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Comparative morpho-anatomical and ecological aspects of desiccation-tolerant vascular plants

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Index

1. Introduction

1.1. Definition and brief history of desiccation tolerance

Desiccation tolerance can be defined as "the ability to dry to equilibrium with moderately dry air and then resume normal function when rehydrated" (Alpert & Oliver 2002). In other words, desiccation-tolerant plants dehydrate, remain in the desiccated state for days or months, turn green upon rehydration and become functional again (Fig. 1). Since its first discovery in rotifers by Leeuwenhoek in 1702 this phenomenon has long been the matter of debate in scientific circles. The controversies over the next 150 years were so substantial that the Biological Society of France convened a commission in 1859 to examine the existence of desiccation-tolerant organisms which finally confirmed the basic results of Leeuwenhoek and his successors (Keilin 1959).

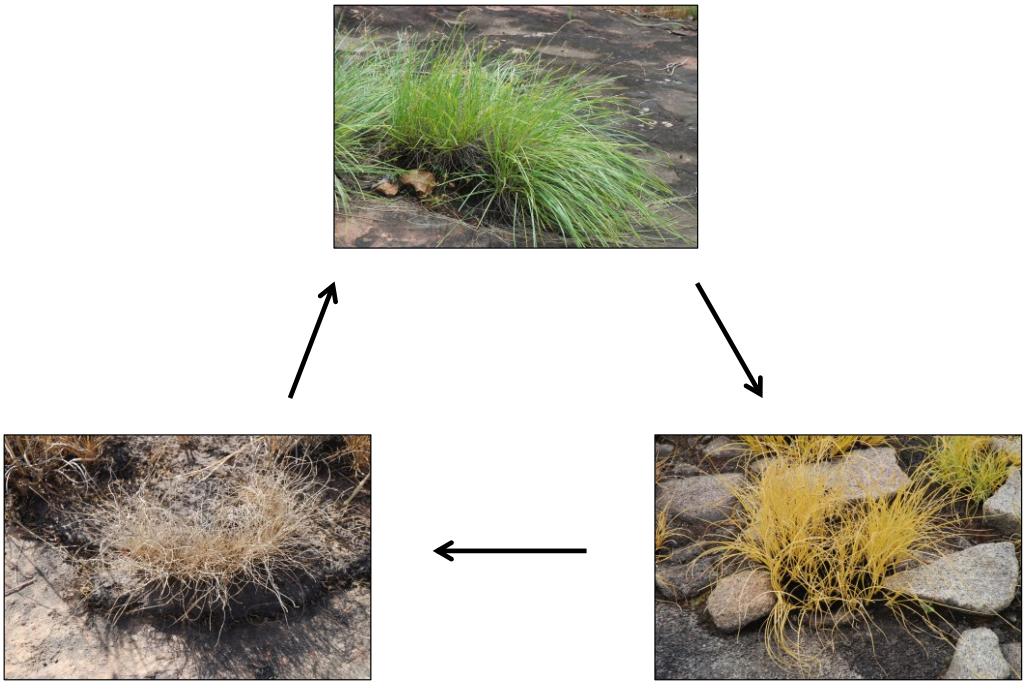

Figure 1 *Afrotrilepis pilosa* (Cyperaceae), a West African desiccation-tolerant species in different stages of the de- and rehydration process (clockwise from top: turgescent, during dehydration, dry) (photographs by S. Poremsbki).

The past 300 years have shown that desiccation tolerance can be found in all three major domains, i.e. Archaea, Bacteria and Eukarya (Wood & Jenks 2007) and is not so rare after all. Within Eukarya protists, fungi, animals and plants harbor desiccation-tolerant species (Alpert 2005). In the plant kingdom algae, lichens, bryophytes and

vascular plants are known to be desiccation-tolerant. These plants are able to tolerate the almost complete loss of water whereas desiccation-sensitive plants only tolerate a partial or moderate loss of water. The latter usually die once their relative water content (RWC) falls below 20-50% (most species even earlier) (Kranner et al. 2002). Most desiccation-tolerant plants on the other hand survive a loss of more than 90% of their cellular water and, depending on the species, water potentials of -650 MPa (Le & McQueen-Mason 2006).

When desiccation tolerance in plants is discussed it is vital to delineate desiccation tolerance of reproductive organs such as pollen, spores and orthodox (i.e. desiccation-tolerant) seeds from desiccation tolerance of vegetative tissues such as leaves and stems. The latter is rare and was the focus of the doctoral thesis presented here. Therefore, the term "desiccation tolerance" usually refers to vegetative organs of plants if not stated otherwise.

The term "resurrection plants" (Gaff 1971) is often used synonymously and usually refers to desiccation-tolerant vascular plants. More importantly, desiccation-tolerant plants are frequently called "poikilohydrous" analogous to poikilothermic in animals (Walter 1931). This is not always correct since poikilohydry implies that a plant has no means of water loss control (Hietz 2010). If desiccation-tolerant bryophytes and algae are considered this term might be used but not for vascular plants because they (with only very few exceptions) possess water-retaining structures. Ergo, poikilohydrous plants are not always desiccation-tolerant and vice versa (Kappen & Valladares 1999). Aside from these basic conceptual aspects a variety of useful but also highly artificial categories exist in the literature which will be briefly discussed in Ch. 4.5.5. Since the term "desiccation tolerance" will mainly be used here, the interested reader may be referred to the corresponding articles (Kappen & Valladares 1999; Oliver et al. 1998; Toldi et al. 2009).

Vegetative desiccation tolerance occurs in a variety of families and can be found in all life forms except trees (for a list of species cf. Porembski 2011). The exact number of desiccation-tolerant species in the plant kingdom is unknown and can merely be based on speculations because only a tiny minority of species, largely omitting algae and lichens, has been tested. However, approx. 420 algae species are considered to be desiccation-tolerant (Davis 1972 in Bewley 1979) but the number is likely to be higher.

Although desiccation tolerance is usually stated to be rather common in bryophytes at most 1% of the 25-30 000 species have been confirmed to be desiccation-tolerant (Wood 2007) although the actual number may be much higher. According to new estimates as much as 1 300 vascular plant species are desiccation-tolerant (Porembski 2011). Ferns and fern allies constitute the majority of this group whereas only 300 angiosperms are known to be desiccation-tolerant. Within the latter group monocots (260 spp.) largely outnumber dicots (40 spp.). Gymnosperms lack desiccation-tolerant species which might be attributed to the fact that this group is dominated by trees (cf. Fig. 2).

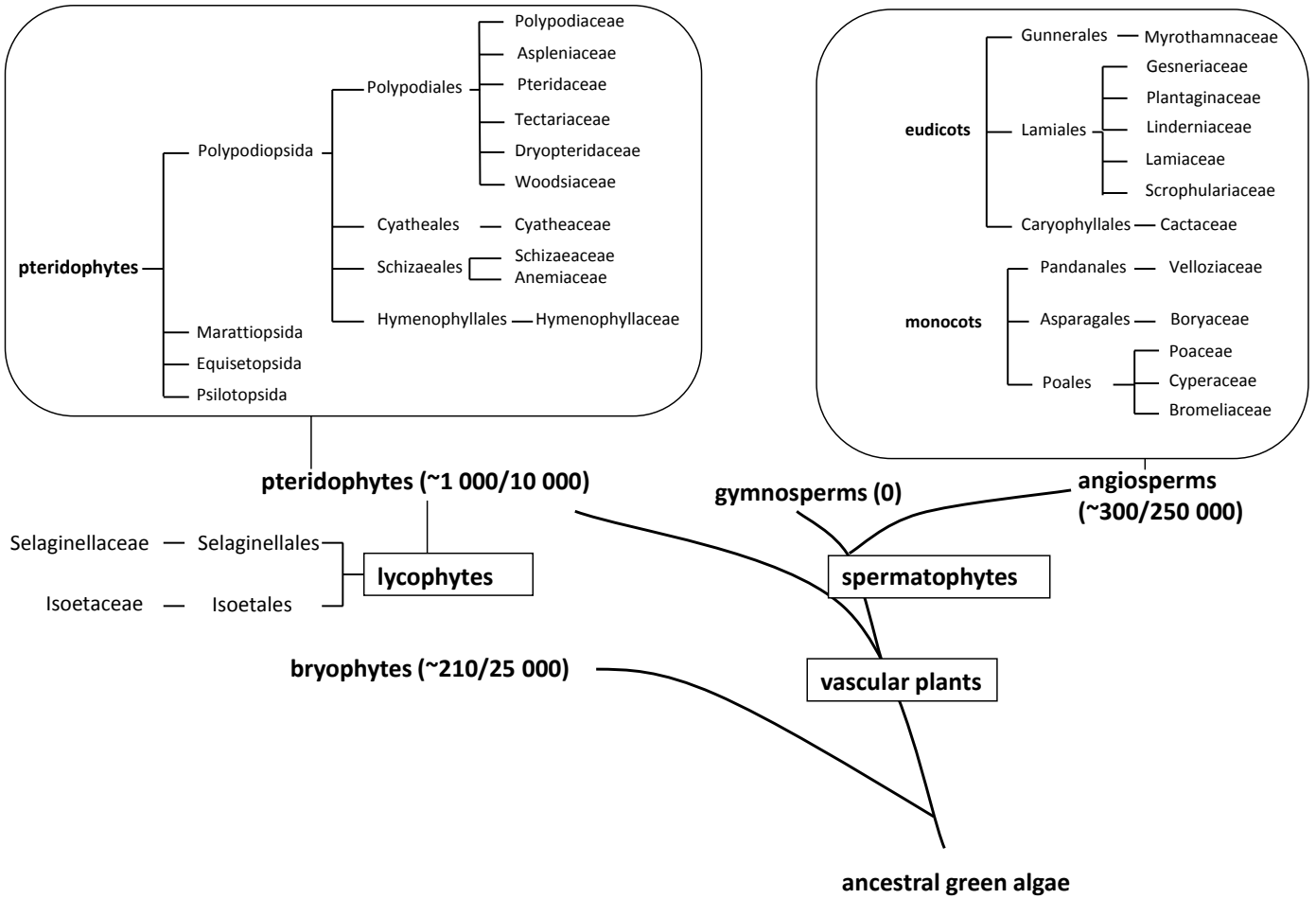

Figure 2 An overview of the distribution of desiccation tolerance in major plant groups and families (Banks 2009; Hietz 2010; Oliver et al. 2005; Porembski 2011; Smith et al. 2006; Wood 2007; Wood & Jenks 2007).

1.2. Geographic and ecological aspects of desiccation-tolerant vascular plants

Desiccation-tolerant plants can be found on all continents, from sea level to 2 800 m (Tuba & Lichtenthaler 2011). Cryptogamic desiccation-tolerant species are cosmopolites; they colonize a wide variety of habitats, even deserts in very arid and cold (Antarctica) regions of the world (Porembski 2011). The majority of vascular desiccation-tolerant plants occur in the tropics and subtropics of West and East Africa (incl. Madagascar), Brazil, Australia, North America and the Western Ghats in India. However, the diversity of desiccation-tolerant plants in Africa is much higher compared to Australia although similar habitats exist. This is probably due to the fact that Africa has been exposed to longer periods of wet-dry cycles than the Australian flora (Cretaceous vs. Tertiary) (Kappen & Valladares 1999).

Not much is known about desiccation-tolerant vascular plants in Asia although recent years have shown that at least a few species seem to be viable candidates, e.g. *Acanthochlamys bracteata* (Velloziaceae) and *Reaumaria soongorica* (Tamaricaceae) (Gao 1993; Liu et al. 2007). However, the latter species should not be categorized as a real desiccation-tolerant plant because it only survives water potentials of max. -15.9 MPa which is relatively low compared to other desiccation-tolerant species. Additionally, Gesneriaceae in south east Asia should be examined since this is a family with desiccation-tolerant species in other parts of the world. A few desiccation-tolerant vascular species can be found in Europe as well; these are mainly ferns and some *Ramonda* and *Haberlea* species (Gesneriaceae) from the Balkan peninsula (Drazic 1999; Porembski 2011).

This broad geographic distribution is in stark contrast to the habitat restrictions. The majority of desiccation-tolerant vascular plants occur on rock outcrops such as inselbergs (Fig. 3) and lateritic plateaus (e.g. ferricretes). Especially pteridophytes colonize the canopy of forests as epiphytes as well. Nevertheless, a few exceptions exist such as *Ramonda serbica* which grows on the more humid northern sides of rocky slopes (Markovska et al. 1994). Rock outcrops and tree canopies are characterized by harsh environmental conditions: unreliable water supply, lack of soil (for the most part), high solar radiation and high temperatures (Szarzynski 2000). The ability to endure long periods of drought is therefore highly necessary for survival but it also a strategy to avoid competition (cf. Ch. 1.3.). It is fairly obvious that desiccation tolerance is one

strategy to cope with periods of extreme water stress (Kessler et al. 2007) but the question whether desiccation-tolerant plants evade, avoid or tolerate drought is difficult to resolve. Although this question seems to be easy to answer via pure terminology some authors consider desiccation tolerance to be a resistance mechanism with reference to Levitt (1972) (Grene et al. 2011; Levitt 1972; Tuba & Lichtenthaler 2011). However, Levitt (1972) clearly considered resurrection plants to be drought-tolerant.

Figure 3 An inselberg in Angola (W. Lobin).

Therefore desiccation tolerance here is regarded as a specific type of drought tolerance in accordance with other authors as well (Alpert 2005; Alpert & Oliver 2002; Bewley 1979).

Interestingly, although desiccation-tolerant vascular plants are common in azonal habitats they cannot be found in deserts. Apparently, desiccation tolerance is not a guarantee for survival in arid habitats; it seems to be difficult for vascular plants to gain a cumulative positive carbon balance under these environmental conditions (Alpert 2000).

Accordingly, as a result of the colonization of extreme habitats and limited periods of photosynthetic activity desiccation-tolerant vascular plants are mainly constituted by herbs and small shrubs. Only very few exceptions such as arborescent Velloziaceae in Brazil and parts of

Africa and taller shrubs like *Myrothamnus flabellifolius* and *Myrothamnus moschatus* exist. Some researchers call this the “desiccation pruning effect” (cf. Ch. 1.3., Farrant

2007). Another reason for this size limitation is that the shrinkage induced by turgor loss during dehydration and the resulting challenges upon rehydration become more complex with increasing size (Alpert 2006). The difficulty of the re-establishment of a water column in the embolized vessels upon rehydration is one example. Root pressure is not always sufficient alone; capillary forces which are generated by transpiration are thought to be the drivers of water movement in xylem vessels in higher plants (Schneider et al. 2000; Sherwin & Farrant 1996).

1.3. Taxonomic and evolutionary aspects of desiccation-tolerant vascular plants

Desiccation tolerance today is regarded as an adaptation to long periods of drought. From an evolutionary point of view it was a necessary asset to colonize the land 400 Mio years ago (Bartels et al. 2011). It has been hypothesized that desiccation tolerance was primitively present in bryophytes (Oliver et al. 2000), then lost during plant species evolution and only maintained in reproductive organs and for stress responses. It re-evolved 3 times in *Selaginella* (Korall & Kenrick 2002). In pteridophytes desiccation tolerance evolved independently in 12 distant lineages and 19 genera (Brodribb & Holbrook 2004), approximately 250 Mio years before the appearance of flowering plants (cf. Fig. 1, Oliver et al. 2000). Desiccation tolerance in pteridophytes is derived as well and often times regarded as an intermediate form because strategies from bryophytes and angiosperms were identified (Oliver et al. 2000). Within angiosperms desiccation tolerance evolved 12 times in angiosperms alone, i.e. it is clearly a polyphyletic trait (Oliver & Bewley 1997). This is underlined by the fact that many similarities and differences in different species and plant groups have become apparent during the examination of mechanisms of desiccation tolerance. Some basic adaptations on the morpho-anatomical, physiological, biochemical and molecular adaptive levels could be identified but almost every species shows at least minimal variations. Poikilochlorophylly, defined as the complete breakdown of the photosynthetic apparatus upon dehydration, is hypothesized to be the latest evolutionary adaptation to longer periods of drought compared to homoiochlorophyllous species which maintain the majority of their chlorophyll and an intact photosynthetic apparatus in the desiccated state (Tuba & Lichtenthaler 2011).

However, other hypotheses for the evolution of desiccation tolerance exist. Oliver et al. (2005) propose that desiccation tolerance might have first appeared in spores of early land plants and possibly their charophyte algae relatives and was later expressed in vegetative tissues (Oliver et al. 2005). Some authors also suggest that desiccation tolerance might have evolved from desiccation-sensitive plants as a response to severe abiotic stresses such as low temperatures, salt and drought. Several studies have shown that a considerable number of genes induced upon dehydration is activated under salt and temperature stress as well (Bartels & Sunkar 2005; Knight & Knight 2001). This assumption is supported by the fact that many species in the dry state are additionally exposed to high temperatures and excessive light which do not seem to have a deleterious effect (Kappen & Valladares 1999). However, this cannot be resolved completely yet and remains subject to speculation.

Current research results indicate that a primary, primitive vegetative desiccation tolerance in cryptogams and a secondary, derived vegetative desiccation tolerance in vascular plants can be distinguished (Bartels et al. 2011). In vascular plants vegetative desiccation tolerance was at least partly regained from reproductive organs because some mechanisms from orthodox seeds are applied to vegetative tissues as well (Farrant & Moore 2011; Illing et al. 2005). The loss of desiccation tolerance and the resulting change to a drought avoiding strategy can be explained by the desiccation tolerance productivity trade-off hypothesis (Alpert 2006). Desiccation-tolerant organisms are relatively small as mentioned earlier. This cannot solely be explained by the difficulty of a re-establishment of a continuous water column upon rehydration. Desiccation tolerance seems to come with a cost, i.e. slow growth (Alpert 2006). This reduced productivity is not advantageous as it reduces competitiveness. In more zonal habitats desiccation-tolerant plants would naturally be outcompeted by desiccation-sensitive plants with a much higher growth rate. Although growth and tolerance seem to be correlated, desiccation tolerance might also have disappeared in now sensitive plants because it was not relevant for selection anymore (Alpert & Oliver 2002). *Lindernia brevidens* (Linderniaceae), desiccation-tolerant itself and a close relative of the desiccation-tolerant model organism *Craterostigma plantagineum*, adds to the complexity of the issue (Phillips et al. 2008). This species is a neo-endemic in east African relict rainforests. Surprisingly, it has not lost its ability to tolerate desiccation

although it seems to be superfluous under the given environmental conditions. This is partly attributed to a high genome stability and a lack of selection pressure, i.e. a higher growth rate coupled with the loss of desiccation tolerance was not necessary in this habitat.

