 ± 89 a	528 ± 44 b	696 ± 89 a	0.0001
SS (mg g^{-1})	124 ± 11 b	182 ± 18 a	169 ± 9 a	120 ± 9 b	0.0002
MDA (nmol g^{-1})	671 ± 38 a	750 ± 61 a	549 ± 29 b	512 ± 61 b	0.003
Chl_a (mg g^{-1})	5.9 ± 0.5 a	3.7 ± 0.5 b	6.9 ± 0.4 a	3.7 ± 0.5 b	0.0001
Chl_b (mg g^{-1})	1.7 ± 0.2 a	1.1 ± 0.1 b	1.9 ± 0.1 a	0.9 ± 0.1 b	0.0001
Chl_{Total} (mg g^{-1})	8.9 ± 0.8 a	5.5 ± 0.7 b	10.3 ± 0.6 a	5.3 ± 0.7 b	0.0001
Carotenoids (μg g^{-1})	2.7 ± 0.2 a	1.5 ± 0.2 b	2.7 ± 0.2 a	1.3 ± 0.1 b	0.0001
Percentage composition of the volatile compounds (%)					
Sabinene	0.33 ± 0.06 b	0.3 ± 0.2 b	0.61 ± 0.05 a	0.4 ± 0.1 b	0.005
Limonene	3.0 ± 0.6 b	3 ± 2 b	6.7 ± 0.5 a	5 ± 1 b	0.0001
β-Ocimene	3.5 ± 0.6 b	4 ± 2 b	6.1 ± 0.5 a	8 ± 1 a	0.006
Dihydrotagetone	83 ± 3 a	89 ± 8 a	10 ± 3 b	1.2 ± 0.5 b	0.0001
β-Citral	0.77 ± 0.06 b	0.7 ± 0.2 b	1.24 ± 0.05 a	1.4 ± 0.1 a	0.0001
α-Thujone	0.04 ± 0.03 c	0.03 ± 0.02 c	0.52 ± 0.03 a	0.23 ± 0.09 b	0.0001
β-Linalool	0.22 ± 0.05 b	0.2 ± 0.1 b	0.34 ± 0.04 b	0.7 ± 0.1 a	0.003
2,4 Dimethyl 2,6 octadiene	0.05 ± 0.03 b	nd	0.42 ± 0.02 a	0.45 ± 0.07 a	0.0001
cis-Epoxiocimene	nd	nd	6.1 ± 0.6 a	2.7 ± 0.9 b	0.0001
cis-Tagetone	3 ± 1 b	3 ± 1 b	38 ± 3 a	26 ± 7 a	0.0001
trans-Tagetone	nd	nd	1.0 ± 0.7	nd	ns
cis-Ocimenone	1.5 ± 0.8 b	nd	7 ± 1 a	9 ± 3 a	0.005
Verbenone	3 ± 2 c	nd	21 ± 2 b	45 ± 6 a	0.0001

Values in each row (ANOVA) followed by the same letter do not differ significantly at $p < 0.05$. nd: not detectable. BM: biomass. [Pb]: Pb concentration. TE: Total extraction. EEP_w: Exudate extraction power relative to water. TF: Translocation factor. TTF: Total transfer factor. FRAP: ferric reducing ability of plasma. SS: Soluble sugars. MDA: malondialdehyde. Chl: Chlorophyll

3.2 Lead Uptake Effect on *T. minuta* Populations

Most publications on heavy metal accumulation in plants and their related physiology have focused on the analysis of heavy metal concentrations in the soil and have considered the heavy metal concentrations in plants only as a response variable. To try to obtain a deeper understanding, a different approach was adopted, taking into account the plants which grew in only Pb-polluted soils, and analysing the relationship between Pb concentrations in plant organs, the physiological variables (Table 3) and the volatile compositions (Table 4).

