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RESEARCH PAPER

Characterization of glandular trichomes of *Minthostachys verticillata*"peperina" from northwest and central Argentina: relation with essential oil content

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Abstract

Minthostachys verticillata is an endemic species in Argentina that shows higher contents of essential oils (EO %, v/w) in plants from the central area than in those from the northwest. Although the reason for this difference is still unknown, studies on species from this family indicate that the variation in essential oil yields could be due to genetic or environmental factors or their interaction, which modifies the number and size of the glandular trichomes, structures where the oil is synthesized and stored. The main objective of this study was to analyze the density and size of glandular trichomes on *M. verticillata* leaves and their relation to the EO in wild plants from northwestern (NA) and central Argentina (CA); and then in a semi controlled environment, we studied the relation between the density and size of these to the leaf size (apical vs basal leaves) and plant origin. For the first experiment, we collected random wild plants in the main natural areas of distribution; and in the second one, we used cloned plants and grew them in a completely randomized design. The analyses of the trichome density and size and the EO% were carried out using a transmission electron microscope and a Clevenger type trap respectively. The wild plants from the central area showed larger glandular trichomes and higher EO concentrations; in addition, under semi-controlled conditions, we also found a greater number of trichomes per mm^2 in both apical and basal leaves in the plant from CA than in that from NA. Thus, the trichome size and number were affected in a different way by the environment.

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Introduction

Minthostachys verticillata is an endemic species in Argentina (Schitd-Lebuhn, 2007) that grows on mountainsides and is normally protected by trees (Elechosa, 2009). It is very commonly used by locals for tea due to its aromatic and medicinal properties. Most of the studies of wild *peperina* plants indicate higher contents of essential oils (EO) in plants from the central area of Argentina, specifically the provinces of Córdoba and northern San Luis (0.8-4.9% EO), than in those from the northwest of Argentina, mainly the Province of Tucuman (0.2-2.2% EO). In addition, qualitative differences in the EO composition have been documented between plants from these two areas (Zigadlo *et al.*, 1996; Ojeda, 2004; van Baren *et al.,* 2014). Also, genetic differences between peperina plants in these two areas have been documented by Ojeda (2004) and Bonafede *et al.* (2014), but the reason for the higher EO contents documented in *in-situ* collections from the central area is still unknown.

Plants in the Lamiaceae family synthetize and store their EO mainly in the peltate glandular trichomes, which are specialized epidermal structures on the surface of leaves, flowers, and stems (McCaskill *et al.*, 1992). Therefore, the essential oil yield is directly related to the number and size of these structures (Gersheshon, 1994; Rios-Estepa *et al.*, 2010). However, glandular trichomes are often different in both numbers and size because of genetic factors, variations in the environmental conditions, and type and growth of the organ - flowers, leaves, or stems - (Werker *et al.*, 1985 and 1993; Wagner, 1991; Wagner *et al.*, 2004; Shilmiller *et al.*, 2008); for example, during the leaf growth, the cell elongation reduces the ratio between the trichome number and the foliar area, and therefore the hair density decreases (Werker, 2000).

Thus, our main objective was to analyze the density and size of glandular trichomes on M. verticillata leaves and their relation to the EO in wild plants from northwestern and central Argentina; and then in a semi controlled environment, we studied the relation between the density and size of these to the leaf size (apical vs basal leaves) and plant origin.

Materials and methods

The first study (a mensurative experiment) was carried out on wild *M. verticillata* plants in the main areas of their natural dispersion; see table 1 and fig. 1.

Fig. 1. Google Earth image with GPS points and meters above sea level (MASL) for sampling sites of *Minthostachys verticillata* in central and northwest Argentina. Horizontal lines = latitude; vertical lines = longitude.

After that, plants from these areas were cloned and grown in the National Institute for Agricultural Technology (INTA) in Hurlingham, Buenos Aires, Argentina (experiment B).

