Horticulture Open Access Journal

Research Article

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Stem Anatomical Differences Among Cacti Species Growing in Wild and Greenhouse Conditions

Volume 1 Issue 1 – 2024

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Received: November 19, 2024 **Published:** December 18, 2024

Citation: Jiménez-Noriega, et al. Stem Anatomical Differences Among Cacti Species Growing in Wild and Greenhouse Conditions. Horti Op Acc Jour. 2024;1(1):09–18.

Abstract

The possible anatomical variation of four cacti, *Coryphantha cornifera*, *C. clavata*, *C. radians* and *Mammillaria magnimamma* from wild and greenhouse sources, is explored as well as the implications of the variation on reintroduction to their natural communities. Three individuals per species per condition were fixed, processed using the paraffin technique, and one-way variance analysis applied to six variables. Results showed differences between wild and greenhouse individuals. Outer epidermal periclinal cell wall thickness had values >4 µm in the wild and <2.5 µm in green-house individuals, whereas for hypodermis values were higher (67-137 µm) in the wild than (34-70 µm) in the greenhouse plants. The epidermal tissue reduction in greenhouse compared to the wild may be related to irrigation and fertilization applied since plants were not exposed to periodic droughts, but vessel tracheids reduction may be related to the higher temperatures in greenhouse. Individuals from the wild showed parenchyma cells in the succulent regions (cortex and pith) whereas in two species of *Coryphantha* grown in greenhouse, the parenchyma cell walls lignified. This lignification of the stem succulent tissue reduces the ability of these collapsible cells to fold during a drought. Probably lignification will be deleterious for re-introductions, because the stems are more rigid. We conclude that special attention should be given to the horticultural practices (supply of nutrients and watering) to avoid structural modifications.

Keywords: *Coryphantha, Mammillaria*, anatomic variation, greenhouse, hypodermis; biominerals

Introduction

Cacti are valued as ornamental plants and play a crucial role in maintaining the arid zones of America. In the Cactaceae family, 669 species have been reported in Mexico,[1] and the tribe Cacteae exhibits the highest diversity in size and growth forms.[2] This tribe includes two genera, *Coryphantha* and *Mammillaria*, known for their high species richness and ornamental value.[3,4] However, one-third of the species in both genera are considered endangered;[5] out of the 43 *Coryphantha* species, 42 are listed in Appendix II (could be endangered), and one more is in Appendix I (endangered species). Of the 175 registered species of *Mammillaria*, 166 are in Appendix II and one is in Appendix I.[6]

The vulnerability of certain cacti is linked to their limited ability to recover after a disturbance process such as land convertion to mining, livestock and agriculture, as well as collection of living plants.[7,8] In addition, rare species that have a limited geographic distribution are more linked to extinction processes[9] and are therefore more suitable for conservation.[10] Cactus conservation in botanical gardens is successful in most cases.[11,12] However, major problems are known to arise when reintroducing them to their natural habitats, since low survival rates have been observed,[13-15] and they are even lower when the individuals come from micropropagation.[16] The low percentages of survival in the reintroduction to their habitat have been related to a decrease in available water,[1] as well as changes in light intensity and humidity.[17]

The environment can influence the variation in anatomical characteristics among individuals of the same species.[18] Anatomical studies on *Mammillaria*[19-28] cover approximately 25% of the species, while in *Coryphan*tha,[29-30, 19, 24-25, 31-33] 11 species in total have been anatomically studied. Most of these studies have focused on describing anatomical characteristics (e.g., epidermis, hypodermis or biominerals) to support systematic interpretations [34]. Studies comparing anatomical attributes under greenhouse and field conditions indicate that the epidermis can be modified as found for in genus *Eriosyce*[35] or as the secondary xylem modified by changes in the intensity of light, water and soil nutrients in *Cereus peruvianus*.[18] The variation in the stem anatomical characteristics of *Coryphantha* and *Mammillaria* under greenhouse and field conditions remains unclear. Thus, the aims of this study were to determine