Finally, and this can either be rated as a specific adaptation or as a current evolutionary process, some species are only partly desiccation-tolerant. This does not refer to seasonal desiccation tolerance such as in the fern *Mohria caffrorum* or *Polypodium vulgare* (Farrant et al. 2007a; Kessler et al. 2007) or life stage-triggered desiccation tolerance as in some ferns (Kappen & Valladares 1999). Some species have organs which are only partly desiccation-tolerant, e.g. the Poaceae *Eragrostis nindenis* (Vander Willigen et al. 2003). In *Eragrostis hispida* a lower percentage of tissue is desiccation-tolerant. Here, only the first few centimeters of the leaf have been shown to be desiccation-tolerant (Gaff & Ellis 1974). Consequently, Gaff & Ellis (1974) suggest that desiccation tolerance might extend to other leaves as well and is an evolutionary new event and less developed compared to completely desiccation-tolerant species. Another example is the well-studied *Lindernia (Chamaegigas) intrepidus* (Linderniaceae) which has desiccation-tolerant and desiccation-sensitive leaves (Heilmeier & Hartung 2011). Aside from this roots of *L. intrepidus* seem to have a velamen radicum-like layer. Since the plant survives the dry season as a rhizome any structure that facilitates water uptake is naturally of high adaptive value. It is, to date, the only desiccation-tolerant dicot with such a structure, formerly reserved for a variety of (desiccation-tolerant) monocots (Porembski & Barthlott 1995). These results again underline the hypothesis that desiccation tolerance evolved several times in angiosperms and similar traits evolved independently several times as an adaptation to comparable environmental constraints.

On a genetic level a response to drought resp. desiccation is multigenic and extremely complex. It has been hypothesized that the majority of required genes and metabolic capacities for desiccation tolerance are present in all plants; merely a few changes in the developmental program are necessary for a plant to achieve desiccation tolerance (Hartung et al. 1997). Recent studies have shown that desiccation tolerance does not merely seem to rely on differential gene expression. These species possess (often few) novel genes as well as “house-keeping” genes which are common in desiccation-sensitive species as well (Grene et al. 2011; Leprince & Buitink 2010). The existence of

the former has been shown for *C. plantagineum* (Linderniaceae) and *Xerophyta humilis* (Cyperaceae) (Collett et al. 2004; Furini et al. 1997; Hilbricht et al. 2008). A comparison of the desiccation-tolerant lycophyte *Selaginella lepidophylla* (Selaginellaceae) with a desiccation-sensitive *Selaginella* species showed that almost two thirds of the genome were only found in *S. lepidophylla*, suggesting a specific adaptation to abiotic stress (Iturriaga et al. 2006). Obviously, general conclusions cannot be drawn from these results; the adaptations on a genetic level are, as for the other adaptive levels as well, very specific, if not species-specific.

1.4. Relevance of desiccation tolerance research

In accordance with the previous chapter detailed knowledge about mechanisms of desiccation tolerance in plants can be used to shed more light on early evolutionary processes of land plants. Studies of adaptive processes in bryophytes and vascular plants have already given some clues but especially comparative studies should be considered to deduce new hypotheses (Cushman & Oliver 2011).

Another reason to study desiccation-tolerant plants lies in the fact that stress responses are usually interconnected (e.g. Bartels & Sunkar 2005). Understanding the responses to drought and desiccation can be complementary to other stress response categories and vice versa, e.g. salt and light stresses. Since these environmental factors are often times relevant for desiccated plants as well extensive research in this area can have valuable synergistic effects. These data are highly relevant for agroecological and biotechnological research efforts.

The vast majority of agriculturally viable land is currently under cultivation, resources are limited but the world population is increasing rapidly (Anonymous 2004). Additionally, climate change has already altered, and will continue to, change precipitation regimes around the globe. This will have fundamental effects on agriculture: yields will be less reliant which does not only have economic but social and ecological effects as well. However, to secure the global food production yields have to be secured. In other words: the productivity of the cultivated land has to be raised albeit, at least regionally, severe weather changes. One aspect to target this fundamental problem is the breeding of better-adapted varieties. Although this practice dates back to

the Neolithic revolution it is extremely time-consuming and not always successful. Biotechnological approaches may be time-consuming as well but should be considered as a viable alternative. Research to generate more drought-tolerant crops is currently at full blast. A few decades ago, the potential of desiccation-tolerant grasses as fodder had already been realized (Gaff 1986; Gaff & Ellis 1974). Today, BASF, Monsanto and other agro-technology companies invest a substantial share of their budget in this part of their research and development branch (Anonymous 2011). Under this respect the elucidation of desiccation tolerance in plants becomes highly relevant. The alteration of specific physiological components such as sugar metabolism, which is known to be a key component in drought- and desiccation-tolerant plants, has led to a higher drought and osmo-tolerance in some species (e.g. Holmström et al. 1996; Kishor et al. 1995; Romero et al. 1997). However, negative side effects such as stunted growth are not unusual. Consequently, more in-depth research is required to understand the exact mechanisms of desiccation tolerance. This includes research on the morpho-anatomical level as well. This aspect has largely been omitted over the past few years although it is a vital part for the complete understanding of desiccation tolerance in plants. Several ultrastructural studies (e.g. Dalla Vecchia et al. 1998; Moore et al. 2007a; Vander Willigen et al. 2003) exist but no extensive comparative studies about morpho-anatomical characteristics of desiccation-tolerant plants are available because only the physiological, biochemical and molecular levels have long been regarded to be essential for our understanding of desiccation tolerance. The exact role, quality and quantity of morpho-anatomical features are therefore an important contribution to our *complete* understanding of desiccation tolerance.

1.5. Objectives of the doctoral thesis

As mentioned earlier adaptations to desiccation tolerance occur on the morpho-anatomical, physiological, biochemical and molecular level. Many researchers deny unique general morpho-anatomical adaptations of desiccation-tolerant plants but they usually integrate several plant groups such as bryophytes and angiosperms. Within one major plant group, e.g. vascular plants, the existence of common morpho-anatomical traits has never been tested extensively in a comparative study. Therefore, the main goal

of the doctoral thesis was to make a fundamental contribution to the morpho-anatomical characteristics of desiccation-tolerant angiosperms (and pteridophytes to some extent) and their possible adaptive links.

The over-arching questions were if, how and to what extent desiccation tolerance is represented on the morpho-anatomical level in desiccation-tolerant vascular plants, especially angiosperms. Consequently, the morpho-anatomical changes of a variety of desiccation-tolerant angiosperms during de- and rehydration were analyzed. First, a suitable method for this analysis and a proper anhydrous fixation method had to be developed. Second, important morpho-anatomical structures vital for the structural maintenance of de- and rehydration cycles were identified and their relative adaptive values were determined. Third, as a summary of the results, the existence or absence of a morpho-anatomical syndrome in desiccation-tolerant vascular plants was discussed extensively.

In addition to research on the morpho-anatomical level an ecological field study was conducted in Côte d'Ivoire to test the desiccation tolerance productivity trade-off hypothesis. As a first step for a large scale study a non-destructive method for the growth measurement of the desiccation-tolerant Cyperaceae *Afrotrilepis pilosa* was developed and tested in the field.

2. Anatomical analysis of turgescent and semi-dry resurrection plants using X-ray micro-computed tomography (µCT)

The following chapter is largely based on the following published article:
Korte, N., Porembski, S. (2011). Anatomical analysis of turgescent and semi-dry resurrection plants: the effect of sample preparation on the sample, resolution, and image quality of X-ray micro-computed tomography (µCT). Microscopy Research and Technique 74: 364-369.

2.1. Abstract

Computer tomography has been used frequently for the 3-D visualization of plant anatomical traits but sample preparation has been widely neglected. Without any preparation smaller (i.e. up to 1x1 cm) turgescent or semi-dry plant samples (especially leaf samples) diminish the image quality of a scan due to gradual water loss and therefore constant movement. A suitable preparation for scans of turgescent and semi-dry plant samples with a high resolution µCT (< 1-5 µm) has to be very thin, heat-resistant (up to 35 °C), have a low attenuation coefficient and should not alter the water content and structure of the sample. Several agents have been tested but only a coating with vaseline conserved the water content of a plant sample efficiently. However, water molecules and vaseline both attenuate the X-ray beam, which decreases the image quality of scans of turgescent or semi-dry plant samples. Therefore, trade-offs between the spatial resolution, sample water content, sample size and image quality have to be considered: larger samples have to be placed further away from the X-ray tube, which leads to a lower spatial resolution; water and preparation agents attenuate the X-ray beam, causing low-quality images which may be accompanied by motion artifacts compared to a scan of a dry sample where no preparation is necessary.

2.2. Introduction

The invention of the microscope enabled researchers to examine fine structures of objects thoroughly. In terms of botanical research turgescent material of plants can

easily be cut, placed in water and examined under a light microscope. Although this has opened a wide range of possibilities classic light microscopic examination methods are only partly useful when desiccation-tolerant plants are involved. This refers to the visualization problem of dry tissue. Starting in the 1970s researchers interested in desiccation-tolerant plants realized that fixation methods were crucial to gain a thorough understanding of morpho-anatomical features of desiccation-tolerant plants in the turgescent and dry state alike (Hallam 1976; Hallam & Luff 1980). With the onset of scanning electron microscopy and its vast distribution and application in various scientific disciplines the problems of fixation arose again. Since water-containing tissues cannot be placed in the sample chamber of an SEM because this would impact the detector negatively and therefore the image quality, water-replacing fixation methods had to be developed. Until today studies still focus on the finding of a decent and preserving anhydrous fixation method (e.g. Platt et al. 1997; Vander Willigen et al. 2003). Aside from immersion oil (e.g. Gaff et al. 1976) these methods usually involve several chemical fixatives or freeze substitution and are usually developed for SEM and TEM as the methods from early studies as well. Although these are undeniably important contributions to examine the ultrastructure of desiccation-tolerant plants in different stages of the de- and rehydration cycle the question whether fixation or hydration comes first remains partly unresolved. One extreme answer to this perpetual dilemma is the use of no fixation at all. Turgescent samples can easily be examined in an environmental scanning electron microscope (ESEM). The detector of an ESEM is less sensitive compared to a traditional SEM. Water vapor from the sample is not a major problem but it comes at a cost as well: images can be less accurate compared to SEM but this is certainly not always the case. Theoretically, a gradual dehydration cycle can be monitored using an ESEM but a severe cooling to 4 °C of the pressure chamber is necessary before the internal relative humidity can be gradually altered. This certainly has a negative effect on the unfixed sample. The sample movement can also be impaired because even fresh leaf or root material has to be fixed on the metal support. This does not necessarily include glue or other chemical components since the sticky surface of the metal support is usually sufficient. Nevertheless, it prevents the natural folding process of the sample which would then not be visible in an ESEM either. However, aside from own unpublished work, an ESEM has not been widely used for the study of

the anatomy of desiccation-tolerant plants. Depending on the research question, the potential of this technology has certainly not been fully tapped. The patchy distribution at universities or research institutes compared to SEM and the technological challenges involved with the continuing alteration of pressure in the pressure chamber during the dehydration process may be blamed for this. However, the use of an ESEM should definitely be kept in mind for future research efforts concerning the alterations of morpho-anatomical traits of desiccation-tolerant plants.

A major disadvantage of all techniques discussed above is that they only allow a 2-D sample analysis. The examination of several continuous thin sections of one sample can result in a 3-D structure but this procedure is very time-consuming and therefore rarely used today. Micro-computed tomography however is an excellent (and fairly new) tool to study the precise 3-D structure of objects, including plants. Since its invention by Hounsfield and Cormack in 1971 computer tomography has become a major tool in diagnostic medicine (Bautz & Kalender 2005). Computer tomographs (CTs) non-destructively generate 2-D digital images of a sample by the use of X-ray radiation. The 2-D digital images are reconstructed and further processed by the application of a 3-D volume rendering software (e.g. Imaris© (Bitplane) or VG Studio Max© (VolumeGraphics)) for the visualization and close-up analysis of the inner and outer 3-D sample structure. Fromm et al. (2001) and Steppe et al. (2004) showed that results of measurements of wood anatomical structures obtained from a µCT (a high-resolution CT, < 1-5 µm, values set for the µCT used in this study) correspond to those obtained by microtomy.

Since its beginnings computer tomography has been applied to many disciplines (for an overview cf. Kaick & Delorme 2005) and it has been perceived as a powerful tool for plant anatomists, especially wood anatomists (Fromm et al. 2001; Hattori & Kanagawa 1985; Steppe et al. 2004; Van den Bulcke et al. 2010; Watanabe et al. 2008). Although studies from plants other than trees have been published as well (Lontoc-Roy et al. 2006; Mendoza et al. 2006; Verboven et al. 2008) the potential of micro-computed tomography for plant (not wood) anatomical studies has not been fully realized.

Nevertheless, there are constraints to the use of micro-computed tomography as well (Wildenschild et al. 2002). Artifacts can lower the quality of the scan. Motion artifacts, for instance, are induced by sample movement during the scan. Beam hardening

artifacts occur if an object consists of different components with different attenuation coefficients (e.g. water and plant tissue). Additionally, the correct sample preparation may pose a problem. Although some researchers (e.g. Stuppy et al. 2003) state that micro-computed tomography of plant material does not require any further preparation; this largely depends on the research question and the tissue type. To circumvent the problem of preparation and the occurrence of artifacts by plant movement due to gradual water loss some researchers propose minimizing the measurement time and adjusting the measurement parameters (Kaminuma et al. 2008). Hereby, the length and quality of a scan are naturally limited and the results not accurate. Over the past few years in vivo μCTs have been developed which allow the scanning of live tissues but the resolution of these scanners is relatively low compared to other μCTs (~ 9 μm for the SkyScan 1176 *in-vivo* hi-res micro-CT) (SkyScan 2010).

However, when using a μCT for the study of plant anatomical traits of turgescent or semi-dry plant material, the sample has to be conserved properly without altering its inner structure and moisture content. Kaminuma et al. (2008) also referred to the interdependence of sample size (i.e. volume measurement) and spatial resolution: the smaller the plant sample and the closer it can be placed in front of the X-ray tube, the higher the resolution (even values less than 1 μm can be reached). Therefore, a suitable sample preparation allows only a thin coat of a heat-resistant (up to 35 °C) fixation material with a low attenuation coefficient. If the sample happens to be longer than approx. 2 cm, several consecutive scans of the same object can be executed.

Since there have not been many satisfactory considerations about plant sample preparations in any study using micro-computed tomography, different chemical and non-chemical fixation methods which have been tested for their suitability and accuracy will be elaborated on. Since one main objective was the examination of anatomical changes during the dehydration process of resurrection plants, which has never been documented, leaf samples of different water contents were to be scanned non-destructively to visualize the successive anatomical changes. As a prerequisite to this a suitable preparation method for turgescent or semi-dry plant samples using micro-computed tomography had to be identified which will be reported in this paper. It should, however, be noted that the effect on the fine structure of each sample was not

examined; merely the size changes were considered in order to deduce the effect of the preparation method on the sample.

2.3. Material and Methods

Leaves of the west African desiccation-tolerant species *Afrotrilepis pilosa* (Boeck.) J. Raynal (Cyperaceae), cultivated in the Botanical Garden of the University of Rostock, were used for this study. For each preparation method, five small pieces (~ 0.2x0.5 cm) of turgescent leaf material were cut (with one oblique end to measure the top and bottom width of the sample, Fig. 4).

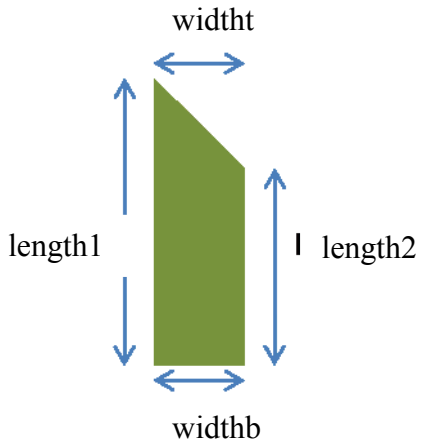

Figure 4 Schematic sketch of a leaf sample.

The length and width of each sample was measured using a Zeiss Stemi 2000C stereo microscope with a measurement ocular. The magnification for all samples remained the same, with one bar in the ocular representing 0.0769 mm. Leaf samples were coated with the following agents for approx. 24 hours:

1. Water-based agents

- water-based acryl polish (C. Kreul, Germany)
- gelatine (RUF Sofort Gelatine, on cold water basis)
- silicone rubber compound (solvent-free, contains acetoxysilane, RS Components, Northants, UK)
- water-based glue (UHU Bastelkleber, UHU, Germany)

2. Solvent-based agents

- glue (UHU hart, UHU Alleskleber, Pattex Blitzkleber, Henkel, Germany)
- clear nail varnish (Manhattan, Manhattan Cosmetics, UK, containing acetone)
- hair spray (which is frequently used to temporarily conserve ornamentals)
- plant impregnation agent (Glorex of Switzerland)
- bio-preparation agent (Glorex of Switzerland)
- xylene-free Canada balm (Merck, Germany)
- liquid cover glass, containing toluene (Merck, Germany)

3. Paraffin-based agents

- hot candle wax
- white vaseline (Bombastus, Germany)
- paraffin (Merck, Germany, solidification point 42-44 °C): samples were coated with paraffin shortly before its solidification point. Variations were the over-coating with acryl polish to prevent a possible softening of the paraffin coat and the wrapping of the sample in thin paraffin plates. Some samples were placed in a contrast agent for 48-72 hours (Imeron© 150, Bracco Imaging, Germany; 1:100 and 1:10 or iodine) to visualize the inner structure.
- thin wax plate: Samples were wrapped in a thin wax plate.

4. Other

- glycerol (85%): after having been treated with glycerol for 24 hours, samples were allowed to air-dry and were measured again to determine the size alteration during the drying process.
- glycerine (99.9%): used as plasticizer in light microscopy (glycerine-water mix, cf. Jansen et al. 1998). After the treatment all samples were dried and measured again.
- sunflower oil: after the treatment all samples were dried and measured again.
- freeze-drying: samples were stored on ice in plastic cubes for one hour and then kept in a lyophylle for 22 hours.
- critical point drying: samples were fixed as follows: 30 min glutaraldehyde, rinsed in sodium phosphate and 30% ethanol, fixed for 10 min in 50% ethanol,

10 min in 75% ethanol, 15 min in 90% ethanol and 20 min in absolute ethanol. The fixed samples were dried in a Polaron CPD 7501 (Polaron, UK).

- shrink-wrapping (Clatronic FS 777, Germany): samples were either shrink-wrapped with or without a thin coat of vaseline.

If the preparation agent did not (or hardly) alter the size of the sample, semi-dry plant samples were examined as well, scanned with a µCT (nanotom© 180 NF, moveable detector and object holder, max. resolution: 0.6 µm as specified by the manufacturer (Phoenix X-Ray, Fig. 5) and measured under the stereo microscope immediately after the scan to monitor possible size effects of the scan (Table 1).

