The effect of rootstock on the anatomy of the *Phaseolus vulgaris* L. scion: graft union, stomatal density, stomatal index, and leaf thickness

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ABSTRACT

Objective: To evaluate the effect of the *Phaseolus acutifolius* A. Gray rootstock on the establishment of the *Phaseolus vulgaris* L. scion, analyzing the anatomy of the graft union, stomatal density, stomatal index, and leaf thickness.

Design/Methodology/Approach: A completely randomized experimental design was used. The experimental unit consisted of one plant per pot. Data were analyzed using Tukey's Mean Comparison Test ($p \leq 0.05$). Some segments of the graft union were fixed in formaldehyde-acetic acid-alcohol (FAA), embedded in paraffin, and stained with safranin and fast green, while others were analyzed fresh. Fragments of the central leaflet, fixed in 3% glutaraldehyde and processed for observation under a Scanning Electron Microscope, were used to describe leaf anatomy.

Results: Five stages were identified during the formation of the graft union: development of the necrotic layer, callus proliferation, vascular cambium differentiation, vascular tissue restoration, and restoration of the epidermis. Grafted plants recorded increases of 20%, 24.7%, and 66% in stomatal density, stomatal index (on the underside of leaves), and in mesophyll thickness, respectively.

Study Limitations/Implications: The *Phaseolus acutifolius* rootstock was used in the anatomical evaluation of the *Phaseolus vulgaris* scion under irrigation.

Findings/Conclusions: The anatomical compatibility between rootstock and scion was confirmed, allowing the development of new plants with foliar micromorphological characteristics that modify their physiological behavior.

Keywords: common bean, heterograft, graft union anatomy, leaf anatomy, stomata.

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INTRODUCTION

Grafting is a vegetative propagation technique that physically joins organs from different plants (heterograft) or from the same plant (homograft) into a single plant (Loupit *et al.*, 2023). The upper section of the grafted plant is known as scion, while the lower

section is called rootstock. The plant that produces the scion is commercially productive and resistant or tolerant to foliar diseases. Meanwhile, the rootstock serves as the root of the grafted plant and usually has no economic value; however, it contains genes that include characteristics such as greater vigor and resistance or tolerance to soil limitations (Bohra *et al.*, 2022). The scion-rootstock interaction results in anatomical changes that impact the characteristics of the grafted plant and improve scion yield, nutrient and water uptake, and vigor (Sharma & Zheng, 2019). These changes are different depending on the rootstocks (Tedesco *et al.*, 2020). The grafted plant should maintain the characteristics and benefits of both plants (Basto-Pool *et al.*, 2021).

Grafting has been used for centuries with perennial plants —mainly fruit trees, forest trees, and ornamental trees. Since the beginning of the 20th century, it has also been practiced on vegetables, such as Cucurbitaceae and Solanaceae (Gautier *et al.*, 2019). However, research has not achieved the same advances regarding the genus *Phaseolus*.

In Latin America and North Africa, bean (*P. vulgaris* L.) is the most consumed species of the genus *Phaseolus*, as a result of its carbohydrate, protein, vitamin, and mineral content (Abdelhafez *et al.*, 2021). This legume is mainly produced in dryland; however, droughts decrease bean yield depending on the intensity, duration, and phenological stage in which the event takes place (Domínguez *et al.*, 2019). *P. vulgaris* cv. 'Pinto Saltillo' has an indeterminate bushy growth habit (type II) (CIAT, 1982). Its yield reaches 2.3 kg ha^{-1}. This legume was chosen because its shelf life is longer than the shelf life of other cultivars. Its testa slow darkens under storing conditions. It contains 22.1% protein and has a short cooking time (Sánchez, 2001). The brown tepary bean (*P. acutifolius* A. Gray) used as a rootstock has an indeterminate prostrate growth habit (type III) (CIAT, 1982). It has a deep and vigorous root system, which enables it to absorb groundwater and adapt to low-rainfall regions (Jimenez-Galindo *et al.*, 2018).

In order to obtain cultivars tolerant to drought stress, plant breeding programs focus on the generation of inter- and intraspecific crosses between cultivars of the genus *Phaseolus*. The tepary bean (*P. acutifolius*) stands out for its high tolerance to drought and extreme temperatures. However, its percentage of introgression with *P. vulgaris* is low; consequently, numerous selection and production cycles are required to obtain tolerant cultivars.

