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Anatomical Studies of Two *Jatropha* Species with Importance for Biodiesel Production

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Abstract

Jatropha curcas L. and *Jatropha macrocarpa* Griseb. (Euphorbiaceae) are perennial species adapted to marginal conditions not suitable for agriculture, and have been recently exploited for oil and biodiesel production**.** The anatomy of different organs in members of this family exhibits a wide range of variations. However, knowledge of anatomical features is still incomplete. The aim of the present work was to analyze the anatomical structure of stem, leaf and root of *J. curcas* and *J. macrocarpa* seedling cultivated in a greenhouse. Fixed samples were properly treated using triple stain hematoxylin, safranin and fast green. Primary roots were diarch and triarch in *J. curcas*, whereas in *J. macrocarpa* were diarch and the cortex showed parenchyma cells, larger in *J. macrocarpa* than *J. curcas*. Stem cortex was thicker in *J. macrocarpa* than in *J. curcas*. Both species had parenchyma cells with cystolith, chloroplasts, laticifers and starch granules, these being more abundant in *J. macrocarpa*. Leaves were characterized by dorsoventral anatomy, with the epiderm showing amphistomatic condition with high stomata density at the lower surface. Both *Jatropha* species had paracytic stomata. Druses and non-articulated branched laticifers were recorded in the mesophyll. Some of the different anatomical features of *J. curcas* and *J. macrocarpa* could explain the different tolerance to abiotic stress.

Keywords: anatomy, environmental adaptation, *Jatropha curcas*, *Jatropha macrocarpa*, stomatal density

1. Introduction

The interest in the cultivation of some species of genus *Jatropha,* suitable for production of biodiesel oil from their seeds (Wassner et al., 2012), is due to their adaptation to marginal conditions for agriculture production. *Jatropha curcas* and *Jatropha macrocarpa* (Euphorbiaceae Fam.) are perennial plants, growing in semi-arid and arid soils, and their non-edible seeds have high oil content (Achten et al., 2008).

J. curcas L. is native to Mexico and Central America and it is cultivated in Central America, South America, Southeast Asia, India and Africa (Heller, 1996). It is a perennial shrub or small tree, reaching a height of 5 m. The leaves are deciduous, and present 5-7 acuminate lobes (Prakash et al., 2007). The fruits are 2.5 cm to 4 cm long, oval in shape, with 2 to 3 black seeds containing 30-35% oil. The present interest in its cultivation is mainly due to its ability to grow in wastelands and desert areas, in this way helping arid land revegetation and carbon dioxide capture in environments where most crop plants cannot survive. In addition, as a second-generation (non-food supply) biofuel crop (Santos et al., 2013), it can be considered a sustainable oil source with minimal environmental impact (Azam et al., 2005; Achten et al., 2008; Datta & Mandal, 2014).

J. macrocarpa Griseb. is endemic to the western Chaco of Argentina, Bolivia and Paraguay. This species is adapted to nutrient-poor soils. It lives in special ecological conditions with arid and semi-arid areas; consequently, as for *J. curcas*, its extensive industrial use does not compete with traditional crops. *J. macrocarpa* is a shrub or small tree, more than two meters high, having soft wood, gray-white bark that exudes translucent latex, and large palmate leaves (Falasca & Ulberich, 2008). Natural populations of *J. macrocarpa* have low seed

production with low oil content in comparison to *J. curcas*; however, their ability to survive at low temperatures (freezing winters) is the main reason to consider its use for biodiesel production (Wassner et al., 2012).

The anatomy of different organs in members of the Euphorbiaceae Fam. exhibits a wide range of variations that correlate with habitat; morphological features like presence of marginal glands on leaves, pubescence on leaf epidermis and exfoliating bark, may contribute to the recognition of different species of the genus *Jathopha* (Jiménez, 1992). *J. curcas* has more lactiferous tissues than other species such as *J. gossypifolia*, *J. glandulifera* and *J. multifida* (Elumalai et al., 2013). Limited work has been done on anatomical characteristics in *J. curcas* and, to our knowledge, no data exist for *J. macrocarpa*; therefore comprehensive studies are needed to understand the potential importance of their anatomical differences in relation to their differential tolerance to environmental factors also in view of the production of oils.