Figure 5 A look inside the nanotom© 180 NF. The sample is glued on the glass rod which is fixed in the moveable CNC (computer numerical control, right). Next to the CNC the X-ray tube is visible, the detector is situated left of the CNC (out of frame).

Table 1 List of the scanning parameters of the most promising preparation methods. FOD = distance of the sample from the X-ray tube [mm], FDD = distance of the detector from the X-ray tube [mm]. The parameters differed slightly but remained in the same range.

	paraffin	wax plate	vaseline
magnification	56.37	61.45	38.68
voxel size	0.00177394 (1.77 µm)	0.00162731 (1.62 µm)	0.00258521 (2.58 µm)
FOD	4.08	4.88	5.17

FDD	229.99	299.99	200.01
no of images	720	1 080	720
binning	1	1	1
voltage/current	30/260	30/250	30/250
mode	0	0	0

The samples were either glued on a glass rod without direct contact to the glue (i.e. fixed in modeling material which had been glued on the glass rod for increased stability) or fixed in a glass capillary (3-4 mm diameter, depending on the size of the leaf sample) in paraffin deep enough to prevent the sample from moving during the scan. The resolution reached from 1.24 µm to 4 µm, depending on the sample size, the distance from the X-ray tube and the position of the detector. For the image acquisition datosacq© (Phoenix X-Ra, Germany), for the image reconstruction datosx© (Phoenix X-Ray), for the 3-D evaluation VG Studio Max© (VolumeGraphics, Germany) and Imaris© 6.3.1 (Bitplane AG, Germany) were applied. The program SAS 9.2. (SAS Institute, USA) was used to compute the arithmetic mean and standard deviation of the size alteration of all five samples per preparation method, expressed as a fraction (%) of the initial size.

2.4. Results

Most of the tested preparation agents altered the size (and therefore presumably the ultrastructure) of the plant sample. Table 2 displays the size-effect of each preparation agent.

Table 2 Size effects of all preparation methods and agents on turgescent samples. All values are given as a fraction (%) of the initial size. Negative values indicate shrinkage, positive values size expansion resp. sample swelling. Widthb=width bottom, widtht=width top, length1, length2= length left resp. right.

treatment	variable	mean	std dev	minimum	maximum
bio-preparation agent	widthb	-32.74	14.04	-53.30	-17.40
	widtht	-27.66	7.65	-40.70	-20.80
	length1	-2.98	2.04	-4.90	0.00
	length2	-3.90	1.08	-5.00	-2.30
glycerine, dry sample	widthb	-2.28	6.14	-9.10	3.20
	widtht	-3.02	10.38	-18.90	9.40

	length1	0.00	0.99	-1.40	1.40
	length2	1.24	2.04	0.00	4.70
glycerine	widthb	-8.86	9.45	-20.00	0.00
	widtht	-8.90	11.61	-27.00	0.00
	length1	-0.94	1.76	-3.30	1.40
	length2	0.92	2.38	-1.60	4.70
glycerol	widthb	-0.12	4.53	-6.70	6.10
	widtht	-3.34	4.72	-10.00	0.00
	length1	0.00	0.00	0.00	0.00
	length2	-0.36	0.80	-1.80	0.00
glycerol, dry sample	widthb	2.54	2.59	0.00	6.10
	widtht	4.32	12.12	-6.70	25.00
	length1	0.98	1.53	-3.50	0.00
	length2	-1.66	1.92	-4.70	0.00
plant impregnation agent	widthb	-29.04	3.19	-32.40	-24.20
	widtht	-32.36	2.19	-35.30	-29.40
	length1	-2.62	1.88	-5.30	0.00
	length2	-3.58	2.52	-7.10	0.00
sunflower oil	widthb	-9.86	9.06	-24.20	0.00
	widtht	-8.18	6.41	-18.80	-3.20
	length1	0.60	0.83	0.00	1.70
	length2	1.36	4.54	-2.10	9.10
sunflower oil, dry sample	widthb	-47.00	5.87	-52.90	-39.30
	widtht	-49.62	4.44	-52.90	-41.90
	length1	-4.78	4.19	-11.30	0.00
	length2	2.36	6.96	-6.30	11.30
critical point drying	widthb	-4.08	4.27	-9.70	0.00
	widtht	-3.52	4.09	-10.00	0.00
	length1	-0.74	1.03	-2.10	0.00
	length2	0.00	0.00	0.00	0.00
UHU glue	widthb	-20.52	4.41	-25.00	-13.60
	widtht	-28.28	10.04	-44.00	-17.40
	length1	-3.72	3.07	-7.40	0.00
	length2	-0.10	3.36	-2.00	5.90
water-based acryl polish	widthb	-11.26	16.98	-21.10	18.80
	widtht	-20.42	3.35	-25.00	-16.70
	length1	-0.52	1.16	-2.60	0.00
	length2	0.00	0.00	0.00	0.00
nail polish	widthb	-25.50	13.45	-41.20	-5.30
	widtht	-29.38	10.25	-41.20	-14.30
	length1	-1.04	1.43	-2.80	0.00
	length2	1.80	2.61	0.00	5.70
warm wax	widthb	-3.60	5.01	-10.30	0.00
	widtht	-3.68	7.83	-12.00	7.40
	length1	-6.18	13.32	-29.70	2.40
	length2	-2.80	2.90	-6.90	0.00
water-based glue	widthb	-8.16	5.43	-12.50	0.00
	widtht	-1.06	4.62	-5.90	5.60

	length1	-0.34	0.76	-1.70	0.00
	length2	-0.34	0.76	-1.70	0.00
cover glass	widthb	-19.48	9.20	-30.40	-5.00
	widtht	-21.54	7.92	-30.40	-10.00
	length1	-2.14	3.06	-4.50	3.20
	length2	-1.64	2.66	-4.80	1.90
freeze-drying	widthb	-20.04	5.60	-26.10	-11.10
	widtht	-31.58	1.52	-33.30	-29.60
	length1	-6.48	2.90	-9.50	-3.20
	length2	-3.48	2.29	-7.40	-1.80
paraffin	widthb	-1.92	2.63	-4.80	0.00
	widtht	-2.12	2.91	-5.60	0.00
	length1	0.00	0.00	0.00	0.00
	length2	0.00	0.00	0.00	0.00
paraffin, semi-dry sample after 45 min	widthb	0.00	0.00	0.00	0.00
	widtht	-2.22	4.96	-11.10	0.00
	length1	-0.94	2.10	-4.70	0.00
	length2	0.00	0.00	0.00	0.00
shrink-wrapping	widthb	-9.90	9.20	-25.50	-4.30
	widtht	-10.74	9.19	-23.40	-2.20
	length1	-0.90	0.83	-1.70	0.00
	length2	-0.38	0.85	-1.90	0.00
vaseline	widthb	0.00	0.00	0.00	0.00
	widtht	0.00	0.00	0.00	0.00
	length1	0.00	0.00	0.00	0.00
	length2	0.00	0.00	0.00	0.00
vaseline, semi-dry sample after 30 min	widthb	0.00	0.00	0.00	0.00
	widtht	0.00	0.00	0.00	0.00
	length1	0.00	0.00	0.00	0.00
	length2	0.00	0.00	0.00	0.00
vaseline, glued with silicone rubber compound	widthb	-7.64	6.19	-16.70	0.00
	widtht	-4.40	4.63	-11.10	0.00
	length1	0.00	0.00	0.00	0.00
	length2	9.68	17.08	0.00	40.00
wax plate	widthb	-0.76	1.70	-3.80	0.00
	widtht	-0.84	1.88	-4.20	0.00
	length1	-0.28	0.63	-1.40	0.00
	length2	0.00	0.00	0.00	0.00

The solvent-based agents such as nail polish or hair spray induced shrinkage of the samples. Therefore, only the results of standard preparation methods and promising agents are described below. The fixation for the critical point drying did not affect the samples severely (maximum deviation was -8.7%, cf. Table 2) but the drying itself did.

Freeze-drying as another exponent of the standard conservation techniques did not conserve the inner structure, either.

The wrapping in cold wax did not have a negative impact but although the sample was closely surrounded by the wax plate, it shrunk during the scan due to a small air slot between the sample and the wax coat (Fig. 6). The formation of this tiny air slot might be prevented by pressing the sample into the wax which on the other hand could impair the sample.

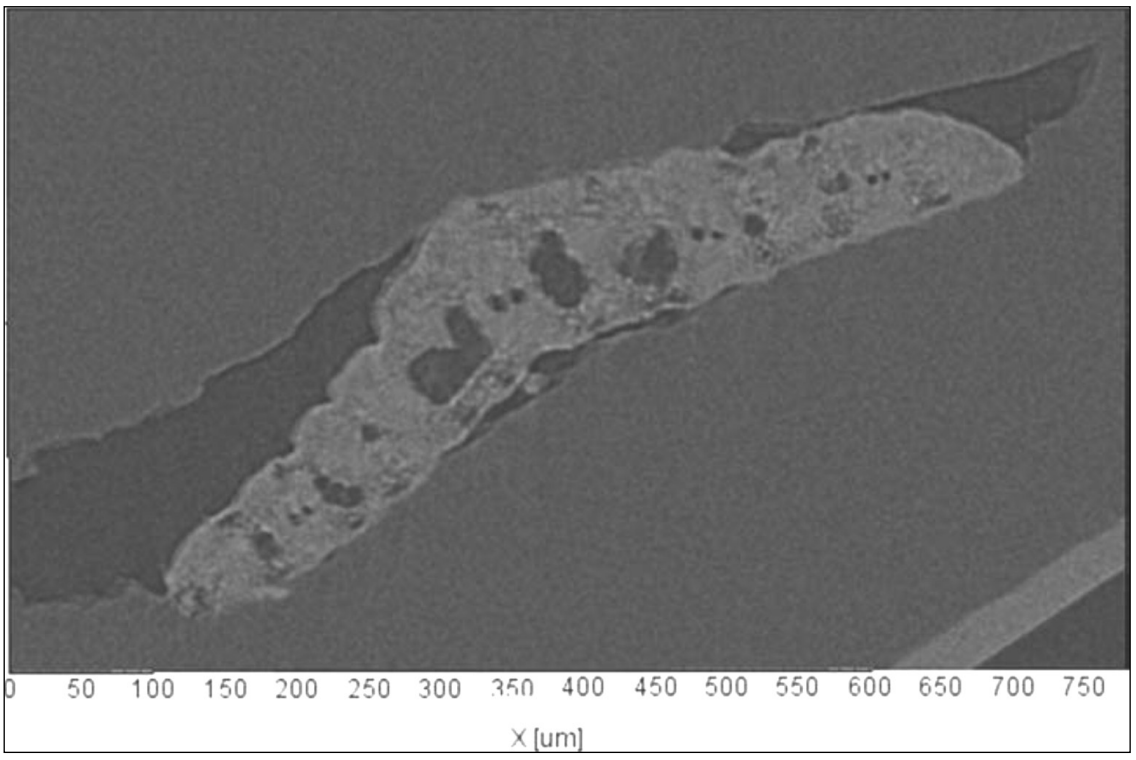

Figure 6 A leaf section of *Afrotrilepis pilosa* wrapped in a thin wax plate (voxel size: 1.62 µm). The air slot mentioned between the sample and the wax is very prominent. The resolution is relatively high but the sample dried during the scan. The low water content explains the good visualization of the inner structure.

Similar to the preparation with wax, the layer around a sample coated with paraffin is relatively thick. An air slot cannot be prevented and the X-ray beam is attenuated which has a negative effect on the image quality: the mesophyll and some vessels are visible but finer structures cannot be distinguished. Figure 7a shows that – compared to a scan of a dry leaf section of *A. pilosa* (Fig. 7b) - even a thin paraffin layer diminishes the image quality. The combination of a contrast agent (Imeron© 150, Bracco Imaging or iodine) and paraffin did not yield better images either.

Shrink-wrapping did not conserve the sample perfectly. Since the samples were not shrink-wrapped too tightly in order to maintain the structure of the plant, a relatively large air space remained. In addition, the plastic surrounding the sample diminished the quality of the scan.

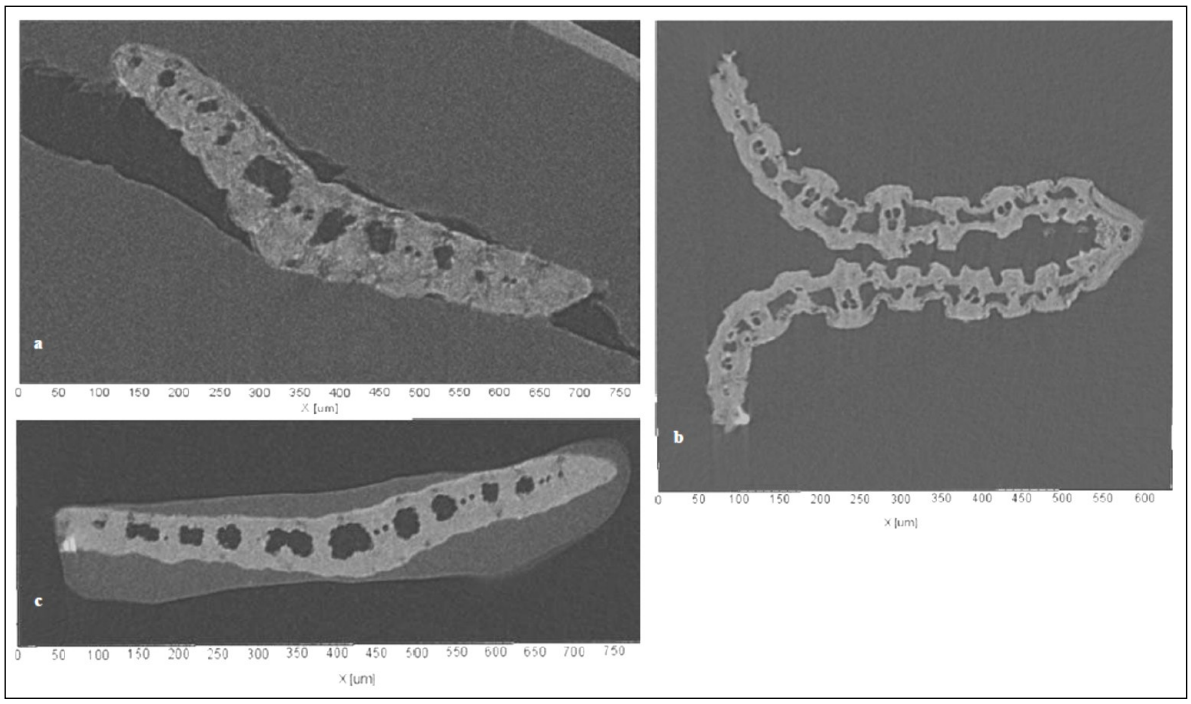

Figure 7a-c a - A leaf section of *Afrotrilepis pilosa* coated; b - A dry leaf section of *Afrotrilepis pilosa*; c - A leaf section of *Afrotrilepis pilosa* immersed in vaseline (grey layer around the leaf) (voxel size: 2.58 µm).

No negative size-effects were detected among samples conserved with a thin coat of vaseline for 24 hours or even after longer scans (i.e. 720 or 1 080 images) (Fig. 7c). Vaseline was the only agent which prevented the plant sample from dehydration and preserved the inner structure (Table 2). Nevertheless, the image quality of a dry sample was much higher compared to a water-containing and vaseline-wrapped sample (cf. Fig. 7b). Similar to the preparation with wax and paraffin structures and tissues are easier to delineate. The mesophyll does not appear blurry and more vessels can be distinguished.

2.5. Discussion

Standard preparation methods such as critical point drying or freeze-drying did not prove to be suitable for the exact conservation of turgescent or semi-dry plant samples. Only the coating with vaseline conserved the exact water content of a sample. However, if a very high resolution is desired, the image quality of dry samples is much higher compared to those of turgescent vaseline-wrapped plant samples. Therefore, for the examination of samples during the de- and rehydration process other methods might be applied if a high image quality is desired and only dry samples could be examined using a µCT.

Paraffin and vaseline have been used successfully for the sealing of soil and plant material before (Groner 1936; Kuroda et al. 2006; Lindgren 1991). In the case of paraffin, the sample has to be coated shortly before the solidification point to prevent a sample size alteration. This lowers the liability of this method. In general, it is also better suited for larger samples, because the coating of small samples needs some training before one can skillfully coat small samples. Therefore, the use of paraffin can only be recommended with certain limitations. The same applies to medical contrast agents since most of them contain varying amounts of muriatic acid. Aside from that, in the dry state a whole leaf could be scanned whereas only half a leaf in the turgescent or semi-dry state was scanned at a similar resolution due to the size increase as a consequence of the coating.

It has to be noted that all agents and methods have specifically been tested under the question of the exact conservation of turgescent or semi-dry plant tissue without altering its ultrastructure and moisture content. Nevertheless, they could be of interest under different research questions and for different species and plant parts as well. However, regardless of the research question, any turgescent or semi-dry plant sample should be conserved prior to a µCT scan. This does not only include the preservation of the actual water content but the mechanical fixation of the sample on the glass rod as well. If a proper preparation is neglected, the sample moves due to gradual water loss and the image quality of the scan is very poor.

All above-mentioned agents and methods have been tested on *Afrotrilepis pilosa*, a Cyperaceae whose leaves contain a large amount of sclerenchyma. Other species with less supporting tissue may not necessarily be suited for this preparation. Here, the

mechanical fixation becomes very important. Samples might have to be stabilized first or only plant parts with more supporting tissue (e.g. leaf blade, stem) should be considered suitable for a preparation with vaseline. In other biological disciplines the coating of contrasted samples with vaseline may even be more promising.

However, the general problem of water-containing samples remains untouched by the preparation method. Water molecules – like any other matter - attenuate the X-ray beam, which can result in low-quality images. This may be worsened by motion artifacts due to sample movements. If a very high spatial resolution (< 1-5 µm) and the exact inner structure of a small sample (i.e. up to 1x1 cm) is intended, scans of dry plant samples yield better results than those of turgescent or semi-dry plant samples. The occurring image artifacts are less prominent with a lower spatial resolution. Therefore, trade-offs between the spatial resolution, sample water content, sample size and image quality of turgescent or semi-dry plant samples have to be considered: larger samples have to be placed further away from the X-ray tube, which can lead to a lower spatial resolution; water and preparation agents attenuate the X-ray beam, causing artifacts and low-quality images. Nevertheless, even if a lower resolution is desired where image artifacts are less obvious, the turgescent or semi-dry sample should be treated with vaseline to ensure a proper conservation and to prevent a constant sample movement.

3. A morpho-anatomical characterization of *Myrothamnus moschatus* under the aspect of desiccation tolerance

The following chapter is largely based on the following published article:
Korte, N., Porembski, S. (2012). A morpho-anatomical characterisation of *Myrothamnus moschatus* (Myrothamnaceae) under the aspect of desiccation tolerance. Plant Biology 14: 537-541.

