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Sexual Differentiation in Yerba Mate Plants: the Role of Stomatal Density

Sandra Patricia Rocha[1]
https://orcid.org/0000-0002-8698-843X

Ana Valeria Morales[1]
https://orcid.org/0009-0008-6821-2639

Pablo Andrés Poszkus Borrero[1]
https://orcid.org/0000-0001-6881-8587

Moeses Andrigo Danner[2*]
https://orcid.org/0000-0002-1159-6546

Fernando Niella[1]
https://orcid.org/0000-0002-3454-3033

[1]Universidad Nacional de Misiones (UNaM), Facultad de Ciencias Forestales, Eldorado, Misiones, Argentina; [2]Universidade Tecnológica Federal do Paraná (UTFPR), Programa de Pós-Graduação em Agronomia, Pato Branco, Paraná, Brasil.

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*Correspondence: moesesdanner@utfpr.edu.br; Tel.: +55(046)32202545 (M.A.D.).

HIGHLIGHTS

- We investigate the stomatal density variability between female and male yerba mate.
- Stomatal density was significantly higher in leaves from female than male plants.
- This secondary sexual dimorphism serves for gender determination in seedlings.

Abstract: Yerba mate (*Ilex paraguariensis* St. Hil., Aquifoliaceae), a dioecious, subtropical, perennial tree found in South America, was hypothesized to exhibit secondary sexual dimorphism in relation to stomatal density (SD). The objective of this study was to examine the variability of SD in yerba mate leaves and its relationship to secondary sexual dimorphism as a first step towards identifying a genetic marker for gender determination in seedlings. Leaves from four male and four female plants were collected from two monoculture plantations, San Vicente and General Alvear, in the Misiones province, Argentina, during both flowering and vegetative latency periods. Results showed that SD was significantly higher in leaves from female than in male plants in both sites. Also, samples collected during the vegetative growth period have higher SD compared to flowering period. The findings indicate that the SD of yerba mate leaves varies based on gender and serves as a secondary marker for sexual determination in this species. We also discussed the impact of SD variability in photosynthesis rate between female and males.

Keywords: *Ilex paraguariensis;* Aquifoliaceae; Sexual dimorphism; Leaf traits; Photosynthesis rate.

INTRODUCTION

Many dioecious plant species show sexual dimorphism (SD), although it is often only recognized in animals. The evolution of dioecy, affects not only flowers and inflorescences, but frequently the physiology and morphology of the plant, sex differences [1]. Only 6% of the 240,000 known species of angiosperms are dioecious [2]. Sexual dimorphism can be interpreted as an adaptive character resulting from evolution, so

each gender can meet its unique reproductive resource requirements [3]. In many perennial plant species, the demand for resources by females is higher, compromising their vegetative growth and long-term survival, as well as a higher mortality rate among females compare to males [4,5].

The yerba mate (*Ilex paraguariensis* St. Hil., Aquifoliaceae) is a subtropical, perennial, dioecious, south American tree of traditional economic importance in Argentina, Paraguay, Brazil, and Uruguay. Its leaves and stems are used as an infusion due to its nutritional and medicinal properties (tonic, choleretic, diuretic, antirheumatic, etc.) [6]. Its natural habitat is the subtropical forests with an understory growth. However, commercial plantations in Misiones Province, mainly, are monocultures, under full-sun light conditions to promote greater biomass production per plant.

Plant leaf is one of the organs with the greatest flexibility in adapting to environmental conditions. Among the structures that make up the epidermal tissue are the stomata, which, for yerba mate plants, has been described as cyclocytic type stomata, 24 µm long and 18 µm wide [7], absent in the upper epidermis and abundant in the lower (hypoestomatal), helping to protect the photosynthetic system from solar radiation, and its adaptability to grow at full sun [8]. As such, yerba mate plant has been classified as shade avoider. Transpiration and respiration intensity are causally related to the number and opening of the stomata, and the amount and distribution of this organ, directly impact chlorophyll assimilation. In this way, increased stomatal density improves photosynthetic capacity [9].

In general, plants characterized by a low density of large stomata typically exhibit reduced stomatal conductance and a diminished photosynthetic rate compared to counterparts with a high density of smaller stomata [10,11]. However, there are species featuring a low density of large stomata display decreased stomatal conductance, yet do not necessarily exhibit a concurrent reduction in photosynthetic rate. This discrepancy may arise from compensatory adjustments in mesophyll conductance and biochemical processes within the leaf, which mitigate the adverse effects of stomatal traits on photosynthesis [12, 13].