2. Methods

Seedlings derived from seeds were cultivated in a greenhouse at 30 °C for 1 month and ten seedlings of each species were examined. Samples were taken from the first internode and hypocotyl; blade and petiole of leaf and roots and fixed in FAA (50 parts 95% ethanol, 5 parts glacial acetic acid, 10 parts 37% formaldehyde, 35 parts water). Root samples were taken at two different levels, comparable in the two species, to analyse the primary structure and secondary structure of these organs. After fixation, samples were dehydrated according Johansen (1940) and embedded in Histowax (highly purified paraffin wax blended with polymer additives). A series of transverse sections 10 μm thick were obtained from the sample blocks using a Minot rotary microtome. The sections were triple-stained with hematoxylin, safranin O and fast green FCF, as described by Johansen (1940). The sections were air-dried and mounted in DPX Mountant (Sigma-Aldrich, St Louis, MO, USA). A standard Zeiss Model 16 microscope was used to assess the histological preparations; microphotographs were taken with a Zeiss Axiophot microscope, equipped with AxioCam HRc camera and an image capturing and digitalization system (AxioVision 4.3).

To study leaf tegumental tissues, epidermis was isolated by manual removal using the technique of "peeling" (D'Ambrogio de Argüeso, 1986) and finely macerated with sodium hypochlorite (Metcalfe & Chalk, 1960). To improve the contrast in microscope analysis, epidermal strips were stained with safranin or fast-green. Five replicas for each species were examined under the light microscope with clear camera, analyzing 5 microscope fields (0.25 mm × 0.25 mm) in each one.

All measurements were performed using the Image-Pro plus 2.0 Program. In the stomata of foliar epidermis the length, width of guard cells and total area of individual stomata (guard cell complex, including pore) in both leaf surfaces were measured. The number of stomata was counted from five microscope fields randomly selected (area: 0.02979 mm^2) at 400x magnification. The leaf stomatal density was expressed as the number of stomata per unit leaf area (Bogani et al., 2015). Also the diameter of xylem vessels through transverse images of ten permanent cuts in different organs was also measured. Mean diameter of the 25 largest vessels of the whole sections was reported (Villar-Salvador, 1997). In this study five replicas from each organ were analyzed for each species and means ± standard error showing different letters are significantly different ($P \leq 0.05$) as determined by Duncan's multiple range test ($n = 5$).

3. Results

3.1 Root Anatomy

Our results about root structure are reported in Figure 1. The young root zones showed a monostratified epidermis. The cortex consisted of several layers of isodiametric parenchyma cells, larger in *J. macrocarpa* than in *J. curcas*. The latest internal layer was the endodermis, in contact with a monostratified pericycle, surrounding the vascular tissues. The primary vascular tissues were represented by phloem alternating with xylem strands. Primary roots were both diarch and triarch in *J. curcas* (Figures 1A and 1B), whereas in *J. macrocarpa* the roots were diarch (Figure 1C). In the secondary structure, a thick periderm formed by suber and phelloderm strata was detectable. In the root, the periderm originated from phellogen, the secondary meristem formed from pericycle. In both species, below periderm secondary phloem was observed, where the fiber component was clearly visible (Figures 1D and 1E). Next, a wide cambial zone and abundant secondary xylem were detectable. Secondary xylem vessels were more frequent and showed significantly larger diameter in *J. macrocarpa* than *J. curcas* (57.50±3.24 μm and 47.16±3.06 μm respectively) with $P \leq 0.05$.

Residues of primary xylem were still present in the inner region with the typical centripetal differentiation, where metaxylem occupied the central region and emitted protoxylem projections (Figures 1D and 1E).