These limitations could be addressed in the short term through the production of grafted bean plants, which could increase crop tolerance to water stress (Basto-Pool *et al.*, 2021).

The level of adaptation to drought stress can be assessed by measuring various anatomical parameters of leaves, including the thickness of the upper and lower epidermis, total leaf thickness, the thickness of the palisade and spongy parenchyma tissues, the size of the vascular bundle, the size and density of stomata, and other structural attributes that are directly linked to drought resistance (Taratima *et al.*, 2020; Sun *et al.*, 2022). Likewise, determining the affinity of the cultivar to be grafted is fundamental to avoid compatibility problems and epidermal modifications of the leaves of the grafted plants. This element is important because the gas exchange of photosynthesis takes place in these organs. Photosynthesis is a chemical process through which photoassimilates are translocated to the plant organs that requires them. When stomatal density increases in the leaves of the grafting, the rate of CO_2 assimilation, transpiration, and stomatal conductance also increases (Ayala-Arreola *et al.*, 2010). Therefore, the objective of this research was to evaluate the effect of the brown tepary (*P. acutifolius*) rootstock on the establishment of the *P. vulgaris*, cv. 'Pinto Saltillo' scion, evaluating the anatomy of the graft union, stomatal density, stomatal index, and leaf thickness.

MATERIALS AND METHODS

Plant material and experimental design

The study was established in a tunnel-type greenhouse at the Colegio de Postgraduados (COLPOS) - Campus Montecillo, Texcoco, State of Mexico (19° 27' 40" N, 98° 54' 19" W, and 2,353 m.a.s.l.). Brown tepary bean plants (*P. acutifolius*) and *P. vulgaris* cv. 'Pinto Saltillo' were used as rootstocks and scions, respectively. Seeds from both species were individually sown in 120 mL propagation tubes with peat moss as substrate. The method proposed by Lee *et al.* (2010) was used to make cleft grafts when the first compound leaf (V3 stage) of the plants appeared (CIAT, 1982). Fifteen days after grafting (15 DAG), the plants were transplanted into 2 L plastic pots with tezontle (≤5 mm particle diameter). Irrigation was carried out for 90 d with 100% Steiner nutrient solution (Steiner, 1961). Treatments consisted of 12 repetitions of grafted and non-grafted beans, distributed into a completely randomized experimental design. The experimental unit was one plant per pot.

Anatomy of the graft union

Four 5×5 mm stem fragments from grafted plants were taken 1 cm below and 1 cm above the graft union; subsequently, they were fixed in formaldehyde-acetic acid-alcohol (FAA) for 48 h under vacuum. They were then washed with deionized water (three 15-minute rinses each) and dehydrated in an ethanol series: 50%, 60%, 70%, 80%, 96%, and 100% (two changes), for 4 h each. Afterwards, they were placed in an ethanol:xylene transition medium, using 3:1, 1:1, and 1:3 ratios, followed by two xylene changes (100%), for 4 h each.

The samples were infiltrated in McCormick Scientific™ Paraplast Plus paraffin (Supplier: Leica Biosystems) in an oven at 60 °C, in two changes: first in paraffin with an intermediate solvent (xylene) for 4 h and the second in pure paraffin for 24 h. An American Optical Company™ rotary microtome (USA) was used to cut 7-10 μm-thickness serial sections (cross-section and longitudinal). Chrome alum adhesive [1% gelatin, 0.1% phenol, and 0.05% chrome alum ($KCr(SO_4)_2\ 12(H_2O)$) in water] was used to adhere the sections to slides. After 12 h on a hot plate (60 °C), sections were deparaffinized with xylene (100%; three changes of 3 min each) and hydrated in an ethanol series (100%, 96%, 70%, and 50%; 3 min each). The sections on the slides were stained with safranin [0.05%; Safranin O (C.I. 50240) for microscopy, Merck] in a saline solution (3% NaCl in water) for 2 h. Subsequently, the sections were washed twice with deionized water for 3 min and dehydrated in an ethanol series (50%, 70%, 90%, and 100%) for 2 min each. Afterwards, the sections were stained with 0.12% Fast Green FCF (C.I. 42053 for microscopy, Merck) in 95% ethanol for 1 min. The dye excess was removed with 96% and 100% ethanol, followed by three xylene changes (100%), for 5 min each. The slides were mounted in synthetic resin and dried on a hot plate at 60 °C.