The stomatal density defined as the number of stomata per unit of leaf surface area is involved in the control of CO_2 and H_2O exchange between leaf and atmosphere, is strongly influenced by the species [14]. In most woody plants, leaf morphological and physiological characteristics are extremely variable across environmental gradients, particularly across altitudinal gradients, demonstrating the climate adaptation strategies of stomatal traits in natural forest communities [15]. Also, the size and frequency of stomata may play a key role in plant adaptation to prevailing environmental conditions, such as viewed in 39 evergreen and deciduous broadleaved subtropical tree species [14]. Besides, it was observed morphological and physiological differences in dioecious species, with females exhibiting higher stomatal density in *Hippophae rhamnoides* [16] and with females of *Populus deltoides* allocating more resources to stems and leaves, whereas males showed no prioritized allocation, demonstrating secondary sexual dimorphism for biomass allocation [17]. Furthermore, stomatal density association to gender, could have potential as genetic marker (morphological or molecular) for gender detection in seedlings of dioecious species.

SD in yerba-mate is not controlled by an XY chromosome system, and up to date, genetic markers have not been developed, being impossible to identify sex in yerba-mate seedlings [18]. At the same time, the photosynthetic leaf rate was higher in female yerba mate plants than in male ones, for various leaf ages that grow in open monocultures [19]. The yerba mate also showed morphological, structural, and physiological SD, and secondary sexual characteristics are even expressed in the chemical composition of the leaf [20]. However, throughout the plant's life, male plants were more productive at earlier harvest ages, whereas the frequency of female plants that were more productive increased as the plants reached later harvest ages, ultimately resulting in equal frequencies of male and female progenies [21].

The functional implications of trait dimorphism are not always apparent when examined in isolation. To fully appreciate their significance, one must consider these traits within the broader context of an organism's physiology, morphology, ecology, and life history [22]. For instance, research on *Silene latifolia* reveals that sexual dimorphism (SD) traits are interconnected rather than isolated. This interconnectivity implies that a change in one trait, triggered by environmental factors or genetic mutations, can influence other traits, thereby affecting the plant's overall form and functionality [23]. This emphasizes the importance of adopting a multi-trait quantitative genetic approach to understand the basis of sexual differences. In some instances, SD traits may be subtle or entirely absent in one gender, complicating their study, particularly in young or non-flowering plants. The use of genetic markers can significantly enhance our ability to identify plant sex before reproductive maturity, facilitating earlier and more effective research. However, the scarcity of these markers has shortened our understanding of when and how these differences manifest in young plants and the extent to which changes in adult plants influence juvenile traits [24]. This gap highlights a critical area for future research, where developing and applying such markers could unravel the complexities of plant sexual dimorphism and its developmental dynamics.

Based on prior literature, it is well-documented that trees typically display smaller stomatal size coupled with elevated stomatal density, alongside the occurrence of SD in dioecious species. Moreover, stomatal density has been implicated in leaf photosynthetic capacity and plant adaptability to diverse environmental conditions. Building upon these established insights, we posited the hypothesis that yerba mate plants would manifest SD in stomatal traits, with female individuals exhibiting greater stomatal density compared to their male counterparts. The primary aim of this study was to assess the variation in stomatal density across yerba mate leaves and its association with secondary SD. This investigation represents an essential initial step towards the identification of morphological genetic markers for gender determination in yerba mate seedlings

MATERIAL AND METHODS

Plant material

For the studies of stomatal density, fully developed yerba mate leaves were taken from the sun-exposed branches of the middle third of the plant. The leaves samples were collected from two productive monoculture plantations, one located in San Vicente (SV) (latitude: -26.617°, longitude: -54.133°, and elevation: 561 m) and the other in General Alvear (GA) (latitude: -27.487°, longitude: -55.120°, and elevation: 333 m), both in Misiones Province. The sites have a subtropical climate that corresponds to a Cfa classification according to Koppen-Geiger climate classification, without a dry season, with both locations characterized by an annual average of minimum temperatures not lower than 9º C, and a maximum average, not exceeding 28º C, and more than 2,000 mm annual precipitation [25]. The leaves samples collection was performed in two periods, at the time of flowering of the plants (FL), and during vegetative latency (VG). During each period, a composite leaf samples were collected from four representative plants of both genders, female (F) and male (M).

Stomatal Density analysis

To determine the stomatal density of Yerba Mate leaves, "epidermis peels" were obtained using the maceration technique [26]. The determination of the stomatal density was carried out by counting the number of stomata present in an area of one mm^2, from the middle section of the leaf, with the help of a Motic® digital microscope at 10 and 40X.

