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Morphological Analysis of Stomata of *Cicer arietinum* L.

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ABSTRACT

Background: Sinaloa is Mexico´s leading producer of Kabuli chickpea, but its cultivation is increasingly constrained by limited water resources due to its dry and semi-arid climate. Stomata, which regulate gas exchange and water loss, play a vital role in plant adaptation to drought and high temperature stress. Studying stomatal traits can reveal plant responses and help identify chickpea cultivars better suited to water-scarce and high-temperature environments.

Methods: The experiment was conducted in Sinaloa, Mexico using three Kabuli chickpea cultivars: Blanco Sinaloa-92, Combo-743 and Sinalomex-2018. Key traits assessed included stomatal density, epidermal and trichome counts, stomatal area, seed yield, grain size and exportable grain percentage. Data analysis involved correlation, principal component analysis, cluster analysis, MANOVA and ANOVA to evaluate trait variability and cultivar differences.

Result: Cultivar Combo-743 was distinguished by its larger stomata, while Sinalomex-2018 exhibited smaller stomata. On both the adaxial and abaxial leaf surface, a significant negative correlation ($P<0.05$; $r = -0.65$ and $r = -0.73$, respectively) was observed between stomatal area and trichome density. This supports the notion of a trade-off between trichome and stomatal development reported in various plant species.

Key words: Leaf anatomy, Plant physiology, Seed yield and size, Stomatal morphology.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an economically significant crop in Mexico, primarily marketed as a dried seed and plays a vital role in the agricultural economy of the Northwest regional (Muy-Rangel *et al*., 2011; Yücel, 2018). Breeding efforts have focused on addressing major challenges such as chickpea wilt, caused by a complex of fungal pathogens including *Fusarium* spp., *Neocosmospora* spp., *Macrophomina phaseolina*, *Rhizoctonia solani, Sclerotium rolfsii* and *Sclerotinia sclerotiorum* (Cota-Barreras *et al*., 2024), as well as improving resistance to terminal drought and, more recently, heat waves (Karimizadeh *et al*., 2021). Strategies to overcome these constraints have included the incorporation of resistance to diseases, drought and cold, alongside efforts to enhance the yield potential of new cultivars (Shivwanshi and Babbar, 2019).

The chickpea genetic improvement program in Sinaloa has developed cultivars such as Blanco Sinaloa-92 (Gómez, 1993), Combo-743 and Sinalomex-2018, which exhibit tolerance to the aforementioned pathogens and demonstrate high seed yield (Valenzuela-Herrera *et al*., 2024). These improved varieties have shown superior performance in the dry-tropical regions of Northwest Mexico, where adverse conditions like drought and elevated temperatures limit agricultural productivity (Gaur *et al*., 2019).

In light of these challenges, understanding the physiological mechanisms that enable chickpea to adapt to such environments is essential. Stomata-microscopic pores located on the atmosphere, controlling carbon dioxide (CO_2) uptake for photosynthesis and water loss through transpiration. Stomatal traits, including density, distribution and size, are critical for evaluating water use efficiency and productivity in crops (Lawson *et al*., 2014).

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Previous studies have demonstrated the relevance of stomatal traits in crop performance. For instance, Cherrepano-Manrique *et al*. (2021) reported that pea (*Pisum sativum*) plants fertilized with 120 kg N ha^{-1} achieved a yield of 13.25 t ha^{-1} and exhibited a stomatal density of 98 stomata mm^{-2}. Similarly, Álvarez-Holguín *et al*. (2018) found that flag grass (*Bouteloua curtipendula*) genotypes with lower stomatal density (152 stomata μm^{-2}) and stomatal index (13.41%), but larger stomatal area (186.7 μm^{-2}) and higher chlorophyll concentration (8), produced greater forage biomass (30 g per plant). These findings underscore the importance of stomatal traits in crop selection and productivity enhancement.

Although chickpea is widely recognized for its adaptation to water-limited environments, detailed information on its stomatal characteristics remains scarce. For this reason, the objective of this study was to characterize the distribution and density of stomata in three chickpea cultivars, as well as to determine their relationship to seed yield and size.