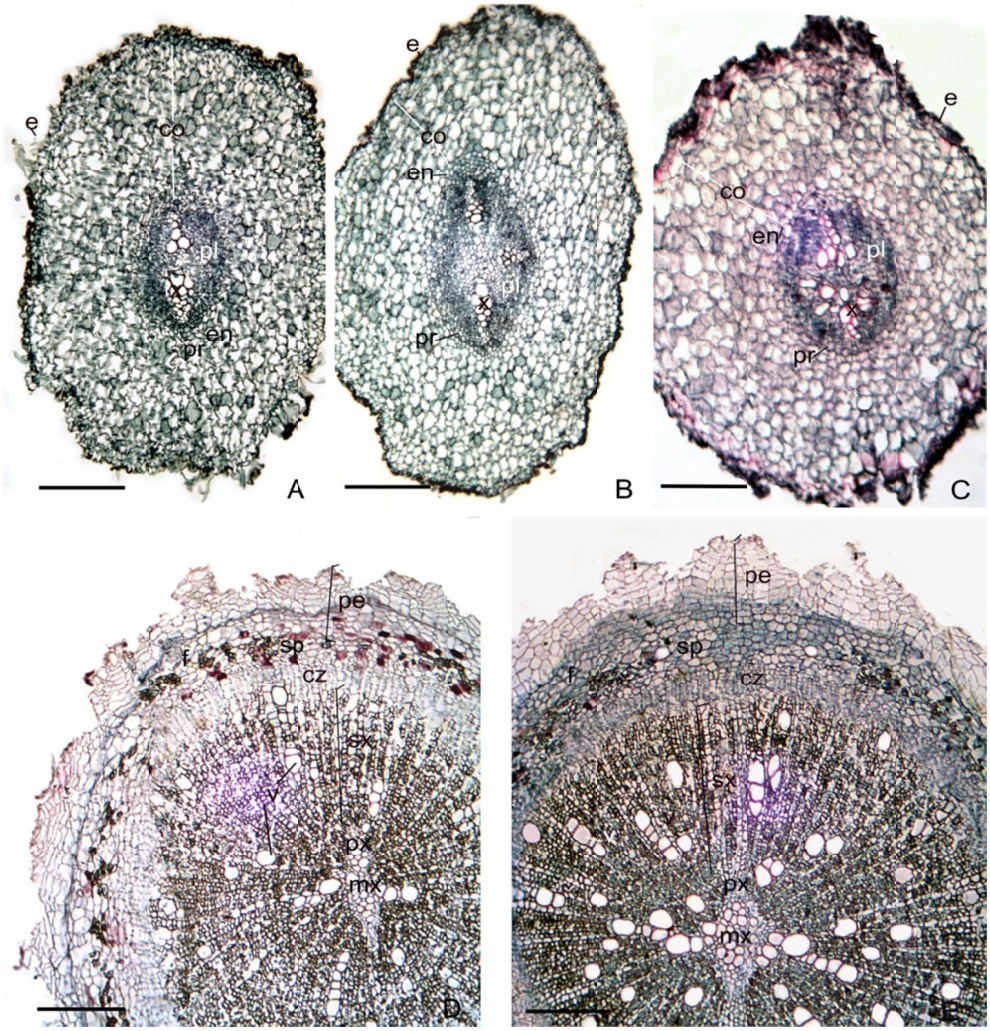

Figure 1. a) Transverse section of the younger root zone. A: *J. curcas*, diarch root. B: *J. curcas*, triarch root. C: *J. macrocarpa*, diarch root, cortex with large parenchyma cells. Scale bar = 150 µm; b) Transverse section of the older root zone. D: *J. curcas*. E: *J. macrocarpa*, secondary xylem vessels with large diameter. Scale bar = 250 µm

Note. co, cortex; cz, cambial zone; e, epidermis; en, endodermis; f, fibers; mx, metaxylem; pl, primary phloem; pe, periderm; pr, pericycle; px, protoxylem; sp, secondary phloem; sx, secondary xylem; x, primary xylem; v, xylem vessel.

3.2 Stem Anatomy

The first internode showed a monostratified epidermis overlying a pluristratified hypodermis; this mechanical tissue, while not presenting particular wall thickenings, could give strength and flexibility to young stems. The cortex, formed by isodiametric parenchyma cells, was thicker in *J. macrocarpa* than *J. curcas* (Figures 2A and 2B). In both species cortex had laticifers, druses and starch granules, while chlorenchyma below the hypodermis was only observed in *J. macrocarpa* (Figures 2C and 2D). The vascular system consisted of collateral vascular bundles separated by wide inter-fascicular areas. The eustele was composed by seven to eight vascular bundles of different size in *J. curcas*, while *J. macrocarpa* had four vascular bundles (Figures 2A and 2B). In *J. curcas* vascular bundles were smaller with xylem vessels showing a significant reduced size if compared to *J. macrocarpa* (9.04±0.99 µm and 11.40±0.51 µm respectively, with $p \leq 0.05$). The laticifers were abundant near the vascular tissue. The pith of *J. curcas* showed large parenchyma cells with starch granules and laticifers. *J. macrocarpa* had parenchyma cells with more abundant starch granules and laticifers (Figures 2E and 2F).

