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Original article

Preliminary study of foliar anatomical changes in *Pinus tropicalis* ***and*** *Pinus Caribbean* ***subsp. caribaea from areas adjacent to the Castellanos mine, Minas de Matahambre, Pinar del Río, Cuba***

Estudio preliminar de los cambios anatómicos foliares en ***Pinus tropicalis*** *y* ***Pinus caribaea*** *subsp.* ***caribaea*** *de zonas colindantes de la mina Castellanos, Minas de Matahambre, Pinar del Río, Cuba*

Estudo preliminar das alterações anatômicas foliares em ***Pinus tropicalis*** *e* ***Pinus caribaea*** *subsp.* ***caribaea*** *de áreas adjacentes à mina Castellanos, Minas de Matahambre, Pinar del Río, Cuba*

Ana Lucia Corrales-García [1*], *Gretel Geada-López* [1]

[1] University of Pinar del Río "Hermanos Saíz Montes de Oca". Pinar del Rio, Cuba.

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ABSTRACT

The leaf constitutes the most sensitive organ for detecting abiotic stress conditions, including heavy metal pollution derived from mining-metallurgical activities. In areas adjacent to the Castellanos mine, Minas de Matahambre municipality, Pinar del Río province, Cuba, natural pine forests of *Pinus tropicalis* and *Pinus caribaea* subsp. *caribaea* grow on soils affected by mining activity. With the aim of characterizing foliar anatomical changes in both taxa, needles were collected from 12 individuals per species (five replicates per individual) and transverse sections were made, measuring 13 anatomical variables. An increase in cuticle thickness was observed in both taxa, along with a generalized decrease in resin canal diameter. In *P. caribaea*, a decrease in chlorophyllous parenchyma thickness was also noted, while in *P. tropicalis* stomatal number, chlorophyllous and transfusion parenchyma thicknesses, and vascular bundle dimensions were reduced. In both species, intracellular concretions were detected in vascular tissues, transfusion parenchyma, and endodermis, suggestive of metal chelates. Additionally, in *P. tropicalis* epidermal modifications in the form of hairs were observed. The anatomical changes present may be adaptive responses to chronic abiotic stress. This study constitutes the first anatomical evidence of the response of Cuban pines to mining contamination and lays the groundwork for future physiological research and ecological restoration programs in the region.

Keywords: foliar anatomy, conifers, abiotic stress, heavy metals, mining.

RESUMEN

La hoja constituye el órgano más sensible para detectar condiciones de estrés abiótico, incluyendo la contaminación por metales pesados derivada de actividades minero-metalúrgicas. En las zonas colindantes a la mina Castellanos, municipio Minas de Matahambre, provincia Pinar del Río, Cuba, se desarrollan pinares naturales de *Pinus tropicalis* y *Pinus caribaea* subsp. *caribaea* en suelos afectados por la actividad minera. Con el objetivo de caracterizar los cambios anatómicos foliares en ambos taxones, se colectaron acículas de 12 individuos por especie (cinco réplicas por individuo) y se realizaron cortes transversales midiendo 13 variables anatómicas. Se observó un aumento del grosor de la cutícula en ambos taxones y una disminución generalizada del diámetro de los canales resiníferos. En *P. caribaea* se constató, además, disminución del grosor del parénquima clorofílico, mientras que en *P. tropicalis* se redujo el número de estomas, el grosor de los parénquimas clorofílico y de transfusión, y las dimensiones del haz vascular. En ambas especies se detectaron concreciones intracelulares en tejidos vasculares, parénquima de transfusión y endodermis, sugestivas de quelatos metálicos. Adicionalmente, en *P. tropicalis* se observaron modificaciones epidérmicas en forma de pelos. Los cambios anatómicos presentes pueden ser respuestas adaptativas al estrés abiótico crónico. Este estudio constituye la primera evidencia anatómica de la respuesta de los pinos cubanos a la contaminación minera y sienta bases para futuras investigaciones fisiológicas y programas de restauración ecológica en la región.

Palabras clave: anatomía foliar, coníferas, estrés abiótico, metales pesados, minería.