3.1. Abstract

Morpho-anatomical traits of the rarely studied dicotyledonous desiccation-tolerant shrub *Myrothamnus moschatus* were examined and compared to *Myrothamnus flabellifolius* under the aspect of desiccation tolerance for the first time. Both species almost exclusively occur on rock outcrops and differ mainly in their geographic range and leaf morphology (fan-shaped in *M. flabellifolius*, lanceolate in *M. moschatus*) but have a very similar leaf and wood anatomy except for the lack of hydathodes in *M. moschatus*. Both species adopt the parallel leaf venation of monocots although this is more pronounced in *M. moschatus*. This is a mechanical and protective advantage over the net venation of most dicots and facilitates the reversible, drought-induced accordion-like leaf contraction. The amount of sclerenchyma as a stabilizing tissue is low and mainly confined to vascular bundles in leaves of both species. Here, mechanical support seems to be less crucial for the survival of long periods of drought compared to other morpho-anatomical traits (e.g. a parallel leaf venation).

3.2. Introduction

Myrothamnus flabellifolius Welw. and *Myrothamnus moschatus* Baill. comprise the Myrothamnaceae (dicots, Gunnerales). Both species can be found on rock outcrops either in southern Africa from Namibia to Angola and eastern South Africa to Kenya (*M. flabellifolius*) or endemic to Madagascar (*M. moschatus*) (Kubitzki et al. 1998). The Myrothamnaceae are exceptional because (a) they constitute two of only approx. 40 desiccation-tolerant dicots (Porembski 2011); the vast majority of the estimated 1 300

desiccation-tolerant vascular plants are monocots and pteridophytes (Porembski 2011). (b) The family has – in comparison to other desiccation-tolerant dicots – a rather basal position in the core-eudicots, close to *Gunnera* (Kubitzki et al. 1998) and (c) both Myrothamnaceae are shrubs (height of up to 1.5 m), which is highly unusual for desiccation-tolerant plants because the elimination of xylem embolism during rehydration becomes more difficult with increasing plant height (Sherwin & Farrant 1996). In contrast to *M. flabellifolius*, *M. moschatus* has rarely been studied (Moore et al. 2007b). Merely its habitat, desiccation tolerance, basic morpho-anatomical descriptions and musk odor have been reported (e.g. Baillon 1870; Carlquist 1990; Niedenzu & Engler 1930). Consequently, morpho-anatomical characteristics of *M. moschatus* are specified here and differences to *M. flabellifolius* are highlighted. Key morpho-anatomical traits of both species were analyzed under the aspect of desiccation tolerance for the first time.

3.3. Material and Methods

3.3.1. Plant material

Material of *M. flabellifolius* was provided by the Botanical Gardens of the University of Bonn (Germany); material of *M. moschatus* was collected near Andranovelona (Madagascar). Stems had a maximum diameter of 0.4 cm.

3.3.2. Rehydration

Three approx. 14 cm long dehydrated shoots of *M. moschatus* were rehydrated for five days at room temperature. Every 24 hours photos were taken to document the rehydration process. The macroscopically visible greening and unfolding of leaves was taken as an indicator for the rehydration process. For the leaf size quantification during rehydration the length and width (top (a few mm below the leaf tip) and bottom of the leaf blade) of five leaves were measured in the dry and rehydrated state with a Zeiss Stemi 2000C stereo microscope (Carl Zeiss Jena, Germany) with a measurement ocular. All leaves were of approx. the same age and were harvested from similar positions on the stem.

3.3.3. Leaf and wood anatomy

For light microscopic studies cross sections of rehydrated leaves of *M. moschatus* were prepared with a Hyrax V50 vibratome (Carl Zeiss Jena) and examined under a Zeiss Axioplan (Carl Zeiss Jena). Dried leaves and stem sections were scanned using micro-computed tomography (= µCT; nanotom© 180 NF, Phoenix X-Ray). For image acquisition datosacq© (Phoenix X-Ray), for image reconstruction datosx© (Phoenix X-Ray), for 3-D evaluation VG Studio Max© (VolumeGraphics) and Imaris© 6.3.1 (Bitplane AG) were applied.

For SEM studies samples of rehydrated leaf material of *M. flabellifolius* and *M. moschatus* were dried (air-dry and critical point drying with ethanol using an Emitech K850 (Emitech, UK). Additionally, small pieces of wood were macerated in equal parts of acetic acid and hydrogen peroxide for 72 hours and left to dry. All samples were glued on a metal support and examined in a SEM (DSM 960 A, Carl Zeiss Optik, Germany, 1991).

3.4. Results

3.4.1. Rehydration

In the dry state the decussate leaves of *M. moschatus* (width and length, dry: c. 0.1x1.5 cm, rehydrated: c. 0.3x1.6 cm) were folded around the stem and had a brownish color (a considerable number of leaves remained green though) like dehydrated leaves of *M. flabellifolius* (width and length, dry: c. 0.3x1.2 cm, turgescent: c. 0.7x1.4 cm) (Moore et al. 2007b) (Fig. 8). 24 hours after rehydration the first leaves turned green and unfolded from the stem. After 48 hours the rehydration process was completed as no further significant changes in morphology and leaf color were observed (Fig. 8).

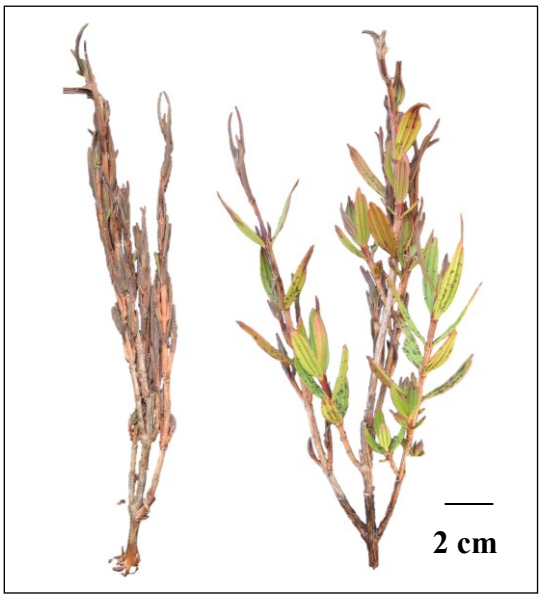

Figure 8 A stem of *Myrothamnus moschatus* in the dry state (left) and rehydrated (right).

3.4.2. Leaf morphology, leaf and wood anatomy

In accordance with Niedenzu & Engler (1930) leaves of *M. moschatus* (and *M. flabellifolius* in that respect) are isolateral with no distinct mesophyll division. Oil cells on the adaxial and abaxial side of the epidermis are very abundant. Some leaves have a reddish color which can be attributed to the occurrence of anthocyanins. In comparison to *M. flabellifolius* leaves of *M. moschatus* are narrower, not fan-shaped but lanceolate as has been pointed out before (cf. Niedenzu & Engler 1930) (c. half the size of *M. flabellifolius* leaves, depending on age and site location). The difference in width at the top and base of the leaf is not significant. Three nearly parallel main veins can be distinguished. During the dehydration process the leaf reduces its width by 40-60%; the reduction in length amounts to only 11% (Figs. 9, 10). This is in contrast to *M. flabellifolius* where the reduction in length is much higher.

Figure 9 Adaxial side of a *Myrothamnus moschatus* leaf (left: rehydrated, right: dry).

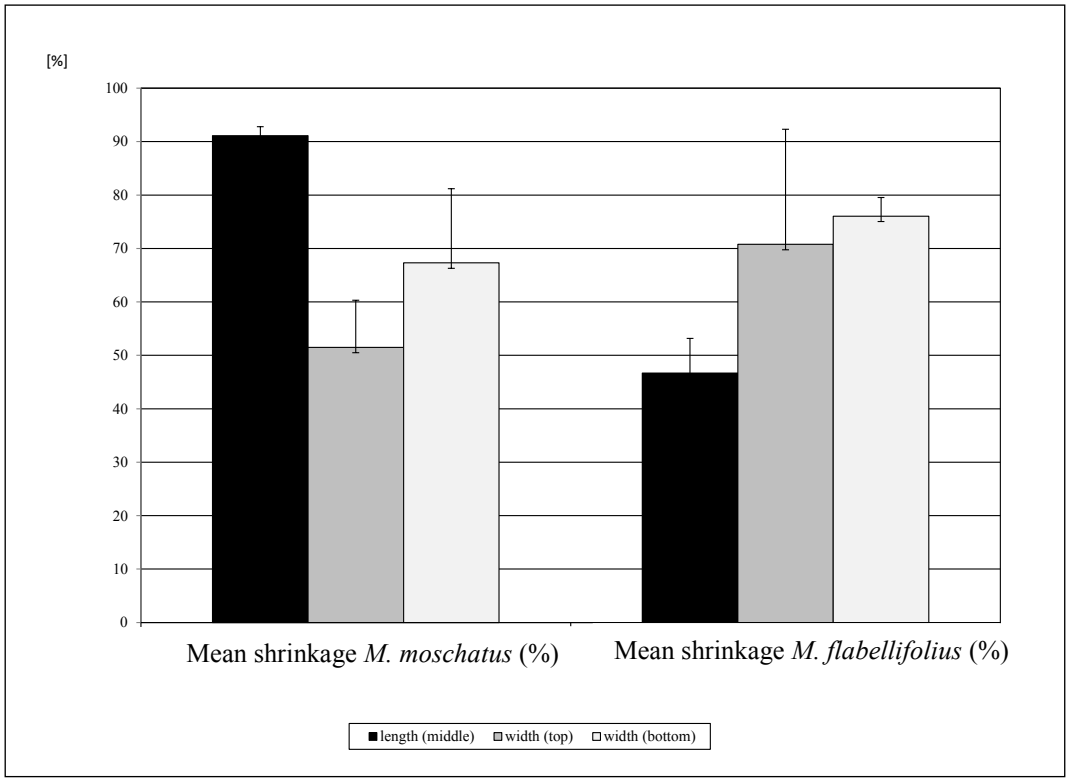

Figure 10 Shrinkage *of Myrothamnus moschatus* and *Myrothamnus flabellifolius* leaves (% of initial size).

Microscopically, an average of 8-10 vascular bundles per leaf can be distinguished. Sclerenchyma is mainly limited to vascular bundles. Neither marginal nor laminar

hydathodes were – in contrast to *M. flabellifolius* – identified using µCT, SEM and light microscopy (cf. Carlquist 1990). As in *M. flabellifolius* bands of horizontal xylem elements, which connect the vertical vascular bundles, were conspicuous in many cross sections (Figs. 11, 12). In contrast to *M. flabellifolius* the stomata are slightly sunken. The distribution is, as in *M. flabellifolius*, relatively even and not confined to furrows (Fig. 13) (Drennan et al. 2009).

The wood shows a very prominent cross-like pith structure which has been pointed out by Niedenzu & Engler (1930) for both Myrothamnaceae. The pith is surrounded by wood which constitutes approx. 90% of the stem. A few layers of secondary phloem follow the cambium (not distinguishable here). The first few layers of wood adjacent to the pith are formed by tracheids and mostly free of vessels, which are abundant in the following layers. Growth rings are inconspicuous. Due to the stem cutting for the µCT sample preparation, the bark appears slightly disrupted (Fig. 14). Images of macerated wood of *M. moschatus* show that the surface of vessels is not – as has been shown for *M. flabellifolius* – equipped with knob-like structures. Table 3 gives a short summary of major morpho-anatomical traits of both species.

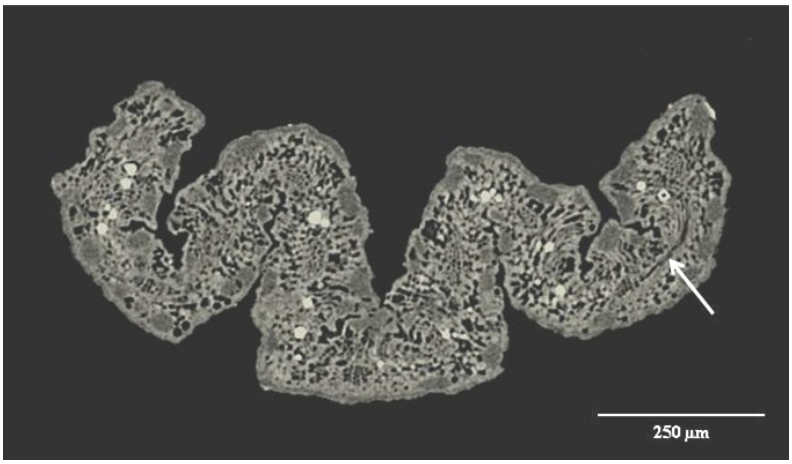

Figure 11 Air-dried leaf of *Myrothamnus moschatus* scanned under a µCT. The arrow indicates horizontal xylem elements.

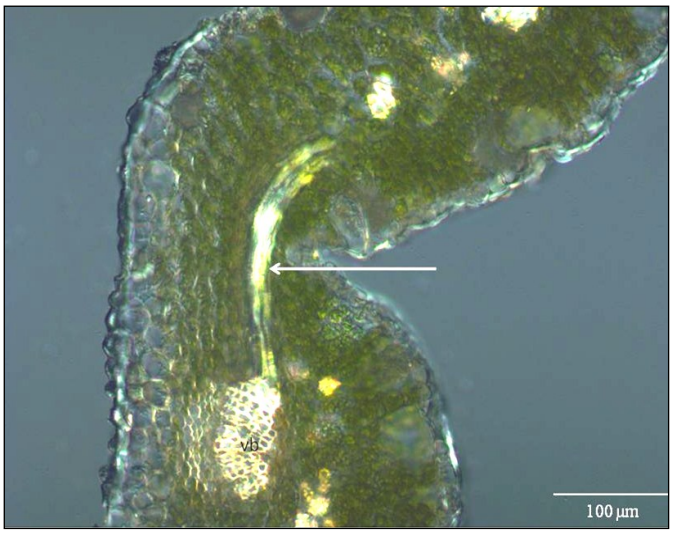

Figure 12 Cross section of a rehydrated *Myrothamnus moschatus* leaf. A vascular bundle (vb) and horizontal xylem elements (arrow) are visible.

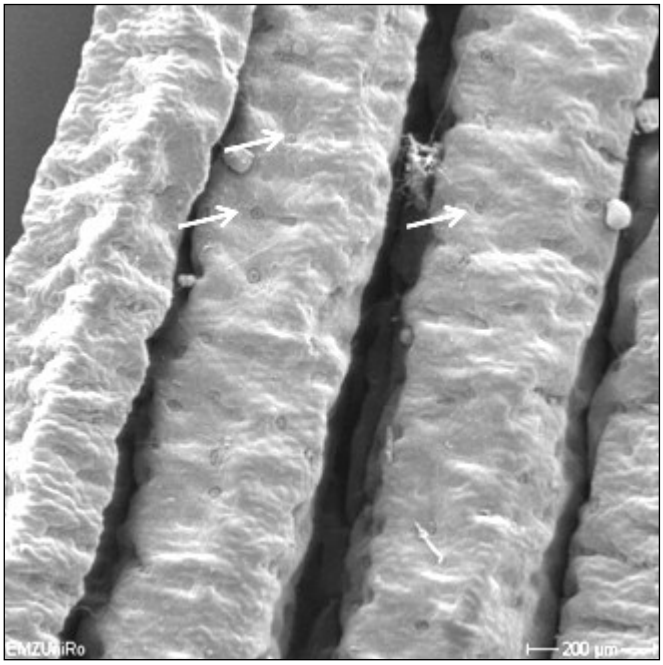

Figure 13 Stoma distribution on the adaxial side of a dry *Myrothamnus moschatus* leaf (SEM). Arrows emphasize a few stomata.

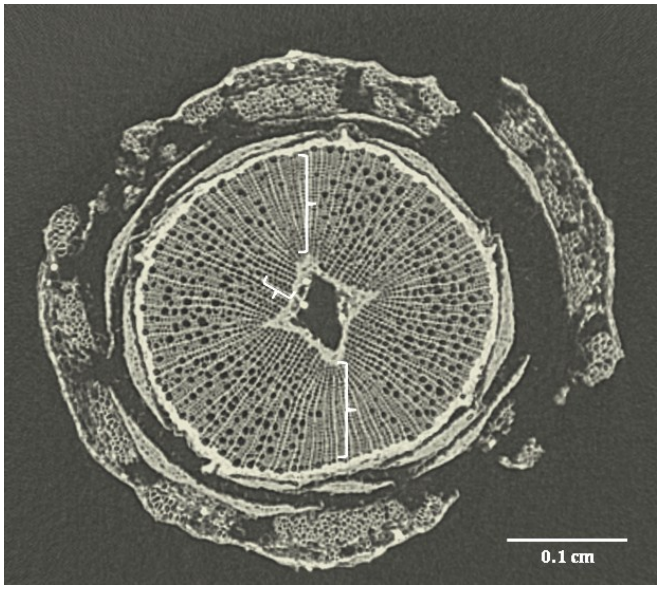

Figure 14 Cross section of a dry stem section of *Myrothamnus moschatus* using micro-computed tomography (µCT). Brackets indicate areas rich in tracheids**.**

Table 3 Summary of major morpho-anatomical traits of *Myrothamnus flabellifolius* and *Myrothamnus moschatus.*

	M. flabellifolius	*M. moschatus*
leaf shape	fan-shaped	lanceolate
leaf venation	parallel	parallel (more pronounced than in *M. flabellifolius*)
hydathodes	yes	no
leaf anatomy	isolateral, little sclerenchyma	isolateral, little sclerenchyma
wood anatomy	primitive, knob-like structures on vessels	primitive, no knob-like structures on vessels
stomata	not sunken	slightly sunken

3.5. Discussion

A potentially important morpho-anatomical adaptation of *M. moschatus* (similarly but not as obvious in *M. flabellifolius*) lies in the adoption of a parallel leaf venation which is very rare among dicots: major vascular bundles dominate and constitute a parallel leaf venation despite the fact that the horizontal xylem elements are very frequent and build

a net of cross connections. Major vascular bundles are interconnected in monocots as well but by minor vascular bundles which are rarely visible in cross sections. Among desiccation-tolerant monocots the parallel assembly of major vascular bundles is a mechanical advantage in the folding process. In many monocots the leaf involution is realized through the reversible collapse of translucent bulliform cells during dehydration located above the midrib or in furrows, minimizing the transpirational surface, preventing the absorption of excess light energy, and increasing the stiffness of the leaf (Metcalfe 1971). Since *M. moschatus* and *M. flabellifolius* do not have a midrib or bulliform cells the leaf just contracts accordion-like.

Another protective mechanism is constituted by the folding of the leaves towards the stem in the dry state, hereby protecting younger leaves which are more sensitive to severe insolation. The surface reduction and protection is therefore attained differently in desiccation-tolerant monocots and dicots. In Myrothamnaceae protection against insolation and damage due to the absorption of excess light is additionally achieved by the occurrence of anthocyanins (Moore et al. 2007a).

