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PRELIMINARY ANATOMICAL STUDY ON SECONDARY THICKENING PARTS OF ENDEMIC *DRACAENA CINNABARI* BALF.FIL. FROM THE SOQOTRA ISLAND

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ABSTRACT

The present study investigates anatomical structure of secondary thickening parts (root-stem-branch) of endemic monocot *Dracaena cinnabari*. The measurement of microscopic structure parameters was carried out using analytical imaging. The differences between vascular bundles were determined. The results show presence of concentric vascular bundles in all investigated plant organs. In general, the parenchyma cells cover majority of the total area (an average of 77%), much less covers xylem (an average of 19%) and phloem (an average of 4%). The results indicate that the plant body is well adapted to sub-tropical climate regimes and specific environmental conditions prevailing on the island such as limited access to soil moisture and sufficient nocturnal dew which is essential for plant growth and survival.

KEYWORDS: Dragon's Blood Tree, monocot, anatomy, secondary thickening, image analysis.

INTRODUCTION

Dracaena genusis a living monocot representative of the Tertiary flora (Habrová et al. 2009). In the APG IV classification system, it is placed in the family *Asparagaceae*, subfamily *Nolinoideae* (The Linnean Society of London 2016). Endemic *D. cinnabari* growing on Soqotra Island is unique for its tree habit as well as other 6 arborescent species out of a total of at least 60 species of *Dracaena* genus. According to Brown and Mies (2012), its closest relatives are found in southern

Arabia (*D. serrulata*), eastern part of Africa (*D. ombet, D. schizantha*), Macaronesia and Morocco (*D. draco*).

Dracaena cinnabari grows in mist-affected areas of the island, usually above 300m up to the highest mountain areas (Brown and Mies 2012). According to some studies (e.g. Hubálková 2011, Miller and Morris 2004), the occurrence and distribution of Dragon's Blood Tree on the island is dangerously limited and seriously affected by excessive livestock grazing. *Dracaena cinnabari* as Soqotra's most iconic plant suffers from lack of regeneration due to a decline in the quality of habitat caused principally by overgrazing.

Dracaena cinnabari has been a focus of conservation efforts and research activities in recent years (e.g. Adolt et al. 2012, Attore et al. 2007, Habrová et al. 2009), but there are very few current studies on the anatomy and physiology (Adolt 2001, Jura-Morawiec and Wiland-Szymańska 2014).

D. cinnabari is a single-trunked tree with umbrella-shaped crown, branches forming a crown consist of sausage-shaped sections (Miller and Morris 2004). Elongated leaves are densely tufted, tipped and scleromorphic as a specialized feature to prevent excessive loss of water (Brown and Mies 2012). Earlier botanists (Scott and Brebner 1893, Tomlinson and Zimmermann 1967 and 1969) in their studies of palms and other arborescent monocotyledons devoted considerable attention to a few forms with secondary vascular tissues. Arborescent Dracaena species achieve stem thickening by means other than due to a fascicular cambium. In Dracaena sp. there is a 'typical' secondary thickening meristem (STM) with derivatives of radially aligned chaos of vascular bundles in a parenchymatous ground tissue, resulting in a 'woody' underground stem (Carlquist 2012, Rudall 1995). The exact definition of STM is problematical. Rudall (1995) mentions a clear relationship between STM and primary thickening meristem (PTM), the latter being almost ubiquitous in monocots.

Very little is known about root system of the *Dracaena*, which is not easily obtained for a study. Based on previous observations, successive growth of *Dracaena* trees is accompanied by vigorous development of horizontal roots which run far beyond the vertical projection of the crown (Hubálková et al. 2014). Their thickness generally exceeds that of the tap-root. Most of the horizontal roots are found within the upper 30 cm (Jeník 2014).