MATERIALS AND METHODS

Description of the area

The experiment was conducted at the Culiacan Valley Experimental Field, part of National Institute of Forestry, Agriculture and Livestock Research (INIFAP, by its Spanish acronym). The site is located at coordinates 24°38′00.4″N and 107°26′17.2″W, with an elevation of 23 meters above sea level. The climate in this region is classified as dry to semi-dry, with highly variable annual rainfall ranging from 500 to 800 mm. The average annual temperature is approximately 25°C, peak summer temperatures range between 36°C and 40°C, while the lowest temperatures, around 10°C.

Soil preparation

For soil preparation, the field was fallowed and laid out with furrows measuring 50 meters in length and spaced 75 cm apart. The soil was classified as sandy clay loam with a pH of 6.20. Assimilable nitrogen was measured at 10.0 ppm. Phosphorus content was 5.0 ppm using the Bray 1 method, while Bray 2 phosphorus was 55.0 ppm. Available potassium was found to be 490 ppm and organic matter content was 0.90%.

Sowing was carried out on 20 November 2023 using a precision seeder, calibrated to a density of 14 seeds per linear meter at a depth of 10 cm.

Plant material

Three Kabuli-type chickpea (*Cicer arietinum* L.) cultivars were evaluated in this study (Table 1). The seeds were sourced from the chickpea improvement program of INIFAP, Mexico.

Experimental design and agronomic management

The experiment was conducted using a randomized complete block design with three blocks (plots). Each block comprised 15 rows, which served as the experimental units. The treatments involved three chickpea cultivars, randomly assigned within each block to ensure that each variety was replicated five-time per block. In total, the trial included 45 rows, with 15 replicates per variety, resulting in a fully balanced design.

No irrigation was applied during the growing season. Fertilization was carried out three days prior to sowing using a physical mixture of 57-52-00 (N-P-K), comprising 300 kg ha^{-1} of monoammonium phosphate and 100 kg ha^{-1} of urea. To manage pod borer infestation, Spinetoram (active ingredient), marketed under the brand name "Palgus", was applied at a rate of 0.1 L ha^{-1}. Two applications were made: the first at 81 days after sowing and the second three weeks later.

Variables evaluated

The following variables were measured in 90 randomly selected plants per cultivar, with six plants sampled from each plot. Stomatal characteristics were assessed on a single sampling date during the seed-filling stage. After harvest and drying, seed yield was also evaluated.

Stomatal characterization

Stomatal density (DE), epidermal cell count (NC), stomatal index (IE) per square millimeter (mm^2) and stomatal area (AE) in square micrometers (μm^2) were determined. For this analysis, one mature leaf was selected from each plant. The method involved placing a drop of Kola Loka® instant adhesive (Kola Loka SA de CV, Mexico) on a microscope slide, then pressing the leaf onto the adhesive for 60 seconds.

Sample were examined under an electron microscope at 40x magnification. Images of three optical fields per leaf, each covering an area of 0.0945 mm^2, were captured. DE and NC were counted in each image, while AE was determined by randomly selecting three stomata from each optical field. Image acquisition and analysis were performed using Zen 2 Core software. The stomatal index (IE) was calculated using the formula:

$$IE = \frac{DE}{NC + DE} * 100$$

The variables DE, NC, AE and IE were calculated separately for the adaxial and abaxial surfaces.

Agronomic variables

At harvest, 90 plants from each cultivar were randomly sampled and placed in paper bags to determine seed yield (REN1), expressed in kg ha^{-1} and later converted to t ha^{-1}. For each cultivar, the number of grains per 30 g was recorded. Exportable grain yield (EXP) was calculated by determining the percentage of seeds exceeding 9 mm in diameter from a representative sample.

Statistical analysis

To explore the relationship between stomatal traits (DE, NC, NT, AE and IE) and seed yield parameters (REN1, CAL

Table 1: Description of the Kabuli chickpea cultivars used in the study.

