



Leaf morpho-anatomical characterization in *Pinus patula* Schiede ex Schltdl. & Cham. progenies

Caracterización morfo-anatómica foliar en progenies de *Pinus patula* Schiede ex Schltdl. & Cham.

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Abstract

Progeny trials are experimental fields where the genetics of selected parent trees are evaluated. They bring together families from different origins that vary due to genetic and/or environmental factors. The objective of this research was to describe and analyze the foliar morpho-anatomy of *Pinus patula* from two progeny trials established at the *Tlatoxca* ranch in the state of *Hidalgo* and the *Ojo de Agua ejido* in the state of *Veracruz*, Mexico. Ten fascicles were collected from 64 trees. Cross-sections were made and stained with safranin and alcian blue. The structures were analyzed and described using an optical binocular microscope. The number of needles per fascicle, the number of stomatal rows (abaxial and adaxial surfaces), and the number of resin canals were subjected to an analysis of variance ($P \leq 0.05$). A single-layered epidermis composed of highly clarified rectangular cells was observed. The hypodermis is single-layered and bi-layered; 54 % of the needles at the *Tlatoxca* ranch exhibited a mostly bi-layered hypodermis, while at the *Ojo de Agua ejido*, 33 % did. Significant differences were found in the number of resin canals ($P \leq 0.0001$); at the *Tlatoxca* ranch, the number ranged from (2-)3(-6) with an average of 3.48, while at the *Ojo de Agua ejido*, the range was (0-)3(-4), with an average of 2.64. The resin canals were frequently located medially and internally, and exceptionally externally and septally. The environmental conditions of each plantation site influenced some morpho-anatomical characteristics of the *P. patula* needles in two two-year-old plantations.

Keywords: Needles, leaf anatomy, resin canals, endoderm, epiderm, hypoderm.

Resumen

Los ensayos de progenie son campos experimentales en los que se evalúa la genética de progenitores seleccionados. Reúnen familias de diferentes procedencias que varían por factores genéticos o ambientales. El objetivo de esta investigación fue describir y analizar la morfo-anatomía foliar de *Pinus patula* de dos ensayos de progenie establecidos en el rancho Tlatoxca en Hidalgo y el ejido Ojo de Agua en Veracruz, México. Se recolectaron diez fascículos de 64 árboles. Se realizaron cortes transversales y se tiñeron con safranina y azul-alcian. Mediante microscopio óptico binocular se analizaron y describieron las estructuras. El número de acículas por fascículo, el número de hileras estomáticas (cara abaxial y adaxial) y el número de canales de resina se sometieron a un análisis de varianza ($P \leq 0.05$). Se observó epidermis monoestratificada formada por células rectangulares altamente esclerificadas. La hipodermis es monoestratificada y biestratificada; 54 % de las acículas procedentes del rancho Tlatoxca exhibieron hipodermis mayormente biestratificada, y solo 33 % del ejido Ojo de Agua. Se verificaron diferencias estadísticas en el número de canales resiníferos ($P \leq 0.0001$); el material de Tlatoxca se ubicó en un intervalo de (2-)-3(-6) y promedio de 3.48; los valores para el ejido Ojo de Agua fueron de (0-)-3(-4), con promedio de 2.64. La posición de los canales de resina, fue media e interna, excepcionalmente externa y septal. Las condiciones ambientales de cada sitio de plantación influyeron sobre algunas características morfo-anatómicas de las acículas de *P. patula*, en dos plantaciones de dos años de edad.

Palabras clave: Acículas, anatomía foliar, canales de resina, endodermis, epidermis, hipodermis.

Introduction

Environmental pressure can affect the morphological traits and phenotype of plants (Rodríguez-Laguna & Capo-Arteaga, 2005). Environmental conditions are directly related to growth and development (Fernández & Salvo, 2003) and play an important role in the morphological and anatomical variation of each organ (Sotolongo-Sospedra et al., 2022). This variation is influenced by abiotic factors such as temperature regimes, light quantity and availability (Tiwari et al., 2013), soil properties (Ghimire et al., 2014; Gianoli, 2004), and plant genetics (Chávez-García et al., 2022; Zhang et al., 2017).