Experimental design and analysis

A completely randomized design was used, with a factorial distribution of treatments: two sampling period, two sampling sites and both genders, with 20 replications per treatment, the experimental unit being the one mm^2 leaf section obtained from a composite sample of four plants. Data were analyzed using InfoStat Professional software [27]. The analysis of variance (ANOVA) was performed at the significance level of 5% ($P < 0.05$). When the ANOVA was significant, treatment means (main effect as well as interaction means) were compared on least significant difference (LSD) at $P < 0.05$ using Fisher test.

RESULTS

The results of the study showed that stomatal density in yerba mate plants exhibited statistically significant differences based on gender and sampling period. Moreover, significant interactions were observed between gender and leaf sampling site, and between site and leaf sampling period. However, there was no significant interaction between gender, sampling period, and site (Table 1).

Table 1. Stomatal density *Analysis of variance* for all the factors and their interaction (leaf sampling period, leaf sampling site and gender) studied in yerba mate plants.

Factors	SS	DF	F	*p*-value
Model	937.128,76	7	35,19	<0,0001
Gender	534.964,37	1	140,62	<0.0001*
Leaf sampling period	267.360,82	1	70,28	<0.0001*
Leaf sampling site	7.541,46	1	1,98	0.1612
Gender*Period	13.658,22	1	3,59	0.0600
Gender*Site	66.586,88	1	17,50	<0.0001*
Period*Site	35.626,91	1	9,37	0.0026*
Gender*Period*Site	11.390,10	1	2,99	0.0856
Error	578.241,28	152		
Total	1.515.370,04	159		

*Significant effect by ANOVA ($p \leq 0.05$).

Leaf samples obtained from female plants showed significantly higher stomatal density (508.9 stomata/mm^2) compared to male plants (393.2 stomata/mm^2) (Table 2, Figure 1), implying that stomatal density could be used as a morphological genetic marker for early sexual differentiation in yerba mate seedlings, nevertheless, further work is needed.

Table 2. Stomatal density summary of mean values and standard error (SE) for gender leaf samples in yerba mate plants.

Gender	N	Means ± SE
Female	80	508.9 ± 8.8 a
Male	80	393.2 ± 8.8 b

Means with a common letter are not significantly different (P > 0.05). N: replications.

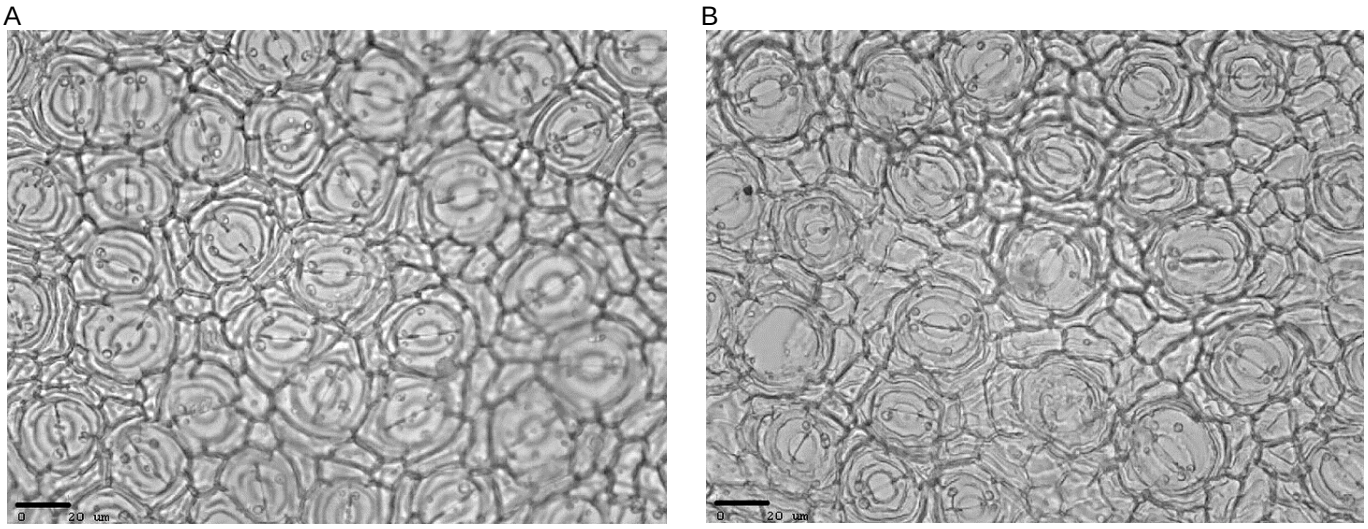

Figure 1. Abaxial epidermis of *Ilex paraguariensis* where the cyclocytic stomatal apparatus is observed. (A): Female sex, and (B): Male sex. Bars: 20 µm.