Cultivar	Grain color	Seed in 30 g^{-1}	Exportable grain (%)	Days to maturity	Yield (t ha^{-1})
Blanco Sinaloa-92	White	46-48	95	140	2.4
Combo-743	White	40-41	96	130	2.6
Sinalomex-2018	White	40-41	94	130	2.5

and EXP), a correlation analysis was conducted using R software (version 4.4.2), employing the Hmisc and corrplot packages. Pearson correlation coefficients and corresponding p-values were calculated using the corr() function. Subsequently, a principal component analysis (PCA) was performed using the FactiMineR, factoextra and ggplot2 packages in R. The variables included in the PCA were DE, NC, NT, AE, REN1, CAL and EXP. The stomatal index (IE) was excluded due to its high correlation with other variables ($p<0.05$), which could introduce multicollinearity into the analysis.

To investigate cultivar-level relationships, a cluster analysis was performed. The dataset was normalized using the scale() function and a Euclidean distance matrix was computed *via* the get dist() function. The optimal number of clusters was determined using pseudo-statistics such as CCC, T^2 and gap statistic to identify the most appropriate cluster solutions. A multivariate analysis

Table 2: Descriptive statistics for stomatal and agronomic variables of three cultivars of chickpea (*Cicer arietinum* L.) Kabuli type.

Cultivar	Leaf part	Variable	Average	Standard deviation	Coefficient of variation
Blanco sinaloa-92	Adaxial	DE (mm^2)	14.6	1.89	12.9
	Abaxial		16.2	2.90	17.9
	Adaxial	NC (mm^2)	121.2	19.5	57.9
	Abaxial		126.1	17.7	14.1
	Adaxial	NT (mm^2)	2.2	1.2	57.9
	Abaxial		2.5	0.8	34.3
	Adaxial	AE (μm^2)	441326.4	62353.1	14.1
	Abaxial		471454.9	73271.4	15.5
	Adaxial	IE	10.9	1.7	16.1
	Abaxial		11.4	2.1	17.6
		REN1 (kg ha^{-1})	2476.5	216.6	8.75
		CAL	37.0	4.6	12.6
		EXP (%)	96.2	1.48	1.54
Combo-743	Adaxial	DE (mm^2)	14.2	2.2	16.1
	Abaxial		18.1	3.6	20.3
	Adaxial	NC (mm^2)	128.7	12.2	9.5
	Abaxial		143.1	18.5	12.9
	Adaxial	NT (mm^2)	4.2	1.7	40.3
	Abaxial		4.6	1.9	42.6
	Adaxial	AE (μm^2)	512437.1	63800.2	12.4
	Abaxial		502981.1	119676.2	23.8
	Adaxial	IE	10.0	1.63	16.2
	Abaxial		11.2	1.68	14.9
		REN1 (kg ha^{-1})	2493.75	90.36	3.62
		CAL	39.2	1.4	3.7
		EXP (%)	97.5	0.5	0.5
Sinalomex-2018	Adaxial	DE (mm^2)	14.4	2.4	16.7
	Abaxial		15.5	3.7	23.9
	Adaxial	NC (mm^2)	127.6	13.9	10.9
	Abaxial		132.7	15.1	11.4
	Adaxial	NT (mm^2)	5.4	2.1	38.2
	Abaxial		4.9	2.1	41.7
	Adaxial	AE (μm^2)	238113.8	35169.2	14.7
	Abaxial		243533.4	41803.8	17.1
	Adaxial	IE	10.2	1.8	17.8
	Abaxial		10.4	1.8	17.2
		REN1 (kg ha^{-1})	2237.5	152.0	6.7
		CAL	37.5	0.5	1.3
		EXP (%)	98.5	0.5	0.5