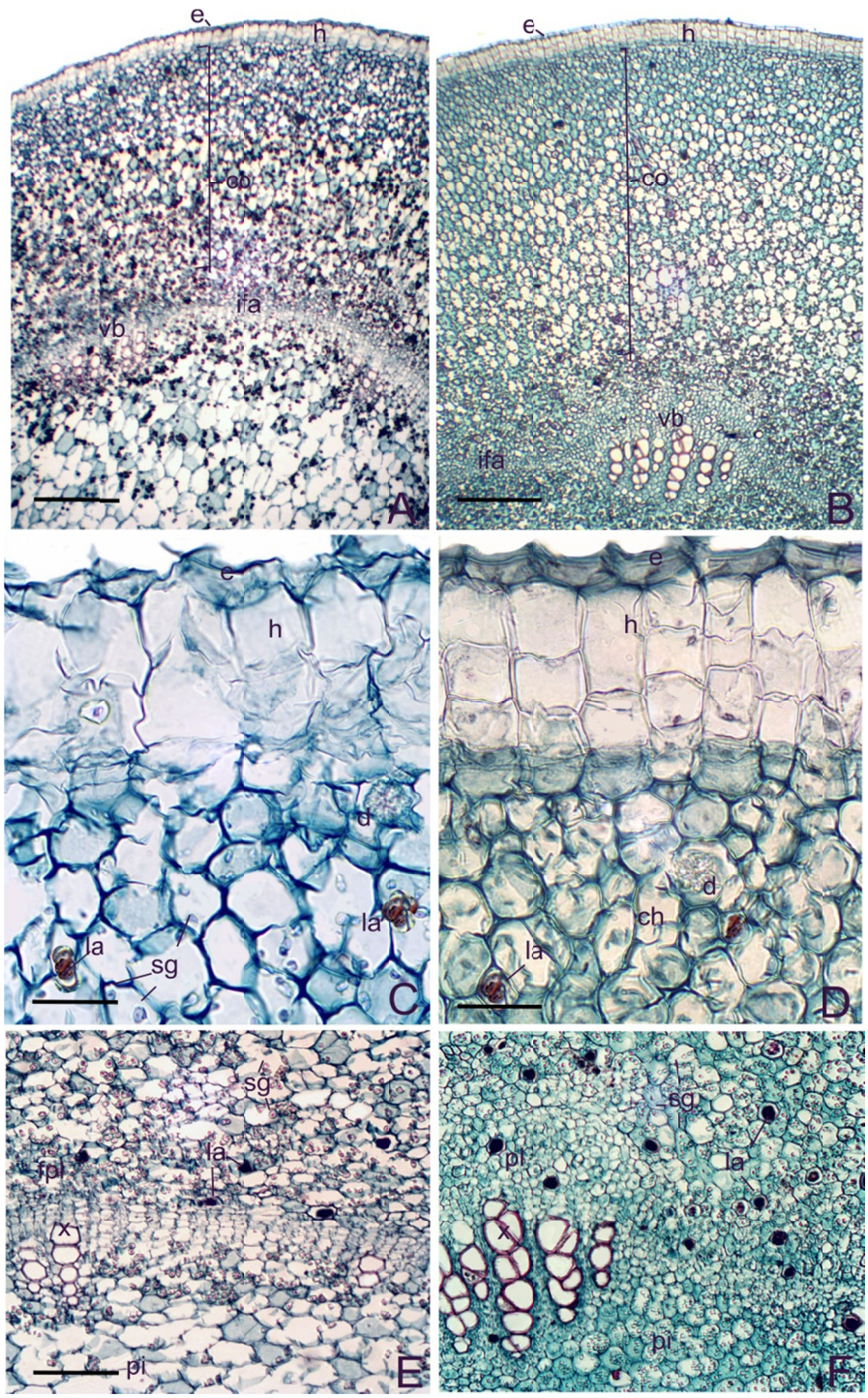

Figure 2. a) Transverse section of first internode. A: *J. curcas*. B: *J. macrocarpa*. Scale bar = 250 µm; b) Detail of hypodermis and cortex. C: *J. curcas*. D: *J. macrocarpa*, with chlorenchyma below the hypodermis. Scale bar = 40 µm; c) Detail of vascular tissue and pith. E: *J. curcas*. Large parenchyma cells in pith. F: *J. macrocarpa*, xylem vessels with large diameter. Scale bar = 40 µm

Note. ch, chlorenchyma; co, cortex; d, druses; e, epiderm; h, hypodermis; ifa, inter-fascicular area; la, laticifer; pl, phloem; sg, starch granules; vb, vascular bundle; x, xylem.