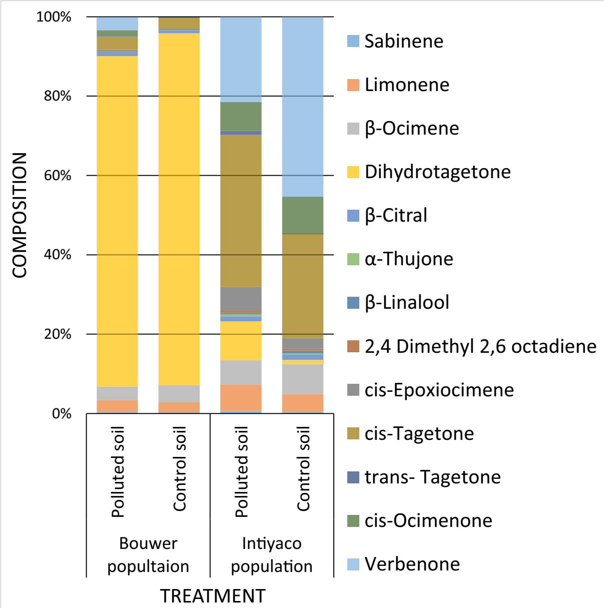


Fig. 1 Average volatile composition profile of *Tagetes minuta* L. essential oils for historically Pb exposed and unexposed populations grown in polluted and in control soil

Physiological variables in leaves were correlated with Pb concentrations in leaves for BP, but not for IP. Nevertheless, for the latter population, physiological parameters in leaves were correlated with Pb concentrations in roots. For both populations, the correlations were positive for pigments and FRAP. For BP, MDA was not correlated with Pb in leaves, while for IP, this parameter was positively correlated with Pb in roots, indicating lipid peroxidation related to the metal presence in the plant. For BP, soluble sugars were positively correlated with Pb concentrations in leaves.

The percentage compositions of some volatile compounds were correlated with Pb concentration, biomass production or total extraction in leaves in both populations (Table 4).

In the case of BP, sabinene, limonene, β-ocimene, β-citral and verbenone were positively correlated with Pb concentrations in leaves, while dihydrotagetone was negatively correlated.

Table 3 Spearman correlation coefficients for Pb concentration in leaves or roots with physiological parameters for *T. minuta* populations

	Bouwer population $[Pb]_{Leaves}$	Intiyaco population $[Pb]_{Root}$
FRAP	0.40*	0.39*
SS	0.43*	ns
MDA	ns	0.39*
Chl_a	0.54**	0.32*
Chl_b	ns	0.31*
Chl_{Total}	0.46*	0.35*
Carotenoids	0.44*	0.38*

ns, not significant.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [Pb]: Pb concentration. FRAP: ferric reducing ability of plasma. SS: Soluble sugars. MDA: malondialdehyde. Chl: Chlorophyll

Table 4 Spearman correlation coefficients for Pb concentration, biomass and total extraction in leaves with volatile compounds for *T. minuta* populations

	Bouwer population			Intiyaco population		
	$[Pb]_{Leaves}$	BM_{Leaves}	TE_{Leaves}	$[Pb]_{Leaves}$	BM_{Leaves}	TE_{Leaves}
Sabinene	0.62***	ns	ns	ns	−0.36*	ns
Limonene	0.53**	ns	ns	0.32*	−0.41*	ns
β-Ocimene	0.61***	ns	ns	ns	ns	ns
Dihydrotagetone	−0.51**	ns	ns	ns	ns	ns
β-Citral	0.4*	0.53*	0.49*	−0.38*	0.67***	0.59***
α-Thujone	ns	ns	ns	ns	ns	ns
β-Linalool	ns	0.43*	ns	−0.38*	0.51**	0.46**
2,4 Dimethyl 2,6 octadiene	ns	ns	ns	ns	ns	ns
cis-Epoxiocimene	ns	ns	ns	ns	ns	ns
cis-Tagetone	ns	ns	ns	ns	ns	ns
trans-Tagetone	ns	0.47*	ns	ns	ns	ns
cis-Ocimenone	ns	ns	ns	ns	ns	ns
Verbenone	0.71***	ns	Ns	ns	ns	ns

ns, not significant.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. [Pb]: Pb concentration. BM: biomass. TE: Total extraction

In addition, β-citral, β-linalool and *trans*-tagetone were positively correlated with leaf biomass production. In this study, for BP, β-citral was the only volatile compound that was correlated with Pb total extraction in leaves, because it was related to both Pb concentration and biomass production.