The speed of recovery of *M. moschatus* can be regarded as moderate since there are homoiochlorophyllous species, which recover more quickly, and poikilochlorophyllous species with a slower rate of recovery (Sherwin & Farrant 1996). The rehydration of *M. moschatus* was (at least visually) completed after 48 hours which is 12 hours faster than values reported for *M. flabellifolius* (measured by increase in water content) (Sherwin & Farrant 1996). However, since macroscopic observations may not be as exact as data derived from measurements of increase in water content, the velocity of rehydration for both species may be fairly similar. It should however be kept in mind that poikilochlorophylly has so far only been attributed to desiccation-tolerant monocots. This matter can only be resolved with the help of physiological studies of fresh plant material.

In both Myrothamnaceae the amount of sclerenchyma is very low although some articles describe the amount as relatively high (for *M. flabellifolius* cf. Moore et al. 2007a). Additional (but probably not as rigid) stabilization is offered by the abundant horizontal xylem elements. Nevertheless, mechanical support seems to be less essential for the successful survival of long periods of drought in Myrothamnaceae and is also known for other desiccation-tolerant dicots (Phillips et al. 2008). Here, other morpho-

anatomical structures such as the parallel leaf venation seem to be crucial for survival. This underlines the assumption that plant species living in dry habitats do not necessarily have to have sclerophylls or many typical xeromorphic features in general (Fahn & Cutler 1992). The stoma distribution in *M. moschatus* supports this. The stomata are sunken, which can be a xeromorphic feature, but not confined to furrows and distributed across the lamina. Sclerenchyma does however, aside from its stabilizing function, play a role in the recovery from water loss. Sclerophylls recover more completely than non-sclerophylls but they do not recover more rapidly (Salleo et al. 1997). It can be hypothesized that the speed of recovery, which may be impaired by a high value of sclerenchyma, is more important in Myrothamnaceae. Under this aspect, the plant height (up to 1.5 m) should be considered. In a relatively high plant the dissolution of embolized xylem elements is more complicated and a complete rehydration may additionally be retarded by a high amount of sclerenchyma. If *M. moschatus* is homoiochlorophyllous the low amount of sclerenchyma can also be attributed to the fact that homoiochlorophyllous species are generally adapted to shorter periods of drought (Proctor & Tuba 2002), rendering a high amount of stabilizing tissue less important.

In *M. flabellifolius* rehydration is thought to be supported by hydathodes (Drennan et al. 2009). These structures are also thought to function as salt secreting organs to elevate the sugar:cation ratio which facilitates the dehydration process (Drennan et al. 2009). As has been noted above, the shrinkage of *M. moschatus* leaves during dehydration is more severe in width than in length. The higher shrinkage in length of *M. flabellifolius* leaves can most certainly be attributed to measurement errors because dry leaves of *M. flabellifolius* tend to bend slightly in the dry state, making exact measurements difficult. In *M. moschatus* and *M. flabellifolius* this shrinkage is achieved through a reversible folding of mesophyll cells due to massive turgor loss (reduction in width amounts to c. 50-70%, depending on the species, cf. Fig. 10).

Anatomical studies of *M. flabellifolius* revealed the primitive character of its wood (Carlquist 1976). Although more research is required to support the preliminary assumption that this is also true for *M. moschatus* the high number of tracheids located in the first layers adjacent to the pith is very distinct. These tracheids certainly have a stabilizing function during long periods of drought and additionally lower the number of

embolisms due to their small diameter. Therefore, adaptive value can be assigned to the wood anatomy as well. In conclusion, the detailed data on morpho-anatomical traits of *M. moschatus* presented here can be regarded as a good supplement to much-needed research on the physiological, biochemical and molecular level of desiccation-tolerant plants.

4. Leaf anatomical traits of desiccation-tolerant vascular plants: a comparative analysis

4.1. Abstract

Desiccation tolerance is rare among vascular plants (c. 1 300 spp.). In contrast to the molecular, physiological and biochemical mechanisms underlying desiccation tolerance the importance and adaptive value of morpho-anatomical traits has lately been neglected. Therefore, in this first and extensive comparative study desiccation-tolerant Cyperaceae, Velloziaceae, Myrothamnaceae, the desiccation-sensitive *Stipa capillata* (Poaceae), *Carex pendula* (Cyperaceae) and literature data about pteridophytes were analyzed. Leaf anatomical traits in the rehydrated and dry state of these desiccation-tolerant and desiccation-sensitive vascular plants were examined. Since the morpho-anatomical traits within and among monocots, dicots and pteridophytes differ substantially a universal morpho-anatomical syndrome could not be postulated for desiccation-tolerant vascular plants. However, several important adaptive morpho-anatomical traits were identified: bulliform cells facilitate the leaf involution in many desiccation-tolerant monocots, hereby protecting the leaf from excess radiation; monocot leaves show a high share of sclerenchyma which stabilizes the leaf in the dry state; the occurrence of a parallel leaf venation is of high adaptive value as well because many desiccation-tolerant dicots mimic this venation type. In addition to this, the dominance of monocots among desiccation-tolerant vascular plants was analyzed and links of morpho-anatomical traits to phylogeny and other adaptive levels are discussed extensively.

4.2. Introduction

Desiccation tolerance is a highly specialized strategy which evolved several times in different lineages (Alpert 2000). Adaptations to desiccation tolerance occur on the molecular, physiological, biochemical and morpho-anatomical level. Due to climate change, predicted and current water shortages the (biotechnological) innovation of drought-tolerant crops is of major importance and associated with an enormous economic potential (e. g. Iyer et al. 2007). Therefore, current research projects mainly focus on the molecular, physiological and biochemical adaptations to desiccation

tolerance. Since it is equally important to uncover adaptations on the morpho-anatomical level to gain a complete and thorough understanding of desiccation tolerance the research presented here focuses on the morpho-anatomical adaptations of desiccation-tolerant vascular plants. It is the first extensive comparative study of morpho-anatomical traits under the aspect of desiccation tolerance over a wide taxonomic range. Consequently, a structure-function relationship is a priori postulated, i.e. a tight connection between the anatomy and morphology of a plant and its environmental conditions. The occurrence of xeromorphic traits such as sunken stomata, a thick cuticle and extended sclerenchyma bands or girders might suggest that this is the case in desiccation-tolerant vascular plants as well (Carlquist 1975; Fahn & Cutler 1992; Molina et al. 2006; Smith & Ayensu 1976). Reality has proven to be more complex, rendering this approach as too broad and not universally true: some species such as *Blossfeldia liliputana* (Cactaceae) exhibit almost hygromorphic traits (Barthlott & Porembski 1996). Apparently, a wide variety of morpho-anatomical adaptations exist among desiccation-tolerant plants, e.g. a velamen radicum in some Cyperaceae and Velloziaceae or cell wall folding on an ultrastructural level (Porembski & Barthlott 1995; Vicré et al. 2004).

Obviously an over-arching, re-occurring morpho-anatomical syndrome, i.e. traits which can be identified in every desiccation-tolerant species, does not exist. This is mainly due to the fact that a variety of groups such as cryptogams, pteridophytes and angiosperms are integrated (e.g. Kappen & Valladares 1999). This hypothesis has never been tested on a smaller scale, i.e. within vascular plant families and groups (i.e. pteridophytes, monocots and dicots) and lead to the following research questions:

(a) Does a re-occurring morpho-anatomical syndrome exist among desiccation-tolerant vascular plants?

(b) Is it, in general, possible to identify decisive morpho-anatomical traits among desiccation-tolerant vascular plants? This includes the array of sclerenchyma girders, vascular parenchyma, mesophyll, and cuticle thickness but the mechanism of leaf involution, leaf size reduction during dehydration and the role of leaf venation as well. Other traits such as trichomes, stomata density and distribution were for the most part neglected.

(c) If these two questions cannot be answered conclusively: are adaptations on the molecular, physiological and biochemical level finally more important than those on the morpho-anatomical level?
(d) Does the comparative anatomy and morphology give clues on why desiccation-tolerant monocots are much more abundant than desiccation-tolerant dicots?
Under this respect desiccation-tolerant monocots from different families (Cyperaceae, Velloziaceae), subfamilies and tribes, a dicot (Myrothamnaceae) and the desiccation-sensitive *Stipa capillata* (Poaceae) and *Carex pendula* (Cyperaceae) were chosen to compare leaf morpho-anatomical traits of desiccation-tolerant and desiccation-sensitive species. Basic anatomical studies about selected species have already been published (for instance Ayensu 1968; Ayensu 1969; Chevalier 1933; Coetzee 1974; Engler & Krause 1911; Hambler 1961; Raynal 1963) but detailed comparative morpho-anatomical studies under the aspect of desiccation tolerance are lacking. Literature (due to the shortage of fresh plant material) about morpho-anatomical accounts of desiccation-tolerant pteridophytes and other dicot families were consulted as well. The results will additionally be discussed in relation to phylogeny. Thus, the relevance of mechanisms and morpho-anatomical structures of desiccation-tolerant vascular plants for the survival of long periods of drought are examined and emphasized.

4.3. Material and Methods

4.3.1. Plant material

Plant material was provided by the Botanical Garden of the University of Rostock and the Botanic Gardens of the University of Bonn (collected in the field before 1980).
The following monocots were examined:
Cyperaceae: *Afrotrilepis pilosa* (Boeck.) J.Raynal, *Microdracoides squamosus* Hua (both west Africa, tribe Trilepideae), *Carex pendula* Huds. (holarctic, desiccation-sensitive, tribe Cariceae)
Velloziaceae: *Xerophyta* spp. (Africa), *Vellozia andina* Ibisch, R. Vásquez & Nowicke, *Vellozia crassicaulis* Mart., *Vellozia glochidea* Pohl, *Barbaceniopsis castillonii* (Haumann) Ibisch (all South America)
Poaceae: *Stipa capillata* L. (holarctic, desiccation-sensitive, tribe Stipeae)

The following dicot was examined:
Myrothamnaceae: *Myrothamnus flabellifolius* Welw. (south and south west Africa)

To enhance the comprehensibility of the anatomical descriptions in the results chapter, species with similar anatomical features within Velloziaceae were summarized into groups.

4.3.2. Visualization of dry plant material

For the study of dry plant material leaves were clipped and dried for several days. X-ray micro-computed tomography (µCT) was used to visualize the 3-D structure of the species. Small leaf sections (c. 0.5x0.5 cm, depending on the initial leaf size, 5 repetitions) of plant material were glued on a glass rod and scanned in an X-ray computer tomograph (nanotom© 180 NF, Phoenix X-Ray). The following parameters were used for the scans (with slight variations from sample to sample): 35 V, 270-300 µA, mode 1, 1 000 ms, 1 080 images, binning=1, resolution: 1.71-3.59 µm, magnification: 27.78-58, average duration per scan: 72 min. For the image acquisition datosacq©, for the image reconstruction datosx© and for the 3-D evaluation Imaris© 6.3.1 (Bitplane AG) and VG Studio Max© (VolumeGraphics) were applied.

4.3.3. Visualization of rehydrated plant material

Since the analysis of rehydrated or semi-dry plant tissue using µCT is only possible with a relatively low image quality and resolution (Ch. 2, Korte & Porembski 2011) light microscopy was applied for the study of rehydrated plant material. Leaves were rehydrated for at least 24 hours (dry material) or clipped from the plant, cut with a vibratome (Hyrax V50 (Carl Zeiss Jena)) and the cross sections were examined under a light microscope (Zeiss Jenaval, Carl Zeiss Jena, magnification: 125-400). For light microscopic pictures and cuticle measurements the software analySIS 3.2. (Soft Imaging, Germany) was applied. The µCT reconstructions and light microscopic pictures were used as templates for representative schematic anatomical prototypes generated with CorelDraw 12 (Corel Corp., USA).

4.3.4. Shrinkage of leaves during dehydration

The amount of shrinkage of rehydrated plant material during the dehydration process was measured under a scanner (Brother DCP 110C, Brother International). The leaf width and length of five samples per species (c. 0.5x0.5 cm, depending on the initial leaf size) were measured using CorelDraw 12 (Corel Corp.). After at least 72 hours of drying the same sample was scanned and measured again.

4.4. Results

4.4.1. Cyperaceae

Rehydrated leaves of *A. pilosa* and *M. squamosus*

The leaf size of *A. pilosa* is naturally dependent on age but also on regional climatic variations. In a relatively dry climate leaves may only be as wide as 0.5 cm. Older leaves growing under more favorable, i.e. more humid, conditions, are 1.5-2 cm wide (personal observation N. Korte). The apical leaf rosette of *M. squamosus* on the other hand consists of several spiky and smaller leaves which usually attain a width of 0.5 cm at the base of the leaf rosette. The impact of climatic variations on leaf size differences have not been studied yet.

In both species all major vascular bundles constitute a parallel leaf venation. In leaves of *A. pilosa*, the size of the vascular bundles differs. The smallest bundles are located adjacent to the midrib and leaf tips whereas the largest can be found in the middle of the leaf (Fig. 15). Two large metaxylem elements and several layers of vascular parenchyma can easily be distinguished. The size differences in leaves of *M. squamosus* among vascular bundles are less obvious (Fig. 16). As in *A. pilosa*, all vascular bundles have two large metaxylem vessels and are surrounded by a one-layered bundle sheath.

In both species the parenchyma is comprised of small isodiametric cells and is only roughly differentiated into palisade and spongy parenchyma (Chermezon 1933, personal observation N. Korte). Thin-walled, large translucent cells between two vascular bundles are very prominent. These cells sometimes collapse and air cavities (lacunae), which are characteristic of Cyperaceae, are formed (Metcalfe 1971). Above the midrib of *A. pilosa* bulliform cells are located which are important for the involution of the leaf (Metcalfe 1971). In *M. squamosus* a midrib and bulliform cells are lacking.

The cuticle thickness of *M. squamosus* and *A. pilosa* varies from 1.2-2 µm. The epidermis is only slightly furrowed. Mechanical support is facilitated by the occurrence of adaxial and abaxial rectangular sclerenchyma girders located below the epidermis on each side of a vascular bundle (Figs. 15, 16). The sclerenchyma distribution differs in both species. In leaves of *M. squamosus* very prominent sclerenchyma caps surround the vascular bundles, extending to the abaxial and adaxial epidermis (Pfeiffer 1927). Additionally, small sclerenchyma strands are located between each vascular bundle. The vascular bundles of *A. pilosa* are surrounded by several cell layers of sclerenchyma but they merely extend to the epidermis as regularly as in *M. squamosus*. The midrib is surrounded by an abaxial crescentiform sclerenchyma girder. To enhance the comprehensibility of this results section the morpho-anatomical traits of all species are summarized in Table 4 (reference to Table 4 will largely be omitted in the following chapters).

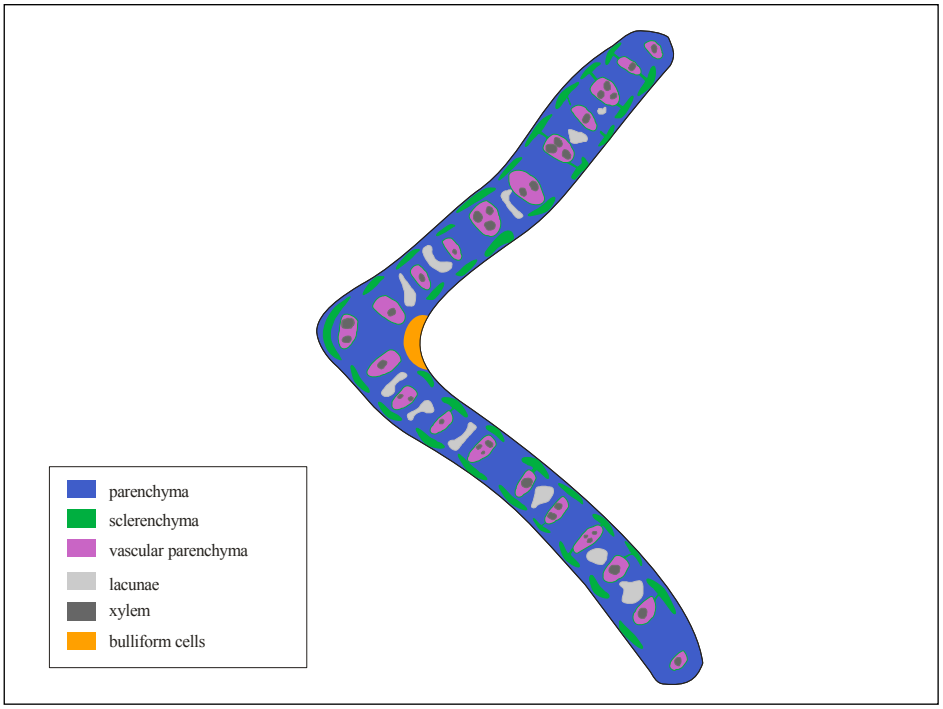

Figure 15 Schematic cross section of *Afrotrilepis pilosa* (rehydrated).

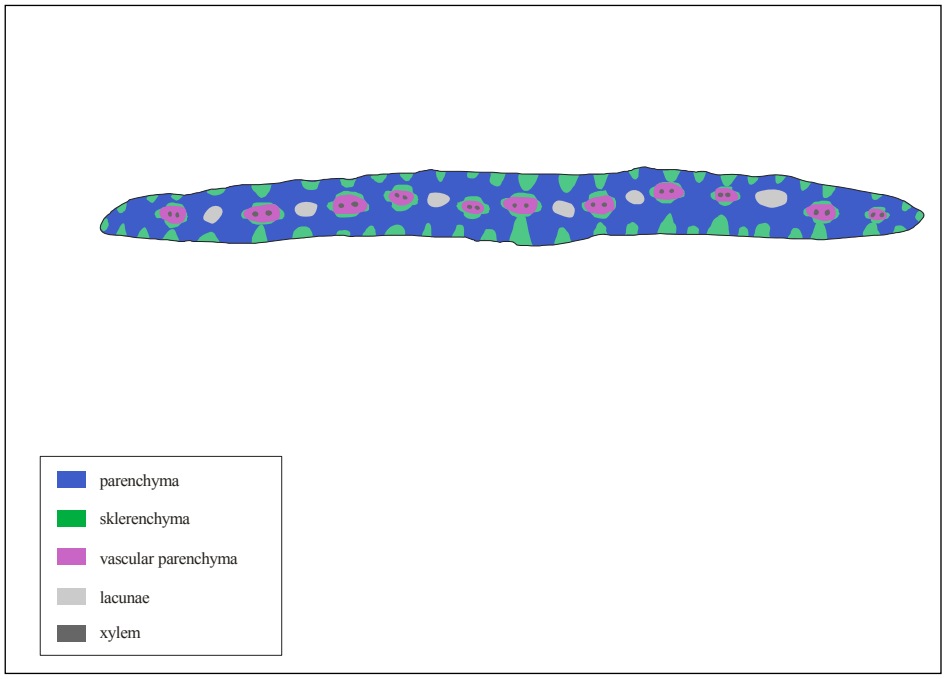

Figure 16 Schematic cross section of *Microdracoides squamosus* (rehydrated).

