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Original article

Effect of the position of the needles in the crown of the tree on the foliar anatomy of Pinus caribaea Morelet var. caribaea Barrett and Golfari

Efecto de la posición de las acículas en la copa del árbol sobre la anatomía foliar de Pinus caribaea Morelet var. caribaea Barret y Golfari

Efeito da posição da agulha no dossel sobre a anatomia da folha de **Pinus** *caribaea Morelet var.* **caribaea** *Barret e Golfari*

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ABSTRACT

Variations in the structure of plant leaves are mainly due to environmental variability. In many cases the way to acclimatize to unfavorable conditions. It is documented that in Cuba the anatomical and morphological characteristics of *P. caribaea* needles differ between ecotopes with different edaphoclimatic conditions. In this study, the effect of the position in the tree crown on the anatomy of the needles is analyzed. For this purpose, needles were collected from the lower, middle and upper part of 30 trees. The variables analyzed were thickness of the chlorophyll parenchyma, thickness of the transfusion parenchyma, height of the conduction tissue and width of the conduction tissue, cuticle thickness, epidermis thickness, hypodermis thickness, number of layers of hypodermis cells, number of channels and number of stomat. The results of the analyzes show a greater differentiation of the morphology of the needles towards the upper part of the tree, which is expressed by the increase in the thickness of the protective tissues and related to the greater exposure to the sun and lower relative humidity than they are exposed. The variables that contribute the most to differentiate the needles according to their position in the tree are the number of stomata and the number of cells in the hypodermis.

Keywords: needle, anatomical variation, resin canal, tree crown.

RESUMEN

Las variaciones de la estructura de las hojas de las plantas se deben fundamentalmente a la variabilidad ambiental. En muchos casos la vía para la aclimatación a condiciones desfavorables. Está documentado que en Cuba las características anatómicas y morfológicas de las acículas de *P. caribaea* difieren entre ecótopos con distintas condiciones edafoclimáticas. En este estudio, se analiza el efecto que tiene la posición en la copa del árbol en la anatomía de las acículas. Para esto se colectaron acículas de la parte inferior, media y alta a 30 árboles. Las variables analizadas fueron grosor del parénquima clorofílico, grosor del parénquima de transfusión, altura del tejido de conducción y ancho del tejido de conducción, grosor de la cutícula, grosor de epidermis, grosor de hipodermis, número de capas de células de la hipodermis, número de canales y número de estomas. Los resultados de los análisis muestran una mayor diferenciación de la morfología de las acículas hacia la parte superior del árbol, que se expresa por el aumento del grosor de los tejidos de protección y relacionado con la mayor exposición al sol y menor humedad relativa a la que están expuestas. Las variables que más contribuyen a diferenciar las acículas según la posición en el árbol son el número de estomas y el número de células de la hipodermis.

Palabras clave: acícula, variación anatómica, copa del árbol, canal de resina.

SÍNTESE

As variações na estrutura das folhas das plantas são devidas principalmente à variabilidade ambiental. Em muitos casos, é o caminho para a aclimatação a condições desfavoráveis. Está documentado que em Cuba as características anatômicas e morfológicas das agulhas de P. caribaea diferem entre ecótones com diferentes condições de solo e clima. Neste estudo, é analisado o efeito da posição do dossel sobre a anatomia das agulhas. Para este fim, as agulhas foram coletadas da parte inferior, média e superior de 30 árvores. As variáveis analisadas foram espessura do parênquima clorofila, espessura do parênquima transfusional, altura e largura do tecido de condução, espessura da cutícula, espessura da epiderme, espessura da hipoderme, número de camadas celulares da hipoderme, número de canais e número de estomas. Os resultados das análises mostram uma maior diferenciação na morfologia das agulhas em direção ao topo da árvore, que é expressa por um aumento na espessura dos tecidos protetores e está relacionada à maior exposição ao sol e à menor umidade relativa a que estão expostos. As variáveis que mais contribuem para diferenciar as agulhas de acordo com sua posição na árvore são o número de estômatos e o número de células na hipoderme.

Palavras-chave: agulha, variação anatômica, canópia, canal de resina.

INTRODUCTION

Four endemic species of pine grow on the island of Cuba: *Pinus tropicalis* Morelet, *Pinus caribaea* Morelet var. *caribaea* Barrett-and-Golfari, *Pinus cubensis* Griseb and *Pinus maestrensis* Bisse, two of them grow in the western zone and the last two in the eastern zone. These species are extremely important, both economically and ecologically, since they occupy large tracts of land in the archipelago and therefore a large amount of timber and non-timber resources and a wide variety of animal and plant species can be found in the Cuban pine forests.