Secondary leaves (needles) in pines (*Pinus* L.) genus are organs exposed to the environment and exhibit variation within and between different trees of the same species (Azcárraga-Rosette et al., 2022). They are responsible for photosynthesis, atmospheric carbon assimilation, and gas exchange (Donnelly et al., 2016). Needles adjust or modify their morphology and anatomical structure to withstand unfavorable habitat conditions (Geada-López et al., 2021; Nikolić et al., 2016).

Pinus patula Schiede ex Schltdl. & Cham. is a tall, evergreen tree that reaches up to 40 m in height and 100 cm in trunk diameter. It usually has a straight, cylindrical trunk. Fascicles of 3-4(-5) thin, lax, and pendulous needles, (11-)15-25(-30) cm long and 0.7-0.9 mm wide. This species is naturally distributed in Mexico, in the states of *Chiapas*, *State of Mexico*, *Hidalgo*, *Oaxaca*, *Puebla*, *Querétaro*, *Tamaulipas*, *Tlaxcala*, *Veracruz*, and Mexico City, on mountainous formations of the *Sierra Madre Oriental* (Dvorak et al., 2000; Farjon & Styles, 1997; Pérez-Luna et al., 2024); at an altitude of 1 500 to 3 100 m (Perry, 1991). *P. patula* has been evaluated through progeny trials due to its wide distribution and forestry importance (Salaya-Domínguez et al., 2012).

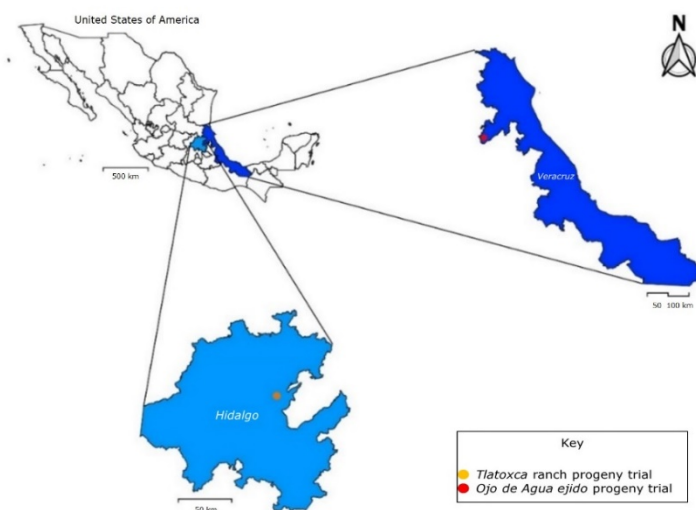
Progeny trials are forest plantations designed to evaluate selected individuals from diverse populations (Flores-Flores et al., 2014; Ipinza-Carmona, 1998). These trials allow for the description and analysis of natural variation and provide information for examining variability (Androsiuk et al., 2011) because they bring together families (progenies) from different origins that can be modified by environmental and/or genetic factors (Rodríguez-Vásquez et al., 2021; White et al., 2007).

Therefore, the research question posed was: What is the morpho-anatomical response of the *Pinus patula* leaf in two progeny trials established at different sites? Thus, the objective of this study was defined as characterizing and comparing the needle anatomy of *P. patula* trees in two progeny trials established at different sites in the central region of its natural distribution.