Dry leaves of *A. pilosa* and *M. squamosus*

Since *A. pilosa* is poikilochlorophyllous (i.e. breakdown of chlorophyll upon dehydration) the leaves lose their green color and turn brownish. The parenchymatic translucent bulliform cells above the midrib collapse and facilitate the symmetrical folding of the leaves. The endings of older leaves may additionally curve (Fig. 17).

In an apical leaf rosette older leaves envelope inner younger leaves to protect them from excess solar radiation. Leaves of *M. squamosus* turn brown as well, do not fold but merely curve due to the lack of a midrib and bulliform cells (Fig. 18). The leaves of *M. squamosus* shrink considerably less than those of *A. pilosa*, the width reduction amounts to slightly more than 40% (Fig. 19). Lacunae, which appear as thin-walled parenchyma cells, fold during the dehydration process and are common in both species but less frequent in young leaves of *M. squamosus* (Engler & Krause 1911).

In contrast to *A. pilosa* where the leaf is deeply furrowed in areas above the lacunae the abaxial and adaxial epidermis of *M. squamosus* is barely furrowed in the dry state. Since small sclerenchyma strands are located below the epidermis on both sides of the vascular bundles, its collapse is prevented.

The amount of sclerenchyma in *M. squamosus* is much higher. It is concentrated below the epidermis, around the vascular bundles and baculiform sclerenchyma girders which extend to the abaxial and adaxial epidermis. The small sclerenchyma strands, which were clearly visible in the rehydrated state, occur as one adaxial and abaxial strand stretching from one end of the leaf to the other. In contrast to *A. pilosa,* the vascular parenchyma in *M. squamosus* cannot be distinguished from the vascular sclerenchyma on µCT images.

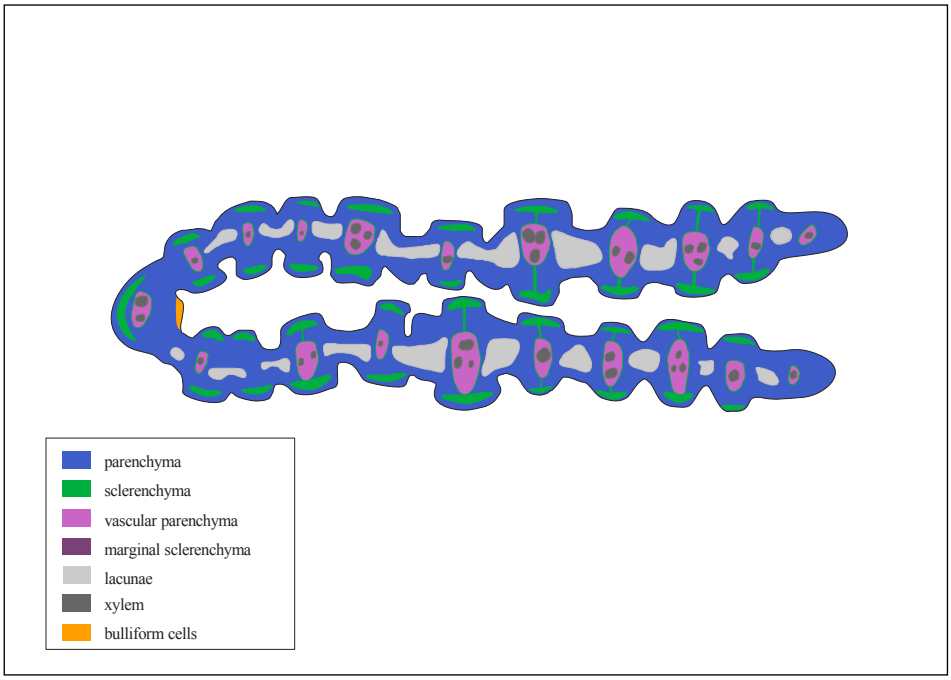

Figure 17 Schematic cross section of *Afrotrilepis pilosa* (dry).

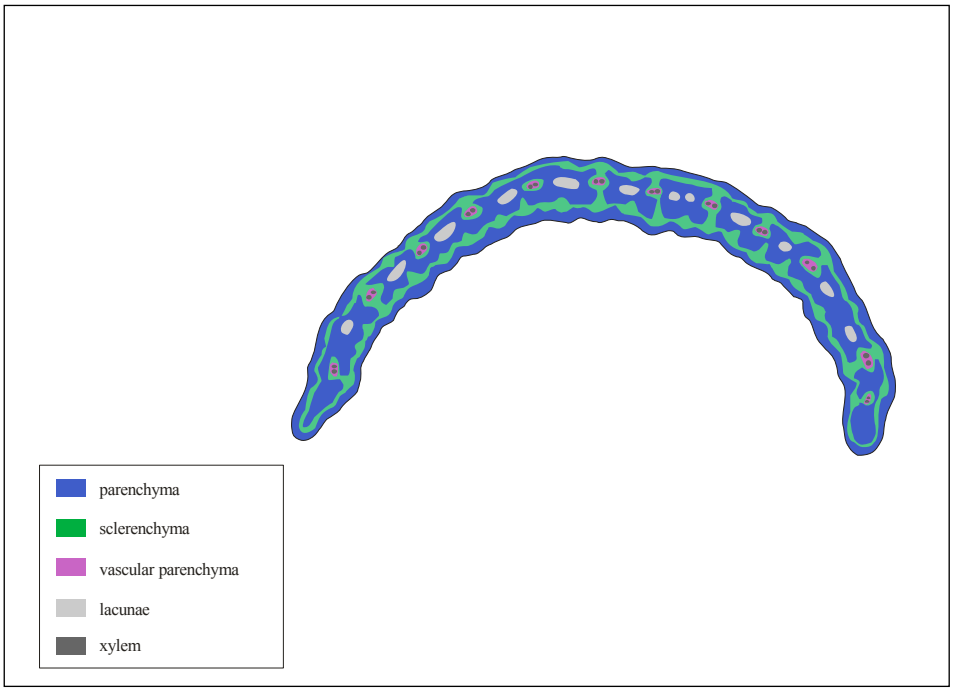

Figure 18 Schematic cross section of *Microdracoides squamosus* (dry).

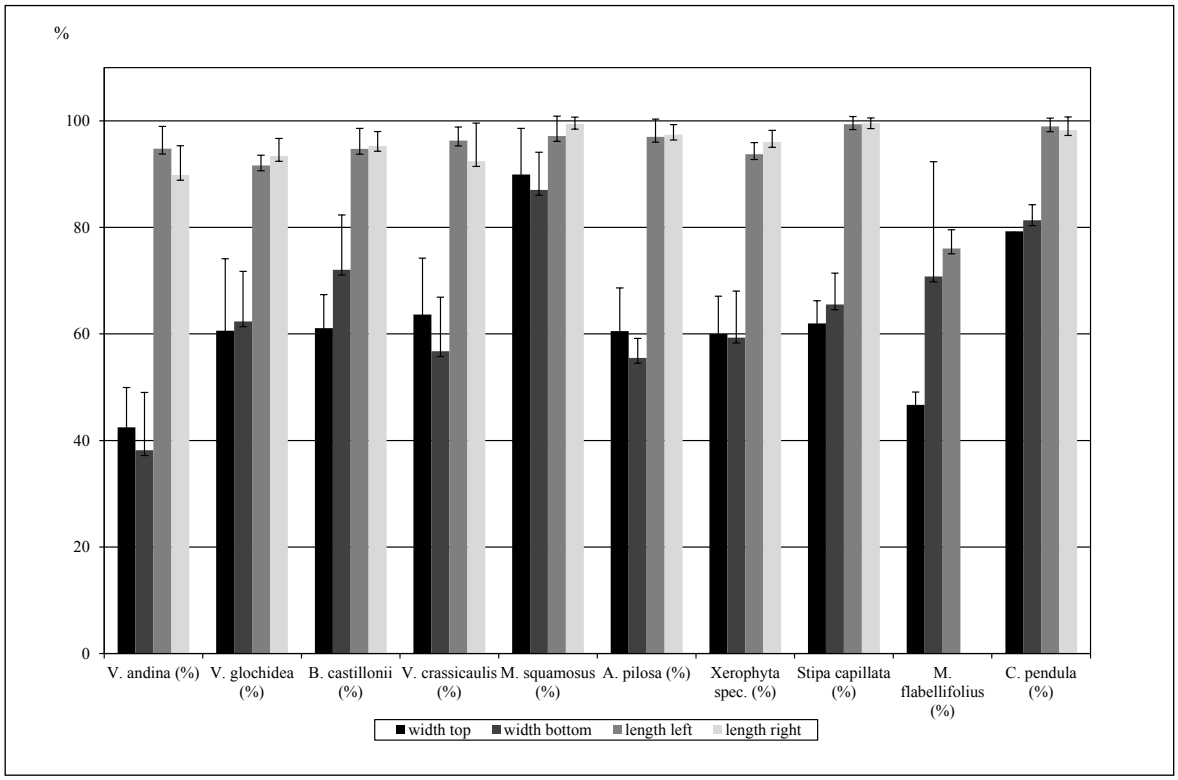

Figure 19 Shrinkage of leaves (width and length) during dehydration (% of initial size).

4.4.2. Velloziaceae

Rehydrated leaves of *Vellozia glochidea* and *Vellozia crassicaulis*

Out of five species two groups of anatomically similar species can be distinguished. The first group comprises *Vellozia glochidea* and *Vellozia crassicaulis*, the second group includes *Xerophyta* spp., *Vellozia andina* and *Barbaceniopsis castillonii*.

Vellozia glochidea and *Vellozia crassicaulis* are native to South America (notably Brazil) (Alves & Kolbek 1994). The epidermis of both species is furrowed on the abaxial side. Both species feature a considerable amount of sclerenchyma around each vascular bundle extending to the epidermis and the midrib but also between vascular bundles where small sclerenchyma strands on the adaxial side are conspicuous (Fig. 20). The cuticle of both species is ~ 1.5 µm thick.

As in *A. pilosa* the smallest vascular bundles are located adjacent to the midrib and tip of the leaf. Each vascular bundle is surrounded by a bundle sheath. Beneath this one-layered sheath two Y-shaped sclerenchyma girders extend from the abaxial and adaxial epidermis and are separated by a few layers of vascular parenchyma (sclerenchyma type I according to Ayensu 1969). In contrast to *A. pilosa* and *M. squamosus* only one large central metaxylem vessel is prominent, which is – in *V. glochidea* – surrounded by a thin layer of sclerenchyma and vascular parenchyma. As in *A. pilosa,* large bulliform cells are located above the midrib. The abaxial side of the midrib is supported by a multi-layered winged crescentiform girder.

In both species the margins of the abaxial furrows have small sclerenchyma strands which are absent from the species of the second group. *V. glochidea* and *V. crassicaulis* both have marginal sclerenchyma at the leaf tip which is a fairly common but not universal trait in Velloziaceae (cf. below).

Dry leaves of *Vellozia glochidea* and *Vellozia crassicaulis*

Due to the reversible collapse of bulliform cells above the midrib the leaf involution is comparable to *A. pilosa*. The leaf size reduction of both species is fairly similar: little more than 40% reduction in width, the length remains almost unchanged (Fig. 19).

The most obvious anatomical change lies in the formation of one adaxial sclerenchyma strand from the formerly separated strands in the rehydrated state situated between the vascular bundles. However, only the abaxial epidermis is, as in the rehydrated state,

furrowed (Fig. 21). This is due to the reversible collapse of mesophyll cells. This collapse of mesophyll cells fused the formerly divided Y-shaped sclerenchyma girders in the vascular bundle into one single girder.

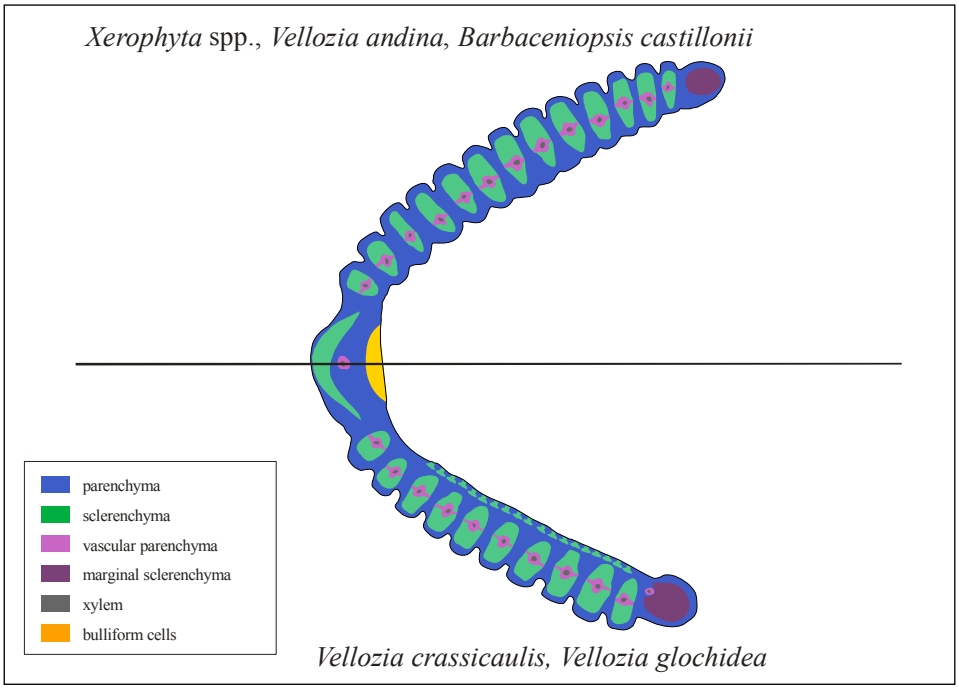

Figure 20 Schematic cross section of all Velloziaceae (rehydrated).

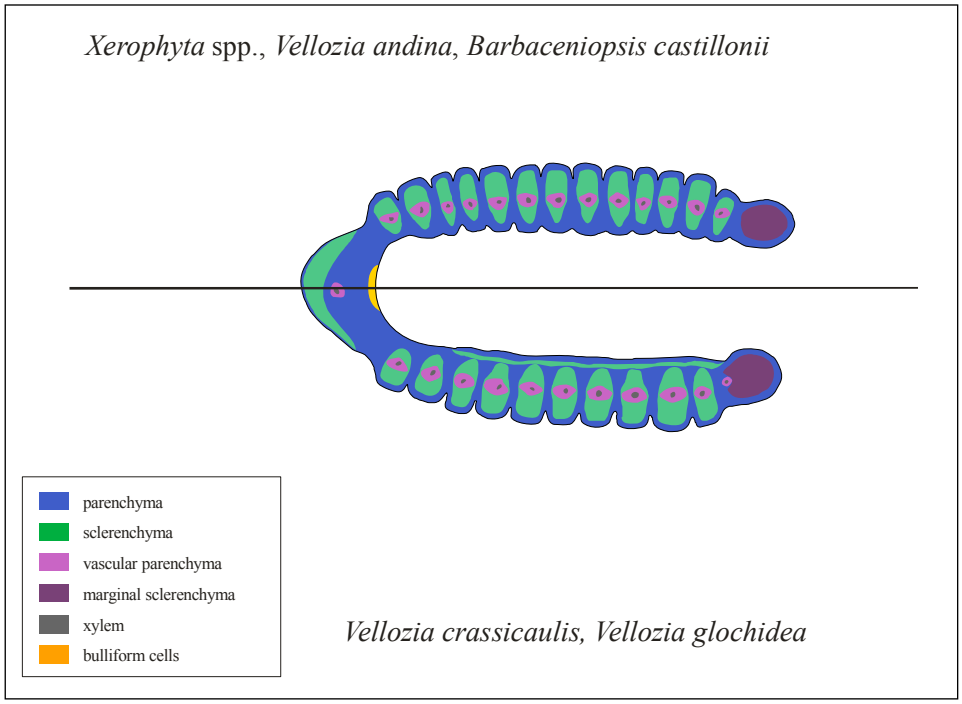

Figure 21 Schematic cross section of all Velloziaceae (dry).

Rehydrated leaves of *Xerophyta* spp., *Vellozia andina, Barbaceniopsis castillonii*

Xerophyta spp. belongs to an Old World genus and shows many anatomical similarities to the New World species *Barbaceniopsis castillonii* and *Vellozia andina*. The abaxial epidermis of all three species is furrowed, the adaxial epidermis only in *B. castillonii* (Fig. 20). All three species have distinct marginal sclerenchyma, a winged crescentiform girder on the abaxial side of the midrib, and Y-shaped sclerenchyma girders which are - as in *V. glochidea* and *V. crassicaulis* - separated by a thin layer of vascular parenchyma. One large metaxylem element is visible, adjacent to some smaller vessel elements. The amount of sclerenchyma in the midrib differs slightly with *B. castillonii* having less sclerenchyma.

Unlike the other species, *Xerophyta* spp. has small sclerenchyma strands on the adaxial epidermis between the vascular bundles. Another difference are translucent elongated cells stretching from the adaxial epidermis to the sclerenchyma, surrounding the vascular bundles, which are typical of Old World Velloziaceae. The cuticle of *B. castillonii* is slightly thicker (3 µm) than those of *V. andina* and *Xerophyta* spp. (1.6 resp. 1.8 µm).

Dry leaves of *Xerophyta* spp., *Vellozia andina, Barbaceniopsis castillonii*

The furrows of all species are very pronounced after dehydration. The lack of an abaxial sclerenchyma strand, which is a major difference to *V. glochidea* and *V. crassicaulis*, however does not have an influence on the severity of shrinkage of all species compared to *V. glochidea* and *V. crassicaulis*. The leaf size reduction is very high in *V. andina* whereas *Xerophyta* spp. and *B. castillonii* are similar to the other species as well (Fig. 19). Due to the collapse of parenchyma cells the leaf contracts, moving the vascular bundles closer together and expanding them after rehydration. The (symmetric) leaf involution of all species is realized through translucent bulliform cells above the midrib (Fig. 21). The vascular parenchyma mainly stays in shape; one large metaxylem element is still clearly visible. In *Xerophyta* spp., the translucent cells stretching from the adaxial epidermis to the vascular sclerenchyma collapse. The adaxial sclerenchyma strands are only clearly visible in the rehydrated state.

4.4.3. Myrothamnaceae

Rehydrated leaves *Myrothamnus flabellifolius*

The desiccation tolerance of the shrubby *M. flabellifolius* is remarkable for two reasons: only very few dicot families contain resurrection plants and a shrubby growth form is very rare among desiccation-tolerant plants. This is mainly due to the difficulty to eliminate embolisms during rehydration which becomes more difficult with increasing plant height. Since much research has been done on *M. flabellifolius* (e.g. Drennan et al. 2009; Grundell 1933; Jäger-Zürn 1966; Moore et al. 2007b; Puff 1978) the anatomical traits will only be briefly summarized.

