Research Article

NaCl Salinization Differentially Affects Protophylls Anatomy in Two Varieties of *Phaseolus vulgaris* L.

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Abstract | *Phaseolus vulgaris* is very sensitive to salinity, however, genotypic differences have been observed in its response to this stress factor. The effect of NaCl salinization on the anatomy of protophylls in two commercial varieties with differences in their sensitivity to salinity (Montalbán: sensitive; Tacarigua: moderately sensitive) was studied, in order to evaluate the possible existence of leaf anatomical traits associated with their differential response. The seedlings grew in a greenhouse in an inert substrate that was irrigated with nutrient solution containing 40 mol m^{-3} of NaCl, while a control group without NaCl solution was maintained in parallel. Seven days after the start of the salinization period, when the plants were approximately 14 days old, samples of the protophylls were fixed in FAA (formaldehyde-acetic acid-70% ethanol) and processed and observed optical microscopy. In both varieties the leaves were amphistomatic, with glandular and tector trichomes in the epidermis and bifacial mesophyll. The most notable changes induced by salinization were an increase in the stomata density and a reduction in their size, as well as an increase in the trichomes density in the adaxial and abaxial epidermis, these changes being of greater magnitude in Tacarigua than in Montalbán. These results suggest that the histological changes in the primary leaves under NaCl stress during the seedling phase, could be related to a better capacity to cope with the water stress effect caused by salinity, mainly in Tacarigua.

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Introduction

The progressive salinization of agricultural soils around the world is one of the main problems facing agriculture since the vast majority of crops are sensitive to this stress factor. Salinity affects seed germination, plant growth and agricultural productivity, requiring the adoption of effective mitigation strategies (Karimi *et al*., 2025). It has been estimated that globally the area of soils affected by salts is approximately 1,000 million ha (Ivushkin *et al*., 2019; Teshale, 2023). This problem is exacerbated by taking into consideration future projections of climate change and human population growth (Hassani *et al*., 2021; Paz *et al*., 2023; Salvucci *et al*., 2023). In Venezuela, it is estimated that the area of agricultural land affected by salts is increasing and it has been recognized that desertification and salinization of soils is an important limitation to crop productivity (Fernández *et al*., 2011; Mogollón *et al*., 2017; Torres *et al*., 2017).

The impact of soil salinity in agriculture is driven by several mechanisms, among which the most critical is the reduction of the soil solution's osmotic potential, thereby restricting the ability of plants to absorb water (Elsheery *et al*., 2025). Salinity affects crop development, mainly due to the effect of osmotic stress, ionic toxicity, nutritional and hormonal imbalance, as well as the oxidative damage caused by salts (Fang *et al*., 2021; Ahmed *et al*., 2024). The degree of sensitivity of crops to salinity depends on the species, duration of the period of exposure to salts and the growth phase in which it occurs (Grieve *et al*., 2012; Wu *et al*., 2018).

An important strategy to reduce the impact of salinity on agricultural productivity is genetic improvement based on the selection of morphological, anatomical, physiological and biochemical characteristics associated with tolerance to salt stress (Tahri *et al*., 2020; Atta *et al*., 2023; Awaad, 2023). Ion exclusion and compartmentalization at the vacuolar level is one of the important mechanisms to mitigate the toxic effect caused by salts (Hualpa-Ramírez *et al*., 2024). Recently, it has been indicated that the use of silicon nanoparticles can activate physiological and genetic repair mechanisms in plants subjected to salt stress, improving their resistance to this stress factor (Ijaz *et al*., 2023).

From an anatomical perspective, several characteristics of vegetative organs have been considered important to cope with the adverse effect of salts on crops. Regarding the leaf, an increase in the lignification of the chlorophyll parenchyma and other leaf tissues, as well as a greater development of aerenchyma and an increase in leaf thickness have been considered important characteristics to mitigate the adverse effect of salt stress (Akhtar *et al*., 2017; Akcin and Yalçın, 2023; Karumanchi *et al*., 2023; Sarath *et al*., 2023; Jiang *et al*., 2024).