Additionally, 1 cm fragments of fresh stem were cross-sectioned with a hand-held microtome. In order to prevent dehydration, the 100-μm thick sections were retrieved with a fine paintbrush and placed in 100×20 mm Petri dishes, with distilled water, at laboratory temperature. Afterwards, they were placed on a slide with a drop of water and covered with coverslips. The anatomy of the graft union was documented with a ZEISS SteREO Discovery V20 optical microscope and a Canon SD digital camera.

Stomatal density, stomatal index, and leaf thickness

Initially, 5×5 mm sections were obtained from the central leaflets of trifoliate leaves —which are located in the middle stratum of grafted and non-grafted *P. vulgaris* plants. Afterwards, they were fixed in glutaraldehyde solution [3% glutaraldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2) for 24 h under vacuum]. Subsequently, the same buffer was used for two rinses of 10 min each. The samples were post-fixed with 2% osmium tetroxide in water for 2 h, followed by two rinses with deionized water for 30 min each. Subsequently, they were dehydrated for 40 min each, in 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol series (two changes). The samples were dried at 31 °C with a Tousimis™ Samdri-780A critical-point dryer and coated with a thin layer of 80:20 gold:palladium, using a metal ionizer (Fine Coat Ion Sputter JFC-1100). A JEOL JSM-6390 scanning electron microscope was operated at 10 kV for observations.

The ImageJ (v. 1.51k, NIH, USA) software was used to establish stomatal density, stomatal index, and leaf thickness. Stomata and epidermal cells were counted on the adaxial (upper) surface and abaxial (lower) surface of four central leaflets, with eight repetitions, respectively. Mesophyll thickness (including palisade parenchyma and spongy parenchyma) was determined in three central leaflets, with four repetitions, using the midrib as a reference.

Using the number of stomata and the number of epidermal cells, stomatal density (SD) and stomatal index (SI) were estimated according to the following expressions:

$$SD = \frac{number\ of\ stomata}{mm^2}$$

$$SI = \left(\frac{SD}{SD + ECD}\right) \times 100$$

where SD=stomatal density, SI=stomatal index, and ECD=epidermal cell density (Willmer & Fricker, 1996).

Statistical analysis

SAS (SAS, 2002) was used to subject the stomatal density, stomatal index, and leaf thickness data to an analysis of variance and a mean comparison test (Tukey: $p \leq 0.05$).

RESULTS AND DISCUSSION

Anatomy of the graft union

Five stages of the graft union formation were identified: 1) development of a necrotic layer, 2) callus proliferation in the graft interface, 3) differentiation of a new vascular cambium, 4) restoration of new vascular tissue, and 5) restoration of the continuity of the epidermis in the graft union. These stages have been described for various plant species and graft types. The difference lies in the time required to complete each stage, as it takes less time for herbaceous plants than for woody plants (Melnyk, 2017; Wang *et al*., 2017).

No cambial activity was recorded during the first hours after the physical union of the rootstock and the scion. However, after 24 h, a necrotic layer (Figure 1a) —characterized by intensely stained cells (Figures 1c-d)— was observed on the rootstock and scion. This layer is the initial response to the damage caused by the cut. It progressively breaks down, facilitating the formation of a common cell wall (Tamilselvi & Pugalendhi, 2017; Adhikari *et al*., 2022).

The polysaccharides accumulation —mainly pectin secreted by cells in the graft interface— plays a crucial role in the initial physical union of the plants, because these polysaccharides are adherents (Sala *et al*., 2019). However, initial adhesion on the graft surface does not guarantee a successful graft union: the functional vascular connection between the scion and rootstock must be established during subsequent stages (Velasco-Alvarado *et al*., 2017; Martínez *et al*., 2020).

The first evidence of cell division was the development of the callus (5 DAG), which formed a bridge at the scion-rootstock interface (Figures 1b and d). Subsequently, differentiation of the new vascular cambium and the restoration of new vascular tissue became evident (Figure 1e). The continuity of the epidermis at the scion-rootstock union (15 DAG) indicated the complete establishment of the union (Figure 1f).