Mensurative experiment A: wild plants in center and northwest of Argentina

We sampled 20 random plants in a similar phenology state (bloom) at different sites in the provinces of Cordoba and northern San Luis (n=10) and in the province of Tucuman (n=10); see table 1 and fig. 1. Before we harvested them, a couple of representative and non-senescent leaves from the same node (nº5) in each plant were herborized and stored for characterization of their trichomes in the laboratory; we only picked leaves from a main branch. The extra leaves from each plant were stored for the quantification of its EO content.

Manipulative experiment B: container-grown plants

Twenty *M. verticillata* plants (geographical origins: Córdoba n=10 + Tucumán n=10) were grown in individual pots and arranged in a completely randomized design in the Biological Resource Institute of INTA, -34° 36 '33.72" -58° 40' 21.38". In order to obtain the 20 plants, two mother plants - one from Cordoba and the other from Tucuman - were selected according to the concentration and quality of their essential oils and then were reproduced asexually. The plant propagation was carried out by the cutting of apical extremes from branches, the aggregation of naphthalene acetic acid (1500ppm) in the form of powder, the placing of the cuttings in propagation trays filled with 2/3 of perlite and 1/3 of peat moss, and acclimatization in a mist system during 30 days. The new plants were grown individually; firstly, they were placed in one liter pots under a greenhouse until the beginning of the austral spring (September); two months later, they were transferred to three liter pots for four months until their harvest. Both types of pots were filled with a homogenous mix of compost and top soil (1:1), and the full water content in the substrate was restored approximately every one to three days during the experiment and once or twice a week before that. The lighting conditions during the experiment were similar to the average of the sampling natural condition (≈50% of the time under direct sun light and the rest of the time under a 75% shade). At the moment of harvest, from each plant we only collected the apical leaves from the second nodes and the basal leaves (fully expanded) from the 11-12th nodes; on two leaves per plant - one apical leaf and one basal leaf - we quantified the density and the average diameter (µm) of the glandular trichomes, and with the remainder of apical or basal leaves we only achieved a quantification of EO content per leaf type and genotype.

Isolation of the essential oil

Leaves were distilled in the laboratory with Clevenger-type trap (IRAM, 1996) for 90 minutes. Previously, subsamples of leaves per plant (experiment A) or per leaf type (experiment B) were weighed and dried in a 70ºC oven until weight stabilization was achieved (≈48 hours). After they were dried, we weighed them again to determine the humidity content in the leaves at the moment of hydrodistillation. The yield of EO was calculated as the rate between the volume of oil and the distillated dry weight of its leaves and expressed in percentage. In three samples from experiment A, the distillated leaf biomass were not enough to measure their EO content.

Counting and measurement of the trichome density and diameter

We only used the abaxial surface of the leaves for our comparisons due to our objective and previous studies of species within this family, which had documented greater numbers of glandular trichomes on the abaxial surfaces than on the adaxial ones (Mafei, 1989; Bosabalidis and Kokkini, 1997). The analyses of the trichomes on the surface of the leaves were carried out using the method described by Nawab *et al.* (2010) with modifications. With a transmission electron microscope, we took ten images per leaf on the abaxial surface. Each image had a measurement of a square millimeter and was taken on the middle area of the leaves and in a perpendicular direction to the midrib. On these areas, we studied the average diameter of the trichomes (µm) and the number-per-square-millimeter. In order to achieve this, we used the Piximetre software (available online in: http://ach.log.free.fr/Piximetre/).

Statistical analysis

In both studies, a statistical analysis (ANOVA, Pearson's correlation and Tukey's test) was carried out. We had previously assessed the assumptions of normality and homogeneity of variance. The analyses were achieved by using the software Infostat (version 2012). We also used the software GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com to find differences between slopes and the software Microsoft Excel (2010) to create the graphs.

Results

Wild plants

In this study, the analysis of leaf glandular trichome density showed a large variation, so no differences were found in the number of trichomes per mm^2 (trich.mm^{-2}) between wild plants in the center of the country (30.5±12.8 trich.mm^{-2}, media±standard deviation) and in the Province of Tucuman (31.7±12.5 trich.mm^{-2}) (fig. 2). However, the trichomes were largerin the samples from central Argentina (49.9±1.9 µm) than in those from the northwest (38.5±1.4µm) (fig. 3A). Likewise, the content of essential oil (EO%) was higher (7.8±1.7% *vs.*6.2±1.2%, respectively) in leaves with larger trichomes (figs. 2A and 3A), so a positive correlation between them was also found (table 2).