RESUMO

A folha é o órgão mais sensível para detectar condições de estresse abiótico, incluindo a contaminação por metais pesados provenientes de atividades de mineração e metalurgia. Nas áreas ao redor da mina Castellanos, no município de Minas de Matahambre, província de Pinar del Río, Cuba, florestas naturais de pinheiros das espécies *Pinus tropicalis* e *Pinus caribaea* subsp. *caribaea* desenvolvem-se em solos afetados pela atividade de mineração. Para caracterizar as alterações anatômicas nas folhas de ambas as espécies, agulhas foram coletadas de 12 indivíduos por espécie (cinco repetições por indivíduo) e seções transversais foram realizadas, medindo-se 13 variáveis anatômicas. Observou-se um aumento na espessura da cutícula em ambas as espécies, juntamente com uma diminuição geral no diâmetro dos canais resiníferos. Em *P. caribaea*, observou-se também uma diminuição na espessura do clorênquima, enquanto em *P. tropicalis* houve redução no número de estômatos, na espessura do clorênquima e do parênquima transfusional, bem como nas dimensões do feixe vascular. Concreções intracelulares sugestivas de quelatos metálicos foram detectadas nos tecidos vasculares, no parênquima transfusional e na endoderme de ambas as espécies. Adicionalmente, modificações epidérmicas na forma de pelos foram observadas em *P. tropicalis*. As alterações anatômicas presentes podem ser respostas adaptativas ao estresse abiótico crônico. Este estudo constitui a primeira evidência anatômica da resposta de pinheiros cubanos à poluição por mineração e estabelece as bases para futuras pesquisas fisiológicas e programas de restauração ecológica na região.

Palavras-chave: anatomia foliar, coníferas, estresse abiótico, metais pesados, mineração, Pinar del Río

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INTRODUCTION

The leaf in conifers is the ideal organ for studying the response to abiotic stress due to its dual function (Huang *et al.*, 2016), and therefore can be modified to ensure the plant's acclimatization, adaptation, and/or survival (Kalugina *et al.*, 2024). This organ is the most sensitive in reflecting soil conditions, including nutrients, heavy metals, and water availability (Çomaklı and Bingöl, 2021; Pérez del Valle *et al.*, 2020).

The leaf faces a fundamental physiological tension: maximizing photosynthesis, which requires a large surface area for exchange and the opening of stomata, while simultaneously minimizing water loss, that requires an efficient barrier against transpiration (Evert, 2006). Its architecture is the result of the expression of a genetic battery that, nevertheless, possesses a significant degree of phenotypic plasticity in response to environmental cues during morphogenesis (Zhang *et al.*, 2017; Tsukaya, 2013). Therefore, any significant and consistent modification in its structural pattern can be interpreted as an adaptive response to specific environmental pressures and not as random variation.

Facing chronic environmental stress, such as that caused by pollution, plants respond not only with visible changes in their external morphology but also with profound restructurings at the tissue and cellular levels (Wronska-Pilarek *et al.*, 2023; Gratani, 2014; Evert, 2006). Therefore, studying changes in leaf anatomy represents an alternative for assessing the impact of pollutants, as it provides a set of highly sensitive structural markers that allow us to infer the type and intensity of abiotic stress (Çomaklı and Bingöl 2021; Leghari and Zaidi, 2013). This approach is especially valuable in forest ecosystems subject to combined stress, such as that resulting from heavy metal loads and soil acidity in mining areas.

Although modifications in leaf anatomy have been documented in the genus *Pinus* as a response to environmental pollution, particularly atmospheric pollution, most of these are concentrated in boreal and temperate species (Delian and Savulescu, 2022; Aricak *et al.*, 2019; Ncube and Phiri, 2015; Kord and Kord, 2011) and there are few studies involving soil contamination.

An example of pollution caused by mining and metallurgical activity is found in the areas adjacent to the Castellanos Mine in the municipality of Minas de Matahambre, Pinar del Río province, Cuba (Bruguera-Amarán *et al., 2022), which could affect Pinus* individuals *tropicalis* and *P. caribaea* (Geada-López and García-Beltrán, 2024; Geada-López *et al.*, 2022; 2021), among other naturally growing species, unless they undergo physiological and/or anatomical changes. Therefore, this study aims to characterize the foliar anatomical changes in *Pinus tropicalis* and *Pinus Caribbean* area adjacent to the Castellanos Mine.

MATERIALS AND METHODS

The needle samples were collected in natural pine forests, where *Pinus* species develop in sympatry. *Pinus caribaea*. *caribaea* and *P. tropicalis,* from mining concession areas of the joint venture EMINCAR SA adjacent to the Castellanos Mine, Pinar del Río, affected by mining-metallurgical activity, although there is no direct mining activity. Needles were randomly selected from 12 individuals between both taxa and from each individual five replicates corresponding to different branches were taken.

For anatomical characterization, cross-sections were made on five randomly selected needles from each individual. To verify the continuity of the resin canals and stomatal rows, cross-sections were made every 1 cm along the needle; since these were continuous, the middle portion of the needles was chosen for measurements.