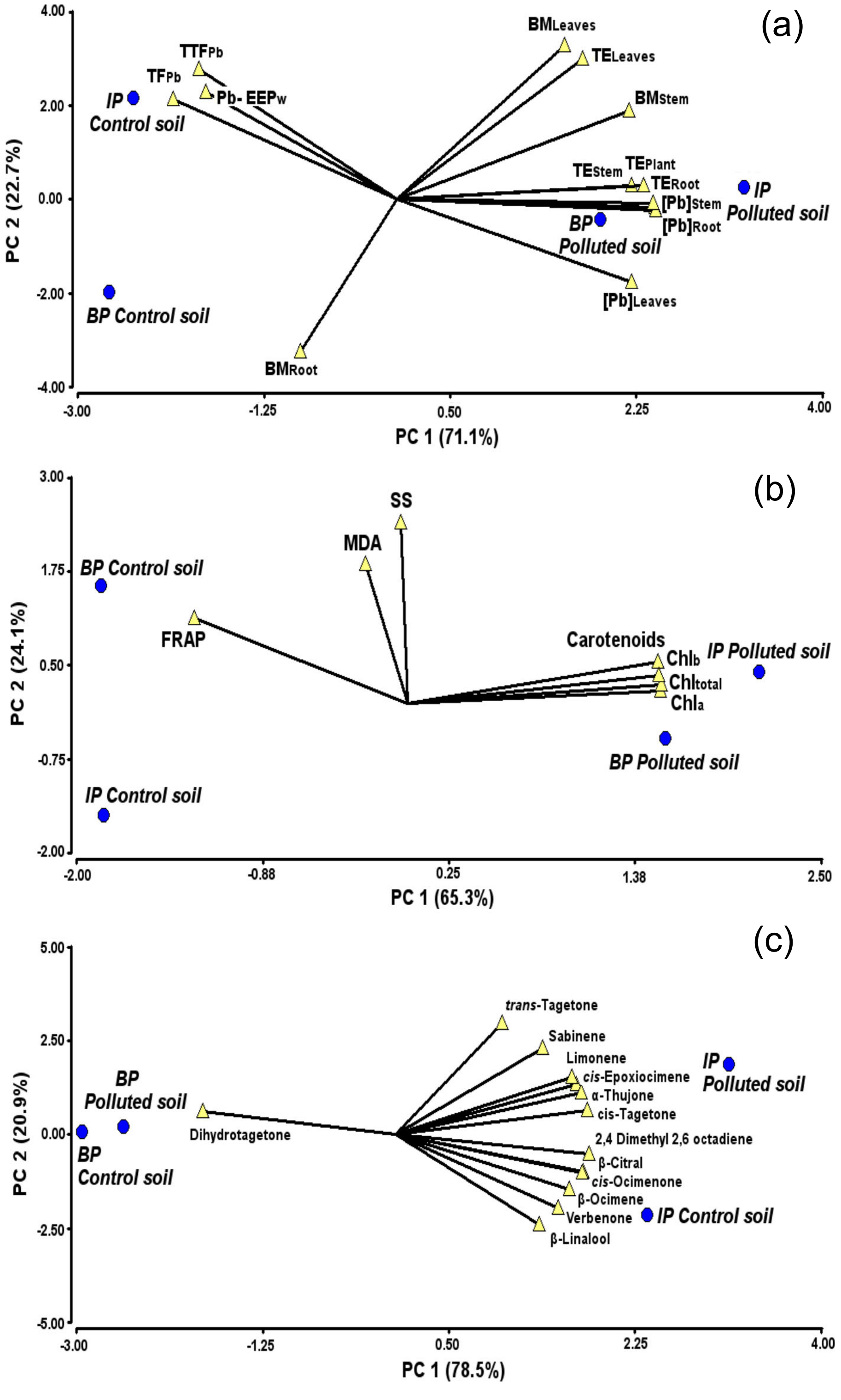
For IP, only limonene was positively correlated with Pb concentrations in leaves, while β-citral and β-linalool were negatively correlated to this variable. As Limonene was also negatively correlated to biomass, then this volatile compound was not related to Pb total extraction. For β-citral and β-linalool, correlation with the biomass was positive, and on balance, these compounds were positively correlated with Pb total extraction.