(1) if the variation in stem anatomical tissues is significant and if it is related to the location the plant came from;
(2) if there are anatomical variations, do they explain the low success of reintroduction to their natural environment; and
(3) if there are constant anatomical characteristics for individuals growing in both field and greenhouse conditions that can be considered attributes with taxonomic

Materials and Methods

The species studied and collected in their natural populations were *Coryphantha clavata* (Scheidw). Backeb., *C. cornifera* (DC.) Lem., *C. radians* (DC.) Britton & Rose, and *Mammillaria magnimamma* Haw (Supplementary S1). The voucher for each species (collector and collector number) were deposited at MEXU herbarium with the following numbers: *C. clavata* (T. Terrazas 886, T. Terrazas 963; S. Arias 1672, S. Arias 1705); C. *cornifera* (S. Arias 1700), *C. radians* (T. Terrazas 877); and *Mammillaria magnimamma* (S. Arias 1693).

The greenhouse material for the species of both genera was obtained from the Botanical Garden of the FES-Cuautitlán (*C. clavata* 93-155, *C. cornifera* 2019-1, *C. radians* 94-92, and *M. magnimamma* 93-174 JB). The specimens were grown in tunnels covered with white plastic, with a milky caliber of 720. Each individual was cultivated in a pot with dimensions of 7.62 × 7.62 × 10.16 cm with a substrate made of 95% tepojal (small stone the 0.3 a 0.5 mm of volcanic origin, covered with clay) and 5% perlite (amorphous volcanic glass contains the 1 a 2 mm). Irrigation was carried out together with fertilization as follows: 500 mg of Peters® formula 20-10-20 (nitrogen, phosphorus y potassium), 70 ml of phosphoric acid 52% (H3PO4), 50 gr of calcium nitrate ($Ca(NO_3)_2$) and 40 gr of micronutrients (zinc 4.8%, iron 4.3%, sulfur 5.7%, magnesium 1.3%, manganese 0.3%, molybdenum 0.017%, cobalt 0.0090%) dissolved in 1000 liters of water. Irrigation was carried out twice a week in the warmer months (April to June) and once a week in the rainy and colder months (July to March). Minimum and maximum temperatures by month are given in Supplementary S2). The greenhouse individuals of the species studied reached the reproductive stage between 2 and 5 years.

2.1 Sampling

Three plants for each of the *Coryphantha* and *Mammillaria* species were collected. Each plant was sectioned into three stem regions: apical, median and base. For each region, blocks 2 to 3 cm high depending on the size of the individuals of each species were cut. The blocks, from epidermis to pith, were placed in flasks with the fixing solution (40% formaldehyde 5 ml, 96% ethyl alcohol 50 ml, glacial acetic acid 5 ml, water 35 ml; [36]) for 24 hours. The fixed samples were washed and processed with the conventional paraffin technique [36], where the dehydration process in ethanol (50-100%) and infiltration in paraplast® was carried out in a tissue processor (Leica TP1020, Wetzlar, Germany). Transverse, longitudinal radial, and serial longitudinal tangential planes were made with a thickness of 12-16 µm on a rotary microtome (Leica RM2125RT, Leica, Wetzlar, Germany) and stained with safranin and fast green to later be permanently mounted on synthetic resin. A total of eight anatomical characters were quantified (10 measurements/plant/species) with an image analyzer with Image-Pro Plus V. 7.1 software (Media Cybernetics, Rockville, MD), and qualitative characters of epidermal, fundamental and vascular tissues were determined.