Materials and Methods

Study area

The study was carried out in two trials made up of 64 families of *P. patula* trees established in 2019 on the private property *Tlatoxca* ranch, in the *Zacualtipán de Ángeles* municipality, state of *Hidalgo* (20°37'49" N, 98°38'10" W) (Figure 1), with an altitude of 1 910 m, and the presence of a temperate humid climate C(m), precipitation of 1 144 mm and an average annual temperature of 17.1 °C (Fick & Hijmans, 2017). The second trial was established in the *Ojo de Agua ejido* property, in *Huayacocotla* municipality, state of *Veracruz* (20°28'21" N, 98°29'56" W), at an altitude of 2 310 m, with the presence of a temperate sub-humid climate C(w₂), precipitation of 1 004 mm and an average annual temperature of 14.7 °C (Fick & Hijmans, 2017) (Figure 1).



The dots indicate the two locations: *Tlatoxca* ranch (orange circle), *Zacualtipán de Ángeles* municipality, *Hidalgo* state, and *Ojo de Agua ejido* (red circle), *Huayacocotla* municipality, *Veracruz* state.

Figure 1. Location of two progeny trials of 64 families of *Pinus patula* Schiede ex Schltdl. & Cham. in the central area of its distribution.

Planting design

The progeny trial at *Tlatoxca* ranch was established on land previously used as pasture, while the trial at *Ojo de Agua ejido* was established on abandoned agricultural land. Therefore, it was necessary to remove shrubby and herbaceous vegetation by harrowing the soil at both sites. The planting design was a square grid pattern; seedlings were planted in common stock with a spacing of 3×3 m between plants, and a row of trees was established around the perimeter as a buffer strip.

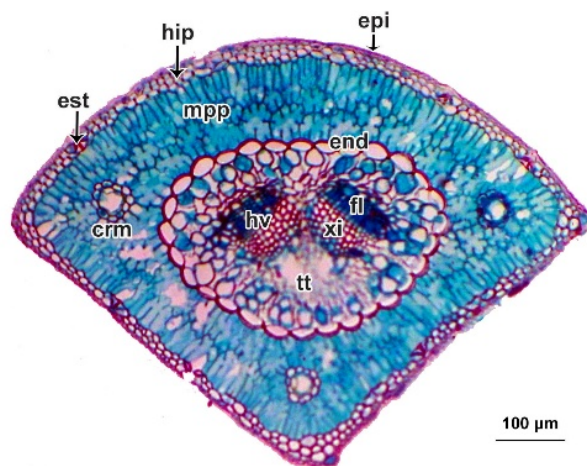
Collection of plant material

Ten fascicles were randomly collected from 40 trees at *Ojo de Agua ejido* and 24 trees at *Tlatoxca* ranch (due to the low survival rate of the families at the latter site). The fascicles were taken from the middle part of the canopy, only from first-order branches and without apparent damage. They were placed in 20 mL bottles with FAA (Formaldehyde:Glacial Acetic Acid:50 % Ethyl Alcohol, in a 5:5:90 ratio) for fixation (indefinitely).

Needle and stomatal row count

The number of needles was counted from ten fascicles per tree, and the number of stomatal rows was obtained by taking stomatal impressions from the abaxial (convex)

and adaxial (triangular) surfaces (Figure 2) of ten needles per tree randomly selected from the progeny trials.



Symbols: crm = Middle resin canal; end = Endodermis; epi = Epidermis; est = Stomata; fl = Phloem; hip = Hypodermis; hv = Vascular bundle; mpp = Splicated parenchyma mesophyll; tt = Transfusion tissue; xi = Xylem. Magnification: 10X.

Figure 2. Cross-section of a *Pinus patula* Schiede ex Schltdl. & Cham. leaf.

To obtain stomatal impressions, a layer of Vogue® enamel was applied to the middle portion of the needles (on the abaxial and adaxial surfaces). These were then kept at room temperature for 10 minutes to dry. Finally, the impressions were transferred with transparent adhesive tape (Scotch®) to microscope slides for viewing under a model WB-3T Iroscope® optical microscope.

Anatomical sections

From the pine needles used for stomatal impressions, five were selected from each tree. Transverse sections were made freehand in the middle of each needle using a double-edged Gillette® razor blade. These sections were then mounted on microscope slides with a mixture of Hycel® synthetic resin and toluene (C₆H₅CH₃) from Sigma-Aldrich® (USA) for observation at different magnifications under a model WB-3T Iroscope® optical microscope to obtain photomicrographs.