Adolt (2001) mentions presence of tracheids in xylem of the stem and presence of tracheas in metaxylem of young roots in *Dracaena* plants. According to Adolt (2001), there are collateral vascular bundles surrounded by sclerenchymatic sheaths and uniformly arranged parenchyma cells in young stems and on the other hand concentric vascular bundles without sclerenchymatic sheaths, surrounded by in-lines arranged parenchyma cells in secondary thickening stem. Jura-Morawiec and Wiland-Szymańska (2014) have studied the issue of the structure of amphivasal secondary bundles of *Dracaena draco* stem. They have described a secondary growth of stem as formation of amphivasal vascular bundles in which a centrally located phloem is surrounded by a ring of xylem cells. Röseler (1889) cit. in Jura-Morawiec and Wiland-Szymańska (2014) pointed out that the type of vascular bundles, their shape, distribution and the type of tracheary elements present become a useful criteria to identify secondary body in a cross section. According to Myburg et al. (2013), the parenchymatous pith is creating in central part of older stem. Mauseth (2009) adheres to the theory of diffuse secondary growth consisted of ground parenchyma cells and additional vascular bundles proliferation near the periphery.

The main goal of this paper is to complement previously published data on *Dracaena*'s anatomical structure by study of the anatomy of secondary thickening organs. Anatomic examination and description of roots, stems and branches of Dragon's Blood Tree is key to clarification yet unexplained questions about thickened organ's features associated with specific

tree habit and ecological conditions. So far, little information is known about secondary thickening in arborescent *Dracaena*'s and no publication pursues all secondary thickening organs in *Dracaena cinnabari* despite its threat and limited distribution. This preliminary study serves as the basis for further ecophysiological research of the species.

MATERIAL AND METHODS

Dracaena cinnabari is a unique endemic plant occurring on Yemeni Island of Soqotra whose floral endemism rate making it one of the most biodiverse islands in the world (Miller and Morris 2004).

Study area

The samples of secondary thickening parts (roots, stems and branches) of *Dracaena cinnabari* were collected from fresh wind-throw on Firmihin plateau on the Socotra Island(N 12°28´8.59´´, E 54°00´6.02´´). On Firmihin, there is the only closed forest stand of Dragon's Blood Treesbeca use the plateau is isolated and protected by two deep valleys. As regards the type of relief, this is an elevated plateau with Tertiary limestone bedrock (Pietsch and Morris 2010). The wind-throw is at the altitude of 650 m ASL. The climate is strongly influenced by the seasonal Monsoon winds, blowing from the North-East during the period November-Marchand from South-West during the period June-September. Nocturnal dew is far more important to the water supply than monsoonal rain. The average annual air temperature is 23.7°C (Král 2005).

Field work

The wind-throws occurs sporadically in localities where *Dracaena* trees grow, especially due to lack of soil and strong fixing of anchor roots in parent material. Moreover, taking of live plant samples is strictly prohibited because of nature protection of the island. Therefore, the increment cores were taken from branch, stem, tap-root of the only found wind-throw on Firmihin. The age of the tree was estimated on 380 years according to the procedure described in Adolt et al. (2012). The basic dendrometric characteristics of the wind-throw, characterized by average values, are presented in Tab. 1.

Tab. 1: Basic dendrometric characteristics of the wind-throw.

Dendrometric characteristics	Breast-height diameter (cm)	Tree height (m)	Crown base height (m)	No. of branch sections
Parameters	62	7.5	4.2	20

Laboratory work

Samples in the form of microcores were taken from all cardinal points of the stem. The increment cores were taken by means of a Haglöf Increment Borer. Sampling was conducted at the height of 1.3 m ± 20 cm. From branches and roots were taken 15 blocks of wood with peel (5 blocks from branches, 5 blocks from buttress roots and 5 blocks from tap-root). The increment cores were located separately in histological cassettes and immersed in fixative solution FAA (formaldehyde-acetic acid-ethanol) for a week. For longer storage, the samples were immersed in the solution of 96% ethanol and distilled water with the proportion of 30:70. Before further processing, the redundant wood and peel were cut off, and then the samples went through an alcohol series consisting of ethanol of various concentrations (70%, 70%, 90%, 90%, 95%, 100%,

100 %) and xylene (triple maceration). The time of soaking the microcores was one and half hours in each solvent. The reason for this step is the preparation for the stage when the samples are impregnated in paraffin so that they could be cut using the rotation microtome. Paraffin is not soluble in water that is why the samples are dehydrated by ethanol. Then the ethanol has to be displaced by xylene which is mixable with paraffin. The samples are left in the paraffin for at least four hours.