2.2 Epifluorescence microscopy

The presence of parenchyma cell lignification in the cortex and pith of the individuals from the greenhouse was verified through epifluorescence microscopy. The transverse sections previously obtained were observed using a wide-field fluorescence microscope (Zeiss Axio Imager Z2) with Apotome 2.0 (Zeiss Apotome 2), an AxioCam MRc 5 (Zeiss) and a microscope metal halide fluorescence light source (Zeiss HXP 120). The multicolor images were obtained with a triple excitation/emission bandwidth: DAPI (blue) with excitation of 365/10 nm and emission of 445/50 nm, FITC (green) with excitation of 470/40 nm and emission of 525/50 nm; TRITC (re2.3 d) with excitation of 546/12 nm and emission of 575–640 nm.[37]

2.3 Statistical analysis

Tests of normality and homogeneity of variance were applied to the data. Tukey's mean comparison analysis was performed to identify significant differences between anatomical characteristics with SAS 9.4 statistical software (SAS Institute, Cary, NC).

Results

Qualitative and quantitative differences were found between greenhouse and field individuals, and they are summarized in Table 1 and Figures 1-3. The differences between wild and greenhouse individuals are described below based on tissue characteristics of the stem from the outer epidermal to the fundamental (cortex and pith) and secondary vascular tissues.

Epidermis and hypodermis

The three species of *Coryphantha* and *M. magnimamma* exhibited a thicker cuticle in greenhouse individuals (Table 1, Figure 1A-D) than in the wild. The epidermis width and outer periclinal wall thickness of the epidermal cells were greater in wild individuals and statistically significant differences, except in *C. clavata* (Table 1, Figure 1). The hypodermis thickness was greater in wild individuals than in greenhouse individuals of all species (Figure 1), and statistically significant differences were observed (Table 1), being more than two-magnitudes wider in *M. magnimamma* (Table 1, Figure 1D, H).

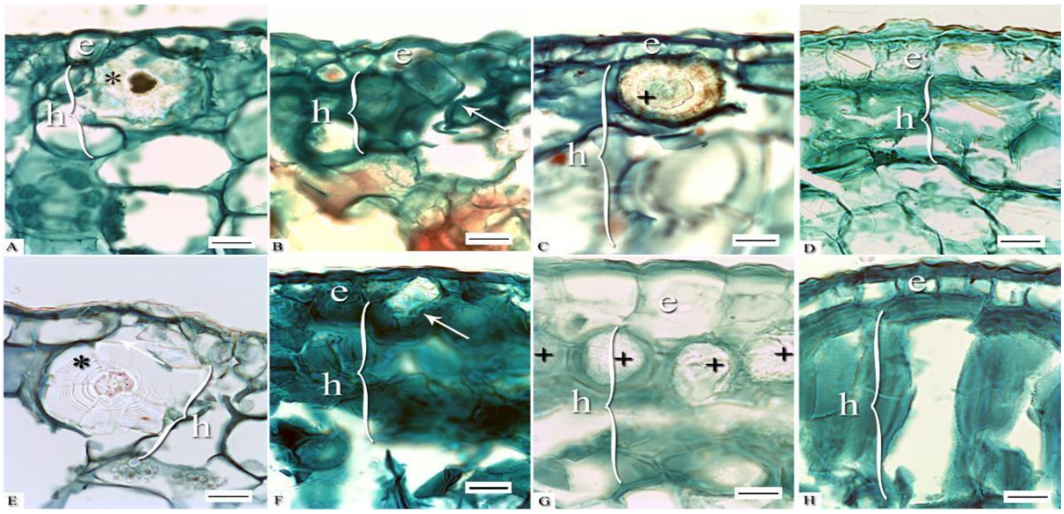

Figure 1. Epidermis and hypodermis of the stems of *Coryphantha* and *Mammillaria* species, transverse sections of individuals from the wild (A-D) and greenhouse (E-H). A, E) *C. cornifera*. B, F) *C. clavata*. C, G) *C. radians*. D, H) *M. magnimamma*. e=epidermis, h=hypodermis, *=druses, +=spherulites, white arrow=prisms. The bar in A-H is 20 µm.

Table 1. Anatomical characteristics of *Coryphanth*a and *Mammillaria* species growing in the wild and greenhouse. Different letters between columns for each species indicate significant differences with α=0.05.