Pinus caribaea var *caribaea*, is geographically distributed in the province of Pinar del Río and Isla de la Juventud (Lopez-Almirall, 1987; Geada-López *et al.*, 2021). To a large extent it associates sympatrically with *Pinus tropicalis* Morelet or in pure stands only on the Cajálbana plateau. From the evolutionary point of view, it is a young species that reports an invasive behavior (Camacho *et al.*, 2018; Gallien *et al.*, 2015).

Anatomical and morphological variations in plants are responses to environmental variability, processes such as adaptation and acclimatization are derived from or associated with these variations. For this reason, the study of variability in leaf anatomy between populations that occupy ecotopes with different edaphoclimatic conditions is of great importance for the conservation and management of the genofondo of the species, especially for the use of provenances for reforestation.

The needles play an important role in the acclimatization or adaptation process, being the organ responsible for photosynthesis, the assimilation of atmospheric CO_2 and gas exchange. Hence, they modify the anatomical structure of the needle, to withstand unfavorable environments as a differential response to these, which in the long term will affect the survival and growth of the plant (Grill *et al.*, 2004, López *et al., 2010*, Huang *et al.*, 2016, Geada-López *et al.*, 2021).

Environmental factors have a direct influence on the structure of the needle tissues, on the pattern and position of the stomata, the number and size of the resin ducts, especially for Pinus species (Tiwari et *al*., 2013 Ghimire *et al.,* 2014; Meng *et al.,* 2018), the thickness of the transfusion parenchyma and the mesophyll area (Meng *et al.,* 2018; Köbölkuti *et al.,* 2017, Huang *et al.,* 2016). Therefore, the anatomy can vary between populations and species, especially within those with wide ranges of continental distribution (Jasiñska *et al.,* 2014; Boratyñska *et al.,* 2015; Zhang *et al.,* 2017).

So far, various studies related to the subject have been carried out, for example: (Pérez-del Valle *et al.,* 2016) demonstrated some atomic variation in the needle of *Pinus caribaea* var, *caribaea* in six localities of Pinar del Río. Later (Pérez-del Valle *et al.,* 2020) found differences in the anatomy of the needle of *Pinus caribaea* var. *caribaea* in three ecotopes of the species where it lives in sympatria with *Pinus tropicalis.* Foliar anatomical differences were recently demonstrated in natural populations of *Pinus caribaea* var. *caribaea* (Pinaceae) in Pinar del Río and Artemisa, Cuba (Geada-López *et al.,* 2021).

In general, the needles of *Pinus caribaea* var. *caribaea* are 13.6 ± 1.0 cm long and 1.3 ± 0.3 mm wide. The equifacial anatomical structure, in the shape of a fan, shows the unistratified epidermis. The stomata are distributed on both surfaces of the needle, the average number of rows of stomata per needle is 17 and these are sunk into the hypodermis. The hypodermis has three to five layers of cells, of the multiform type and with sclereids. Chlorophyll parenchyma averages three layers of folded-edge cells, followed by several layers of transfusion parenchyma tissue cells. The type of resin canal is endonal within the chlorophyll parenchyma, sometimes reaching the transfusion parenchyma (Pérez-del Valle *et al.,* 2020).

For *P. caribaea* up to now, the study factor that has prevailed is the influence of the ecotope on leaf anatomy (Pérez-del Valle *et al.*, 2020, Geada-López *et al.*, 2021). The position in the tree has not been addressed and the variability under this factor can be an indicator of the maturity of the individual, of the stand and the potential for resin production. The study hypothesizes that the height at which the needle develops in the tree influences its anatomical and morphological characteristics. Taking into account that this variability has been demonstrated in other species of the genus *Pinus,* the objective of this work is to characterize the anatomical variation of the needles of *Pinus caribaea.* var. *caribaea* depending on its altitudinal position in the tree.

MATERIALS AND METHODS

Sampling

The samples were taken from 30 trees from a stand of *P. caribaea* located in areas of the Viñales experimental station, Pinar del Río province, Cuba. Needles were collected in the lower, middle and upper part of young individuals of approximately three meters in height.