This process matched the descriptions of Rasool *et al.* (2020) and Tedesco *et al.* (2023), who identified the connection of vascular tissues as the key event for a successful graft.

Auxins seem to play a critical role in vascular differentiation in the graft union. Authors such as Nanda and Melnyk (2018) and Wang *et al.* (2017) highlighted that polar auxin transport —temporarily interrupted by the cut— accumulates at the base of the rootstock and influences cell growth and vascular tissue differentiation, especially in the graft union (Habibi *et al*., 2022).

Stomatal density, stomatal index, and leaf thickness

The leaflets of grafted and non-grafted plants displayed dorsiventral characteristics, with a uniseriate adaxial (upper surface) and abaxial (lower surface) epidermis of irregularly shaped cells and wavy cell walls. Paracytic stomata were found on both leaf surfaces. They had a pair of subsidiary cells parallel to the ostiole, covered by a striated cuticle. The results of this study confirm the amphistomatic nature of bean leaves. Two main types of trichomes were identified: a unicellular, hooked trichome with an enlarged base and a multicellular trichome with an enlarged, glandular distal end. Both were located on the primary vein, at the branching point of the veins, and on both leaflet blades (Figure 2 a-d). The type of trichomes found in bean plants is consistent with those found by Jiménez

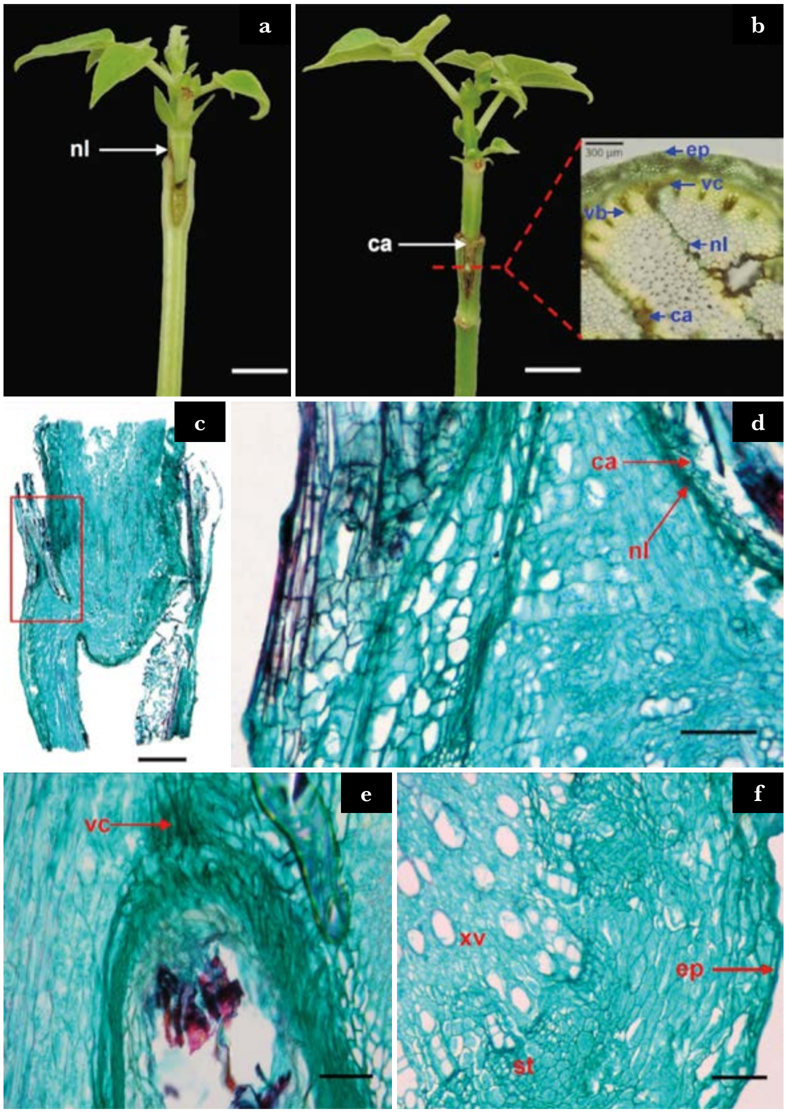