Anatomical description and analysis

For the description of the epidermis, hypodermis, endodermis, vascular bundles, and resin canals, photomicrographs were taken using a model VF Evolution™ camera (MediaCybernetics by QImaging, Canada) attached to a model BX41-TF Olympus® binocular microscope (Olympus Corporation®, Tokyo, Japan). The photomicrographs were processed using ImageJ® version 1.50i (Ferreira & Rasband, 2012) and Photoshop® version 13.0.121 (Adobe, 2012).

Statistical analysis

The quantitative variables —number of needles per fascicle, number of stomatal rows (abaxial and adaxial surfaces), and number of resin canals— were subjected to an analysis of variance, with a significance level of $P \leq 0.05$ (Geada-López *et al.*, 2022; Rodríguez-Laguna & Capo-Arteaga, 2005; Zhang *et al.*, 2017), using the following linear model:

$$Y_{ji} = \mu + A_j + e_{ji}$$

Where:

Y_{ji} = Response variable

μ = Population mean

A_j = Random effect of the j -th tree ($j=1,2$)

e_{ij} = Random error

Statistical analyses were performed separately for each progeny trial using Statistical Analysis System software version 9.00 (SAS Institute, 2022).

Results

Number of needles per fascicle

The analysis of variance for the number of needles per fascicle showed significant differences ($P \leq 0.0001$) between trees within the progeny trial (Table 1). At the *Tlatoxca* ranch, the fascicles had a lower number of needles (3-5) (Table 2); while at the *Ojo de Agua ejido*, the number was higher (between 3-6 needles).

Table 1. Analysis of variance of the quantitative variables of *Pinus patula* Schiede ex Schltdl. & Cham. progenies.

Variable	Site	MS	F value	Pr>F
NAF	Tlatoxca ranch	2.79	23.48	<0.0001
	Ojo de Agua ejido	2.56	14.02	<0.0001
HAB	Tlatoxca ranch	8.71	13.73	<0.0001
	Ojo de Agua ejido	4.96	6.81	<0.0001
HAD	Tlatoxca ranch	9.58	16.41	<0.0001
	Ojo de Agua ejido	3.45	6.43	<0.0001

NAF = Number of needles per fascicle; HAB = Stomatal rows on the abaxial surface;
HAD = Number of stomatal rows on the adaxial surface; MS = Mean square.

Table 2. Descriptive statistics of quantitative variables in *Pinus patula* Schiede ex Schltdl. & Cham. progenies.

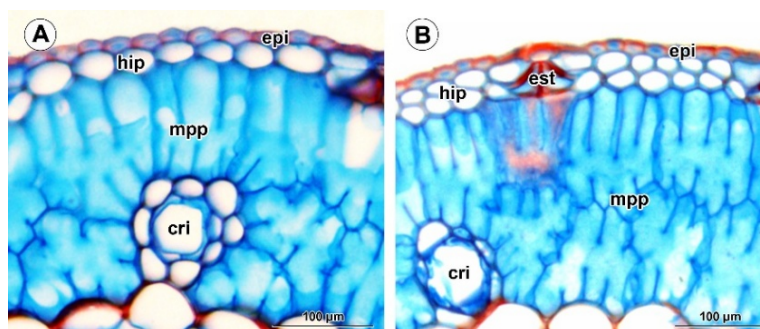
Variable	Tlatoxca ranch			Ojo de Agua ejido		
	NAF	HAB	HAD	NAF	HAB	HAD
Minimum	3.00	4.00	3.00	3.00	3.00	2.00
Mean	3.51	5.44	4.73	4.13	5.75	4.90
Maximum	5.00	7.00	7.00	6.00	11.00	7.00

NAF = Number of needles per fascicle; HAB = Stomatal rows on the abaxial surface;
HAD = Number of stomatal rows on the adaxial Surface.