Variable	***C. radians***		***C. cornifera***		***C. clavata***		***M. magnimamma***	
	wild	**greenhouse**	**wild**	**greenhouse**	**wild**	**greenhouse**	**wild**	**greenhouse**
Cuticle thickness (µm)	3.00 ± 0.2^{b}	3.51 ± 0.1^{a}	2.75 ± 0.2^{b}	3.02 ± 0.1^{a}	2.49 ± 0.3^{b}	3.83 ± 0.2^{a}	2.30 ± 0.2^{a}	2.35 ± 0.1^{a}
Epidermal cell widht (µm)	42.05 ± 1.9^{a}	22.34 ± 0.9^{b}	35.24 ± 4.3^{a}	27.9 ± 1.24^{b}	20.9 ± 2.0^{a}	19.3 ± 0.8^{a}	13.81 ± 0.9^{a}	14.32 ± 4.08^{a}
Outer epidermal periclinal cell wall thickness (µm)	11.84 ± 0.5^{a}	1.99 ± 0.2^{b}	$5.55\pm.0.2^{a}$	2.18 ± 0.15^{b}	4.68 ± 0.1^{a}	2.17 ± 0.18^{b}	8.40 ± 0.4^{a}	2.20 ± 1.0^{b}
Hypodermis thickness(µm)	95.76 ± 4.8^{a}	70.55 ± 2.4^{b}	73.37 ± 2.6^{a}	47.70 ± 1.5^{b}	67.84 ± 2.7^{a}	53.96 ± 1.9^{b}	137.79 ± 3.5^{a}	34.50 ± 1.4^{b}
Hypodermis number of strata	2	2	2	2	3	3	1	1
Radial vessel diameter (µm)	27.88 ± 1.8^{a}	21.42 ± 1.6^{b}	33.42 ± 0.8^{a}	34.53 ± 1.1^{a}	31.73 ± 1.3^{a}	22.81 ± 0.9^{b}	28.31 ± 2.4^{a}	23.10 ± 1.8^{b}
Tangential vessel diameter (µm)	29.26 ± 3.2^{a}	20.18 ± 1.0^{b}	31.68 ± 1.4^{a}	33.87 ± 1.1^{a}	31.25 ± 0.9^{a}	24.16 ± 1.0^{b}	30.13 ± 2.5^{a}	22.23 ± 2.2^{b}
Number of phloem sieve tubes/0.153 mm^2	13^{a}	13^{a}	5^{a}	5^{a}	7^{a}	8^{a}	9^{a}	10^{a}

Biominerals

The occurrence of crystals was constant in the hypodermis, between wild and greenhouse species; druses were observed in *C. cornifera* (Figure 1A, E), spherulites in *C. radians* (Figure 1C, G), and prisms in *C. clavata* (Figure 1B, F). *M. magnimamma* lacked crystals in the epidermal and hypodermal tissue in both wild and greenhouse individuals (Figure 1D, H).

Cortex and pith

The cortex and pith had collateral vascular bundles in the individuals of the four species. Notably, greenhouse individuals of *C. clavata* and *C. cornifera* exhibited lignification of cortex and pith parenchyma cells (Figure 2A, C, G) but wild individuals did not (Figure 2B, D, H). No modification of the parenchyma of either region was observed in the individuals of *C. radians* and *M. magnimamma*, but an increase in mucilage cells was observed in the greenhouse individuals, in both the cortex and the pith of *C. radians* compared to wild individuals (Figure 2E, F). Druse like crystals accumulated in cortical cells (Figure 2D, yellow arrows) except in *M. magnimamma* individuals regardless of their origin.