The samples were placed in Petri dishes, embedded in paraffin by dispenser Leica EG 1120 and heated in oven at 60°C for 4 hours. After that, the samples were put in metal moulds and the moulds were filled by means of paraffin dispenser. When this cooled down, the paraffin block was taken out of the mould and cut using the rotation microtome (Leica RM 2235) so that a part of the microcore was uncovered. The microcores were then immersed in water overnight for repeated hydration so that they could be more easily cut on the microtome. Subsequently, microsections of 14 μm thickness were produced using the rotation microtome; these were laid on water surface (40°C) in Leica HI 1210. This straightened the microsections, which were then taken out and mounted on glass slides with special glue Albumin. The slides with specimens were dried for 5 minutes in the temperature of 60°C and then dried completely in the air. Further, the specimens went through another alcohol series, this time connected with dying. To highlight the non-lignified parts, Astra Blue stain was used and to highlight lignified parts safranin was used. The times of sample soaking are presented in Tab. 2.

Tab. 2: Times of microsection soaking before closing the specimens.

Bioclear	30 min
Bioclear	30 min (displacement of paraffin)
Ethanol (96 %)	15 min
Ethanol (96 %)	15 min (displacement of Bioclear)
Safranin	5 min (dyeing of lignified parts)
Ethanol (96 %)	soaking, rinsing
Astra Blue	3 min (dyeing of non-lignified parts)
Ethanol (96 %)	soaking, rinsing
Ethanol (96 %)	soaking, rinsing
Xylene	soaking, rinsing

The specimens were closed with Canadian balsam and a cover slip. Cover slips of the resulting microscopic specimens were loaded with small magnets to dry (Vichrová et al. 2011).

Image analysis

The measurement of microscopic structure parameters was carried out using NIS – Elements AR image processing tools consisting of digital 5 Mpix camera Nikon DS – Fi 1 connected with a microscope Nikon Optiphot 2.

Prepared microscope slides were imaged using Nikon 4x objective.

Analytical Imaging can be summarized as follows:

- Taking a microphoto (displaced area ca. 2480 x 1860μm).
- Creating a mask (range of interest), so-called sections in the image area where the measurements will be done. Three square masks (each with an area of 608481 μm2) have been created for each analysed image.
- Creating the binary image of the area of xylem, phloem and parenchyma cells. Sequential measuring the areas of binary images (Fig. 1).

- Measuring internal diameter (semi-major and semi-minor axis) of all vessels (tracheae) within the masks and cross-sectional area calculation.

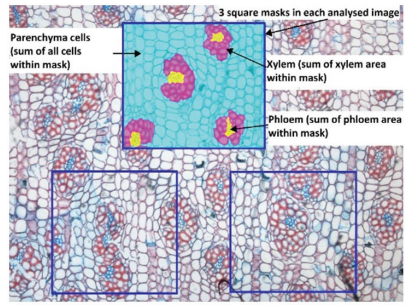

Fig. 1: Image area with 3 identic masks with binary image of xylem, phloem and parenchyma.