Variables

For anatomical measurements, cross sections were made in the middle part of the needle. The following structures were measured at 100x magnification: chlorophyll parenchyma thickness (CPt), transfusion parenchyma thickness (TPt), conduction tissue height (CTh) and conduction tissue width (CTw), the number of stomata (NS) and the number of channels (NC); and with 400x magnification: the thickness of the cuticle (ThC), the thickness of the epidermis (ThE), the thickness of the hypodermis (ThH) and the number of cell layers of the hypodermis (NCLH).

Statistic analysis

The effect of the needle position factor in the crown is determined, with three levels: high (A upper third of the crown), low (B, lower third) and medium (M the central part of the crown). For this, a Kruskal-Wallis analysis of variance by ranges ($\alpha = 0.05$) and a multiple pairwise comparison test between the means of the ranges of the treatments were performed, as described in Conover (1999), since the data did not fit a normal distribution.

Discriminant analysis was used in which the three zones where the high (A), medium (M) and low (B) needles were collected are assumed as a priori groups with the aim of maximizing the differences in leaf anatomy, as well as distinguish the variables that contribute the most to differentiation. The statistical analysis was carried out with the *InfoStat v.2015 program.*

RESULTS

Anatomical characterization of the needles according to their position in the cup

Table 1 shows the mean values of the anatomical variables and their standard deviation according to the position in the crown. This factor has a significant effect, according to the Kruskal -Wallis test (P< 0.05), on the variables number of stomata, the number of cells in the hypodermis, the thickness of the transfusion parenchyma, and the length of the conduction tissue.

Table 1. *- Mean values and standard deviation of the evaluated anatomical variables of Pinus caribaea in three positions on the tree*

Variable		TO			m			B.		h	P
NC	2.17	±	0.53	2.03	±	0.18	2	±	0	0.47	0.16
NS	15.43	±	2.87B -	13.13	±	2.24A -	12.27	±	2.02A -	18.6	0.00
CTh	0.73	±	0.33	0.73	±	0.33	0.78	±	0.3	0.68	0.71
ETh	15.48	±	2.68	15.11	±	3	14.48	±	1.99	2.08	0.35
HpTh	41.35	±	10.47	39.59	±	7.9	37.98	±	9.63	2.17	0.33
NCH	2.63	±	0.72B -	2.1	±	0.76A -	1.93	±	0.87A -	10.28	0.00
CPTh	109.67	±	15.34	105.74	±	18.09	105.53	±	18.04	1.73	0.42
TPTh	129	±	19.26B -	121.35	±	14.19 AB	112.44	±	19.15 A	9.62	0.00
CTw	248.55	±	49.64	252.13	±	52.11	238.68	±	59.74	1.52	0.46
LCt	506.71	±	69.42B -	480.14	±	81.8 AB	439.28	±	87.18A -	10.78	0.00

NC: Number of channels, NS: Number of stomata, CTh: Cuticle thickness, ETh: Epidermis thickness, HpTh: Hypodermis thickness, NCH: Number of cells of the hypodermis, CPTh: Chlorophyll parenchyma thickness, TPTh: Transfusion parenchyma thickness, CTw: Conducting tissue width, LCt: Length of conduction tissue.Position A-high, B-low, M-medium

Figure 1 shows the behavior trend in the variables in which the position of the needle in the crown had a significant effect for a<0.05 in the Kruskal-Wallis test.