Figure 1. Formation of the *Phaseolus vulgaris* L. (scion)-*P. acutifolius* A. Gray (rootstock) heterograft union. a) Union showing the necrotic layer (24 h); b) Callus development in the graft interface and cross-section of the union (5 DAG); c), d), e) Longitudinal sections, and f) cross-section of the graft union stained with safranin-fast green; d) Close-up of the union shown in the red box in c); e) Differentiation of vascular cambium (15 DAG); f) Continuity of the epidermis (ep) in the scion-rootstock union (15 DAG). Callus (ca), vascular cambium (vc), necrotic layer (nl), vascular bundle (vb), xylem vessel (xv), and sieve tube (st). Bar: 1 cm (a, b), 1 mm (c), 50 μm (d), 100 μm (e), and 50 μm (f).

et al. (2012) in the adaxial and abaxial epidermis, including unicellular trichomes and multicellular trichomes, both with an enlarged base.

The main vascular bundle has developed in the midrib; the less prominent veins laterally arranged form the vascular bundle system of the reticulate venation (Figure 3a, b).

In grafted and non-grafted *Phaseolus* plants, the stomatal density (SD) and stomatal index (SI) of the upper surface did not record significant differences (Tukey, $p \leq 0.05$). However, the SD and SI of the underside of leaves of grafted plants were 20% and 24.7% higher than in non-grafted plants (Table 1).

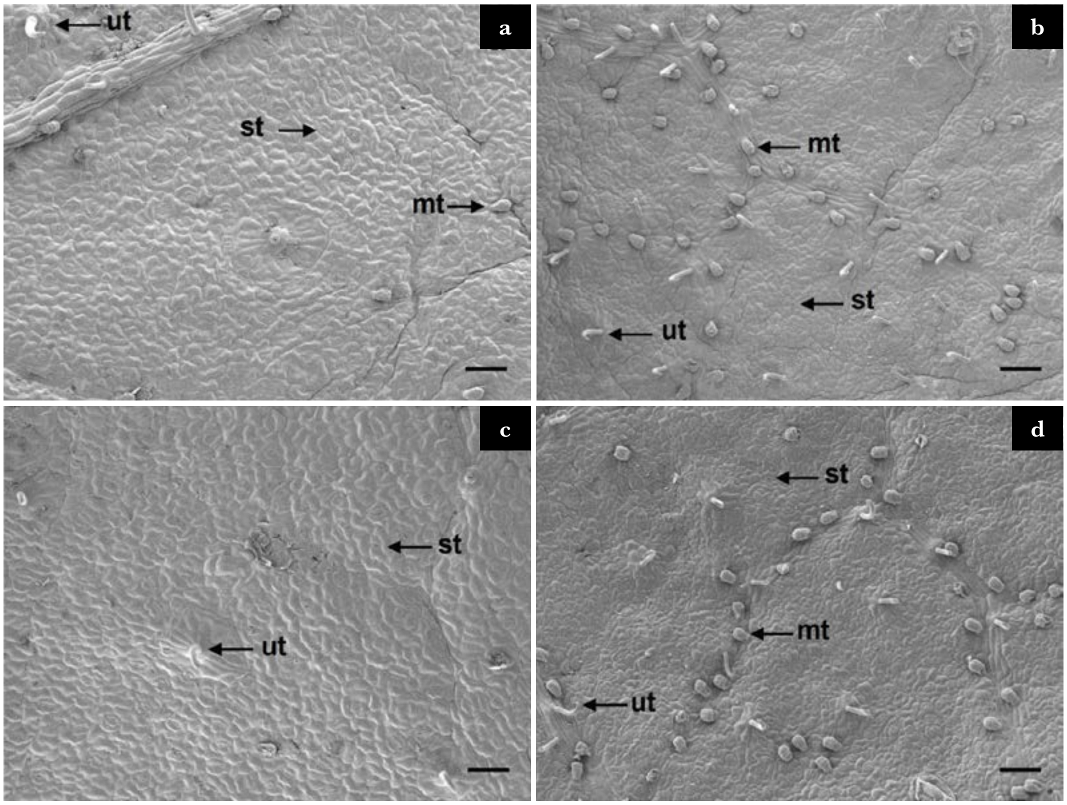

Figure 2. Leaf epidermis. Non-grafted *Phaseolus vulgaris* L. plant, upper surface (a) and lower surface (b). Heterograft *P. vulgaris* L. (scion)-*P. acutifolius* A. Gray (rootstock), upper surface (c) and lower surface (d), observed in a JEOL JSM-6390 microscope. Stoma (st), unicellular trichome (ut), multicellular trichome (mt). Bar: 100 μm.