The tissue of protection in the hypocotyl was the periderm, the suber consisting of six or seven layers of radially flattened rectangular cells with lenticels (Figure 3). *J. macrocarpa* had cells with thick walls. The cortex was

thicker in *J. macrocarpa* than in *J. curcas*. In *J. macrocarpa* subepidermal strata of chlorenchyma and below parenchyma with druses laticifers and starch granules were observed (Figures 3A and 3B). In the cortex of *J. curcas* the chlorenchyma was absent. Both species displayed a well-developed secondary vascular tissue. Secondary xylem vessels showed larger diameter in *J. curcas* (19.60±0.85 µm, $p \leq 0.05$) than *J. macrocarpa* (14.04±0.67 µm, $p \leq 0.05$) (Figures 3C and 3D).

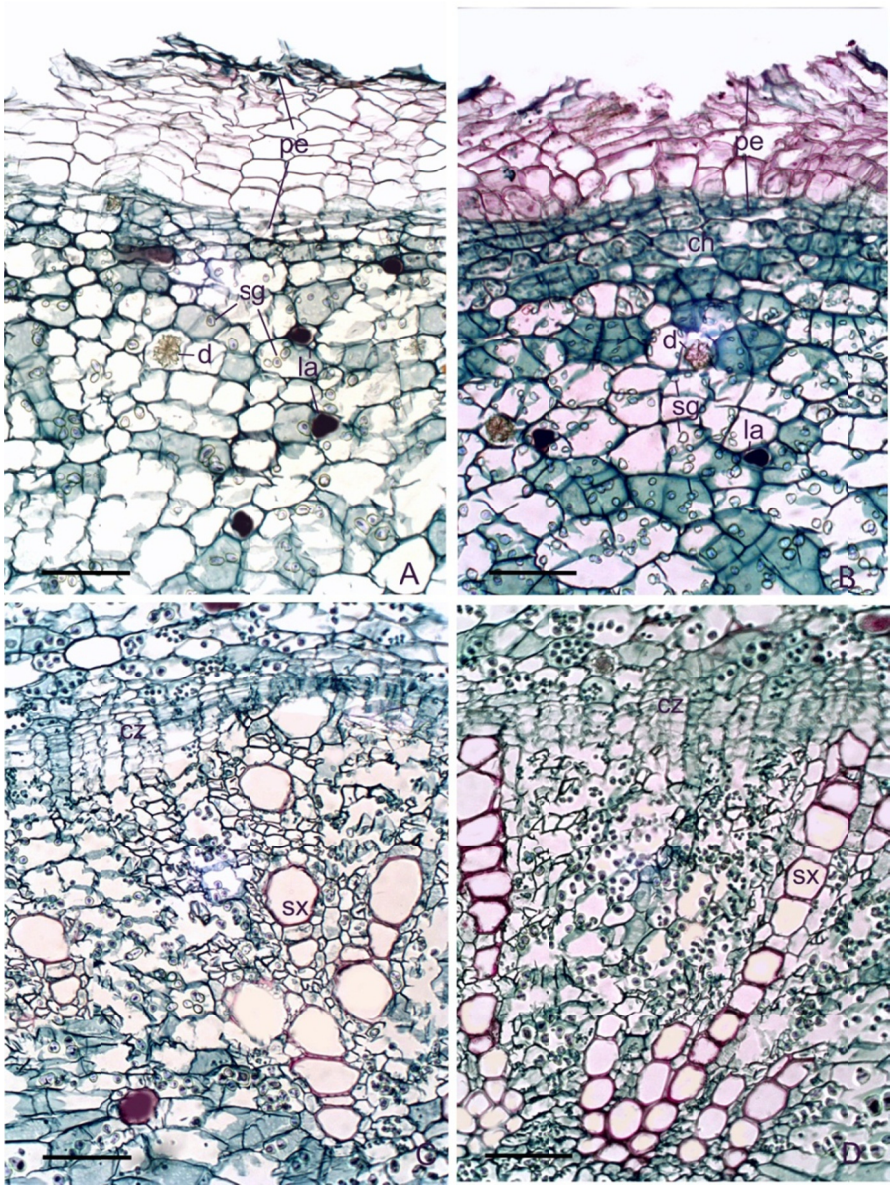

Figure 3. Transverse section of hypocotyl. a) Detail of periderm and cortex. A: *J. curcas*. B: *J. macrocarpa*, periderm with thickened walls cells, cortex with chlorenchyma. Scale bar = 40 µm; b) Detail of vascular tissue, showing well developed secondary xylem. C: *J. curcas*. D: *J. macrocarpa*. Scale bar = 40 µm

Note. ch, chlorenchyma; d, druses; la, laticifer; pe, periderm; sg, starch granules; sx, secondary xylem; zc, cambial zone.