Leaves of *M. flabellifolius* are fan-shaped and not linear as in Cyperaceae and Velloziaceae (Fig. 22). Every leaf has prominent furrows and ridges on the abaxial resp. adaxial side. Since they run almost parallel a monocotyledonous leaf nervature is mimicked. The stomata are not sunken and not confined to furrows (Grundell 1933; Zemke 1939), the cuticle thickness is 1.6 µm. The occurrence of hydathodes is a remarkable feature. These structures are thought to operate as water and salt secreting organs (Drennan et al. 2009). In general, few xeromorphic anatomical traits are present and sclerenchyma is limited to vascular bundles (Zemke 1939).

Dry leaves *Myrothamnus flabellifolius*

The leaves itself do not fold as in most monocots, they contract accordion-like and envelope the stem, hereby protecting the inner younger leaves (Gaff 1981; Grundell 1933) (Fig. 23). The ridges and furrows complement each other during the contraction process. The shrinkage is slightly but not significantly higher compared to most other species (Fig. 19). However, since leaves curved slight inaccuracies during measuring might have occurred.

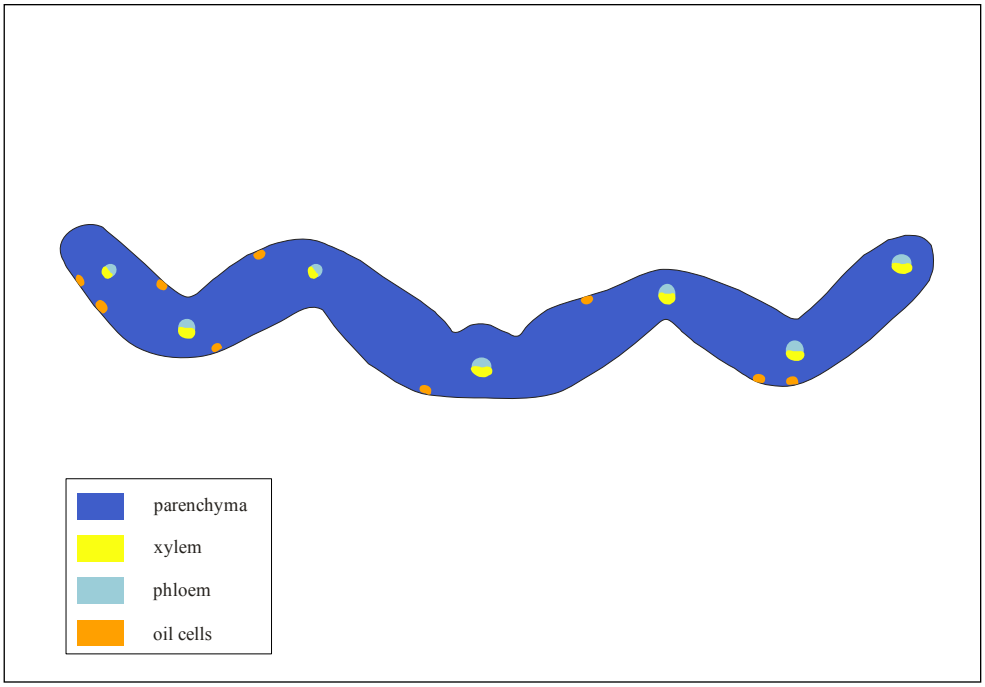

Figure 22 Schematic cross section of *Myrothamnus flabellifolius* (rehydrated).

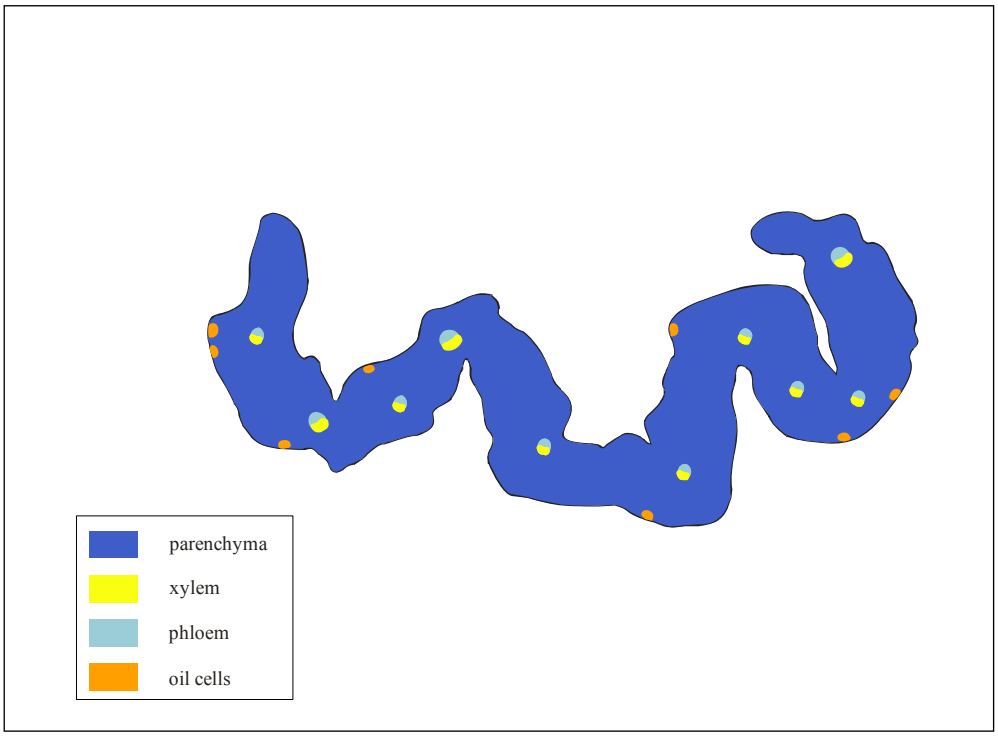

Figure 23 Schematic cross section of *Myrothamnus flabellifolius* (dry).

4.4.4. The desiccation-sensitive species

Rehydrated leaves of *Stipa capillata* (Poaceae) and *Carex pendula* (Cyperaceae)

Leaves of *S. capillata* are much smaller (width: ~ 3 mm) compared to all other species. They are deeply furrowed on the adaxial side (Fig. 24). Several layers of sclerenchyma are located around each vascular bundle and at the leaf tips (similar to the marginal sclerenchyma in Velloziaceae) but it is absent in furrows. In each vascular bundle 1-3 large metaxylem vessels can be distinguished. The sclerenchyma girders usually extend to the abaxial and adaxial epidermis, leaving a relatively small share of parenchyma (Nikolaevsky & Nikolaevskaya 1967). This is even more pronounced than in some of the desiccation-tolerant monocots described above.

The abaxial epidermis is composed of translucent cells. In the adaxial furrows large bulliform cells are centered around the stomata. Trichomes are mainly located on the adaxial epidermis above the vascular bundles. A similarly distinguishable midrib as in most desiccation-tolerant monocots is not present although the leaf is folded symmetrically in the rehydrated state. The cuticle is thicker than those of the other species (~ 3.5 µm).

Leaves of *C. pendula*, which prefers wet habitats such as river banks, on the other hand are relatively large with an average of 30 vascular bundles (the schematic prototype therefore shows an unusually small leaf, Fig. 25). Within each vascular bundle two large metaxylem vessels can be distinguished. The amount of sclerenchyma around the vascular bundles is fairly high but is reduced across the whole leaf compared to *S. capillata* and other monocots examined here. The girders extending to the abaxial and adaxial epidermis are less pronounced (if at all). Large bulliform cells are confined to the midrib, the adaxial epidermis is composed of smaller translucent cells. A remarkable difference to other examined species is the large vascular bundle in the middle of each lamina. Consequently, the lamina sections right and left of the midrib curve. Additionally, the lacunae are very large, dissecting the parenchyma regularly. The thickness of the cuticle is similar to that of *S. capillata* (~ 3 µm).

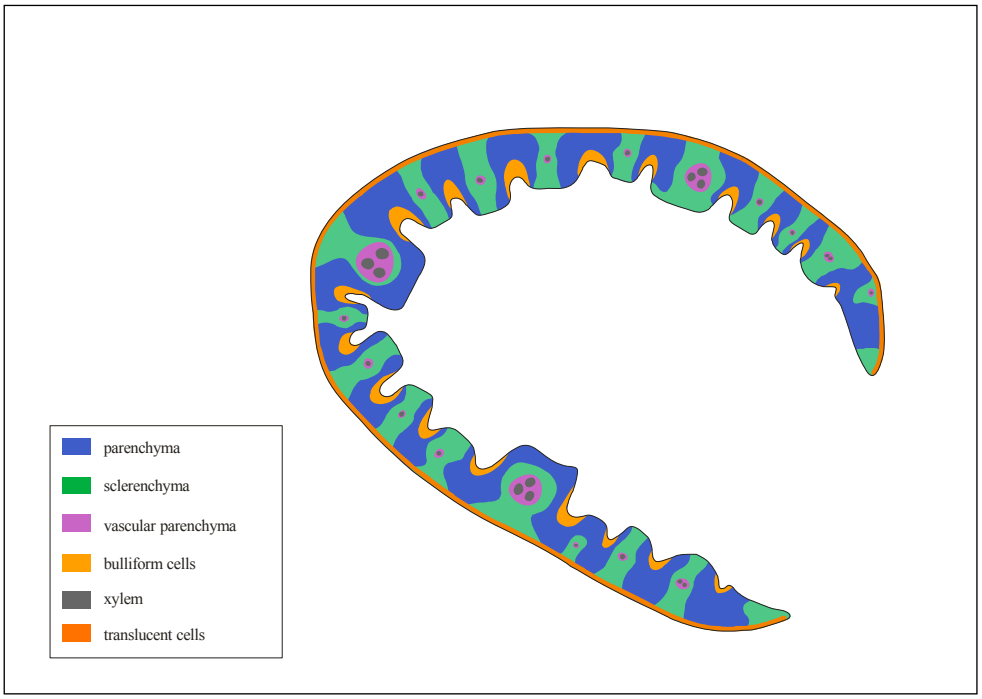

Figure 24 Schematic cross section of *Stipa capillata* (rehydrated).

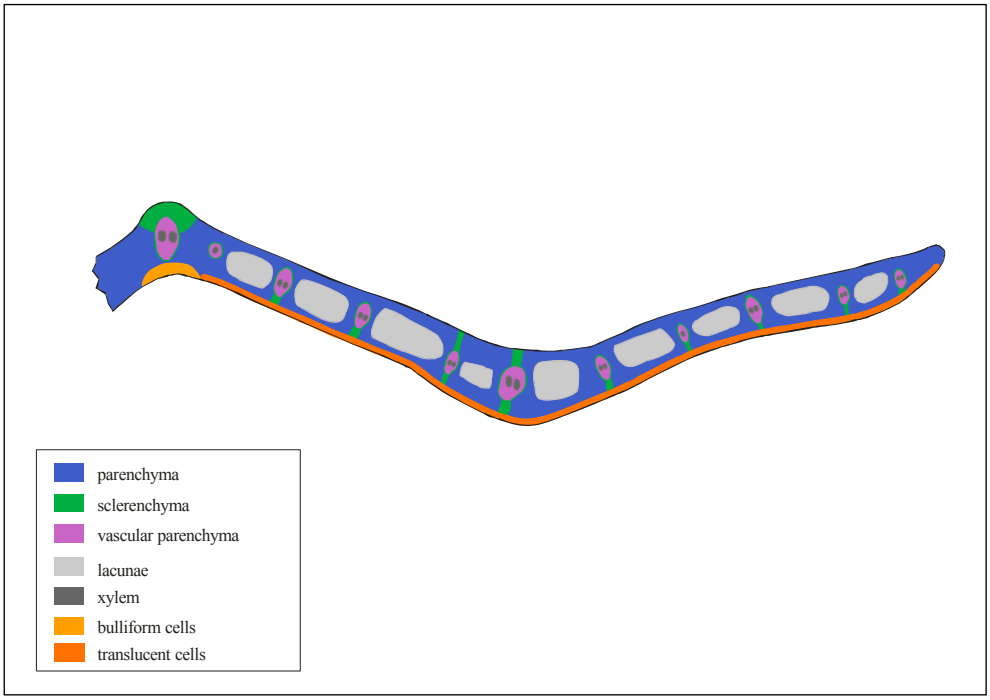

Figure 25 Schematic cross section of *Carex pendula* (rehydrated). Original leaves are usually larger (up to 30 vascular bundles).

Dry leaves of *Stipa capillata* (Poaceae) and *Carex pendula* (Cyperaceae)

Dry leaves of *S. capillata* only curl inwards which is facilitated by the collapse of bulliform cells in the furrows. The involution is therefore not symmetrical and induced by cells above the midrib but by the collapse of parenchyma and bulliform cells in each furrow (Fig. 26). The two large vessels in each vascular bundle are still clearly visible in the desiccated state. Due to the massive turgor loss upon dehydration abaxial furrows develop. The values for shrinkage are similar to those of the desiccation-tolerant species (Fig. 19).

Dry leaves of *C. pendula* roll towards the abaxial side, exposing the adaxial side (Fig. 27). This peculiar folding pattern is induced by the collapse of parenchyma cells, the translucent epidermis cells and the large vascular bundle in the middle of each lamina which prevents a folding towards the adaxial side. Lacunae are now dominant since the parenchyma cells collapse. Leaves of *C. pendula* shrink considerably less than most other species with values are similar to those of *M. squamosus* (Fig. 19). To enhance the comprehensibility of this results section the morpho-anatomical traits of all species are summarized in Table 4.

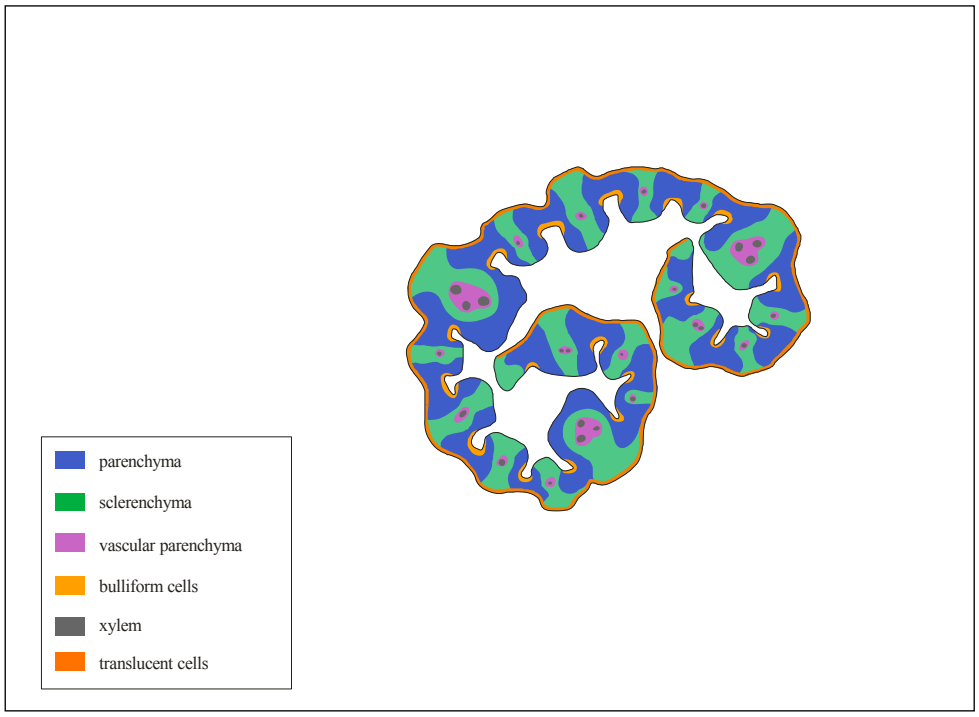

Figure 26 Schematic cross section of *Stipa capillata* (dry).

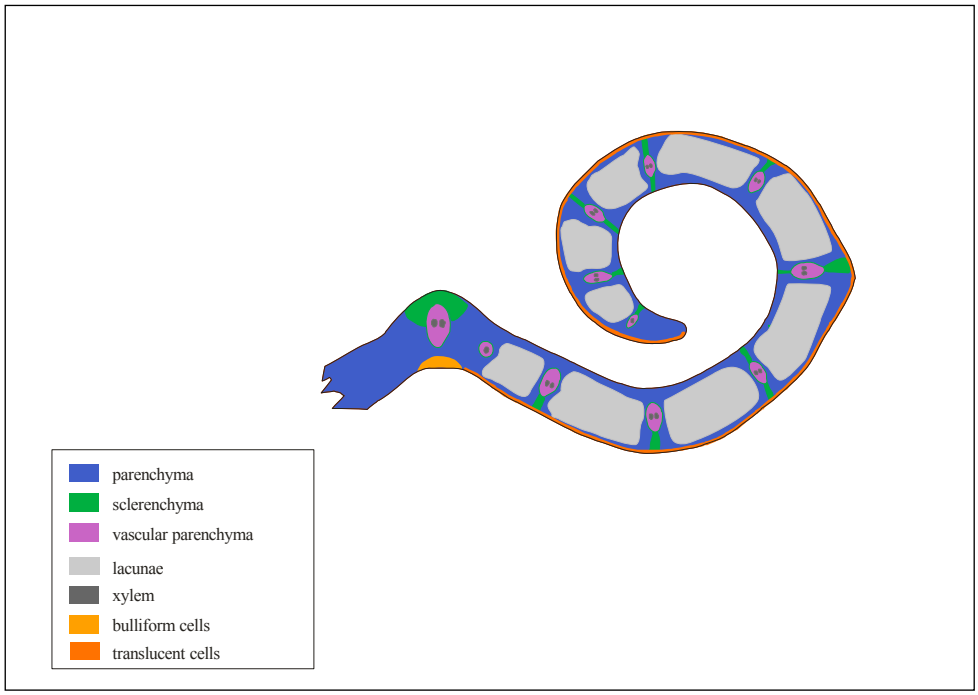

Figure 27 Schematic cross section of *Carex pendula* (dry).