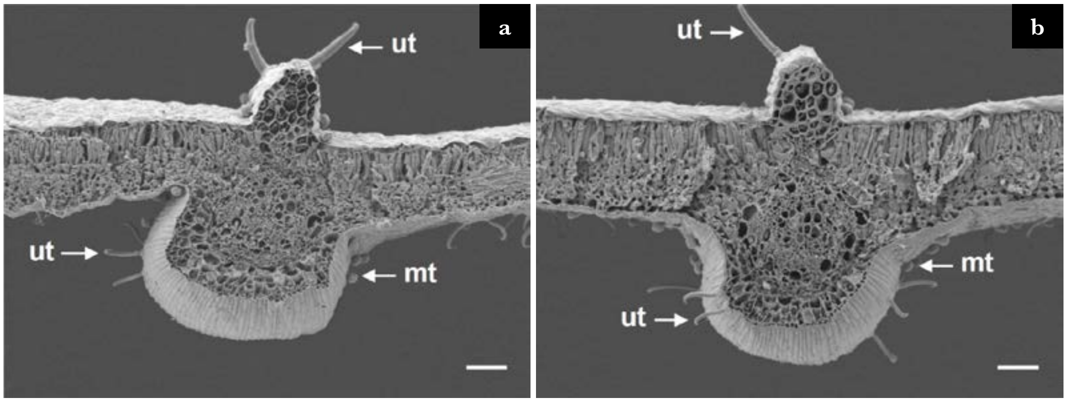

Figure 3. Cross-sections of the midrib of the central leaflet. a) Non-grafted *Phaseolus vulgaris* L. plant. b) Heterograft of *P. vulgaris* L. (scion)-*P. acutifolius* A. Gray. (rootstock) observed in the JEOL JSM-6390 microscope. Unicellular trichome (ut), multicellular trichome (mt). Bar: 100 μm.

These results matched the findings of Camposeco-Montejo *et al.* (2018), who reported similar increases in grafted bell pepper plants, associated with higher rates of CO_2 assimilation, transpiration, and stomatal conductance, as well an efficient water use and stress tolerance (Hernández *et al.*, 2021). However, the results were different from those

Table 1. Effect of *Phaseolus acutifolius* A. Gray (rootstock) on the stomatal density and stomatal index of *P. vulgaris* (scion).

	Stomatal density (SD) (stomata/mm^2)		**Stomatal index (SI) (%)**	
	Upper surface	**Underside**	**Upper surface**	**Underside**
Grafted	89.06±9.83 a	279.96±26.63 a	16.31±0.90 a	22.33±1.02 a
Non-grafted	107.32±10.96 a	233.24±22.00 b	17.09±1.47 a	17.90±1.74 b

Data are means ± SE. Different letters in the columns indicate statistical differences (Tukey, $p \leq 0.05$), n=8.

recorded by Peralta-Manjarrez *et al.* (2016) for grafted cucumber (*Cucumis sativus* L.) plants: the upper and lower surfaces of leaves recorded a reduction in SD and SI.

Stomatal density in bean leaves of both grafted and non-grafted plants fell within the range described for angiosperms: 100-300 stomata/mm^2 (Esau, 1977). This situation suggests that bean leaves follow general patterns of stomatal development, similar to other angiosperm species. Specific variations could be the result of species-specific genetic information and the environmental conditions in which the plants develop. Plants adjust their stomatal density under water stress (Bishnoi *et al.*, 2022). Low stomatal density in *Triticum aestivum* L. is associated with higher yield under water stress (Li *et al.*, 2017), while, in *P. vulgaris*, SD increases under drought conditions (Polania *et al.*, 2020). Apple trees (*Malus domestica* Borkh.) grafted onto dwarfing rootstocks recorded a lower SD than apple trees grafted onto vigorous rootstocks (Zhou *et al.*, 2020).