Phaseolus vulgaris L. is a very sensitive species to salt stress and it has been reported that in substrates where chloride is the predominant anion, the growth and yield are significantly affected from an electrical conductivity (EC) of 1 dS m^{-1} (Maas, 1990). Several studies have documented intraspecific differences in the morphophysiological response of this crop to salt stress (Çirka *et al*., 2022; Hussain *et al*., 2022; Turner *et al*., 2022; García *et al*., 2024); and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis has revealed that salt stress induced the expression of specific *PvNHX* genes in both leaves and roots, suggesting their potential role in regulating salinity tolerance in *P. vulgaris* (Mhadhbi and Boubakri, 2025). However, there is little information documented regarding the anatomical characteristics of vegetative organs that could be linked to the differential responses to salts in this crop. In this research, the effect of NaCl salinization on the anatomy of protophylls in two varieties of *P. vulgaris* (Montalbán and Tacarigua) that differ in their response to salinity was evaluated, in order to determine whether there are leaf anatomical characteristics of possible interest in their differential response.

Materials and Methods

Plant material

The experiments were carried out at the Institute of Physiology and Plant Genetic Resources of INTA in Córdoba, Argentina. Seeds of the commercial varieties of *P. vulgaris*: Montalbán and Tacarigua were used, which were supplied by the Germplasm Bank of the Plant Genetic Resources Conservation Unit of the National Institute of Agricultural Research in Aragua state, Venezuela. Both varieties are widely accepted among farmers in Venezuela due to their good productive performance and resistance to diseases

(De Gouveia *et al*., 2014), but they are sensitive to drought (Domínguez *et al*., 2014) and have shown different behavior to salinity caused by NaCl during the juvenile phase of the crop, with Tacarigua being less affected by this condition than Montalbán (García *et al*., 2010). There is also documented information regarding the behavior of Montalbán as a variety sensitive to salinity during the seedling phase (García *et al*., 2024).

Growth conditions, treatments and experimental design

Seeds of both varieties were placed in trays moistened with distilled water and taken to a growth chamber at a temperature of 30 °C and 90 % relative humidity. After 4 days, seedlings of uniform size were transplanted by transferring them to 200 cm^3 plastic containers containing sterile sand moistened with 0.5 × Hoagland nutrient solution (Hoagland and Arnon, 1950). Then, they were moved to a greenhouse with semi-controlled conditions (average temperature of 26 °C, photoperiod of 16 h light and 8 h dark and photosynthetically active radiation of 250 µmol m^{-2} s^{-1}).

Three days after transplanting, when the protophylls emerged in both varieties, the plants were separated into two groups. In the first group, 40 mol m^{-3} NaCl was gradually added to the irrigation solution and to prevent the seedlings from suffering osmotic shock, the NaCl concentration was gradually increased at a rate of 10 mol m^{-3}, following the procedure used by García *et al*. (2024) in *P. vulgaris* seedlings subjected to salt stress. The second group was left untreated and irrigated with nutrient solution only. The trial was established under a completely randomized design in a 2 × 2 factorial treatment arrangement (two varieties and two types of irrigation solution) for a total of four treatments with six replicates each and six seedlings for each replicate.

Sampling and anatomical processing

Seven days after the salinization period began, when the seedlings were approximately 14 days old, one plant of each replicate was randomly selected from each treatment (six plants/treatment) and 1 cm^2 segments were cut from the middle third of each protophyll and fixed in FAA (formaldehyde-acetic acid-70% ethanol) until processing. Freehand cross sections of the leaf blade were made; additionally, macerations were made for the study of the epidermis in paradermal view, using commercial sodium hypochlorite (5.25%) diluted in water in a 3:1 ratio. All sections were stained with 0.5% aqueous toluidine blue and mounted in water: glycerin (v:v) to obtain semi-permanent slides (Johansen, 1940). The sections were observed in a Leitz optical microscope with a built-in camera for capturing digital images. Additionally, the following quantitative anatomical variables were determined in the leaf blade using a micrometer: Thickness of the epidermis, thickness of the palisade and spongy chlorophyll parenchyma, density and length of stomata, and density of trichomes. In each replicate 10 measurements were made per variable in different randomly selected microscopic preparations.