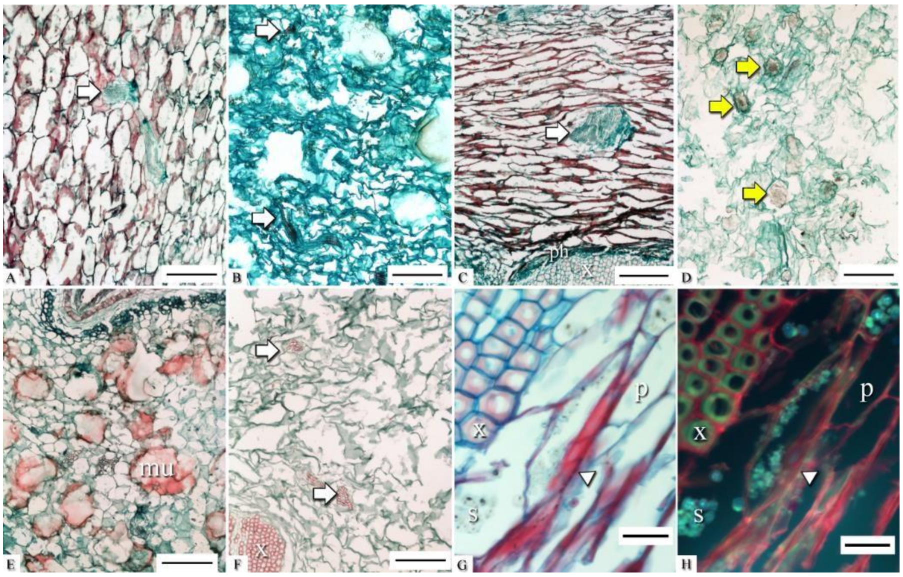

Figure 2. Stem transverse sections of the cortex of *Coryphantha* species. A, B) *C. clavata*, C, D) *C. cornifera*, E. F) *C. radians*, G, H) *C. cornifera*. Greenhouse (A, C, E, G, H) and wild (B, D, F) individuals. G, H) Comparison of a greenhouse individual, (G) bright-field, red color indicates lignified parenchyma cells, pink color lignified secondary walls and (H) fluorescence, green color indicates lignified secondary walls; red tones indicate cellulose in the cell walls. The arrowhead indicates the lignified parenchyma cell wall. mu=mucilage cell, p=parenchyma, s=starch, x=secondary xylem, white arrow=cortical bundle, yellow arrow=druse. The bar in A-F is 300 µm, G and H is 20 µm.

Secondary vascular tissues

In the secondary xylem, the arrangement of the tracheary elements (vessels and wide band tracheids) and the nonlignified axial and radial parenchyma was similar between wild and greenhouse individuals (Figure 3). However, growth marks, reflected by a more abundant axial parenchyma, were less evident in greenhouse individuals (Figure 3A-D). Few ray cells began to lignify in *C. clavata* and *C. cornifera* (Figure 3A, B). The tracheary elements have secondary walls with annular or helical thickenings. Significant differences were found for tracheary elements diameters, except in *C. cornifera*. The largest diameters were found in individuals growing in the wild (Table 1). The vessel diameter frequency distribution showed different patterns of variation among species (Supplementary 1); narrower ranges occurred in *C. radians* both in plants from greenhouses and the wild, whereas wider ranges were distinctive of *C. clavata* and *C. cornifera*. In the conductive phloem (Figure 3I, J), the number of sieve tubes and companion cells did not vary between the wild and greenhouse individuals (Table 1), while the nonconductive phloem presented an inverted cone shape in all individuals (Figure 3I, J).