Observations were made using an NLCD-307B optical microscope. The following anatomical variables were measured at 400× magnification: cuticle thickness, epidermis thickness, hypodermis thickness, and number of hypodermis cell layers; at 100× magnification, the following were measured: transfusion parenchyma thickness, chlorophyll parenchyma thickness, left resin canal diameter, right resin canal diameter, vascular bundle height and width, number of stomata, number of canals, and their type. All variables were defined as described by Pérez-del Valle *et al.* (2020), and endodermis thickness was also included.

RESULTS

Pinus caribaea subsp. *caribaea*

Equifacial, fan-shaped anatomical structure of the needle sections reveals a single-layered epidermis. Stomata are arranged similarly on the abaxial and adaxial surfaces; the average number of stomatal rows per needle was 18. These are sunken, and the stomatal chambers extend into the hypodermis. The hypodermis consists of two to three layers of multiform cells. This is followed by the chlorenchyma, which averages two layers of cells with folded edges, and then several layers of transfusion parenchyma cells. All examined individuals of this taxon exhibited endonal canals embedded in the chlorenchyma and in contact with the endodermis.

In the evaluated samples of *P. caribaea,* images of concretions suggestive of chelates appear in the vascular tissues, transfusion parenchyma, and endodermis. They have different shapes and colors, ranging from dark gray to black and reddish orange (Figure 1).

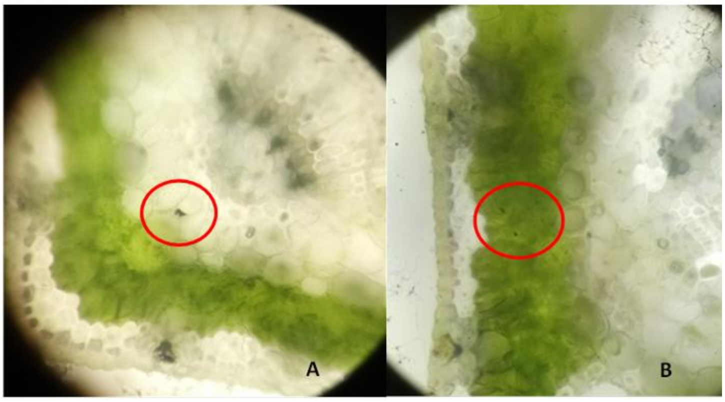

Figure 1. *Sections with suggestive images of black chelates in Pinus needles caribaea. A. in transfusion parenchyma tissue and B. in chlorophyllous parenchyma tissue with a magnification of 100X*

Pinus tropicalis

The anatomical structure is equifacial and semicircular, with a single-layered epidermis. The stomata are arranged uniformly along the cross-section; the average number of stomatal rows per needle is 12. These are characterized by being deeply embedded in the hypodermis, and their substomatal chamber extends into the chlorenchyma. Beneath the epidermis lies the hypodermis, composed of one to two layers of uniform cells; this zone contains a large amount of sclerenchyma. The chlorenchyma is well-defined; large resin canals subdivide it, forming bundles of ground tissue. Towards the interior is the transfusion tissue, and in the center there are two vascular bundles. The two resin canals located at the ends of the adaxial surface are septal, as they are in contact with both the endodermis and the hypodermis, while the rest are marginal.

The sections showed images of concretions suggestive of chelates, which appeared in the vascular tissues, transfusion parenchyma, and endodermis. In most of the *P. tropicalis sections,* a hair-like modification was also observed in the epidermal tissue (Figure 2). The mean values for each of the anatomical variables by taxon are shown in Table 1.

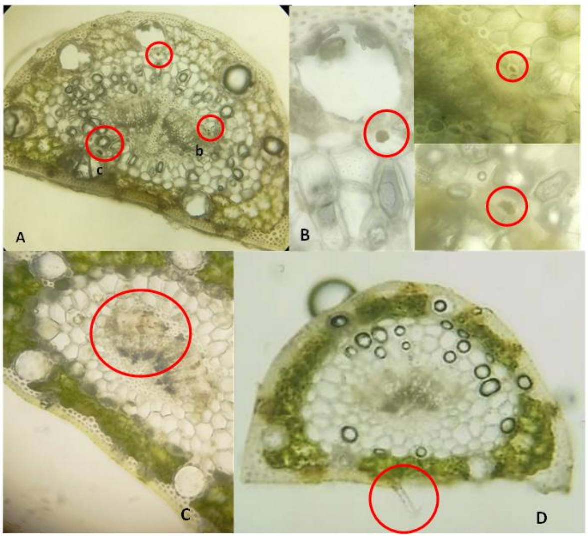

Figure 2. - *Cross-sections with images of concretions suggestive of reddish orange chelates in Pinus needles tropicalis. A. in transfusion parenchyma tissue at 100X magnification. B. Details of A at 400X magnification. C. in vascular bundle tissue and D. unicellular hair: epidermal modification at 100X magnification*