Statistical analysis

The data obtained were subjected to analysis of variance after checking the assumptions of normality and homogeneity of variance using the Shapiro-Wilk and Hartley tests and in the case of the variables that resulted in significant differences, the Tukey means test was applied. The analysis was carried out using the Infostat statistical software (Di Rienzo *et al*., 2019).

Results and Discussion

Salinization with NaCl caused a notable reduction in the size of the protophylls in both varieties tested (Figure 1). The epidermis is unistrata and in the control seedlings the cells have a strongly sinuous outline in paradermal view of both surfaces being the same less pronounced on the adaxial surface (Figure 2A, C) than in the abaxial surface (Figure 3A, C). Salinization induced a notable reduction in the size of the epidermal cells of both leaf surfaces (Figures 2B, D, 3B, D) and additionally the outline of the cells was observed to be straight to slightly sinuous, which confirm what was suggested by Roth (1984) indicating that this character is strongly influenced by the environmental conditions in which the plant grows. In the cross section of the leaf blade, the epidermal cells were tabular or elliptical in both varieties and conditions (Figure 4A, B). The thickness of the epidermis plus cuticle increased slightly with salinization in both varieties but the 'genotype versus condition' interaction was not significant (Table 1). Previous studies have documented the negative impact of salinity on cell development due to osmotic stress and ionic toxicity (Yu *et al*., 2019). Similarly, to what was observed in this research, Wignarahan *et al*. (1975) also observed a notable decrease in the size of

epidermal cells of the first trifoliate leaf in *P. vulgaris* seedlings subjected to NaCl salinization (48 mol m^{-3}). The decrease in cell size could be interpreted as an adaptive mechanism to maintain turgor pressure under salt stress conditions (Saddhe *et al.*, 2021; Srivastava, 2022) and has also been linked to a deleterious effect of salts on the rate of cell expansion due to the decrease in water uptake with the consequent decrease in cell turgor pressure (Taïbi *et al.*, 2012; Yu *et al.*, 2019). This response varies according to the species and in those more sensitive to salinity the inhibition in growth is usually more pronounced than in tolerant species (Calvo-Polanco *et al.*, 2014; Al-Hassan *et al.*, 2016; Sorial *et al.*, 2022).