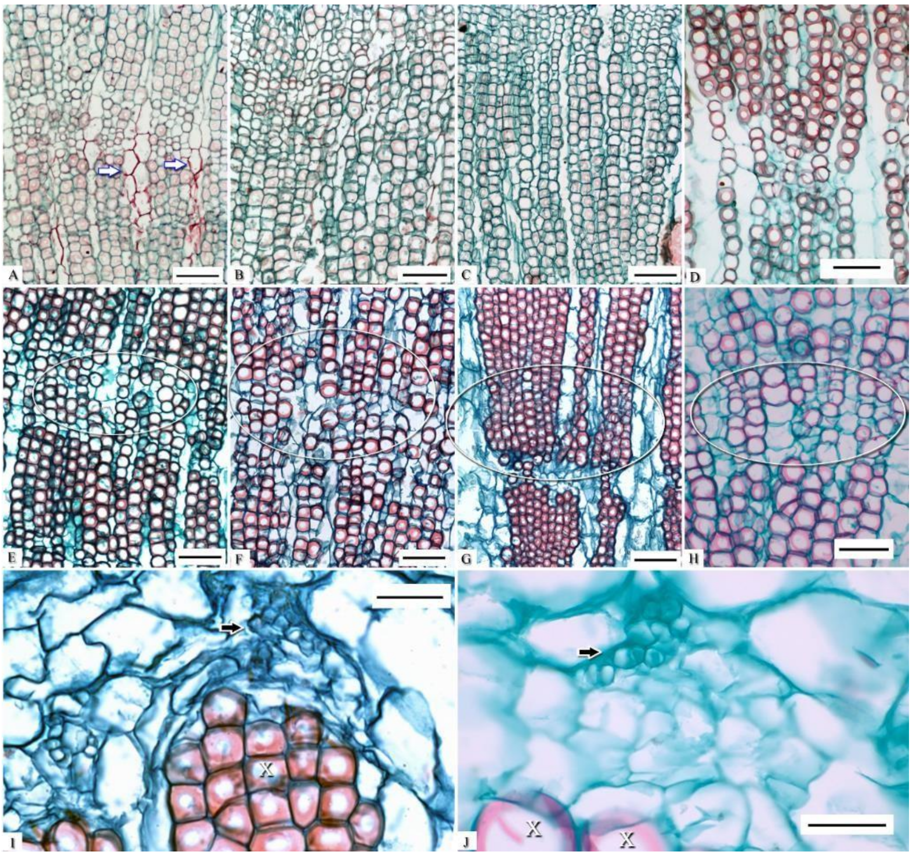

Figure 3. Secondary xylem transverse sections of the stems of *Coryphantha* and *Mammillaria* species. Greenhouse (A-D) and wild (E-H) individuals. A, E) *C. clavata*, B, F) *C. cornifera*, C, G) *C. radians*, D, H) *M. magnimamma*. I) *C. radians*. J) *M. magnimamma*. white arrow=lignified rays, black arrow=phloem, x=secondary xylem. Bar in A-C, E-G is 100 µm, D, H, I is 50 µm, J is 20 µm

Discussion

The most contrasting differences between wild and greenhouse-grown individuals were found in the epidermal and ground tissues.

In various species, a thicker cuticle and thicker outer periclinal wall of the epidermis are associated with individuals who grow in places of high light intensity[38] and low water availability, which promote these xeromorphic characteristics to cope with the environment.[38] In this sense, although the cuticle of the wild individuals was less thick, the sum of the cuticle and outer periclinal wall thickness together turned out to be greater than those of the greenhouse individuals. A similar phenomenon was recorded in species of the genus *Eriosyce*, where wild individuals presented thicker external periclinal walls.[35] It is possible that the greater thickness of the outer periclinal wall is advantageous for cacti in the wild because it is also a water storage site between the cellulose microfibrils and favors the loss of turgor. Both the thickening of the outer periclinal wall of the epidermis and the cuticle thickness have been interpreted as the first defense in the penetration of UV-B radiation in *Arbutus*, *Euphorbia*, and *Cistus* leaves,[38,39] and this interpretation could also apply to the wild plants studied here. Both attributes show the plasticity of the epidermis to respond to the environmental conditions where it develops.[35,40] The thickening of the primary walls of the epidermis and hypodermis in wild individuals may be due to the fact it allows for growth regulation during drought processes for adaptation to water deficit.[41] The cell primary wall response is due to a rapid rearrangement of microfibrils and structural plant proteins[42] and, in turn, cell expansion as a product of pressure by cell turgor.[43] These thicker primary walls may retain water due to their chemical wall composition of abundant pectin, hemicellulose (xyloglucans, mannans and glucomannans) and cellulose[44] and may contribute to maintain cell shape during a severe drought. Individuals grown under greenhouse conditions are not exposed to drought events thus the epidermis and hypodermis maintain thinner walls.