The leaf mesophyll was different between palisade parenchyma near the upper surface and spongy parenchyma near the lower surface (Figure 4).

The interaction of the scion with the rootstock increased palisade parenchyma and spongy parenchyma ratios by 70.5% and 61.7%, respectively (Table 2). This increase resulted in a 66% thicker mesophyll.

Ayala-Arreola *et al.* (2010) reported an increase of SD, ECD, and palisade parenchyma thickness in grafted avocado (*Persea americana* Mill.) plants; their results are similar to those

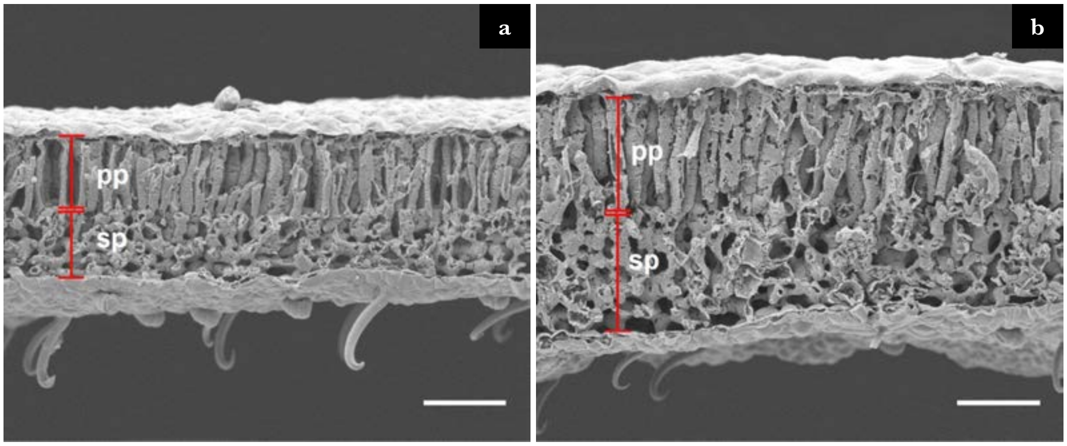

Figure 4. Cross-sections of the central leaflet. a) Non-grafted plant of *Phaseolus vulgaris* L. and b) Heterograft of *P. vulgaris* L. (scion)-*P. acutifolius* A. Gray (rootstock) observed in a JEOL JSM-6390 microscope. Palisade parenchyma (pp), spongy parenchyma (sp). Bar: 100 μm.

Table 2. Effect of *Phaseolus acutifolius* A. Gray (rootstock) on the palisade parenchyma, spongy parenchyma, and mesophyll thickness of *P. vulgaris* L. (scion).

	Palisade parenchyma (μm)	**Spongy parenchyma (μm)**	**Mesophyll thickness (μm)**
Grafted	151.36±5.36 a	147.53±5.83 a	298.89±9.01 a
Non-grafted	88.76±1.11 b	91.23±2.78 b	179.98±3.20 b

Data are means ± SE. Different letters in the columns indicate statistical differences (Tukey, $p \leq 0.05$), n=8.

recorded in this study. However, Zhou *et al.* (2020) reported different results in apple trees grafted onto dwarfing rootstocks, where the palisade parenchyma:spongy parenchyma ratio decreased.

Grafting can have similar effects in different plant species and promote anatomical modifications that potentially improve the efficiency of the physiological processes of plants. The increase in mesophyll thickness associated with a higher SD could improve gas exchange and photosynthetic capacity, favoring the growth and productivity of grafted plants. These results highlight the importance of grafting to adapt to adverse conditions and to optimize key anatomical characteristics for the physiological efficiency of plants.

CONCLUSIONS

The union between the scion and the rootstock formed a strong and functional connection that facilitated the continuous flow of nutrients and water in the grafted bean plants. The scion-rootstock interaction increased the thickness of the mesophyll, as well as the SD and SI on the underside of the leaves, resulting in the modification of gas exchange and transpiration and photosynthetic rates.

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