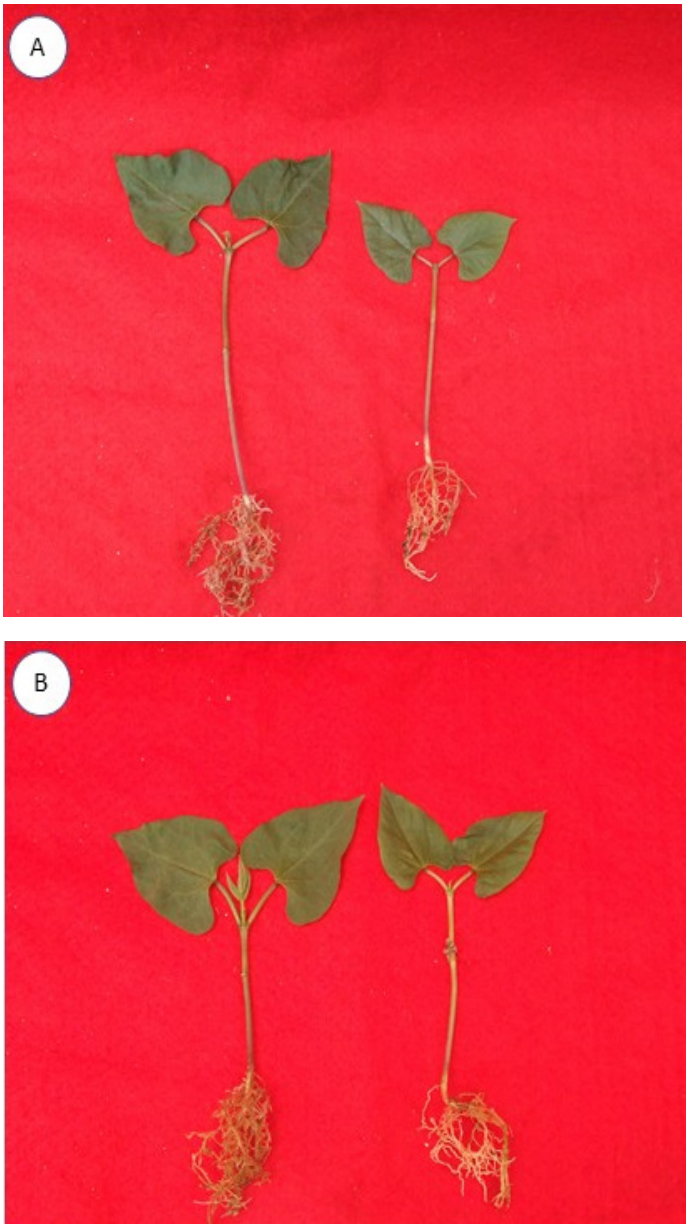

Figure 1: *View of seedlings of two varieties of P. vulgaris, control (left) and subjected to salt stress (right). A: Montalbán B: Tacarigua.*

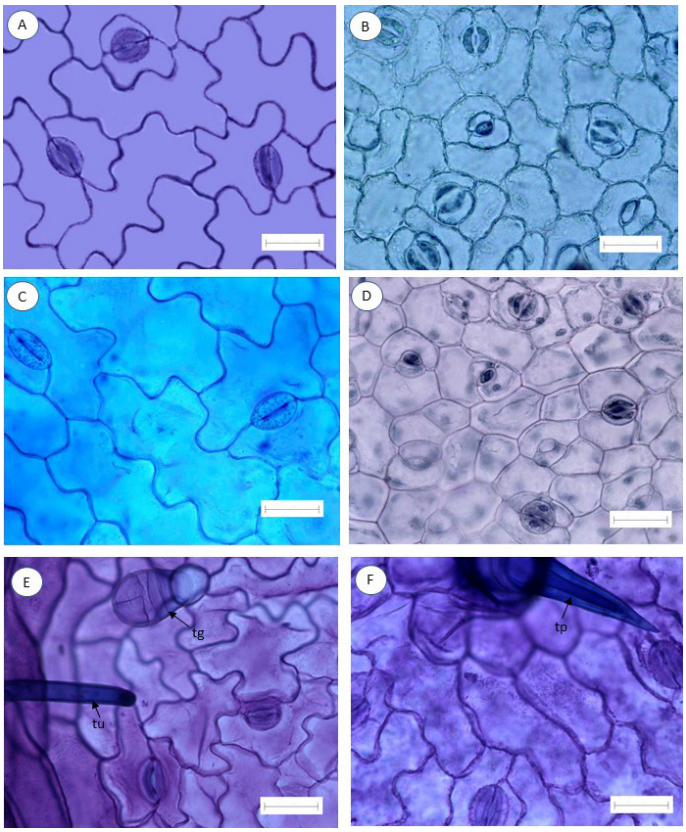

Figure 2: *View of the adaxial epidermis in seedlings of two varieties of P. vulgaris, control and subjected to salt stress. A-B: Montalbán variety, control (A) and saline (B) treatment; C-D: Tacarigua variety, control (C) and saline (D) treatment; E-F: Adaxial epidermis of the Montalbán variety, highlighting different types of trichomes. utt: uncinate tector trichome; tt: tector trichome; gt: glandular trichome.*