Epidermis and hypodermis

The cross-sections of the needles showed a single-layered epidermis with rectangular and elliptical cells, with thick, lignified, and highly clarified walls (Figure 3). Regarding the hypodermis, 67 % (27 trees) of the samples from the *Ojo de Agua ejido* presented mostly single-layered hypodermis (Figure 3A) and 33 % (13 trees) showed two-

layered hypodermis (Figure 3B). In contrast, at the *Tlatoxca* ranch, where 46 % (11 trees) of the samples exhibited a mostly single-layered hypodermis and 54 % (13 trees) a two-layered hypodermis.



A = Mostly single-layered hypodermis; B = Two-layered hypodermis. Symbols: cri = Internal resin canal; epi = Epidermis; est = Stomata; hip = Hypodermis; mpp = Mesophyll of plicated parenchyma. Magnification: 40X.

Figure 3. Transverse anatomical section of the *Pinus patula* Schiede ex Schltdl. & Cham. leaf.

Number of stomatal rows

Significant differences ($P \leq 0.0001$) were found in the number of stomatal rows on the abaxial and adaxial surfaces. The needles exhibited an average of 5.44 (abaxial surface) and 4.73 (adaxial surface) in the *Tlatoxca* ranch (Table 2); while in the *Ojo de Agua ejido*, the average number of stomatal rows was higher, at 5.75 (abaxial surface) and 4.90 (adaxial surface).

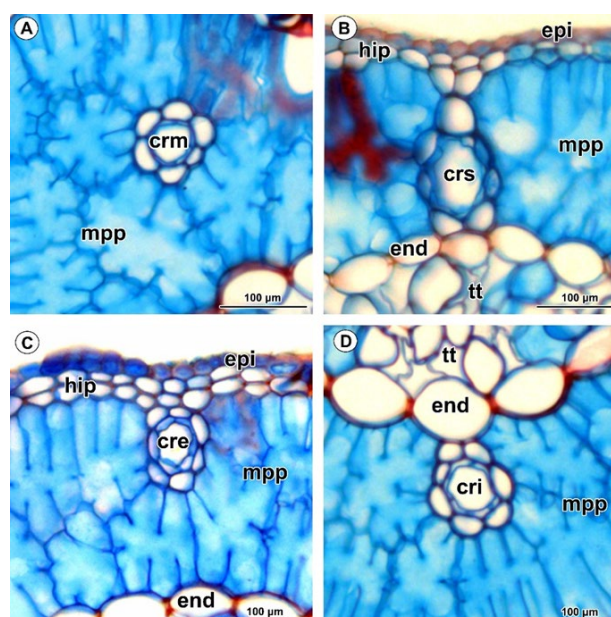
Number and position of resin canals

The number of resin canals showed statistically significant differences ($P \leq 0.0001$) among the trees within the progeny trials (Table 3). The needles of the trees from the *Tlatoxca* ranch presented resin canals in midline (100 %), internal (100 %), rarely septal (16.6 %), and outer (8.3 %) positions (Figure 4); with a number of resin canals of (2-)3(-6) and an average of 3.48. The samples from the progeny trial at the *Ojo de Agua ejido* exhibited less variation in position, which was only midline (100 %) and internal (100 %); with a number of resin canals of (0-)3(-4), and an average of 2.64 (Table 4). In this study, resin canals in outer and septal positions (though not frequent) in *P. patula* leaves are reported for the first time.

Table 3. Results of the analysis of variance of the number of resin canals in *Pinus patula* Schiede ex Schltdl. & Cham. progenies.

Site	MS	F value	Pr>F
<i>Tlatoxca</i> ranch	3.05 0.43	7.06	<0.0001
<i>Ojo de Agua ejido</i>	1.85 0.48	3.85	<0.0001

MS = Mean square.