Table 1. *- Mean values and standard deviations of the evaluated anatomical variables of Pinus caribaea and Pinus tropicalis*

Anatomical variables	***Pinus Caribbean***	***Pinus tropicalis***
Cuticle thickness (µm)	3.30 ± 0.32	3.65 ± 0.22
Epidermis thickness (µm)	13.01 ± 0.75	19.79 ± 2.61
Number of cell layers in the hypodermis	2.33 ± 0.33	2.33 ± 0.17
Hypodermis thickness (µm)	38.25 ± 4.86	33.30 ± 1.99
Number of channels	2.67 ± 0.67	6.11 ± 0.61
Thickness of chlorophyllous parenchyma (µm)	90.43 ± 8.66	96.45 ± 6.8
Transfusion parenchyma thickness (µm)	99.04 ± 14.45	125.91 ± 8.63
Vascular bundle width (µm)	194.40 ± 26.34	333.85 ± 62.53
Height of vascular bundle (µm)	364.72 ± 48.45	266.90 ± 35.09
Number of stomata	18.00	12.00
Endodermis thickness (µm)	25.46 ± 3.31	31.49 ± 2.88
Left channel diameter (µm)	42.90 ± 9.94	86.25 ± 9.21
Right channel diameter (µm)	61.30 ± 6.07	91.89 ± 11.14

DISCUSSION

Pinus Caribbean subsp. *caribaea*

In general, no changes were observed in the arrangement of tissues in the anatomical structure of *P. caribaea* compared to that reported by Pérez-del Valle *et al.* (2020). That study compared the anatomy of *P. caribaea.* and *P. tropicalis* as a response to extreme edaphoclimatic conditions in the same ecotope and the differences found are between the dimensions of the tissues and the secretory structures.

Geada-López *et al.* (2021) analyzed the effect of ecotope on the anatomical structure of the species *P. caribaea,* examining samples from various sites representing all the ecotopes where the species grows in natural pine forests. Among these was the Valle Ancón locality, which, due to its proximity and geological characteristics, could be compared to the sampling area of the present study, and one might expect the results to coincide. However, differences were found between the two studies. The present study shows an increase in cuticle thickness and vascular bundle height, as well as a decrease in the diameter of the right and left canals, the thickness of the chlorophyllous parenchyma, and the width of the vascular bundle compared to what was reported by those authors.

Changes in cuticle thickness have been previously analyzed by Le Provost *et al.* (2013) in *Pinus pinaster* Ait. The study evaluated the role of cuticle waxes in the response to drought conditions and showed that wax thickness and quantity increased with higher levels of drought, suggesting an adaptation to water stress. Occasionally, low soil pH conditions are perceived by the plant as a stressful situation, triggering responses analogous to those given to drought (Ahmed *et al.,* 2018).

Chemical analyses conducted in the studied area revealed extremely acidic conditions in the soil and surface waters, with a pH between 1.5 and 2.0 (Project PS104LH002-022). This acidification significantly increases the bioavailability of heavy metals in the soil solution. For plants, metal toxicity induces physiological stress analogous to water deficit (Kim *et al.* 2015), triggering common adaptive responses, such as thickening of the leaf cuticle.

Regarding the number of hypodermis cell layers and the thickness of the chlorenchyma, a decrease was found compared to that documented by Pérez-del Valle *et al.* (2020) and Geada-López *et al.* (2021) for the ecologically similar ecotope to the one studied. However, for other species, with increased water stress, the number of hypodermis layers increases and the thickness of the chlorenchyma decreases (Nikolić *et al.,* 2014; 2019; Grill *et al.,* 2004).

In the number and diameter of resin canals is part of the adaptive response to the low availability of water and nutrients in different Pinus ecotopes *sylvestris* L. (Tyukavina *et al.,* 2019). Kalugina *et al.* (2024) analyzed the impact of environmental pollution from aluminum emissions on the foliar anatomical structure of this species. Among the most significant changes documented was a decrease in the number and diameter of resin canals. In the present study, no variation in the number of canals was detected compared to that reported by Pérez-del Valle *et al.* (2020) and Geada-López *et al.* (2021). However, a decrease in the diameter of both canals was observed.

Pinus tropicalis

In *P. tropicalis,* the anatomical structure and arrangement of tissues coincides with that reported by Pérez-del Valle *et al.* (2020), but some differences appear in the number of stomata, the number of cell layers of the hypodermis, the number of canals and the thickness of the chlorophyllous parenchyma.