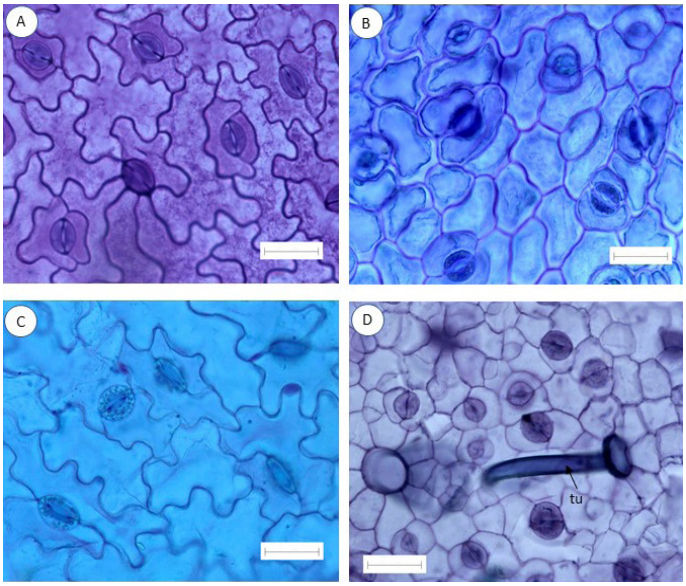

Figure 3: *View of the abaxial epidermis in plants of two varieties of P. vulgaris, control and subjected to salt stress. A-B: Montalbán variety, control (A) and saline (B) treatment; C-D: Tacarigua variety under control (C) and saline (D) treatment. utt: uncinate tector trichome.*

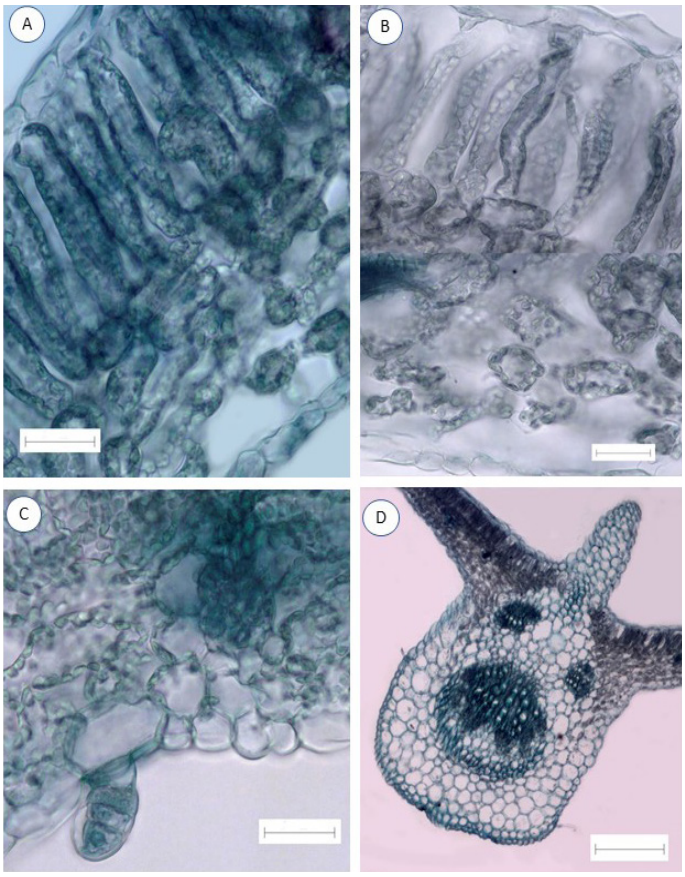

Figure 4: *View of the anatomy of the cross section of the leaf blade in P. vulgaris variety Montalbán. A-B: leaf blade under control (A) and saline (B) treatment; C: detail of the abaxial portion of the leaf blade in the control plant; note the glandular trichome on the abaxial epidermis; D: view of the central rib. de: adaxial epidermis; be: abaxial epidermis; pp: palisade parenchyma; sp: spongy parenchyma.*

Table 1: *Tissue thickness in the cross section of the protophylls of two varieties of P. vulgaris subjected to salinization with NaCl during the seedling phase.*