Data analysis

To assess differences in the proportional area of xylem, phloem and parenchyma between three studied cross-sections (root, stem and branch), we used Multivariate ANOVA (MANOVA) with three dependent variables (proportional area of xylem, phloem and parenchyma), one fixed factor (organ including central part of stem, branch and root) and one random factor (masks) nested within a fixed factor. Wilks lambda was used as a test statistics. Planned vector comparisons between pairs of organs were done after significant effect of global test. Once a multivariate test had found a term significant, a nested ANOVA was used to determine which of the variables and factors were responsible for the significant effects. Data on proportions were log-ratio transformed before analyses (Aitchison 1986). Anested ANOVA was used to assess differences in area of vessels between central part of stem, root and branch as well as in case of various parts at cross section of stem (stem I – a pith; stem II – central part between stem I and stem III; stem III – peripheral part).Area of vessels was dependent variable, fixed factor comprised different organs or different zone of stem, and two random factors comprised the mask nested within the snap nested within the zone. Data were log transformed before analysis to improve normality and homoscedality. Tukey multiple comparison test was used to identify the differences in proportional area of respective organs between secondary growing parts of the plant. Box-and-whisker plots were used for visualization of untransformed data.

RESULTS

The results show differences between the anatomical structures of secondary growing parts. Similar concentric vascular bundles have been detected in all studied plant organs.

Xylem-phloem-parenchyma in root-stem-branch

Relative area proportions of xylem, phloem and parenchyma significantly differed between each other studied cross-sections (Tab. 3, planned comparisons all $P< 0.05$). Parenchyma cells in central part of root, stem and branch always occupied on average more than 74% of area but their proportional representation was significantly higher in root and branch in comparison with stem(Tab. 3, Fig. 2).

Xylem occupied on average from 17 to 23%of area and occupied significantly higher area proportion in stem in comparison with both root and branch (Tab. 3, Fig. 2). Phloem occupies

from 3 to 4% of area on average with significantly higher area in branch in comparison with root (Tab. 3, Fig. 2). Xylem and phloem positively correlated each other for each organ (all r> 0.88 and P < 0.05).

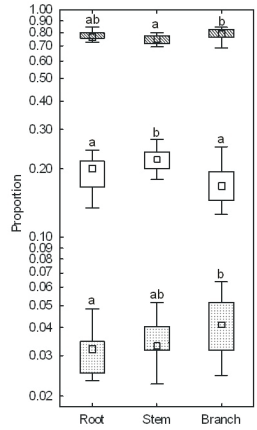

Fig. 2: Box-plots of the proportional area of parenchyma, xylem and phloem (top-down) in three studied cross-sections (root, stem and branch). Different letters suggest significantly different mean proportions of the respective tissue between organs (Tukey multiple comparison test at P ≤ 0.05).

Xylem-phloem-parenchymain stem III-stem II-stem I

Relative area proportions of xylem, phloem and parenchyma were similar between stem I and II (planned comparison, P = 0.459) and both significantly differed from stem-III (Tab. 3, planned comparisons, both P< 0.02). Parenchyma cells occupied on average from 74 to 78% of area in various parts at cross section of stem with significantly lower parenchyma area in stem-II in comparison with stem-III (Fig. 3, Tab. 3).Xylem occupied from19.1% area in stem I (i.e. a pith area) to 22.1% area in stem II (i.e. between pith and cork) and stem-II had significantly larger

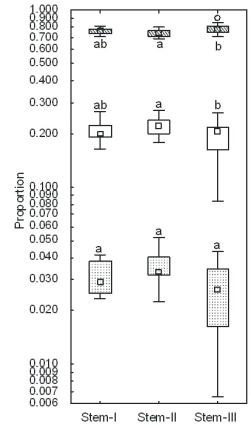

Fig: 3. Box-plots of the proportional area of parenchyma, xylem and phloem (top-down) in the cross section of a stem (stem- I, stem- II, stem- III). Different letters suggest significantly different mean proportions of the respective tissue between organs (Tukey multiple comparison test at P ≤ 0.05).

relative area in comparison with that in stem-III (Fig. 3). Phloemoccupied3.0% area in stem I, 3.5 % area in stem II and 2.7 % area in stem III. There were no significant differences between the area of phloem across the stem (Fig. 3, Tab. 3).