3.3 Relationship among Variables

Principal component analysis was performed to compare populations by integrating groups of variables (Fig. 2). Figure 2 (a and b) shows that both populations had a similar behaviour with respect to variables related to Pb extraction efficiency and physiological parameters, respectively. Aerial biomass, carotenoid and chlorophyll content, Pb extraction, and Pb concentration were associated with plants grown in polluted soils for both populations. Figure 2c reflects the differences in volatile composition between populations, indicating a stronger effect of Pb on IP.

4 Discussion

Many investigations have shown that terrestrial plants are sensitive to the presence of heavy metals in soil, showing a reduction in chlorophyll a and b contents (Moustakas et al. 1994) due to an inhibition of the biosynthesis of these pigments and the enzymes involved in the process (Rai et al. 2016). Even in the case of *Brassica juncea*, a rapidly growing species recommended in the literature for use in heavy metal phytoremediation, a decrease in photosynthetic


(a)
PC 2 (22.7%)
PC 1 (71.1%)
4.00
2.00
0.00
-2.00
-4.00
-3.00
-1.25
0.50
2.25
4.00
TTFPb
TFPb
Pb- EEPw
IP Control soil
BP Control soil
BMRoot
BMLeaves
TELeaves
BMStem
TEStem
TEPlant
TERoot
[Pb]Stem
[Pb]Root
[Pb]Leaves
IP Polluted soil
BP Polluted soil
(b)
PC 2 (24.1%)
PC 1 (65.3%)
3.00
1.75
0.50
-0.75
-2.00
-2.00
-0.88
0.25
1.38
2.50
SS
MDA
BP Control soil
FRAP
Carotenoids
Chlb
Chltotal
Chla
IP Polluted soil
BP Polluted soil
IP Control soil
(c)
PC 2 (20.9%)
PC 1 (78.5%)
5.00
2.50
0.00
-2.50
-5.00
-3.00
-1.25
0.50
2.25
4.00
trans-Tagetone
Sabinene
Limonene
cis-Epoxiocimene
α-Thujone
cis-Tagetone
2,4 Dimethyl 2,6 octadiene
β-Citral
cis-Ocimenone
β-Ocimene
Verbenone
β-Linalool
IP Polluted soil
IP Control soil
BP Polluted soil
BP Control soil
Dihydrotagetone

◀ **Fig. 2** Principal components analysis for *Tagetes minuta* L. from Bouwer population (BP) and Intiyaco population (IP). **a** Variables related to Pb extraction efficiency. **b** Variables related to physiological parameters. **c** Volatile composition of essential oils. Abbreviations: EEPw: exudate extraction power relative to water; FRAP: ferric reducing ability of plasma; MDA: malondialdehyde; SS: soluble sugars; Chl: chlorophyll; TF: translocation factor; TTF: total transfer factor; $[Pb]_{organ}$: concentration of Pb in the plant organ indicated in the subscript; TE: total extraction. BM_{organ}: biomass (dry weight) in the organ indicated