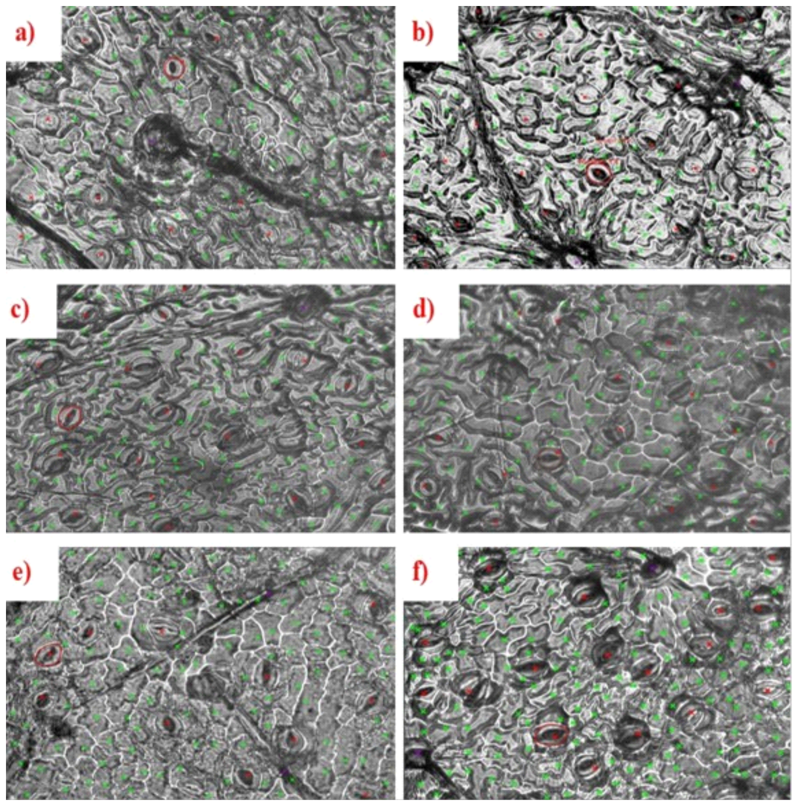
DE = Stomatal density, NC = Number of cells, NT = Number of trichomes, AE = Stomatal area, IE = Stomatal index, REN1 = Seed yield, CAL = Seed size, EXP = Export grain yield.

of Variance (MANOVA) was performed using manova() function was used to assess the overall influence of clusters, followed by individual ANOVAs for each variable using the aov() function.

RESULTS AND DISCUSSION

Stomatal characterization

Table 2 and Fig 1 present the values of DE, NC, NT, AE, IE, REN1, CAL and EXP measured on both the adaxial and abaxial leaf surfaces of the chickpea cultivars. The average stomatal density (DE) across the three cultivars was 14.4 stomata mm^{-2}, with values ranging from 10 to 20 stomata mm^{-2} on the adaxial surface. In terms of stomatal area (AE), the cultivar Combo-743 exhibited the highest values, averaging 512,437.1 μm^2 on the abaxial surface and 502,981.1 μm^2 on the adaxial surface. Conversely, Sinalomex-2018 recorded the lowest AE values, with averages of 238,113.8 μm^2 on the adaxial surface and 243,533.4 μm^2 on the abaxial surface. Notably, Sinalomex-



Chickpea leaves observed under a phase contrast microscope showing: adaxial (a) and abaxial (b) side of cultivar Blanco Sinaloa-92, adaxial (c) and abaxial (d) side of cultivar Combo-743, adaxial (e) and abaxial (f) side of cultivar Sinalomex-2018.

Fig 1: Green mark = Epidermal cells, Red mark = Stomatal density, Purple color = Number of trichomes, Red circle = Stomatal area.

Table 3: Matrix of pearson correlation coefficients (r) between the variables analyzed in three kabuli chickpea cultivars (Combo-743, Sinalomex-2018 and Blanco Sinaloa-92).

Variable	DE	NC	NT	AE	REN1	CAL	EXP
DE	-	-0.11	-0.08	0.12	-0.40	0.31	-0.39
NC	0.54*	-	-0.16	0.09	-0.23	0.09	0.01
NT	-0.34	0.27	-	-0.65**	-0.59**	0.15	0.64**
AE	0.36	-0.16	-0.73**	-	0.56*	0.19	-0.46
REN1	-0.03	-0.25	-0.47	0.60**	-	-0.45	-0.21
CAL	0.51*	0.50*	-0.14	0.08	-0.45	-	-0.01
EXP	0.23	0.48	0.39	-0.56*	-0.21	-0.01	-

DE = Stomatal density, NC = Number of cells, NT = Number of trichomes, AE = Stomatal area, REN1 = Yield, CAL = Caliber, EXP = Export. Pearson correlation coefficients (r) of the lower leaf (underside) below the diagonal, Pearson correlation coefficients (r) of the upper leaf (upper side) above the diagonal. **Correlations not equal to zero ($P<0.05$). *Correlations not equal to zero ($P<0.10$).