Geada-López *et al.* (2022) analyzed the effect of different ecotope conditions on the anatomical structure of the species. In that study, the Manaja locality represents an ecotope similar to the study area, and therefore, a similar behavior in tissue dimensions would be expected. However, differences were found such as: increased cuticle thickness and decreased number of stomata, thickness of chlorophyllous and transfusion parenchyma, and diameter of resin canals, which could then be attributed to the effect of pollution from mining and metallurgical activity.

As in the previous case, low soil pH and high metal concentrations are interpreted as water stress. This species exhibits an increase in cuticle thickness along with a decrease in the number of stomata, which is consistent with the species' standard behavior in extreme ecotopes with low water availability, as studied by Geada-López *et al.* 2022.

Kalugina *et al.* (2024) reported a reduction in foliar anatomical variables such as cross-sectional area and vascular bundle area, leaf mesophyll tissue thickness, and number and diameter of resin canals, as a function of atmospheric pollution ingredient, in *Pinus individuals* This behavior was similar to that observed in this study, but for mining and metallurgical contaminants. However, these changes were not previously observed by Geada-López *et al. (2022)* in any of the species' natural ecotopes.

Cahuana and Aduvire (2019) and Gómez-Bernal *et al.* (2014) have documented that heavy metals available in the soil of sites abandoned by mining and metallurgical activity can be found in plant tissues. They appear as aggregates, and where the metals chelate with starch grains in parenchyma tissue or phenolic compounds such as tannins, these changes immobilize the heavy metals, many of which are toxic to the plant. Although this type of histological study has been very limited in the genus *Pinus,* the more common approach focuses on analyzing the tolerance or bioaccumulation potential of *Pinus sylvestris* L. (Comakli and Bingöl, 2021; Kandziora-Ciupa *et al.*, 2016; Pundyté *et al.*, 2011), *P. halepensis* Mill. (Kharazian *et al.,* 2023), *P. nigra* JF Arnold (Delian and Săvulescu, 2022), *P. brutia* Ten. (Nuhoglu *et al.,* 2015), among others, through the chemical determination of heavy metals in leaves, bark and root.

In the evaluated samples of both *P. caribaea* and *P. tropicalis,* suggestive images of chelates appear in the vascular tissues, transfusion parenchyma, and endodermis. They have different shapes and are dark gray, black, and reddish orange in color. These tissues play a fundamental role in the concentration and movement of solutes from the soil to the aerial parts of the plant (Gómez-Bernal *et al.*, 2014; Geada-López *et al.,* 2021).

Finally, in most of the *P. tropicalis* sections, a hair-like or bud-like structure was observed in the epidermal tissue. The presence of trichomes or similar structures on the needle epidermis is not a previously described feature in the reference anatomy for this genus (Pérez-del Valle *et al.*, 2020; Geada-López *et al.*, 2021). Their presence could be interpreted as a stress-induced morphogenetic response, possibly aimed at increasing radiation reflectivity, reducing transpiration, or creating an additional physical barrier against particle penetration or direct metal toxicity on the leaf surface (Evert, 2006; Vollenweider and Günthardt - Goerg, 2005). This finding, along with the concretions suggestive of chelates in the vascular and transfusion tissues of both species, represents the main changes in their leaf architecture to counteract stress.

Taken together, the anatomical changes observed in *P. caribaea* and *P. tropicalis* point toward a reduction in leaf tissue and the appearance of differentiated epidermal structures and internal concretions, forming a response profile consistent with chronic abiotic stress. While some of these modifications, such as cuticle thickening, are common responses to various stresses (water stress or air pollution), their co-occurrence with alterations in vascular and transfusion tissues, in a soil context of high metal load and acidity, strengthens the interpretation that they are specific adaptations to mining and metallurgical pollution in the study area.

CONCLUSIONS

The study reveals that both taxa exhibit foliar anatomical modifications associated with mining-metallurgical pollution conditions in the Castellanos mine area, which constitutes the first evidence of this type for these species in Cuba.

In *Pinus caribaea*In subsp. *caribaea,* the main changes were the increase in cuticle thickness, the decrease in the thickness of the chlorophyllous parenchyma and the diameter of the resin canals, along with the presence of intracellular concretions in vascular tissues, transfusion parenchyma and endodermis.

In *Pinus Tropicalis,* the most notable modifications include increased cuticle thickness, reduced number of stomata, decreased thickness of mesophyll and vascular bundle parenchyma, as well as the presence of trichome-like epidermal modifications and intracellular concretions in conducting and transfusion tissues.

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The authors have participated in the writing of the work and analysis of the documents.