Plants grown in a semi-controlled environment

The glandular trichome density was higher^a in the apical leaves (56.9±6.0 trich.mm^{-2}) than in the basal ones (37.6±4.9 trich.mm^{-2}). Despite this, the genotype from Cordoba had a higher* density than the one from Tucuman on the apical leaves (62±3.3 *vs.* 51.8±2.9 trich.mm^{-2}, respectively) and on the basal ones (42±1.9 *vs.* 33.2±1.9 trich.mm^{-2}, respectively) (fig. 2). Although no differences in the trichome diameters due to the age of the leaves were found, they were larger^a in the replicas from Cordoba than in the other ones (71.5±3.3 *vs.* 64.9±2.4 µm, respectively) (figs. 3 and 4). The single essential oil yields, which were obtained by a single distillation per treatment, were higher in the apical leaves than in the basal ones, although both single samples from Cordoba were superior in EO% both for the apical leaf sample (14.4 *vs.* 11.7%) and for the basal one (7.5 *vs.* 5.2%) (figs. 2 and 3). For the regression of the EO% and sizes of trichomes, no differences were found between the slopes of the wild plant model and the general model.

Table 1. Geographic coordinates of *Minthostachys verticillata* sampling sites in central and northwest Argentina.

Latitude	Longitude	MASL
26 29,300	64 59,500	982
26 29,500	64 59,500	976
26 29,300	64 59,500	979
26 26,400	64 58,500	964
26 26,400	64 58,500	980
26 26,400	64 58,500	971
26 26,400	64 58,500	975
26 26,400	64 58,500	980
30 46,000	64 27,600	1277
30 46,000	64 27,600	1292
30 46,000	64 27,600	1293
30 46,000	64 27,600	1289
30 46,300	64 27,400	1321
30 46,300	64 27,400	1259
32 11,100	64 23,300	531
33 11,100	64 23,300	548
32 29,100	64 58,400	1047
32 19,100	64 58,800	1094

Discussion

In our studies, the differences in essential oil content corresponded both to differences in the trichome size of the *in situ* samples and to trichome size and density differences in the plants grown in a semi-controlled environment. This could be due to three factors and their interactions and also have an ecological reason.

Table 2. Pearson's correlation: coefficients and probabilities of the density and diameter of trichomes, and the essential oil yield in *Minthostachys verticillata* wild plant leaves from the northwest and center of Argentina. The coefficients are under the diagonal line, and the probabilities or p-values are above the line in cursive numbers.

	Diameter of trich. (µm)	N° of trich.mm^{-2}	EO%
Diameter of trich. (µm)	1.00	*0.78*	***0.05***
N° of trich.mm^{-2}	-0.07	1.00	*0.97*
EO%	**0.49**	0.01	1.00

Effects due to the growth and development of the leaves

The reduction in trichome density from the apical to the basal leaves of *M. verticillata* that was found in this study follows the same pattern as in other species from the same family (Venkatechalam *et al.*, 1984; Werker *et al.*, 1985 and 1993; Maffei *et al.*, 1986 and 1989); this is mainly due to the fact that the cell elongation reduces the ratio between the trichome number and the foliar area (Werker, 2000).