3.3 Leaf Anatomy

Leaves of the two species of *Jatropha* had a petiol characterized by eustelic structure. The eustele was composed by seven vascular bundles and central pith in *J. macrocarpa*, while *J. curcas* had a hollow petiol and ten vascular bundles. The pith showed parenchyma cells in *J. macrocarpa*. In *J. curcas*, the pith was hollow and surrounded by remains of parenchyma cell walls (Figures 4A and 4B).

The cross sections of *J. curcas* showed a leaf blade with a monostratified epidermis covered by a thin cuticle. In the central nervure area there was colorless parenchyma with abundant laticifers, two or three layers of subepidermic collenchyma in adaxial surface and a primary vascular bundle. There was not difference in diameter of the vessels between the two species: 31.55±0.74 μm in *J. curcas* and 34.94±1.01 μm in *J. macrocarpa*. In the wings area, palisade tissue, which constituted approximately 50% of the mesophyll, occurred on adaxial surface (dorsiventrally differentiated leaf) and consisted of two layers of long-narrow cells. Spongy parenchyma arranged in several layers of irregular cells separated by large intercellular spaces (Figures 4C and 4E). The anatomical features of leaf blade obtained from *J. macrocarpa* were similar to those of *J. curcas*. Mesophyll thickness increased gradually from the central nervure towards the wings. In the wing area, palisade parenchyma was formed by three layers and constituted approximately one third of the mesophyll thickness (Figures 4D and 4F).

The leaf epidermis of both the species showed amphistomatic condition. Stomata had two guard cells and two subsidiary cells parallel to the guard cells (paracytic stomata) with surrounding epidermal cells. Subsidiary cells were larger in *J. macrocarpa* than in *J. curcas*.

Stomata showed greater length in *J. macrocarpa* than in *J. curcas*, but no significant differences were observed in their width. In both species stomatal length, width and stomatal area were higher in the lower surface than in the upper surface of leaf. The greatest stomatal area was observed in the lower surface of *J. macrocarpa*. Stomatal density was about half compared to that recorded in the lower surface of *J. curcas* (Table 1). Epidermal cells were polygonal in both, upper and lower surface in *J. curcas* and they were more flattened and with thicker walls in *J. macrocarpa*.

High density of cystolith was recorded in the mesophyll tissues of leaf, near midvein. In addition, there were large irregularly scattered druses, rhombohedral and prismatic calcium oxalate crystals. Laticiferous tissues with nonarticulated branched structure were also detectable (Figures 4E and 4F).