pigments was observed in response to Pb exposure (John et al. 2012), which was the main cause of biomass reduction (Rai et al. 2016). A similar effect was reported for two other Pb accumulator species, *Coronopus didymus* (Sidhu et al. 2017) and *Chrysanthemum indicum* (Mani et al. 2015). In contrast, in our present study, neither population of *T. minuta* exhibited this toxic effect, while enhanced pigments and consistently enhanced biomass production were observed. This indicates that *T. minuta* is tolerant to Pb, independent of the transgenerational history of exposure, and capable of responding to Pb with a hormesis response (Pinto et al. 2004). In this regard, Rodriguez et al. (2015) found similar results in a study on a *Pisum sativum* cultivar resistant to Pb. The increase in chlorophyll may have occurred due to enhanced carotenoid values, which are produced in order to neutralise the reactive oxygen species (Foyer and Harbinson 1994) and have a protective function on photosystems (Rai et al. 2016).

The results of the present study suggest that pigment improvement as a response to Pb toxicity could be related to the uptake of other heavy metals (Table 5). Both populations exuded compounds with negative EEP_w for Fe, Cu and Zn when grown in control soil, which implies that exudates dissolve smaller amounts of these metals than water. In Pb-polluted soil, plants exuded compounds that increased the dissolution of these elements (essential micronutrients) in water. Under stress, some plants may respond by releasing compounds to solubilise the nutrients necessary for their development (Sterckeman et al. 2005; Neumann 2007). In fact, some Ni-accumulating species release exudates that aid the incorporation of this contaminant, thereby gaining tolerance to its toxicity by the incorporation of other nutrient metals (Wenzel et al. 2003). Moreover, our interpretation of our findings is coherent with Venkatachalam et al. (2017), who demonstrated that the Zn supplement alleviates Pb toxicity in the phytoremediation plant *Leucaena leucocephala*, thus enhancing pigment content.

Mn EEP_w was not affected by exposure to the Pb contaminant. Mn is one of the most abundant elements in soil, with its solubility being linked to pH changes (Kabata-Pendias and Pendias 2001). The fact that Pb exposure did not modify Mn EEP_w suggests that *T. minuta* exudates regulate element uptake by organic compounds and not by pH changes, a strategy associated with accumulator plants (Jones and Darrah 1994). Additionally, Mn EEP_w was positive and relatively high in control soil, with an increase in its solubility being probably not necessary to improve tolerance.

Regarding Mn, Fe, Cu and Zn uptake, the concentrations of these metals were significantly higher in plants exposed to Pb, suggesting that these essential elements were required by the plant against this stress factor. This enhancement was also found in leaves for Mn, Cu and Zn. This demand for these elements is coherent with the modification in EEP_w. Singh and Agrawal (2007) studied metal uptake of *Beta vulgaris* growing in soil amended with contaminated residues, and demonstrated competition between Pb and Mn with respect to their uptake, which is in agreement with the findings of Kabata-Pendias and Pendias (1984). It is remarkable that the species studied by Singh and Agrawal (2007) was physiologically affected, with a decrease occurring in photosynthetic pigments. Thus, these authors concluded that this species is not suitable for growth in polluted soils. In the present study, *T. minuta* incorporates a greater

Table 5 Mean values ± SE and results of analysis of variance (ANOVA) for heavy metal (Mn, Fe, Cu and Zn) concentration in leaves, stem and root and exudate extraction power for *T. minuta* populations grown in Pb-polluted and in control soil