2018 also showed the highest total number of trichomes (NT) per mm^2, with 5.4 on the adaxial surface and 4.9 on the abaxial surface.

Pearson correlation analysis

Table 3 presents the correlation coefficients (r) between stomatal traits (DE, NC, NT, AE) and yield-related variables (REN1, CAL and EXP) on both the adaxial and abaxial. On the adaxial side, a significant negative correlation (r = -0.65, p<0.05) Was observed between trichome number (NT) and stomatal area (AE), similar to the abaxial side where the correlation was even stronger (r = -0.73). Additionally, a moderate and significant negative correlation was found between NT and REN1 on the adaxial surface (r = - 0.59, p = 0.04), suggesting that a higher trichome density may be associated with reduced seed yield.

Principal component analysis

For the abaxial leaf surface, Principal Components 1 (Dim1) and 2 (Dim2) explained 70.2% of the total observed variability with Dim1 accounting for 39.1% and Dim2 for 31.1% (Fig 2b). The most influential variables in Dim1 were NT, contributing 21.37% with a correlation of r = 0.76 and AE, contributing 25.47% with a strong negative correlation (r = -0.83). In Dim2, the key contributors were seed caliber (CAL), with 26.60% contribution and r = 0.76 and NC, contributing 17.81% with r = 0.62. On the adaxial surface, Dim1 explained 37.3% of variance, while Dim2 accounted for 25.5%, totaling 62.8% of the variability (Fig 2a). NT was the most significant contributor to Dim1 (31.86%, r = 0.91), followed by AE (25.85%, r = -0.82). In Dim2, CAL contributed 29.72% (r = 0.73) and REN1 contributed 19.12% (r = -0.58). These results suggest that Dim2 is associated with cultivars producing large seeds (CAL) but slightly lower yields (REN1), indicating a potential trade-off between seed size and yield.

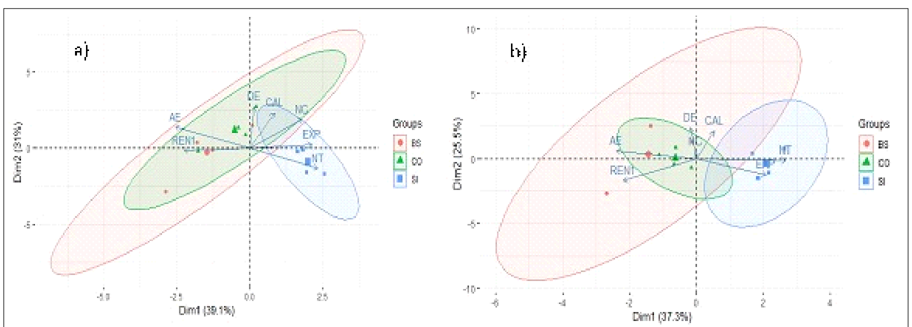
Cluster analysis

Hierarchical clustering divided the chickpea cultivars into two distinct groups on both the adaxial and abaxial leaf surface. This classification was based on clustering criteria including CCC (-0.0722), Pseudo T^2 (1.6987) and Gap Statistic (-1.0859) for the abaxial surface and CCC (-0.8156), Pseudo T^2 (0.2666) and Gap Statistic (-0.0446) for the adaxial surface. This clustering structure accounted for 74% of the total estimated variance, as indicated by the coefficient of determination (R^2 = 0.74). On the abaxial surface (Fig 3a), two clusters were identified: Cluster 1 (C1; red) grouped Blanco Sinaloa-92 and Combo-743, while Cluster 2 (C2; blue) consisted solely of Sinalomex-2018. Cultivars in C1 exhibited higher values for DE, CAL, NC and EXP, whereas C2 was characterized by elevated NT, AE and REN1 values. A similar two-cluster pattern was observed on the adaxial surface (Fig 3b), though with a different distribution. Conversely, Cluster 2 (C2; blue) comprised the cultivars Blanco Sinaloa-92 and Combo-743. These cultivars exhibited higher values for DE, CAL, NC, AE and REN1.