Factors	E+C Adx	E+C Abx	P emp. (µm)	P esp.	Leaf
Genotype					
Montalbán (Mont.)	18.09 ± 2.28 b	16.81 ± 2.48	73.48 ± 8.94 b	159.56± 20.07	267.94 ± 27.46
Tacarigua (Taca.)	21.92 ± 2.57 a	17.89 ± 2.07	80.97 ± 8.63 a	151.35± 22.16	272.13 ± 30.02
Condition					
Control	18.49 ± 2.75 b	15.87 ±1.64b	72.37 ± 7.48 b	138.68± 10.46 b	245.41 ± 6.32 b
NaCl	21.52 ± 2.69 a	18.84 ±1.88a	82.09 ± 8.81 a	172.22± 14.41 a	294.66 ±17.37a
Genotype × Condition					
Mont. control	16.60 ± 2.12	15.54 ± 2.15	68.06 ± 5.19	144.45± 10.30	244.64 ± 6.69
Mont. NaCl	19.58 ± 1.26	18.09 ± 2.23	78.90 ± 8.84	174.67± 15.25	291.24 ± 17.63
Taca. Control	20.39 ± 1.88	16.20 ± 1.02	76.68 ± 7.18	132.92 ± 7.42	246.18 ± 6.45
Taca. NaCl	23.45 ± 2.33	19.59 ± 1.19	85.27 ± 8.26	169.78 ± 14.50	298.08 ± 18.03
Genotype (*p*)	0.0001	0.1446	0.0237	0.1174	0.4537
Condition (*p*)	0.0011	0.0005	0.0048	<0.0001	<0.0001
Genotype × Condition (*p*)	0.9627	0.5563	0.7155	0.5156	0.6341
CV	9.69	10.03	9.71	7.9	4.98

+ C Adx: epidermis plus adaxial cuticle; E + C Abx: epidermis plus abaxial cuticle; P emp: palisade parenchyma; Pesp: spongy parenchyma; different letters in the same column within each factor and for the interaction between them indicate significant difference according to Tuckey's means test.

In both varieties the leaves were amphistomatic with paracytic stomata (Figures 2A, D and 3A-D), these two characteristics being typical in the subfamily Papilionoideae (Metcalfe and Chalk, 1979) and particularly in *P. vulgaris* (Ojeda *et al.*, 2013). The stomatal density was higher in the abaxial epidermis of both varieties, a common characteristic in amphistomatic leaves (Volenikova and Ticha 2001; Camargo and Marenco, 2011; Muir, 2019).

In control plants, stomatal density was similar on both leaf surfaces of the two varieties (Table 2). Salinization induced a significant increase in stomatal density on both leaf surfaces in Tacarigua, while in Montalbán this response was only observed on the adaxial surface; conversely, stomatal size was reduced by salinization on both epidermises in Tacarigua and only on the adaxial epidermis in Montalbán (Table 2).

The changes caused by salinity in terms of stomatal density and stomata size suggest a better ability in Tacarigua than in Montalbán to cope with the effect of water stress caused by salinity, considering that a higher density of smaller stomata is usually related to an increase in the diffusive resistance of the leaf blade and a reduction in foliar transpiration, a key mechanism to optimize water balance in plants exposed to salt stress (Roth, 1990; Raza *et al.*, 2019; Haworth *et al.*, 2021). Similarly, Bray and Reid (2002) observed a significant increase in stomatal density and a reduction in their size in the abaxial epidermis of the first trifoliate leaf of *P. vulgaris* plants subjected to salt stress, highlighting the importance of this response in controlling water loss and CO_2 absorption. It has also been suggested that smaller stomata are able to better control opening and closing (Raven, 2014), which allows them to better cope with environmental variations.

Table 2: *Quantitative anatomical variables in the frontal view of the protophylls of two varieties of P. vulgaris subjected to salinization with NaCl during the seedling phase.*