	Bouwer Population		Intiyaco Population		ANOVA (p)
	Polluted Soil	Control Soil	Polluted Soil	Control Soil	
$[Mn]_{Leaves}$ (μg g^{-1})	107 ± 10 a	37 ± 3 c	123 ± 8 a	60 ± 4 b	0.0001
$[Mn]_{Stem}$ (μg g^{-1})	24 ± 3 a	10 ± 2 c	19 ± 2 b	17 ± 2 b	0.0007
$[Mn]_{Root}$ (μg g^{-1})	48 ± 4 a	22 ± 2 b	34 ± 4 a	24 ± 3 a	0.0001
Mn- EEP_w (μg g^{-1})	4 ± 3	6 ± 4	3 ± 2	7 ± 4	ns
$[Fe]_{Leaves}$ (μg g^{-1})	238 ± 22	205 ± 22	226 ± 18	172 ± 5	ns
$[Fe]_{Stem}$ (μg g^{-1})	71 ± 27	47 ± 8	331 ± 100	60 ± 9	ns
$[Fe]_{Root}$ (μg g^{-1})	741 ± 57 a	421 ± 45 c	582 ± 45 b	393 ± 51 c	0.0001
Fe- EEP_w (μg g^{-1})	5 ± 5 a	−27 ± 7 b	15 ± 4 a	−37 ± 8 b	0.0001
$[Cu]_{Leaves}$ (μg g^{-1})	11 ± 1 a	7.1 ± 0.5 b	9.8 ± 0.9 a	7.0 ± 0.6 b	0.003
$[Cu]_{Stem}$ (μg g^{-1})	5.8 ± 0.7 b	6.8 ± 0.7 b	6.9 ± 0.6 b	10.7 ± 0. a	0.0001
$[Cu]_{Root}$ (μg g^{-1})	26 ± 8	28 ± 20	27 ± 6	94 ± 23	ns
Cu- EEP_w (μg g^{-1})	0.4 ± 0.1 a	−0.3 ± 0.2 b	0.1 ± 0.1 a	−0.6 ± 0.2 b	0.002
$[Zn]_{Leaves}$ (μg g^{-1})	70 ± 3 a	50 ± 3 b	71 ± 2 a	37 ± 3 c	0.0001
$[Zn]_{Stem}$ (μg g^{-1})	25 ± 6	98 ± 56	28 ± 5	23 ± 6	ns
$[Zn]_{Root}$ (μg g^{-1})	41 ± 3 a	25 ± 2 b	35 ± 2 a	22 ± 2 b	0.0001
Zn- EEP_w (μg g^{-1})	0.4 ± 0.2 a	−2.8 ± 0.3 b	0.4 ± 0.2 a	−4.4 ± 0.4 b	0.0001

HM: Heavy metal. $[HM]_{organ}$: heavy metal concentration in each organ. HM-EEPw: Exudates extraction power relative to water for each heavy metal. ns: not significant

amount of Mn in the whole plant in Pb-polluted soil, which is in agreement with the increase in the photosynthetic pigments.

Regarding the FRAP results, Pb exposure reduced the *T. minuta* antioxidant capacity, probably because this is a species with a high content of essential oils which, under normal conditions without stress, have a high antioxidant capacity (Karimian et al. 2014). In agreement, this was reported in a study using the FRAP technique (Mlala 2015). Nevertheless, within exposed individuals, Pb uptake and FRAP were positively correlated, indicating an antioxidant response against Pb toxic effects after uptake.

For MDA, a positive correlation was found with Pb uptake for IP, but not for BP, suggesting that the Pb exposure history has prepared the latter to avoid cell membrane damage. Reinforcing this hypothesis, BP presented a positive correlation between the concentrations of Pb in leaves and SS. In this context, it can be suggested that the exposed population was able to accumulate Pb without experiencing lipid peroxidation, possibly due to the protective effect of soluble sugars (Rodriguez et al. 2015).