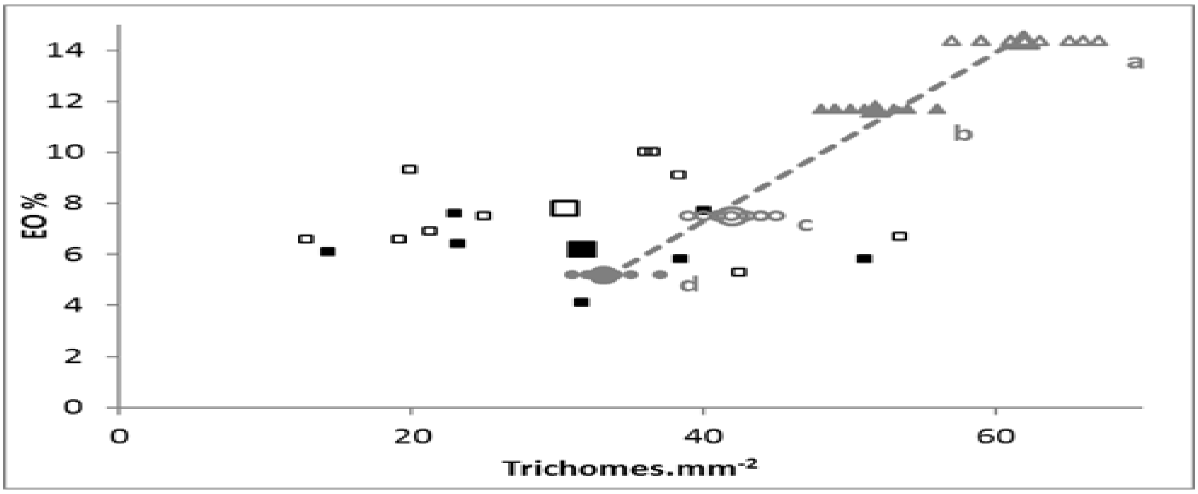

Fig. 2. Relationship between the essential oil content (%) and the trichome density in *Minthostachys verticillata* leaves from northwest (full symbols) and central (empty symbols) Argentina's plants in two experimental situations: (A) an *in situ* sampling (squares), where middle position leaves from wild plants were harvested from the mountains of Tucuman, Cordoba, and San Luis provinces; and (B) an *ex situ* experiment, where plants from Cordoba and Tucuman were gown under similar conditions, and their apical (triangles) and basal (circle) leaves were studied. In B, no repetitions of EO% were reached. Small symbols = single samples; large symbols and trend line is made of and with the averages of the treatments; and Tukey's test: different letters mean statistical differences with p ≤ 0.05.

However, the final number of trichomes is not determined when the leaf first appears, but instead there is a potential number that is formed by an initial amount of trichomes when the leaf appears, plus those which may appear during its development in the residuary meristematic areas (Gershenzon *et al.*, 1992; McCaskill *et al.*, 1992; Gershenzon, 1994; Werker *et al.*, 1993; Tissier, 2012). For example, Maffey *et al.* (1989) found that the absolute number of trichomes increased with the leaf expansion in *Mentha x piperita* L. while the density decreased. In our study, the lack of differences in the size of glandular trichomes between apical and basal leaves could be due to an early synthesis and storage of the essential oils in these structures (Turner *et al.*, 2000). Under this whole framework, therefore, a higher content of essential oil in apical leaves than in basal leaves should be found, such as was the case in our experiment.