A = Midline resin canal; B = Septal resin canal; C = Outer resin canal; D = Internal resin canal. Symbols: cre = Outer resin canal; cri = Internal resin canal; crm = Midline resin canal; crs = Septal resin canal; end = Endodermis; epi = Epidermis; hip = Hypodermis; mpp = Mesophyll of plicated parenchyma; tt = Transfusion tissue. Magnification: 40X.

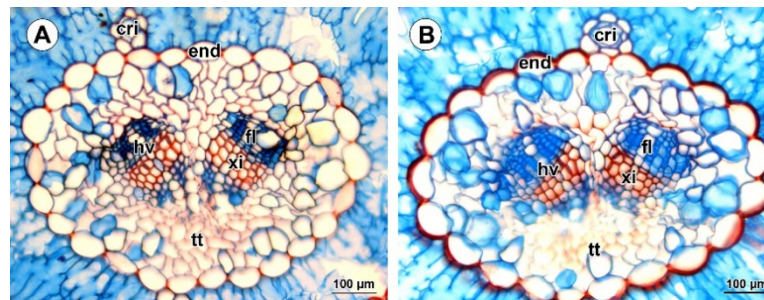
Figure 4. Cross-section of the resin canals of *Pinus patula* Schltdl. & Cham.

Table 4. Descriptive statistics of the number of resin canals in *Pinus patula* Schiede ex Schltdl. & Cham. progeny.

Variable/ Statistics	Number of resin canals	
	<i>Tlatoxca</i> ranch	<i>Ojo de Agua ejido</i>
Minimum	2.00	0.00
Mean	3.48	2.64
Maximum	6.00	4.00

Endodermis

Endodermal thickening was absent in some samples (Figure 5A), but it was present in most needles in both progeny assays (Figure 5B); in the *Tlatoxca* ranch trial, 67 % of the needles showed thickening, while in the progeny trial at the *Ojo de Agua ejido*, it was 55 %.



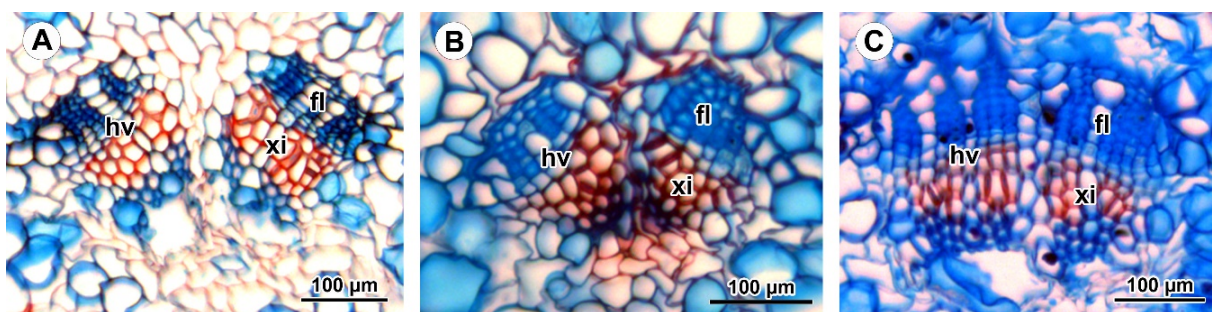
A = Slight thickening of the outer periclinal walls of the endodermis; B = Thickening of the outer periclinal walls of the endodermis. Symbols: cri = Internal resin canal; end = Endodermis; fl = Phloem; hv = Vascular bundle; tt = Transfusion tissue; xi = Xylem.

Magnification: 40X.

Figure 5. Anatomical cross-section of the *Pinus patula* Schiede ex Schltdl. & Cham. leaf.

Vascular bundles

The needles from the trial at the *Tlatoxca* ranch showed vascular bundles that were separate (21 %; Figure 6A), close together via the xylem (67 %; Figure 6B), and fused (12 %; Figure 6C); while in the *Ojo de Agua ejido*, only vascular bundles close together via the xylem (63 %) and separate (37 %) were observed.