Multivariate analysis (MANOVA) and univariate analysis (ANOVA)

Multivariate analysis (MANOVA) and mean comparisons (ANOVA) between the identified clusters (C1 and C2) on both the abaxial and adaxial leaf surfaces revealed significant differences (p<0.05) in stomatal and seed yield traits among the chickpea cultivars. On the abaxial side, clusters differed notably in NT and AE. Cluster C2 exhibited a higher NT (5.4±0.5) compared to C1 (2.9±0.4), suggesting enhanced resistance to environmental stressors such as drought and solar radiation. In contrast, C1 showed significantly greater AE (5504,777.6±11,327.6) than (241,589.7±160,119.7), indicating potentially higher gas exchange efficiency and, consequently, greater seed yield (C1: 1988.1±50.1 vs. C2: 1790.0±70.8).

Similar trends were observed on the adaxial leaf surface, though with notable differences. C1 exhibited a higher NT (5.8±0.4) compared to C2 (3.2±0.3), suggesting enhanced protection against environmental stressors. However, C1 also showed a significantly smaller AE (235,618.4± 18,945.0) than C2 (479,528.6±13,396.2), indicating that cultivars in C2 may possess greater gas exchange efficiency. Unlike the abaxial surface, C2 on the



DE = Stomatal density, NC = Number of cells, NT = Number of trichomes, AE = Stomatal area, REN1 = Yield, CAL = Size, EXP = % export.

Fig 2: Grouping of kabuli chickpea cultivars (CO = Combo-743; SI = Sinalomex-2018; BS = Blanco Sinaloa-92) according to the stomatal and productive variables evaluated, based on principal components 1 and 2 (Dim), in the lower (underside) and upper (top) leaves.

adaxial side demonstrated higher REN1 (1988.1±50.1) compared to C1 (1790.0±70.1).

Stomatal characterization

Stomatal characterization is a vital tool for understanding plant physiological adaptations to environment conditions (Álvarez-Holguín *et al*., 2018). Traits such as DE and AE are key indicators of gas exchange capacity and transpiration regulation (Márquez-Godoy *et al*., 2024). The cultivar Sinalomex-2018 exhibited the smallest AE values, suggesting efficient gas exchange and improved water use efficiency. Smaller stomata limit water vapor loss to the atmosphere, making them a valuable trait for improving drought tolerance (Karabourniotis *et al*., 2020). In a related study, Noperi-Mosqueda *et al*. (2025) evaluated physiological traits, photosynthetic efficiency and stomatal activity in three maize (*Zea mays*) hybrids (P1898, P1445 and P1382) under drought-pone conditions. They found that hybrid P1382, which had smaller stomatal sizes (1000 μm^{-2}), achieved higher seed yield and better water use.

Additionally, Sinalomex-2018 showed higher NT on both leaf surfaces. Trichomes are specialized epidermal structures that provide protection against insects and herbivory and contribute to abiotic stress tolerance (Karabourniotis *et al*., 2020). According to Karabourniotis *et al*. (2020), trichomes also shield plants from UV-B radiation through the deposition of phenolic compounds, acting as optical filters that protect leaf tissues and preserve photosynthetic function.

Pearson correlation analysis

A negative correlation between NT and AE was observed on both the adaxial and abaxial leaf surfaces, suggesting that an increased number of trichomes is associated with reduced stomatal size. This pattern may reflect an adaptive strategy to mitigate water stress. Trichomes, as specialized epidermal structures, can enhance water retention by acting as physical barriers that limit transpiration, particularly under conditions of intense solar radiation (Adebooye *et al*., 2012). Supporting this, Galdon-Armero *et al*. (2018) conducted a comparative study on tomato (*Solanum lycopersicum*) under well irrigated and water-deficit conditions, demonstrating that water stress led to increased trichome density and reduced stomatal size. The presence of more trichomes, especially on leaf surface, reduces direct exposure to wind and light, thereby limiting stomatal expansion. However, this protective mechanism may come at a cost: excessive trichome density can reduce gas exchange and photosynthetic efficiency (Amada *et al*., 2023), which may explain the observed negative correlation between NT and REN1.