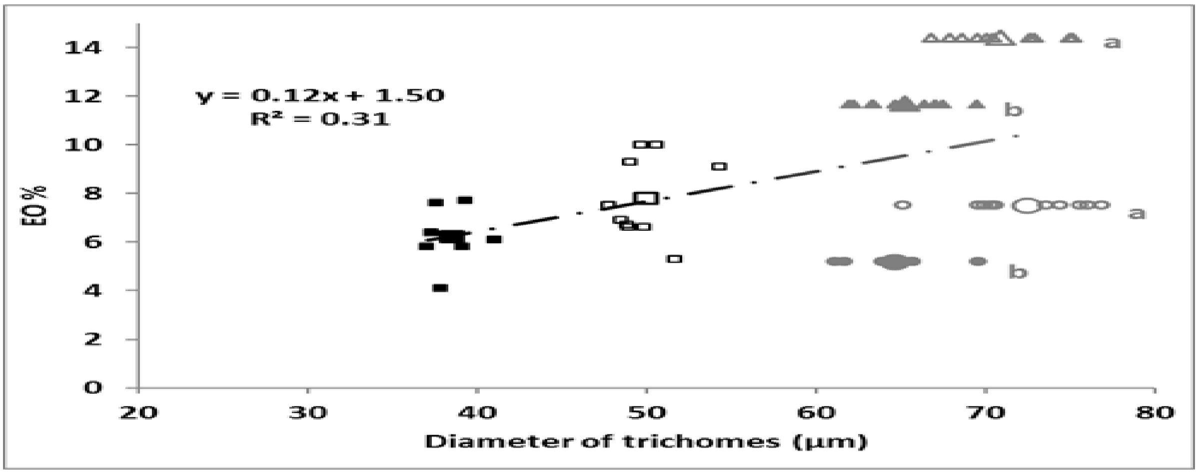

Fig. 3. Relationship between the essential oil content (%) and the trichome size in *Minthostachys verticillata* leaves from northwest (full symbols) and central (empty symbols) Argentina's plants in two experimental situations: (A) an *in situ* sampling (squares), where middle position leaves from wild plants were harvested on the mountains of Tucuman, Cordoba, and San Luis provinces; and (B) an *ex situ* experiment, where plants from Cordoba and Tucuman were gown under similar conditions, and their apical (triangles) and basal (circle) leaves were studied. In B, no repetitions of EO% were reached. Line = trend line of the general model; small symbols = single samples; large symbols = averages of the treatments; and, Tukey's test: different letters mean statistical differences with $p \leq 0.05$.

Environmental effects

In *Mentha* x *piperita,* variations in the amount of radiation, photoperiod, and diurnal and nocturnal temperatures often have effects on the number, size, and maturity level of the trichomes, so the yield and quality of the essential oils could be altered (Burbott and Loomis, 1967; Werker *et al.*, 1985 and 1993; Maffei *et al.*, 1986 and 1989; McCaskill *et al.*, 1992; Voirin and Bayet, 1996; Liakoura *et al.*, 1997; Turner *et al.*, 2000; Croteau *et al.*, 2005). In our study we found considerable differences in the trichome sizes between wild plants and container-grown plants; though these differences could be due at least in part to the genetic factor, it seems to be mainly due to the environment. For example, Rios-Estepa *et al.* (2010) found that whereas the maximum and minimum trichome sizes in two genotypes of *Mentha* × *piperita*under stress conditions were not altered, the proportions of large, medium, and small trichomes were changed. The broad dispersion in the *in situ* density data could be due to a larger variability in the foliar expansion at least partly due to the environmental heterogeneity of where the plants were growing; on the other hand, in the container-grown plants the heterogeneity was less, at least for the experimental and environment conditions.

Genetic effects

Differences in the carbohydrate importation from photosynthetic cells to the glandular trichomes were suggested to explain the greater proportion of the larger trichomes and thus to explain the higher essential oil content in a transgenic plant in contrast with the non-transgenic plant of *Mentha* × *piperita* L.

(Ríos Estepa *et at.,* 2010). In addition, genes that regulate the size and amount of trichomes were found in *Arabidopsis thaliana* and other species (Schwab *et al.*, 2000; Hauser *et al.,* 2001). The higher trichome density in the genotype from central Argentina could be due to a higher differentiation of trichomes per leaf in this plant and/or a wider foliar expansion in the genotype from Tucuman.