Tagetes minuta is a species of commercial interest because it produces a large quantity of essential oils that are used in various industries (Patel and Patra 2014). The importance of studying the essential oils in relation to their ability to tolerate and accumulate Pb lies in their potential action against ROS and free radicals by protecting plant tissues and structures from toxicological damage (Shirazi et al. 2014). According to Gil et al. (2000), *T. minuta* populations can be grouped into three chemotypes (ChT): ChT 1: related to β-ocimene, dihydrotagetone, Z-tagetone, E- and Z-tagetenones and limonene; ChT 2: dominance of dihydrotagetone; and ChT 3: dominance of α-phenandrene and E- β-ocimene. Our results indicate that BP fits into the ChT 2 chemotype, while IP would require a new ChT to be defined. High volatile composition variability has repeatedly been reported for *T. minuta*, with important changes occurring from one year to the next. Therefore, it is considered to be a species of great phenotypic plasticity in this aspect (Mihaliak et al. 1989; Gil et al. 2000). Figure 2c indicates that there is a marked difference in volatile composition according to the population, which could be related to the Pb exposure history, but the natural variability of this species requires further studies to reach a stronger conclusion. Nevertheless, the association between Pb in leaves and β-ocimene for BP coincides with the findings of Sosa et al. (2016) for the same population studied in situ. This compound participates in the activation of defence genes (Arimura et al. 2002) and is an intermediate compound in the dihydrotagetone metabolic pathway (Singh et al. 2016). In our study, it was the dominant compound in BP, which was coherently negatively correlated with the concentration of Pb in leaves. This may explain the detection of a ChT 2 population in a polluted area, with a relatively stable volatile composition. In addition, ChT 2 overexpressed the dihydrotagetone metabolic pathway, with the possibility of dihydrotagetone being derived to β-ocimene production.

For IP, limonene, a compound reported in plant stress conditions (Sánchez-Osorio et al. 2013), was positively correlated with leaf Pb concentration. The accumulation of secondary metabolites is generally a defence mechanism and plays a major role in adapting plants to environmental variations. Consequently, stress situations usually favour the production of these compounds (Figueiredo et al. 2008).

Our findings show that *T. minuta,* independently of its population origin, increases essential nutrient uptake, photosynthetic pigments and carotenoids (and biomass in the case of BP) in response to soil Pb. A change in the composition of the essential oils associated with the increase of Pb in leaves was also observed (more sabinene, limonene, β-ocimene, β-citral and verbenone but less dihydrotagetone for BP, and more limonene for IP). Similar results have also been reported by Gautam and Agrawal (2017) for *Cymbopogon citratus* grown in soils polluted with several heavy metals, and by Patel and Patra (2014), who cultivated *T. minuta* on soils amended with residual sludge (Cr enriched) in an increasing proportion from 0 to 100%. Patel and Patra (2014) observed that as the percentage of sludge increased to up to 50%, the plant responded by increasing chlorophyll a and b, carotenoids, antioxidant enzyme activity and concentration, the concentrations of all metals in the aerial parts, and the percentage compositions of limonene, cis-ocimene (one type of β-ocimene) and tagetone. When the sludge content in the soil exceeded 50%, all these parameters decreased, thereby losing the capacity to accumulate contaminants in the aerial parts. On the other hand, the percentage content of dihydrotagetone was reduced as that of sludge increased, even for the lower percentages of sludge amendment. Patel and Patra (2014) were able to show that when the metal concentration in the soil falls below a certain threshold, the plant has the ability to respond physiologically by increasing the contents of some volatile compounds, thus favouring pollutant translocation. Our present study corroborated this same strategy for the same species, for Pb being the pollutant rather than Cr. However, in order to determine the Pb threshold concentration for which this mechanism is no longer useful, further experiments are required.

5 Conclusions

Tagetes minuta showed favourable characteristics for its use in the remediation of Pb-polluted soils. The species has the capability of responding to Pb pollution by modifying its root exudates, thereby enhancing the solubility and uptake of micronutrients. Consequently, a physiological improvement was observed and the amount of chlorophyll and carotenoids were increased, resulting in a higher biomass (hormesis). Although tolerance and Pb extraction efficiency did not differ between exposure times, the defence response against Pb toxicity was more successful for the long-term exposed population, which achieved a zero lipid peroxidation, a superior biomass production, and presented a volatile composition of its essential oil that was associated with the activation of defence genes.

Tagetes minuta is suitable for Pb phytoremediation, with no differences being found in efficiency between the populations used for seed collection. Nevertheless, phytoextraction efficiency presented a high variability among individuals, which suggests that a selection process is necessary to obtain a cultivar with an enhanced average Pb uptake rate.

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