This trend was observed in both San Vicente and General Alvear sites, with female plants having higher stomatal density (522.4 and 495.3 stomata/mm^2, respectively) than male plants (366.0 and 420.5 stomata/mm^2, respectively) (Table 3).

Table 3. Stomatal density summary of mean values and standard error (SE) for gender and leaf sampling site interactions in yerba mate plants.

Gender	**Site**	**N**	**Means ± SE**
F	SV	40	522.4 ± 9.9 a
F	GA	40	495.3 ± 14.3 a
M	GA	40	420.5 ± 9.6 b
M	SV	40	366.0 ± 13.6 c

Means with a common letter are not significantly different (P> 0.05). N: replications; Leaf sampling site: SV (San Vicente); GA (General Alvear); Gender: F (Female); M (Male)

Samples collected from San Vicente during the vegetative growth period showed a significantly higher stomatal density (500.0 stomata/mm^2) compared to samples collected at the beginning of the flowering period (388.4 stomata/mm^2). A similar trend was observed in General Alvear, with samples collected during the vegetative growth period having a higher stomatal density (483.9 stomata/mm^2) compared to samples collected during the flowering period (432.0 stomata/mm^2). Non-significant differences were observed between sites for samples collected during vegetative latency (Table 4).

Table 4. Stomatal density summary of mean values and standard error (SE) for leaf sampling period and site interactions in yerba mate plants.

Period	Site	N	Means ± SE
VG	SV	40	500.0 ± 12.6 a
VG	GA	40	483.9 ± 15.7 a
FL	GA	40	432.0 ± 9.4 b
FL	SV	40	388.4 ± 16.7 c

Means with a common letter are not significantly different ($P > 0.05$). N: replications; Leaf sampling period: VG (vegetative latency); FL (Flowering); Leaf sampling site: SV (San Vicente); GA (General Alvear).

DISCUSSION

The photosynthetic rate at the leaf level is primarily predicted by the leaf surface's porosity, which is determined by the quantity and aperture of the stomata on the leaf. A significant correlation exists between stomatal porosity (or the diffusive conductance to water vapor) and the rate of CO_2 assimilation. This correlation is applicable to all major vascular plant lineages and is predictable enough to form the foundation for the most used model for forecasting water and CO_2 fluxes from leaves and canopies. These processes are the result of over 400 million years of coevolution among stomatal, vascular, and photosynthetic tissue [28]. Studies have shown that stomatal conductance and transpiration are determined by the size and density of stomata [29]. The lower stomatal density observed in male yerba mate plants in the present study is consistent with previous studies conducted in other dioecious species. In *Acer negundo*, for example, a positive correlation between stomatal density and conductance was presented, and that male plants had lower levels of stomatal conductance and higher water use efficiency compared to female plants [30]. Similarly, *Populus cathayana* showed that males had lower stomatal density than females and lower stomatal density increased stomatal resistance, limiting excessive transpiration and increasing male plant adaptability to drought stress [31]. However, small stomata open faster than larger stomata in tropical plant species [11, 32], which can result in sub-optimal gas exchange, water loss through transpiration, and lower photosynthetic rates [11, 33]. The present study suggests that the higher stomatal density in female yerba mate plants is related to their smaller stomatal size, which leads to faster opening and closing of stomata, optimizing gas exchange, water loss, and photosynthetic rate. This is supported by previous research [19] in which higher photosynthetic rates in female yerba mate plants compared to males was observed. Besides, the lack of differences in stomatal density values in female yerba mate plants between sites could indicate their greater adaptive flexibility.

CONCLUSION

In conclusion, the results of this study suggest that stomatal density in yerba mate plants is influenced by gender and sampling period. Female plants have significantly higher stomatal density compared to male plants, while samples collected during the vegetative growth period have higher stomatal density compared to samples collected during the flowering period. This difference in stomatal density may affect the plant's photosynthetic rate, water loss through transpiration, and water use efficiency. Further research is necessary to understand the impact of these findings on the growth and productivity of yerba mate plants, as well as to develop a morphological genetic marker for early sexual differentiation in yerba mate seedlings.

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