The hypodermis is a protective tissue that provide support and elasticity.[45] The number of strata did not vary between wild and greenhouse individuals. However, a greater wall thickness in the hypodermis was observed in all four species of plants growing in the wild. An increase in the hypodermis wall thickness was also reported for *Eriosyce strausiana* (Cactaceae), where it was attributed to a greater accumulation of pectins in wild individuals, which is associated with xeromorphic environmental conditions.[35] The greater accumulation of pectins in the cell wall of the hypodermis may increase its hydrophobic properties.[44] Regarding the number of layers of the hypodermis, this attribute seems to be a character with taxonomic value. *C. radians* and *C. cornifera* showed two strata, which was also reported in *C. pallida* and *C. retusa*.[24-25,29] In the case of *C. clavata*, it exhibited three layers, while *M. magnimamma* only had one layer, which is common for the hypodermis in the *Mammillaria* genus.[46]

The pattern of secondary xylem (tracheary elements and unlignified radial and axial parenchyma) described for other species of *Coryphantha* and *Mammillaria*[2] was retained in the greenhouse plants. The helical structure of secondary walls in the tracheary elements (wide-band tracheids and vessel elements) allowed contraction and expansion during droughts in their environments.[47] This secondary wall pattern retained under greenhouse conditions suggests that it is genetically fixed, whereas vessel diameter responds to water availability and cultural practices such as fertilization.[18] The mean vessel diameter was wider in plants growing in the wild than in plants growing in greenhouse having a wider or similar range variation (Supplementary 3), which favors rapid water movement when this is available, but also having vessel elements with narrower diameters. Reduction of vessel diameter in plants grown in greenhouse may be related to higher temperatures in the greenhouse compared to their natural populations (Suplementary 2) during the growing periods from May-September. It is important to mention that rays had lignification in two species of *Coryphantha* in which also cortex and pith lignified, reducing the potential to fold together with the tracheary elements. Moreover, the differences in lignification in secondary xylem that occurred in the four studied species whose individuals were grown under greenhouse conditions may be the result of cultural practices (Fig 3A-H). The cultural practices implemented in the botanical garden of the FES-Cuautitlán as the double fertilization with irrigation twice a week, plus the nutrients that were added, could double the effect of some macronutrients, such as N. This effect was recorded in two varieties of *Eucalyptus*, where it was found that high concentrations of N decrease the concentration of lignin and the syringyl/guaiacyl ratio. While, low concentrations of N allow a constant availability of lignin; because genes involved in the metabolism of lignin and phenylpropanoids respond negatively to N.[48] This could be related to a weaker staining of the tracheary elements of greenhouse individuals compared to wild collected individuals (Fig. 3A-H) where the red staining was stronger in response to a higher concentration of lignin. On the other hand, the secondary phloem was anatomically consistent in the four species growing under both conditions and similar to that reported for other Cactoideae members.[49] We were unable to find differences between plants from both conditions; supporting the reports that phloem appears to be less influenced by environmental conditions.[50]

The presence or absence of biominerals in the epidermis and hypodermis was confirmed for both wild and greenhouse individuals. The biominerals in the hypodermis also have diagnostic value, as previously suggested for members of Cacteae,[51] and may be helpful for differentiating between species and taxonomic groups.[52] Although crystals were also present in the cortex and pith, their occurrence was highly variable, and they are considered to be the byproduct of metabolism. The role of crystals has not yet been fully determined, and several authors have proposed that they may be a mechanism developed to reduce the concentration of oxalic acid and calcium that precipitates into calcium oxalate (an inactive salt) inside plants.[53]