Conversely, the positive correlation between AE and REN1 indicate that larger stomatal size is associated with higher chickpea yield, likely due to improved gas exchange efficiency that enhances photosynthesis (Harrison *et al*., 2019), leading to greater biomass accumulation and seed production. Previous studies in grasses and maize species (Álvarez-Holguín *et al*., 2018; Noperi-Mosqueda *et al*., 2025) have similarly shown that genotypes with larger stomata tend to exhibit higher photosynthetic rates, provided water availability is sufficient.

Finally, the negative association between AE and EXP suggests a trade-off, while large stomata may enhance seed yield, they may limit seed size. This implies differential allocation of photo assimilates within the plant. Greater stomatal area supports higher photosynthetic activity, which can increase the number of seeds (Roche, 2015), but may reduce carbohydrate allocation for grain filling, thereby limiting seed size (Lawson *et al*., 2014). In a similar study, Li *et al*. (2023) show that a higher photosynthetic rate increased yield ($r = 0.41$, $P<0.01$) in wheat (*Triticum aestivum*) but limited plant height ($r = -0.63$, $P<0.01$), flag leaf growth ($r = -0.49$, $P<0.01$) and number of ears per m^2 ($r = -0.22$, $P<0.01$). This balance implies that, under certain conditions, the plant must decide how to allocate available resources, which can affect grain number size and quality (Mora-Ramirez *et al*., 2021).

Cluster analysis

The clustering pattern of the evaluated chickpea cultivars (Fig 3) reflects underlying genetic differences, aligning with findings reported by Choudhary *et al*. (2012), (Naghavi

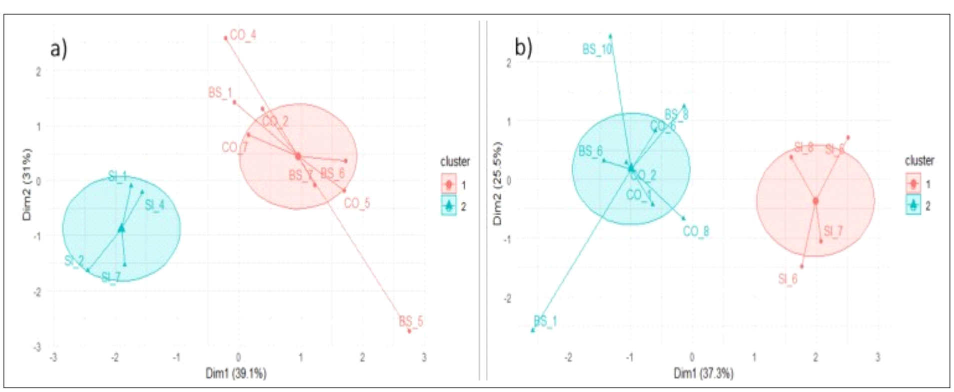


Fig 3: Grouping and classification of chickpea cultivars (CO = Combo-743, SI = Sinalomex-2018, BS = Blanco Sinaloa-92) for the lower leaf (a; abaxial) and upper leaf (b; adaxial).

et al., 2012) and De Giovanni *et al*. (2016). Notably, Blanco Sinaloa-92 and Combo-743 clustered together, suggesting a shared genetic origin likely resulting from the use of closely related parental lines during their development. This contrasts with Sinalomex-2018, which formed a separate cluster, indicating a distinct genetic background. Such divergence is partly attributed to the limited genetic diversity within germplasm used in local breeding programs (Valadez-Moctezuma *et al*., 2020). Blanco Sinaloa-92 was developed through crosses between Spain cultivars and lines from Mexican breeding programs and it saves as the maternal parent of Combo-743, further confirming their genetic relationship. This highlights the high degree of relatedness among Kabuli chickpea cultivars in Mexico, which may explain the moderate variation observed in stomatal traits.

CONCLUSION

The observed morphological variation in chickpea stomata underscores the physiological differences among the three cultivars studied. These findings highlight the importance of selecting cultivars that strike and optimal balance between trichome density and stomatal area on both leaf surfaces to enhance physiological performance, seed yield and seed size. Sinalomex-2018, characterized by smaller stomata, appears to be more resilient to water and hear stress-an increasingly valuable trait in the context of climate change.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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