Tab. 3: Effects of zone and mask nested within zone on proportions of xylem, phloem and parenchyma in a cross-section (root-stem-branch, and stem I-stem II-stem III). (a) Results of MANOVA, (b) results of separate nested ANOVA on each trait. Significant effect (P≤0.05) have P-values in bold.

Group/ trait	(a) MANOVA						(b)	Xylem		Phloem		Paremchyma	
	Source of variation	Wilks lambda	F	DF effect	DF error	P	DF effect	F	P	F	P	F	P
Root-stem-branch	zone mask (zone)	0.242	9.3	6	54.0	**<0.001**	2	12.0	**<0.001**	2.5	**0.010**	6.0	**0.007**
		0.253	1.2	39	80.7	0.221	12	1.5	0.177	0.5	0.916	1.0	0.429
Stem I, II, III zone	zone mask (zone)	0.592	2.80	6	56.0	**0.019**	2	4.3	**0.038**	3.1	0.080	4.9	**0.028**
		0.295	1.19	36	83.5	0.259	12	0.6	0.828	1.5	0.198	0.7	0.761

Area of vessels in root-stem-branch

Except for higher area of vessels in branch in comparison with root, no significant differences were found in area of vessels between cross sections (Tab. 4, Fig. 4A). Area of vessels comprised 39.3 % of total area of vessels in the branch section while only 30.5 % and 30.1 % of total area of vessels in the root and stem cross sections, respectively.

Area of vessels in stem cross section

The area of vessels significantly increased from the inner to the outer parts of a cross section of the stem (Tab. 4, Fig. 4B). Area of vessels in the stem-III section thus comprised 54.5 % of total area of vessels in the stem while that in the stem-I section comprised only 18 %.

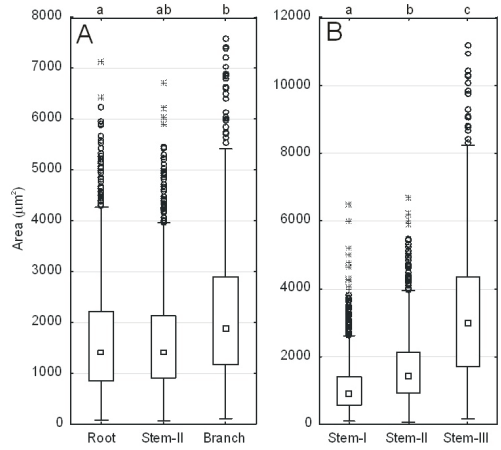

Fig. 4: (A) Box-plots of the area of vessels in three studied cross-sections (root, stem-II and branch). (B) Area of vessels in the cross section of a stem (stem- I, stem- II, stem- III). Different letters suggest significantly different group means (Tukey multiple comparison test at P ≤ 0.05).

Tab. 4: Effects of zone and snap nested within zone and mask nested within snap on area of vessels in a cross-section (root-stem-branch, and stem I-stem II-stem III) tested by nested ANOVA. Significant effect (P≤0.05) have P-values in bold.

Group		**Root-stem-branch**		**Stem I, II, III zone**	
Source of variation	DF	F	P	F	P
zone	2	4.7	**0.034**	96.9	**< 0.001**
snap (zone)	12	2.7	**0.014**	2.1	**0.047**
mask (snap(zone))	30	5.7	**< 0.001**	4.4	**< 0.001**

DISCUSSION

We present a unique data on structure of secondary thickening parts (roots, stems and branches) of *Dracaena cinnabari*, an endemic single-trunked monocot tree growing on Soqotra Island.The study material was sampled from one fresh wind-throw which guarantees similar growth conditions and thus allows direct comparison between anatomical structures. Significant differences in anatomical structure between secondary thickening parts and organs of *Dracaena* mature tree have been found. On the other hand, similar concentric vascular bundles were found in stem, branch as well as in the roots as an adaptation to specific climatic and environmental conditions. Thus the anatomical structure differs considerably from the vast majority of monocots and dicots (Rudall 1995; Tomlinson and Zimmermann 1969).