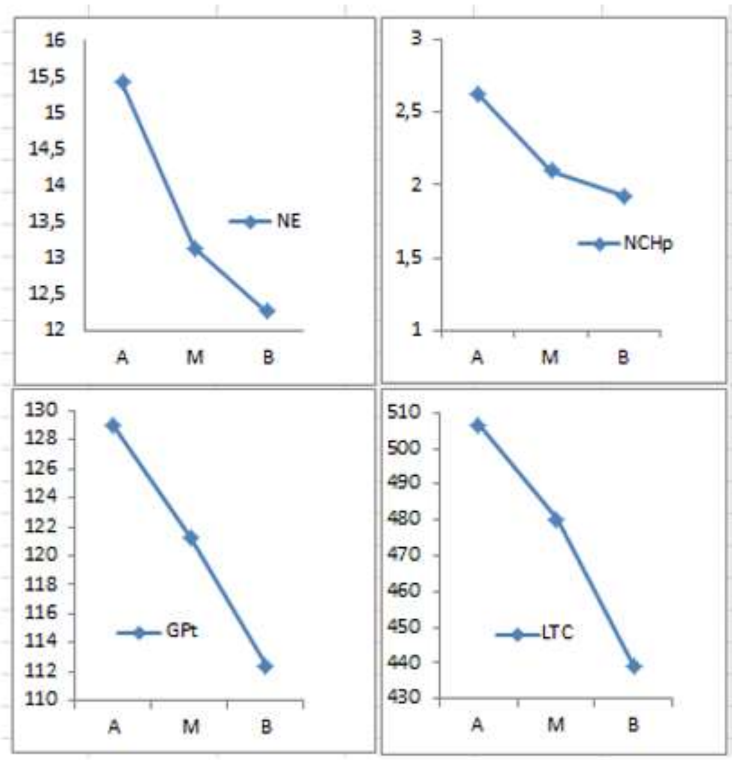

Figure 1. *- Behavior of the variables number of stomata (NS), the number of cells in the hypodermis (NCHp), the thickness of the transfusion parenchyma (GPt) and the length of the conduction tissue (LTC) in the three positions of the cup (high A, medium M and low B)*

Anatomical differentiation between the positions of the needles in the tree

Table 2 shows the results of the discriminant analysis. The two functions obtained from the analysis allowed explaining 100 % of the variations between the three groups defined *a priori.*

Table 2. *- Result of the discriminant analysis, standardized coefficients of each variable and centroids of the three positions of P. caribaea in the two discriminant functions*

	Function	
	1	**2**
Eigenvalues	0.57	0.05
%	91.34	8.66
% accumulated	91.34	100
variables	**standardized coefficients**	
Number of channels	0	0.26
Number of stomata	-0.78	0.35
Cuticle thickness	0.31	0.02
Epidermal thickness	-0.02	-0.39
Hypodermis thickness	0.17	-0.32
Number of cells in the hypodermis	-0.63	0.61
Chlorophyll parenchyma thickness	0.27	0.46
Transfusion parenchyma thickness	-0.15	-0.72
Width of the driving tissue	0.08	-0.39
Conduction tissue length	-0.17	-0.41
	Centroids	
Group (Position in the cup)	**axis 1**	**axis 2**
Upper third (A)	-1	0.1
Lower third (B)	0.79	0.21
Central part (M)	0.21	-0.32

According to the standardized coefficients, the most important variables in the discrimination between the three positions are, in the first function, the number of stomata and number of cells in the hypodermis. In the second, the thickness of the epidermis and the thickness of the transfusion parenchyma.

According to the values of the centroids and the location in the plane defined by the two discriminant functions (Figure 2), there is greater differentiation in the anatomy of the needles collected in the upper part of the cup with respect to the other two, than Although well defined, they have a certain degree of overlap.

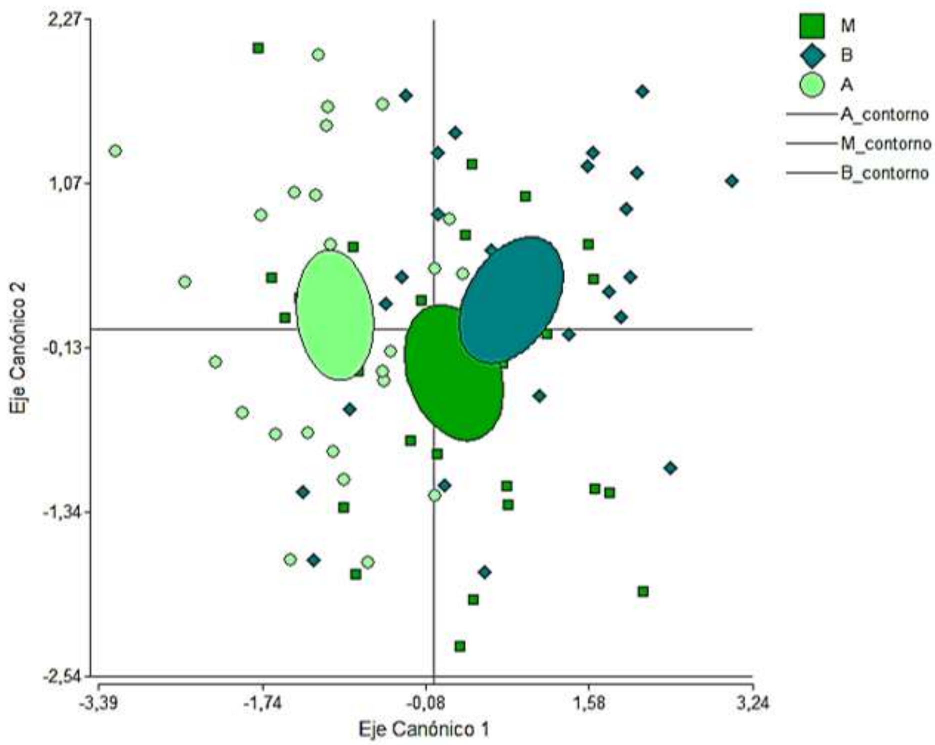

***Figure 2**. - Grouping of the positions in the first two discriminant functions, based on anatomical variables of Pinus caribaea represented in three a priori groups*