As mentioned before, we applied fertilization and irrigation throughout the year, and these practices may have favored lignification in the cortex and pith in two of the four species studied. Lignification decreases the ability of individuals to lose turgidity when there are droughts. If these plants are reintroduced to the field, they may be unable to resist drought, since the lignification of the parenchyma cells would not allow them to lose turgidity and could be detrimental to their survival. Additionally, this lignification also limits the loss of turgidity of the cortical and pith bundles. The lignification of the primary walls of parenchyma cells of the fundamental tissue in both cortex and pith was another contrasting difference between wild and greenhouse-grown individuals. This lignification occurred in greenhouse individuals of two of the four species studied here. In cacti, because of their succulence, a large proportion of the stem is constructed of parenchyma cells, which is considered a storage region that also regulates water during prolonged periods of drought[49] when most of the cells lose turgor. Notably, this parenchyma that have collapsible cell walls favor the reversible changes during dehydratation and rehydratation cycles[54] in adult individuals of different taxonomic groups. Losing the dehydration-rehydration capacity in succulents would be disadvantageous for species exposed to periodic droughts. Our results suggest that the lignin accumulated in the primary walls of the parenchyma cells in *C. clavata* and *C. cornifera* provides rigidity and represents a barrier to cell wall folding (contraction). Cell wall lignification would not be a limitation in these plants grown under greenhouse conditions where irrigation is constant and nutrient solution is added. However, plants with lignified walls will not be able to withstand droughts in their natural distribution areas since their walls will not be able to fold with the cyclical changes in turgor. A possible explanation for the presence of lignification was the amount of calcium nitrate in the applied fertilization[55] that differentially affected species.

The four species grew under the same irrigation and fertilization conditions, so experiments with other concentrations calcium nitrate will allow us to confirm whether this product was responsible for the lignification of parenchyma cells in the cortex and pith of the stem. Modifications in the number and size of cells due to the variation in the macronutrient composition of the nutrient solutions have already been reported in leaves of *Fragaria virginiana*,[56] and an effect contrary to what was indicated was presented in *Cereus peruvianus*.[18] Modifications in the thickness and lignification of the cortical and pith cells suggest that the cell wall exhibits plasticity and adjusts and adjusts rapidly to stressful environmental changes.[41] An acclimatization process of the plants prior to their reintroduction to the wild[57] would be necessary for these species through the transfer to outdoor conditions with the purpose of gradually modifying the relative humidity, substrate conditions, nutrient solution, temperature and light.[58]

Conclusion

The anatomical characteristics that presented variation (epidermis and hypodermis) between the individuals from the wild and greenhouse are those in which the environment is suggested to have a strong interaction. The lignification of parenchyma cells of fundamental tissue (cortex and pith) needs to be further study under different greenhouse conditions as well as in other species. However, these modifications under semi controlled environments may be a disadvantage to recommend these plants for their reinsertion into natural environments. This could be a problem since a large number of species of the family Cactaceae are at risk. The attributes that were stable included the number of layers of the epidermis and hypodermis, and crystals in the hypodermis, between wild and green-house individuals. These attributes could be used for the delimitation of *Coryphantha* and *Mammillaria* species.

Acknowledgement

To the Faculty of Higher Education Cuautitlán UNAM Botanical Garden for providing the biological material in greenhouse conditions and to Madel Jimenez Romano curator collection.

Funding

Funding was provided by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (Program to support Research, Technological and Innovation Projects), UNAM PAPIIT IA202622 to MSJN and IN224307 to TT.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, M.S.J.N. and T.T.; methodology, T.T. and M.S.J.N.; formal analysis, M.S.J.N.; investigation, M.S.J.N. and A.D.T.; writing—original draft preparation, M.S.J.N. and T.T.; writing—review and editing, M.S.J.N., T.T., A.D.T. and D.G.V.; funding acquisition, M.S.J.N. and T.T. All authors have read and agreed to the published version of the manuscript.

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