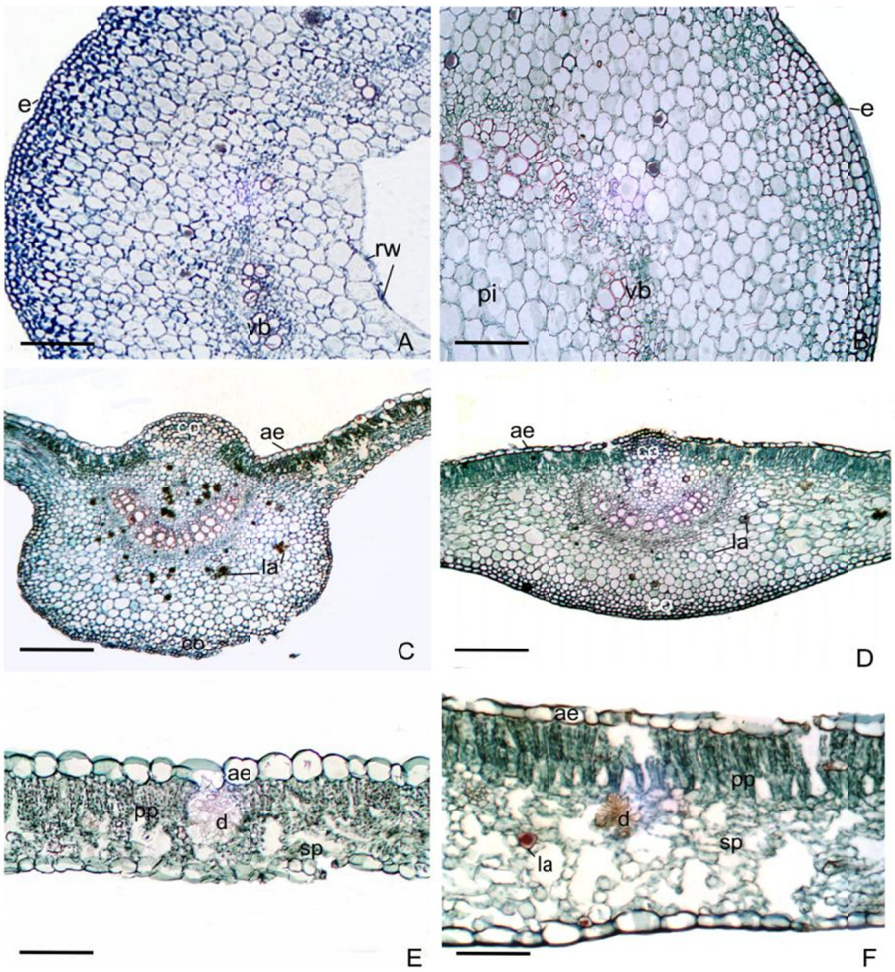

Figure 4. Transverse section of leaf. a) Transverse section of petiol. A: *J. curcas*, hollow petiol. B: *J. macrocarpa*, petiol with parenchyma cells. Scale bar = 200 µm; b) Transverse section of blade, dorsiventrally differentiated leaf. C: *J. curcas* D: *J. macrocarpa*. Scale bar = 250 µm. E: *J. curcas*, palisade tissue constituted approximately 50 % of the mesophyll. F: *J. macrocarpa*, palisade tissue constituted one third of the mesophyll thickness. Scale bar = 40 µm

Note. ae, abaxial epiderm; d, druses; e, epidermis; la, laticifer; pi, pith; pp, palisade parenchyma; sp, spongy parenchyma; vb, vascular bundle; rw, remains walls.

Table 1. Stomata length, stomata width, stomata area and stomatal density in upper surface and lower surface of leaf blade of *J. curcas* and *J. macrocarpa*. Means ± standard error showing different letters are significantly different ($P \leq 0.05$) as determined by Duncan's multiple range test (n = 20)

Specie	surface	Stomata length (µm)	Stomata width (µm)	Stomata area (µm^2)	Stomatal density(mm^2)
J. curcas	upper surface	35.60±0.95c	22.79±0.52b	644.96±20.29c	15±1.93c
	lower surface	40.72±0.82b	26.69±0.53a	794.59±28.51b	68±3.86a
J. macrocarpa	upper surface	42.28±0.97b	21.78±0.48b	671.58±26.80c	14±0.87c
	lower surface	45.67±1.06a	27.58±0.73a	929.45±43.86a	38±2.52b

4. Discussion

Plant capacity to survive to specific environmental conditions is a complex process that involves, at different levels, all aspects of the plant life. Amongst other things, anatomical traits may depict a high adaptive value and the studies on this subject can be useful to understand the biology of a species as well as its potential utilization as a crop species. In this report we have focused on the anatomical features of *J. curcas* and *J. macrocarpa*,

whose specific features were still incomplete, trying to identify in their vegetative organs specific traits useful for the better exploitation of these species for revegetation of wastelands and desert areas.

Considering the root system, the main difference of the two plant species in the primary structure was the mean size of cortex parenchyma cells, which were larger in *J. macrocarpa*. Large cortical cell size has been demonstrated to improve drought tolerance in maize (Chimungu et al., 2014) and, as a consequence, the larger parenchyma cells detected in *J. macrocarpa* could help to explain its performance in arid environments. In addition, the stele in *J. macrocarpa* was diarch, while *J. curcas* had a variable number of archs (2 or 3): this last finding is in contrast to previous studies (Manço de Melo et al., 2011) which indicated a constant number of archs, 2, in the same species. In the secondary structure the thick periderm with suberized cells could be an important trait to rule the inverse flux of water that in severe drought conditions could move from the root to the soil (Hose et al., 2001). In addition, the well-developed xylem comprised, both in root and in stem, vessels with larger diameter in *J. macrocarpa* than in *J. curcas*. Plants with bundles characterized by vessels with large diameter have higher water conductivity; however in dry conditions they may be more susceptible to cavitation in comparison with plants with small diameter vessel (Comas et al., 2013). In fact, vessels with smaller diameter were detected next to the vessels with greater diameter, and thus, the presence of both wood types could provide the right balance between efficiency and safety (De Micco & Aronne, 2012). Krishnamurthy et al. (2012) reported that the phloem in root of *J. curcas* is surrounded by two to three layers of lactiferous cells; nevertheless, the present study shows that there were laticifers also in other organs. The widespread presence of these secreting structures in both species indicates a well-developed defense system against environmental constraints.