In general, the parenchyma cells cover majority of the total area (an average of 77 %), much less covers xylem (an average of 19 %) and phloem (an average of 4 %). Our outcomes confirm previous study (Adolt 2001) showing that most of the space inside stem and roots is filled by parenchyma cells. Concerning individual parts of plant body, we observed significantly largerproportional parenchyma area in branch and slightly also in root compared to stem. Parenchyma is the most common plant tissue and plays a major role as a water reservoir (Mauseth 2009). Function of drought resistance through parenchymatous tissues for storing water and starch (as found in our images)is essential for plants growing in dry subtropics. High moisture absorption in the form of dews and fogs is very important for *Dracaena* trees (Scholte and De Geest 2010). The plants are well adapted to interception of atmospheric humidity by extremely long horizontal skeletal roots, wide crowns and branches with sausage-shaped sections (Miller and Morris 2004).According to our study, these sections are filled with parenchyma cells as an important storage element.

Xylem occupied larger proportional area in cross-section of stem compared to root and branch. This interesting outcome indicates less importance of root system of Dragon´s Blood Tree as the epidermis, Stem-III) and inner parts of the stem: there is significantly largest relative parenchyma area in cross-section of the stem III. In young plants, the tissues link all the parts of the plant, allowing water, nutrients, and other compounds to be carried throughout the plant. Our results support the hypothesis (Myburg et al. 2013, Mauseth 2009) that changing the position of vascular bundles depends on age. Specialized processes occur during secondary thickening when the secondary thickening meristem (STM) is developed outside the primary vascular bundles (Carlquist 2012, Rudall 1995). The activity of STM leads to production of secondary vascular bundles and "filling" parenchyma on its inner side only and densified parenchymatous cells on the outer side. As stated by Adolt (2001), most of the stem mass consists of parenchyma and scattered concentric vascular bundles. Our field observation and results of the measurements proved changes of "initial" parenchymatous tissue in the central part of stem (stem I) depending on plant age. The density is decreasing and the parenchymatous tissue decays gradually, until the central

cavity is formed (Hubálková et al. 2014, Myburg et al. 2013). This hypothesis is also supported by the changes of xylem and phloem relative area at cross section of stem showing their largest proportional area in stem II, i.e. the part between initial and densified parenchymatous layers. It is important to note that cork cambium activity is normal and produces cork and secondary cortex at the outer region.

In the case of individual parts of plant body, there is significantly larger area of vessels in branch compared to root (Fig. 4A). It is probably one of the physiological adaptations to help *Dracaena* with low levels of soil water (Myburg et al. 2013). If a large amount of parenchymatous tissue in stem serves as a water reservoir, water transport rate from the stem into the branches and then into the leaves is one of the crucial plant strategies to cope with drought in the arid region. It is much more important for the plant to have the vessels in the branches than in the roots due to lack of soil water for most of the year (Myburg et al. 2013).

In the case of various parts of cross section of stem, there is significantly largest area of vessels in stem III compared to stem II and stem I (Fig. 4B). We can assume that this phenomenon is associated with age-related formation of vascular bundles and its concentration in the peripheral part of the stem, tightly on the inner side of secondary thickening meristem.

CONCLUSIONS

Because of the strict protection, island isolation and problematic export of plant samples, the anatomical features of *Dracaena cinnabari* have received little attention. This study reports preliminary results presenting anatomical structure and function of secondary thickening organs of such a unique arborescent monocot. The results obtained in this study indicate interesting differences of proportional area of xylem, phloem and parenchyma between root, stem and branch. The achieved results lead us to conclusion that plant body of *Dracaena cinnabari* is well adapted to specific environmental conditions of Soqotra Island. Based on this study, we can determine whole-plant hydraulic conductivity and other properties which will help us to better understand the principles of ecophysiological adaptation of the species. Nevertheless, further studies on the species are advisable with respect to its importance and level of endangerment.

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