Contours correspond to prediction ellipses at 95 % probability NC: Number of channels, NE: Number of stomata, GC. Cuticle thickness, GEp: Epidermis thickness, GHp: Hypodermis thickness, NCHp: Number of cells of the hypodermis, GPc: Chlorophyll parenchyma thickness, GPt: Transfusion parenchyma thickness, ATC: Conducting tissue width, LTC: Length of conduction tissue.

This is confirmed in Table 3, where the classification of each case is presented according to the discriminant functions obtained. It is shown that there is greater uncertainty regarding the anatomical differences (error 60 %) of the needles between the middle part, especially with respect to the lower part of the crown.

Table 3. *- Cross-classification of each case according to the discriminant functions and classification error*

Cluster	High	Low	Half	Total	Mistake (%)
High	23	2	5	30	23.33
Low	6	twenty	4	30	33.33
Half	7	eleven	12	30	60
Total	36	33	twenty-one	90	38.89

DISCUSSION

Anatomical variation of the needles according to the position in the cup

The morphological anatomical variability observed in a species can be determined by geographical, climatic, site variations, between trees in the same site and even variations within the same tree (Cattuse 1991).

The anatomical variation of the needles in the three positions in the crown of the tree that were studied is mainly reflected by four variables: number of stomata, number of cells of the hypodermis, thickness of the transfusion parenchyma and length of the conduction tissue. All of them are linked to processes of water regulation and support, as is the case of conduction tissue.

Geada-Lopez *et al.* (2022) reports that the species *P. caribaea* compared to *Pinus tropicalis* can develop greater number of stomata, greater number of hypodermal cells, and greater thickness of transfusion tissue in response to differences in environmental conditions.

This study confirms, in the four needle anatomy variables in which a significant statistical effect of the position in the crown was tested, a slight increase in their values towards the upper part, which is the most exposed to the drying effect of the wind and the light. These characteristics help to avoid the collapse of the elements of the vascular bundle in conditions of loss of turgor (Larcher 2003, Grill *et al.,* 2004), behavior verified in the anatomy of *P. canariensis* when subjected to tests of drought (Grill *et al.,* 2004) and between provenance tests established in dry and xeric places (López-Rodríguez 2009). However, Dörken and Stützel (2012) state that it is commonly assumed that the representatives of *Pinus* subg. *Diploxylon* have two vascular bundles, when these are actually a single bundle separated by bands of parenchyma cells that can be more or less numerous and lignified or not. Changes in the size of this structure seem to be related to the availability of water in the soil (Boratyñska *et al.,* 2011, 2015b, Ghimire *et al.,* 2014).

In the study, it was verified that in the upper part of the crown, in addition to increasing the number of cells of the hypodermis, there is also a small increase in the thickness of the hypodermis (Table 1). Nevertheless, it is necessary to clarify that the increase in the thickness of the hypodermis it is not always accompanied by a greater number of cell layers of the hypodermis, although both characteristics regulate water loss, especially if it is accompanied by sclereids as an element of the supporting tissue (Grill *et al.,* 2004). Nikolic *et al.,* (2014 and 2019) in studies of the natural populations of *Pinus heldreichii* in the Balkans reported that, towards drier sites, due to the slope and the substrate, the thickness of the hypodermis and cuticle were greater, but with a fewer layers of the hypodermis.

The transfusion parenchyma has its highest values in the upper part, where a greater reserve of water is necessary, since these needles will be more exposed to solar radiation. Lápez *et al.* (2010) state that this behavior is common where it is necessary to store a large amount of water together with reserve materials, since it represents savings in the synthesis of compounds and storage of metabolisms under these conditions (López-Rodríguez 2009, Esteban *et al.,* 2010). This characteristic is advantageous for plants that experience prolonged periods of water stress, since it allows the leaf tissues to be kept alive in the face of prolonged droughts (López *et al.*, 2010, Hodžiæ *et al.* 2020).