A = Separate vascular bundles; B = Vascular bundles close together or fused via the xylem; C = Fused vascular bundles. Symbols: fl = Phloem; hv = Vascular bundle; xi = Xylem. Magnification: 40X.

Figure 6. Cross-section of *Pinus patula* Schiede ex Schltdl. & Cham. needles, vascular bundles.

Discussion

Number of needles per fascicle

The fascicles of *P. patula* consisted of 3-4 and 4-5 needles in the progeny trials, which corresponds to the studies carried out by Perry (1991) and Farjon and Styles (1997), who reported fascicles consisting of 3-4(-5) needles for the species. According to Iglesias-Andreu and Tivo-Fernández (2006), this trait (number of needles) is highly genetically controlled; however, it is affected by environmental factors. Furthermore, Rodríguez-Laguna and Capo-Arteaga (2005) found a positive correlation (0.785) between altitude and the number of needles per fascicle, which corresponds to the findings of this study, since the trees in the trial at the *Tlatoxca* ranch were located at an altitude of 1 910 masl. They showed 3-4, while in the *Ojo de Agua ejido* with an altitude of 2 310 masl they showed a greater number of needles per fascicle (4-5).

Epidermis

According to Farjon and Styles (1997), the shape of epidermal cells varies among species and can be square, rectangular (transversely oblong), or elliptical. For *P. patula*, the epidermis was composed of rectangular and elliptical cells. Bozkurt et al. (2023) state that the epidermis is an anatomical feature that can vary in terms of the shape and size of its constituent cells due to environmental factors such as temperature and precipitation, and Hengxiao et al. (1999) report that altitude affects epidermal thickness.

Number of stomatal rows

According to Toral et al. (2010), stomata are sensitive to changes in environmental conditions, Donnelly et al. (2016) report that its (intraspecific) variation can be influenced by genetic factors and Pérez-Del Valle et al. (2019) by abiotic factors of the plants' origin in the genus *Pinus*. Furthermore, Tiwari et al. (2013) reported that the number of stomatal rows plays an important role in plant adaptation to altitude, a variable with which it is correlated ($r=0.9815$). Regarding the number of stomatal rows, the differences found in this study could be due to the environmental conditions (at altitude) and genetic makeup of the progeny's origin (natural populations). When these factors interact in a homogeneous site such as the planting location, each progeny may express itself differently.

Hypodermis

According to Farjon and Styles (1997) and Martínez (1992), the leaves of *P. patula* have a hypodermis composed of one (monostratified), usually two (bistratified), or three (tristratified) rows of unevenly distributed cells. These results were similar to those obtained in this study; however, the number of cell rows differed among the progeny trials, as most of the trees from *Tlatoxca* ranch exhibited a predominantly bistratified hypodermis. Nikolić et al. (2016) and Grill et al. (2004) reported that the hypodermis varies among individuals of the same species and increases in the number and cell rows when plants are affected by drought stress, as reported in other studies such as Pérez-Del Valle et al. (2019) and Geada-López et al. (2022) in natural populations of *Pinus tropicalis* Morelet where the number of cell layers that make up the hypodermis increase in drier sites.

Position of resin canals

Resin canals are structures specially developed by conifers and are related to plant protection against pathogens (Pérez-Olvera & Ceja-Romero, 2019). According to Kim *et al.* (2014), they are suspended in the mesophyll and can vary in their position (external, internal, medial, and septal). Furthermore, Tiwari *et al.* (2013) suggest that the position of resin canals may be a genetically controlled foliar anatomical characteristic in conifer species. For *P. patula*, Farjon and Styles (1997) reported resin canals frequently in the medial position, followed by internal. This coincides with the results found at *Ojo de Agua ejido*; however, it differs from the results found at the *Tlatoxca* ranch, where resin canals were frequently found in the medial and internal positions, but also rarely in the external and septal positions. The above suggests that the predominant or primary positions of the resin canals in this species are medial and internal, which agrees with the findings of Farjon and Styles (1997), who reported that the position of the resin canals varies among species and has therefore been used as a diagnostic and taxonomic classification characteristic in the genus *Pinus*. This suggests a greater degree of genetic than environmental control of this condition.