Factors	DE Adx (No mm^{-2})	LE Adx (µm)	DE Abx (No mm^{-2})	LE Abx (µm)	DTr Adx (No mm^{-2})	DTr Abx (No mm^{-2})
Genotype						
Montalbán (Mont.)	169.6 ± 52.1	21.7 ± 3.3	258.48 ± 26.87 b	19.85 ± 1.90	10.84 ± 3.82	22.30 ± 12.56 b
Tacarigua (Taca.)	162.5 ± 61.0	20.3 ± 6.7	294.29 ± 74.10 a	19.41 ± 4.43	10.20 ± 3.63	29.96 ± 19.95 a
Condition						
Control	115.71±18.19b	25.31±1.91a	234.95 ± 21.46 b	21.99 ± 1.76 a	7.61 ± 1.33 b	13.17 ± 2.39 b
NaCl	216.41 ± 24.04 a	16.74±3.64 b	317.82 ± 52.02 a	17.27 ± 2.85 b	13.42 ±2.79a	39.1 ± 14.67 a
Genotype × Condition						
Mont. - Control	124.68 ± 13.65 b	24.45±1.82a	239.84±17.16bc	21.00 ± 1.59 b	8.06 ± 1.77	14.87 ± 1.97 c
Mont. - NaCl	214.49 ± 30.59 a	19.03±1.71 b	277.11±21.45 b	18.70±1.50bc	13.61 ± 3.24	29.74 ± 14.51 b
Taca. - Control	106.73 ± 18.68 b	26.17±1.72 a	230.05 ± 25.71 c	22.97 ± 1.40 a	7.16 ± 0.55	11.47 ± 1.30 c
Taca. – NaCl	218.33 ± 18.07 a	14.44±3.69c	358.52 ± 38.94 a	15.84 ± 3.28 c	13.23 ± 2.57	48.46 ± 7.26 a
ANOVA						
Genotype (p)	0.4247	0.1576	0.0041	0.612	0.4963	0.0331
Condition (p)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Genotype × Condition (p)	0.02227	0.0042	0.0005	0.0104	0.7849	0.0035
CV	12.77	11.37	9.80	10.66	21.54	31.38

DE Adx: adaxial epidermis stomatal density; DE Abx: abaxial epidermis stomatal density; LE Adx: adaxial epidermis stomatal length; LE Abs: abaxial epidermis stomatal length; DTr Adx: adaxial epidermis trichome density; Dtr Abx: abaxial epidermis trichome density. Different letters in the same column within each factor and for the interaction between them indicate significant difference according to Tuckey's means test.

In both varieties the leaf blade is pubescent, but the density of trichomes on the abaxial surface was higher than on the adaxial surface, mainly in the Tacarigua in which the density of trichomes on the abaxial epidermis was significantly higher than in Montalbán (Table 2). Salinization caused a significant increase in the abaxial trichome density of the two varieties but this effect was of greater magnitude in Tacarigua than in Montalbán (Table 2), which could play an important role in the lower sensitivity to salinity in the first variety, since the presence of a dense indumentum decreases the absorption of radiant energy and improves the dissipation of the absorbed energy, helping to regulate leaf temperature and avoiding leaf overheating (Johnson, 1975; Fambrini and Pugliesi, 2019), all of which can contribute to reducing the transpiration rate, an important aspect to face the effect of water stress caused by salinity (Srivastava, 2022) and which in turn can favor the photosynthesis process under salt stress conditions (Drake *et al.*, 2019; Narahayaan *et al.*, 2022).

Regarding the morphology of the trichomes in both, the control seedlings and those subjected to salinity of the two varieties three types of trichomes were detected: (i) uniseriate, bicellular, uncinate, thin-walled tector trichome with a short basal cell that continues with a long hook-shaped cell in the terminal portion (Figure 2E, 3D); this type of trichome was the most abundant in both varieties; (ii) uniseriate, tricellular tector trichome, with two short, thin-walled cells and a conical, thick-walled terminal cell (Figure 2F) and (iii) uniseriate glandular trichome with a short pedicel and a globose multicellular head with dense content (Figure 2E, 4C).

The described trichome types are common in the subfamily Papilionoideae (Metcalfe and Chalk, 1979; Leelavathi and Ramayya, 1983). In *P. aconitifolius* Jacq. and *P. trilobus* Ait., uniseriate glandular trichomes with globose heads and uniseriate tector trichomes with a conical terminal cell have also been reported (Leelavathi and Ramayya, 1983). In *P. vulgaris*, Silva *et al.* (1999) observed uncinate tector trichomes on the leaf blade of six cultivars of this species. Some studies with cultivated (Nassar *et al.*, 2010) and wild genotypes (Ojeda *et al.*, 2013) of *P. vulgaris* have documented the presence of the two types of protective trichomes described in this investigation, however, the uniseriate glandular trichome observed

in the two varieties tested in this research has not been reported in these studies; it is possible that this type of trichome plays a role in salt secretion, helping to counteract their toxic effect (Hagemeyer, 1997).