There is also very little variation regarding the number of channels, only a small increase is observed towards the upper part, which behaves as the driest part due to strong exposure to sun and wind. In preliminary studies, it has been detected that the higher density of channels is associated with areas of lower moisture availability (Jankowski *et al.*, 2017, 2019) and their increase in number and size is correlated in *Pinus yunnanensis* with decreases in precipitation and increase in temperature, which contribute to reducing the influence of extreme drought and heat (Huang *et al.* 2016).

In Figure 2, the result of the discriminant analysis, it can be seen that the needles in the upper part of the crown differ from those collected in the other two positions, this disjunction is given by the influence of the environmental variables to which the needles are subjected according to their position, since environmental factors have a direct influence on the structure of the needle tissues, on the pattern and position of the stomata, the number and size of the resin ducts, especially for the species of the genus *Pinus* (Tiwari *et al.*, 2013, Ghimire *et al.*, 2014, Meng *et al.*, 2018), transfusion parenchyma thickness and mesophyll area (Meng *et al.*, 2018, Huang *et al.*, 2016, Köbölkuti *et al.*, 2017).

The discriminant analysis highlights in the first function the variables number of stomata and number of hypodermis cells as the most important variables, deviating towards the negative quadrant of Figure 2, whose needles develop in an environment with higher light intensity and lower relative humidity.

Dörken and Stützel (2012) and Grill *et al.* (2004) describe that *Pinus* species that develop in extreme environments generally develop a prominent, thick cuticle and a hypodermis made up of several layers of thick circular cells. However, *P. caribaea* develops a greater number of layers and thickness of the hypodermis (multiform type) as an adaptation to the same conditions. Kivimäenpää *et al.*, 2017 suggest that a species adapted to sunny habitats can have a thicker mesophyll tissue and a higher stomatal density to achieve higher photosynthetic yields. This also explains the framed differences between the High versus Low position and the Medium position.

Regarding the second discriminant function, the greatest importance corresponds to the transfusion parenchyma, whose tissue is present only in conifers, especially in the genus *Pinus* (Canny 1993 and Liesche *et al.*, 2011) whose function is to store a large amount of water, but which also has a complex post-xylem, pre-phloem functioning mechanism that transports water, mineral nutrients together with simple photoassimilates (Canny 1993, Liesche *et al.*, 2011) that, if necessary, use a symplastic pathway to deliver energy to the assimilation tissue.

In this regard, López *et al.*, (2010) and Liesche *et al.*, (2011) suggest that in dry or xeric ecological conditions the development of a thick transfusion tissue would also mean an advantage with water storage and savings in the synthesis of compounds (López *et al.*, 2010, Esteban *et al.*, 2010, Hodzic *et al.*, 2020).

Forestry implications

Until now, many of the studies carried out are directed mainly towards the selection of trees with superior production capacities, and identify the adequate number of individuals with sufficient genetic variability (Barrett, 1980). The characterization of the anatomical variability along the stem of the *P. caribaea* tree can be an indicator of the potential for resin production. However, few studies have addressed the relationships between resin production and the ecological and climatic aspects where the trees grow (Reyes-Ramos *et al.*, 2019, Abdillah *et al.*, 2020, Rodríguez-García *et al.*, 2015).

This study is not focused directly to the production of resin, regardless of whether the species *P. caribaea* is included in the resination plans of the country, but rather focuses on the importance of knowing the place within the tree where the greatest amount of channels could be found. For this, knowledge of the anatomical variation of the needle makes it easier to diagnose the net production capacity of the species, knowing that the number and size of resin canals are related to resin flow (Westbrook *et al.*, 2015). For example, Reyes-Ramos *et al.*, (2019) demonstrated the relationship between temperature and resin production in *P. oocarpa*, then the needles in the upper part of the crown are exposed to higher solar radiation and temperature, therefore resin production would have to be higher. In this sense, identifying the existence of differences in the channels and dimensions in the tissues is crucial to estimate the resin production.

The foliar anatomical characteristic is a point to take into account not only when it comes to obtaining great results in production for economic purposes but also in the selection of the propagation material, so that the variation in terms of dimensions of the variables studied could be linked to strategies of the defense system of conifers to face different biotic and abiotic disturbances, which integrate a complex defense response in the short and long term.

CONCLUSIONS

In the species *Pinus caribaea var caribaea* there is variation in the anatomy of the needle determined by its position in the crown, an increase in the thickness of the protective tissues is manifested towards the upper part. The variables that most contribute to differentiating the needles according to their position in the tree are the number of stomata and the number of cells in the hypodermis.

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