In relation to stem anatomy, the cortex was thicker in *J. macrocarpa,* which gave deeper and more protected vascular bundles. In addition, the higher cortex thickness along with the larger vessel diameter could contribute also to enhance the adaptability of this organ to arid conditions (Gan et al., 2013). Likewise, parenchyma tissue is very important for a plant response in case of wound and may act as a tampon to attenuate environmental stress (Appezzato-de-la Gloria & Hayashi, 2006). A particular characteristic observed in the cortex and stem pith, in both species, was parenchyma cells with starch granules, these being quite abundant in *J. macrocarpa*. This finding is distinct from other studies, in which starch granules have been identified in root cortex of *Jatropha isabelliae,* species widely studied for its medicinal properties (Riveros et al., 2009). The apparent higher starch content in *J. macrocarpa* stem could help this species during cold periods, maintaining intracellular osmotic concentration by catabolism of this macromolecule (Cavender-Bares et al., 2005; Morin et al., 2007). It is worth noting that also *J. curcas* may show cold tolerance when associated with an arbuscular mycorrhyza, *Glomus intrarradices* (Pedranzani et al., 2015). In young stem, the vascular bundles that make up the eustele were separated by wide interfascicular-areas. This structure is characteristic of herbaceous Dicotiledons (Esau, 1982) and not of woody species such as *J. curcas* or *J. macrocarpa*.

The presence of parenchyma tissue in these species could be an adaptive character to withstand harsh environments, considering that parenchyma cells may operate as active site of carbon reserves in primary and secondary tissues (Morris et al., 2016). This adaptive trait also occurs in the older parts of the stem (hypocotyl) as the secondary xylem, coming from interfascicular cambium activity, presents more parenchyma than conduction element.

Moreover, young stems of xerophytic species often exhibit hypodermis, that takes over and reinforces the protective functions of the epidermis mechanical protection (Esau, 1982; Fahn & Cutler, 1992).

In *J. macrocarpa*, the suber, in the periderm of hypocotyl had significantly less flattened cells with thick walls which can contribute not only to a better adaptation to arid conditions, but also to a thermic isolation against freezing winters.

Foliar anatomy of mesophyll in both species shows a typical dorsiventral arrangement (Fahn, 1978; Esau, 1982). The present study shows that *J. curcas*, presented two layers of palisade parenchyma toward adaxial surface, confirming the observation of Manço de Melo et al., 2011 on the same species and similarly to the anatomy of *J. molissima* and *J. mutabilis* (Sá et al., 2013). This finding is distinct from previous studies in which a four-layered palisade parenchyma was found in both abaxial and adaxial surface (Idu et al., 2009). These differences can be explained by a plasticity response of leaf cells during differentiation, depending on plant cultivation and/or growth environmental condition (Rodrigues et al., 2014). In *J. macrocarpa* the palisade parenchyma consisted of three layers: the higher number of palisade layers is generally associated to a more efficient adaptation to high irradiance levels (Yano & Terashima, 2001) even if in some plant species it can be linked to frost-hardiness (Palta & Li, 1979).

The leaf anatomy in both species showed an amphistomatic condition with paracytic stomata present in a higher number at the lower surface (Idu et al., 2009). The lower stomatal density detected in *J. macrocarpa*, probably makes better balancing transpiratory activity and may characterize winter-hardy genotypes as reported for olive trees (Roselli & Venora, 1990).

In leaves of both species, calcium oxalate crystals of different shapes were observed. The function of calcium oxalate crystals in plant tissues is not yet ultimately elucidated, but results of several studies suggest that calcium oxalate crystals can build up a reservoir to ensure calcium supply for metabolic processes when its absorption and translocation are hindered due to environmental stresses such as salinity (Hunsche et al., 2010) or heavy metals (Tyagi et al., 2013). As typical trait of Euphorbiaceae, there were also in leaves numerous laticifers for translocation of latex, dispersed in the parenchyma (Metcalfe & Chalk, 1960). Laticifers with circular lumen and nonarticulated branching have been also described in other *Jatropha* species such as *J. gossypiifolia* L. and *J. marginata* (Dehgan & Graig, 1978), where their branches, rich in granular content, formed a network in the mesophyll.

This study provides evidences on the economically important species *J. curcas* and *J. macrocarpa*, both adapted to wastelands and desert areas, showing distinct anatomical features, although for several of the characteristics observed there were no or only small differences between these species. However, *J. macrocarpa*, combining both drought and cold hardiness traits shows a good adaptability to different extreme environments and may be considered a better candidate for sustainable biofuel production in spite of the lower oil yield.

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