The anatomy of the mesophyll was similar in both varieties and conditions. The leaf is bifacial with one layer of elongated chlorophyll-like palisade parenchyma cells and 5 to 6 layers of spongy parenchyma with globose to elliptical cells. The vascular system of the leaf blade is made up of closed collateral bundles located at the boundary between the palisade and spongy parenchyma or immersed in the latter tissue (Figure 4A, B). The only difference detected between the two varieties was in the thickness of the palisade parenchyma, which was greater in Tacarigua than in Montalbán. Salinity caused an increase in the thickness of both the palisade and spongy parenchyma and in the leaf thickness but the interaction between variety and condition did not show a significant difference for any of these variables (Table 1).

The histological arrangement of the leaf mesophyll of the two varieties studied is in agreement with that previously reported in *P. vulgaris* (Wignarahan *et al.*, 1975; Silva *et al.*, 1999; Bray and Reid, 2002). Similar to what was observed in this study, Wignarahan *et al.* (1975) found that salinization with NaCl (48 mol m^{-3}) induced an increase in the thickness of the mesophyll tissues of the first trifoliate leaf of *P. vulgaris* mainly due to an increase in the thickness of the spongy parenchyma and a similar effect was observed by Bray and Reid (2002) in the second trifoliate leaf of this crop when the plants were stressed with NaCl (90 mol m^{-3}). Under salt stress, many plants develop thicker leaves, which has been associated with a greater water storage capacity and a more efficient distribution of solutes thereby reduce ionic toxicity and maintain photosynthetic function under saline conditions (Kuster *et al.*, 2019; Lu *et al.*, 2021), however, this response did not differentiate the behavior of the two varieties tested to salinity.

The anatomy of the midrib was similar in both varieties and conditions. In this zone of the leaf blade the epidermis was also unistrata with few trichomes of the same types already described; beneath the epidermis globose parenchyma cells were distinguished, some of them with chloroplasts. The vascular tissue occupies central position and it is represented by a larger open collateral bundle that is arranged towards the abaxial side of the midrib and two small open collateral bundles that are located adaxially.

Conclusions and Recommendations

The anatomy of the protophylls was similar in the two varieties of *P. vulgaris* studied. The most important leaf anatomical changes caused by NaCl salinization during the seedling phase were evident in the leaf epidermis, specifically an increase in stomatal density and a reduction in stomata size on both leaf surfaces in Tacarigua and only in the adaxial epidermis in Montalbán, as well as an increase in the density of trichomes of the abaxial epidermis, of greater magnitude in Tacarigua. These results suggest that the lower sensitivity of Tacarigua to salinity could be associated with histological adjustments to cope with the effect of water stress caused by salinity. It is recommended to explore the behavior of these *P. vulgaris* varieties under saline stress, exploring other physiological parameters (e.g. carbon and water exchange rates) to verify the correlation between these variables and the structural characteristics evaluated in this study. It is also important to evaluate the response of these varieties in other growth phases and using other salts common in saline soils.

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Novelty Statement

Salt stress increased stomatal density while reducing stomatal size in both Montalbán and Tacarigua varieties of *Phaseolus vulgaris* L. Likewise, the trichome density on the abaxial leaf surface increases under saline stress, mainly in Tacarigua.

Author's Contribution

Marina Coromoto García: Devised the idea, supervised the research work and wrote the draft and final version of the manuscript.
Adriana Beatriz Sánchez-Urdaneta: Participated in

the research trial and contribute in the writing of the final version of the manuscript.

Naga Raju Maddela: Provided practical assistance and helped in the write-up of the final version of the manuscript.

Gisela Rivero-Maldonado: Participated in the research trial and in the write-up of the manuscript.

Freddy Zambrano-Gavilanes and Rolando León Aguilar:: Participated in the data analysis and write-up of the manuscript.

Each author equally aided in analysis, feedback, revising, and endorsing the manuscript.

Data availability statement

All data supporting the findings of this study are available within the paper.

Generative AI and AI-assisted technology statement

The authors have declared no Generative AI and AI-assisted technologies in the writing process

Conflict of interest

The authors declare that they have no conflicts of interest related to any stage of the development of this article, including its conception, execution, data analysis, or publication. No financial, personal, or institutional relationships influenced the research process or its findings.

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