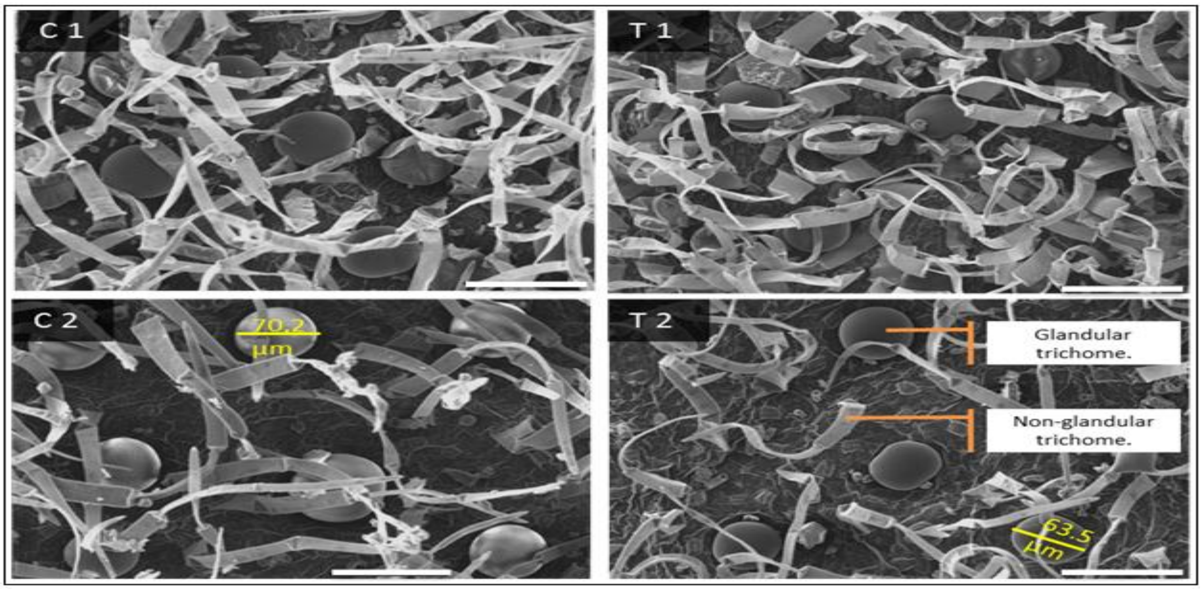

Fig. 4. Images of trichomes on the abaxial surface of *Minthostachys verticcillata* apical (1) and basal (2) leaves from two genotypes, Cordoba (C) and Tucuman (T); the pictures were taken with a transmission electron microscope. White bar = 100 µm.

Ecological approach

Ecologically, glandular trichomes play a protective role against herbivores and pathogens, yet they could have also other roles such as protection against UV rays, temperature regulation, reduction of water loss, and/or attraction of pollinators, so genetic differences for the trichomes and essential oils can be found between plants from the same species (Glas *et al.*, 2012). A very similar pattern to that which we found in *M. verticillata* plants was studied in wild collections of the three *Origanum vulgare* subspecies that grow in Greece; likewise, the subspecies with larger and denser glandular trichomes corresponded to areas of higher radiation and drier summers (Bosabalidis and Kokkini, 1997).

ANOVA:

Mensurative experiment A:

EO%

S.V.	SS	Df	MS	F	p-value
Model.	10.354	1	10.354	4.588	0.049
Area	10.354	1	10.354	4.588	0.049
Error	33.849	15	2.257		
Total	44.202	16			

Density

S.V.	SS	Df	MS	F	p-value
Model.	5.535	1	5.535	0.034	0.855
Area	5.535	1	5.535	0.034	0.855
Error	2407.751	15	160.517		
Total	2413.286	16			

Diameter

S.V.	SS	Df	MS	F	p-value
Model.	538.423	1	538.423	180.746	<0.001
Area	538.423	1	538.423	180.746	<0.001
Error	44.683	15	2.979		
Total	583.106	16			

Manipulative experiment B:

Density

S.V.	SS	df	MS	F	p-value
Model.	4632.3	3	1544.100	234.349	<0.001
Area	902.5	1	902.500	136.973	<0.001
Type of leaf	3724.9	1	3724.900	565.331	<0.001
Area*Type of leaf	4.9	1	4.900	0.744	0.394
Error	237.2	36	6.589		
Total	4869.5	39			

Diameter

S.V.	SS	df	MS	F	p-value
Model.	448.941	3	149.647	17.492	<0.001
Area	437.582	1	437.582	51.147	<0.001
Type of leaf	1.056	1	1.056	0.123	0.727
Area*Type of leaf	10.302	1	10.302	1.204	0.280
Error	307.994	36	8.555		
Total	756.935	39			

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