Number of resin canals

In species of the *Pinus* genus, canals are specialized structures for synthesizing, secreting, and storing resin (Yi *et al.*, 2021). They can vary in number within the mesophyll (Pérez-Olvera & Ceja-Romero, 2019), and according to Farjon and Styles (1997) for *P. patula*, they have reported the presence of (1-)2-3(-4); this is within the range documented in this study, but a larger variation range of 0-6 is reported.

On the other hand, Geada-López et al. (2021) report that environmental factors such as low water availability and high temperatures cause an increase in the number of resin canals within the mesophyll, and Donnelly et al. (2016) observed in *Pinus sylvestris* L. needles that the density (number of channels) increases in sites with lower humidity. Therefore, it can be deduced that the higher temperature at the progeny trial plantation site on the Tlatoxca ranch (17.1 °C) and the slope of the land (25%) hinder water retention in the soil. This results in lower water availability in the soil, which could have caused an increase in the number of resin canals. In contrast, the trees established at Ojo de Agua are located at a site with an average annual temperature of 14.7 °C and a slope of 3%, which favors water retention in the soil and consequently results in a lower number of resin canals.

Endodermis

According to Roden et al. (2009), the possible function of the endodermis is to act as a barrier to regulate or contain the passage of water (preventing loss) to the mesophyll and prevent plant dehydration during critical seasons (low temperatures and droughts), as well as to prevent ice formation on the leaf. Furthermore, García-Esteban et al. (2010) mention that the endodermis is a variable affected by the origin of the progeny, as well as the environmental and soil conditions of each origin site. In *P. patula*, it was observed that some progeny developed thickening in the external periclinal walls, possibly because they originate from different populations with different climates (temperature, precipitation, and humidity), soils, and exposures along the species' biological distribution corridor.

Vascular bundles

In both progeny assays of *P. patula*, needle cross-sections showed two vascular bundles (containing phloem and xylem) embedded in the transfusion tissue under the following conditions: separate, connate (closed by the xylem), and fused. According to Martínez (1992), hardwood pines (*diploxylon* section) such as *P. patula* have two vascular bundles. Farjon and Styles (1997) report that they can be connate (closed by the xylem), and Espinoza-Pelcastre et al. (2018) mention that they can sometimes be separated. In this study, the absence of sclerenchyma in the transfusion tissue region was consistently observed in all analyzed samples, unlike other species in which rows of sclerenchyma cells intermittently surround and usually divide the vascular bundles (Farjon & Styles, 1997).

Conclusions

Based on the observed information, the characteristic features of *P. patula* anatomy in the studied progeny trials can be considered to be: a single-layered epidermis composed of elliptical cells, a mostly single-layered but commonly bi-layered hypodermis, resin canals predominantly located in the medial position, followed by internal and exceptionally external and septal positions. Thickening of the outer walls of the endodermis was either present or absent, and vascular bundles were frequently close together via the xylem, with an absence of sclerenchyma in the transfusion tissue.

The environmental conditions of each planting site promoted modifications in the needles in terms of the number of stomatal rows, the number of hypodermal cell layers, and the number and position of resin canals and vascular bundles. The planting site established at *Tlatoxca* ranch recorded higher temperatures, a 25 % slope, and lower altitude, factors that resulted in a greater range of variation in the foliar morpho-anatomical traits of *P. patula*.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

Contribution by author

Tomás Escobedo Luna and Sergio Hernández León: research work, permanent slides in the laboratory; Tomás Escobedo Luna, Diana Gómez García and Rodrigo Rodríguez Laguna: collection and classification the plant material (fascicles) for the progeny trials; Oscar Arce Cervantes: data analysis. Finally, the entire team reviewed and edited the manuscript.

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