Table 4 Short overview of the occurrence of decisive morpho-anatomical traits in desiccation-tolerant species (exceptions: *Stipa capillata* and *Carex pendula*). Abbreviations: p=parallel.

	leaf involution	**bulliform cells**	**venation type**	**vascular sclerenchyma**	**non-vascular sclerenchyma**	**scales/ hairs**	**mean leaf size reduction (width/length)**
Afrotrilepis pilosa	symmetric	x	p	x	x	x	~ 58% / 83%
Microdracoides squamosus	-	-	p	x	x	x	~ 88% / 98%
Vellozia andina	symmetric	x	p	x	x	x	~ 41% / 92%
Vellozia crassicaulis	symmetric	x	p	x	x	x	~ 60% / 94%
Vellozia glochidea	symmetric	x	p	x	x	x	~ 61% / 92%
Barbaceniopsis castillonii	symmetric	x	p	x	x	x	~ 66% / 95%
Xerophyta spp.	symmetric	x	p	x	x	x	~ 59% / 94%
Myrothamnus flabellifolius	accordion-like (no middle rib)	-	p	x	- (low)		~ 45-70% / 75%
Stipa capillata (desiccation-sensitive)	leaves curl	x (in furrows)	p	x	x	x	~ 63% / 99%
Carex pendula (desiccation-sensitive)	leaves curl		p				~ 80% / 98%

4.5. Discussion

4.5.1. Lack of a morpho-anatomical syndrome in desiccation-tolerant vascular plants

The results clearly demonstrate that the leaf anatomy and morphology of desiccation-tolerant monocots and dicots differs substantially; within monocots (even within the same tribe) anatomical differences are present. This direct comparison shows that a morpho-anatomical syndrome in desiccation-tolerant angiosperms does not exist. Further evidence can be drawn from the anatomy of the desiccation-sensitive *S. capillata* and *C. pendula*. The former has a high amount of sclerenchyma, bulliform cells and a parallel leaf nervature. *C. pendula* shows similar traits compared to desiccation-tolerant plants, e.g. lacunae, sclerenchyma girders, a midrib with bulliform cells. Some traits are less pronounced, e.g. the amount of sclerenchyma is lower compared to desiccation-tolerant monocots and the lacunae much larger than those in the Cyperaceae presented here. However, the differences to desiccation-tolerant species are not so distinct that it would justify the postulation of a morpho-anatomical syndrome in desiccation-tolerant vascular plants. Aside from this, desiccation-sensitive *Carex* species and Cyperaceae in general show similar morpho-anatomical features (Metcalfe 1971).

An in-depth literature review about the anatomy and morphology of desiccation-tolerant pteridophytes revealed a wide variety of adaptations to desiccation tolerance as well. However, before these are discussed some interesting results concerning the restricted nature of desiccation tolerance research in pteridophytes are presented in the following paragraph.

Most pteridophytes are restricted to humid habitats. Since they need water for a successful reproduction because the gametes originate in distant parts on the gametophyte the colonization of drier habitats is naturally limited (Page 2002). However, as mentioned earlier (Fig. 2), the large majority of desiccation-tolerant vascular plants is constituted by pteridophytes which is surprising under these stark reproductive limitations and the periodically dry habitats of many desiccation-tolerant plants, e.g. rock outcrops or tree canopies. Most results concerning desiccation tolerance of pteridophytes refer to the sporophyte with its well-developed vascular system, stomata and cuticle as a water-retaining structure. The gametophyte as the decisive reproductive generation has been widely neglected in terms of desiccation tolerance

because it is thought to be extremely dependent on water and thus not likely to be desiccation-tolerant. The fern gametophyte itself is indeed extremely prone to water loss due to a lack of a cuticle (in most cases) and a vascular system. It can therefore be classified as poikilohydric because no means of water control exist and de- resp. rehydration occur very quickly and largely uncontrolled. Recent studies however reported that desiccation tolerance of gametophytes is apparently not as rare as initially suspected (Watkins et al. 2007). In a study comparing several fern species from tropical lowlands (Costa Rica) from different habitats a link between habitat and recovery from desiccation could be demonstrated. Gametophytes of species usually found in the forest understory were less desiccation-tolerant compared to the epiphyte *Microgramma reptans*. A similar finding was reported for Hymenophyllaceae: terrestrial species exhibited a lower degree of desiccation tolerance compared to epiphytic or epilithic species (Nitta 2006) which are often times exposed to extreme environmental conditions such as water shortage, high insolation and nutrient deficiency. Watkins et al. (2007) showed that gametophytes of *M. reptans* had a more complex morphology with a better water holding ability compared to those species growing in relatively humid areas. In another article it was also shown that the vulnerability to cavitation, a crucial parameter when water resources are scarce, is lower in epiphytic species (Watkins et al. 2010). These are extremely interesting findings for several reasons. The widespread misconception that fern gametophytes are desiccation-sensitive is corrected; a relationship between the habitat, its environmental conditions and the respective morphology (and presumably also other adaptive levels of desiccation tolerance) is drawn (cf. Ch. 4.5.5.) and, finally, it can be hypothesized that a different morphology and therefore a better water holding ability and desiccation tolerance was a crucial asset to colonize environmentally challenging habitats (Watkins et al. 2007). From an evolutionary point of view the high degree of epiphytism within leptosporangiate ferns can be regarded as a strategy to avoid growing competition with angiosperms from the Czenozoic onwards. Apparently, a growing fern diversity can be correlated to the evolution of epiphytism (Schuettpelz & Pryer 2009). Here, morpho-anatomical adaptations allowing a higher degree of desiccation tolerance of the sporo- and gametophyte might have evolved as a prerequisite for the colonization of tree canopies. After having discussed the need for research of the sporo- and gametophyte other

pteridophyte species are discussed in the context of the existence of a morpho-anatomical syndrome. One epiphytic species which has been researched extensively, although not mainly under the aspect of desiccation tolerance, is *Platycerium stemaria* (Polypodiaceae). Aside from its interesting leaf dimorphism many *Platycerium* species have very leathery leaves and several xeromorphic characteristics such as a thick cuticle and sunken stomata. This mainly applies to the sporotrophophylls (Froebe & Strank 1981) and is usually regarded as an adaptation to survive periods of drought or desiccation outside humid habitats. Although *P. stemaria* shows some degree of succulence and xeromorphism xerophytic species are relatively rare among pteridophytes, especially compared to angiosperms. Sclerenchyma, as one typical xeromorphic trait, in general can be found frequently in pteridophytes but not in high amounts. Exceptions are constituted by several genera such as *Cheilanthes*, *Notholaena* and *Anemia* which can be classified as xeromorphic with a considerable amount of sclerenchyma and scales or hairs to increase water absorption upon rehydration (Brodribb & Holbrook 2004; Kramer et al. 1995; Stuart 1968). The genus *Anemia* (Schizaeaceae), can frequently be found on rock outcrops. A recent study showed that the leaf (frond) anatomy from two Brazilian *Anemia* species could be classified as mesomorphic whereas a high share of xeromorphic characteristics were identified in the petiole (Ribeiro et al. 2007). These results are obviously not conclusive but although some morpho-anatomical traits which can be attributed to desiccation tolerance (e.g. sclerenchyma accumulation, leaf rolling, hairs and scales for water absorption) occur in several pteridophytes this does not take desiccation-tolerant filmy ferns (mainly Hymenophyllaceae) into account (Porembski 2011). Leaves of Hymenophyllaceae consist of only 1-4 cell layers, a cuticle and epidermis are often missing (Mettenius 1865; Proctor 2003). Most species prefer shady, relatively humid habitats which is why they are often compared to desiccation-tolerant cryptogams, especially bryophytes. Filmy ferns can be, in stark contrast to desiccation-tolerant angiosperms, classified as poikilohydric because they do not exhibit any water-retaining structures. If this wide variety of morpho-anatomical adaptations (or lack of it) to desiccation tolerance is considered the existence of a morpho-anatomical syndrome cannot be confirmed for pteridophytes. Since this has already been denied for angiosperms it has to be denied for vascular plants in general. Similar to desiccation-tolerant angiosperms it should be

noted that important adaptive morpho-anatomical traits to desiccation tolerance exist (cf. below) but compared to angiosperms a larger number of species (especially poikilohydric Hymenophyllaceae) seems to rely only on molecular, physiological and biochemical mechanisms. This does certainly not imply that morpho-anatomical traits do not matter (e.g. Watkins et al. 2007); the importance of the four adaptive levels may simply differ from species to species.

4.5.2. Important adaptive morpho-anatomical traits

Although a general morpho-anatomical syndrome in desiccation-tolerant vascular plants could not be confirmed relevant morpho-anatomical traits were identified for the examined species (Ch. 4.4.). These include the occurrence and role of bulliform cells, leaf rolling and involution (Dinakar et al. 2012), the leaf venation type and sclerenchyma distribution (cf. Table 4).

Leaf venation, bulliform cells and their role during leaf involution

A proper leaf involution in general reduces the transpirational surface and the speed of dehydration, thus allowing the induction of cellular protection mechanisms. Further advantages are the protection of the leaf from excess radiation and leaf stabilization. Between monocots and dicots only the mimicry of a parallel leaf venation by dicots due to the parallel alignment of the major vascular bundles is a morphological similarity. It facilitates the leaf involution during dehydration. Surprisingly, it is common in other desiccation-tolerant dicots as well such as *Myrothamnus moschatus* (Myrothamnaceae, Ch. 3), *Craterostigma plantagineum*, *Craterostigma wilmsii* and *Lindernia brevidens* (all Linderniaceae) (Dalla Vecchia et al. 1998; Farrant et al. 2007b; King et al. 1996; Phillips et al. 2008; Scott 2000; Sherwin & Farrant 1996) but not ubiquitous since the desiccation-tolerant Gesneriaceae *Ramonda myconi*, *Ramonda serbica* and *Haberlea ferdinandi-coburgii* exhibit a net venation.

The benefits from leaf involution are universal and it is also a decisive trait (and with the same benefits) in the monocots examined but the folding mechanism is completely different. In monocots the midrib, which divides the leaf in two equally sized portions, and the translucent bulliform cells (also called motor cells) above the midrib are important. If they are located in every adaxial furrow (as in *S. capillata*) they induce the

leaf rolling but if the bulliform cells are located above a midrib they form a hinge-like structure and span the lamina in the rehydrated state (Duval-Jouve 1875). If the turgor of the bulliform cells examined in this study decreases along with the turgor of parenchyma cells upon dehydration and finally the cell walls collapse, the leaf cannot be expanded anymore and folds symmetrically. Leaf involution can therefore clearly be classified as a hydronastic movement since it is regulated by the severity of water loss.

However, bulliform cells are apparently multifunctional: leaves of *S. stapfianus*, a rock outcrop dweller from Southern Africa, are usually described as xeromorphic due to their stiffness caused by a high share of sclerenchymatic fibers and many silicized cells (Dalla Vecchia et al. 1998). A marked difference to many other monocots is that the leaf does not fold or curl extensively although bulliform cells are present throughout the leaf. They are not restricted to the midrib (which is not present in *S. stapfianus*). Apparently leaves do not fold upon dehydration as in many other monocots; they merely bend at the leaf tips. Here, bulliform cells have been hypothesized to act as a water storage (Dalla Vecchia et al. 1998).

Bulliform cells cannot be found in dicots; among monocots *M. squamosus* is an exception. These cells are not necessary for a successful leaf involution in all grasses (Shields 1951), however, leaf involution in monocots via bulliform cells is one adaptive trait among many.

As in desiccation-tolerant angiosperms leaf involution or rolling of fronds upon dehydration has been reported several times (Lebkuecher & Eickmeier 1993) and is an important adaptation of desiccation-tolerant pteridophytes. The protective function of leaf rolling has been shown for *Selaginella lepidophylla* (Selaginellaceae): recovery of leaves is significantly lower if leaf rolling is impaired (Gratani et al. 1998). The mechanism seems to differ from desiccation-tolerant angiosperms because a parallel leaf venation has not been reported and bulliform cells are very rare in leaves of pteridophytes – one known exception is *Cheilanthes persica* which folds via bulliform cells and reduces its surface by 51% upon dehydration (Gratani et al. 1998).

Sclerenchyma distribution

Among dicots the photosynthetic tissue is barely interrupted by sclerenchyma. This has not only been demonstrated for *M. flabellifolius* but for *L. intrepidus*, some

Gesneriaceae and *M. moschatus* as well (personal observation N. Korte, Heil 1924; Korte & Porembski in press; Schiller et al. 1999).

Sclerenchmya is abundant in monocot leaves as a supporting tissue during the de- and rehydration process. This has been shown in other studies as well (Balsamo et al. 2006; Hedderson et al. 2009). Desiccation-tolerant monocots seem to be xeromorphic whereas desiccation-tolerant dicots are rather meso- or hygromorphic. This first trend should be subjected to an even wider comparative study.

The high amount of sclerenchyma increases the tensile strength of dehydrated leaves which underlines the importance of this tissue in the function of mechanical support. Ergo, the sclerenchyma distribution in leaves of the monocots described above can be attributed to a higher need for stability. For instance, the adaxial and abaxial sclerenchyma strands in rehydrated leaves of *M. squamosus*, *V. glochidea* and *V. crassicaulis* appear as one in the dry state due to the reversible folding of mesophyll cells. That these strands play a major role in the mechanical support of the leaf is underlined by a relatively low amount of shrinkage during dehydration, especially compared to *V. andina* (Fig. 19). However, other monocots shrink very little in length and width, too – except *M. squamosus* where almost no shrinkage was measured (Fig. 19).

If the mesophyll:sclerenchyma ratio was higher the leaf would shrink considerably. This is supported by the relatively severe amount of shrinkage of *M. flabellifolius*. There are other examples for the stabilizing function of sclerenchyma: the fusion of the Y-shaped girders in vascular bundles, the small sclerenchyma islands located in the mesophyll between the vascular bundles which keep them in place, and marginal sclerenchyma, which does not occur in Cyperaceae and Poaceae but in all Velloziaceae described here. The function of the latter has not been fully clarified but it can be assumed that it has a stabilizing function as well. If less sclerenchyma was present leaves would probably not be able to sustain their stiff form in the dry state and would curl and not fold.

Sclerenchyma is widely regarded as a xeromorphic trait which facilitates the survival in a dry environment (Dalla Vecchia et al. 1998; Fahn & Cutler 1992; Turner 1994). But adaptations to desiccation tolerance are obviously more complex. Some species are xeromorphic whereas others are meso- to hygromorph. Among desiccation-tolerant vascular plants morpho-anatomical features (e.g. epidermis, sclerenchyma) might retard

water loss as mentioned earlier (Dalla Vecchia et al. 1998; Oliver 1996; Salleo et al. 1997). They are therefore important to induce cellular protection mechanisms which are a widespread strategy in desiccation-tolerant angiosperms. Filmy ferns on the other hand (probably similar to cryptogams such as bryophytes) mainly rely on repair mechanisms upon rehydration (Proctor & Tuba 2002). Therefore, xeromorphic traits are not a decisive feature of all desiccation-tolerant vascular plants and not necessarily vital for the survival of periods of drought. This also implies that the analysis of morpho-anatomical traits alone cannot give reliable answers about the desiccation tolerance (and its degree) of a certain species (Farrant et al. 2007a; Oliver et al. 2000; Tuba et al. 1998). Apparently different and complementary molecular, physiological and biochemical strategies are involved in meso-, hygro- and xeromorphic. This can only be resolved conclusively with a large comparative study integrating all adaptive levels.

4.5.3. The dominance of monocots among desiccation-tolerant angiosperms

From a purely morpho-anatomical perspective the majority of Poaceae and Cyperaceae has the potential to be or become desiccation-tolerant. But since morpho-anatomical traits are only a piece in the puzzle of desiccation tolerance the analysis of leaf anatomical traits alone cannot explain the dominance of monocots among desiccation-tolerant angiosperms. Anatomical features such as a high share of sclerenchyma and a parallel leaf venation are certainly a decisive trait since at least the latter is mimicked by some dicots. Molecular, physiological and biochemical aspects have to be taken into account as well. Under this respect the uneven distribution of desiccation tolerance among monocots and dicots can be explained. The major reason for the dominance of monocots can most likely be accredited to a different physiological strategy. Poikilochlorophylly, i.e. the complete dismantling of thylakoid membranes and breakdown of chlorophyll during dehydration, in contrast to homoiochlorophylly, is a strategy which has only been attributed to monocots (Chaw et al. 2004). From a phylogenetic perspective the evolution of homoio- and poikilochlorophylly might be explained by the divergence of monocots and dicots 140-150 Mio years ago (Chaw et al. 2004) and is regarded as the newest evolutionary development within plant desiccation tolerance.

A poikilochlorophyllous species compensates the disadvantage of a longer recovery period by the ability to spend longer periods in the desiccated state due to the fact that the photosynthetic apparatus cannot be damaged anymore. For this, extensive supporting tissues are advantageous to maintain the leaf structure in the desiccated state. Consequently, sclerenchyma and a parallel leaf involution are phylogenetic legacies with a high adaptive value, allowing desiccation-tolerant monocots to endure longer periods in the desiccated state.

Vice versa, it is certainly debatable whether the morpho-anatomical phylogenetic legacy of Cyperaceae, Poaceae and Velloziaceae did not facilitate the evolution of poikilochlorophylly in the first place and can be classified as a purely opportunistic strategy. Unfortunately, this question cannot – at least without a more profound knowledge of the molecular, physiological and biochemical mechanisms of desiccation tolerance – be answered conclusively.

4.5.4. Phylogeny and morpho-anatomical adaptations to desiccation tolerance

Vegetative desiccation tolerance is ancient in plants and was a crucial step for the colonization of the land as already mentioned in Ch. 1.3. (Proctor & Tuba 2002). It is classified as a primitive trait in bryophytes but highly derived in vascular plants (Proctor & Pence 2002; Schuettpelz & Pryer 2008). The phylogenetic distribution pattern of vegetative desiccation tolerance is erratic in vascular plants. Due to the existence of desiccation tolerance in distant clades in the plant kingdom a variety of adaptations to desiccation tolerance can be assumed. This has already been postulated for the physiological, biochemical and molecular level (Leprince & Buitink 2010) and is consistent with the lack of a morpho-anatomical syndrome in desiccation-tolerant vascular plants on a smaller and wider scale. Although the common appearance of a leaf involution (i.e. leaf rolling here) is a similarity to other desiccation-tolerant vascular plants this is generally not confined to desiccation-tolerant species. As has been noted above, the occurrence of bulliform cells is very sporadic and apparently not always crucial for the leaf rolling or folding process. The morpho-anatomical traits of desiccation-tolerant pteridophytes are very diverse which may be attributed to the fact that the range of species and their respective habitats is much more diverse, too (from epiphytes to rock outcrop colonizers). Within angiosperms vegetative desiccation

tolerance is more common in some families than in others (e. g. Ingram & Bartels 1996; Le & McQueen-Mason 2006). Under this respect the widespread occurrence of a parallel leaf venation in desiccation-tolerant dicots underlines its importance for a functional leaf involution, its protective advantages and indicates its independent evolution in different families which can be postulated for the other morpho-anatomical traits discussed earlier. For monocots a parallel leaf venation trait is a phylogenetic legacy with high adaptive value.

4.5.5. Links between morpho-anatomical traits and other adaptive levels

Adaptations to desiccation tolerance are, as mentioned earlier, very complex. Several mechanisms in orthodox seeds and desiccation-tolerant plants are similar and responses run parallel on different adaptive levels (cf. Ch. 1.3., Farrant & Moore 2011; Illing et al. 2005). Although the aforementioned authors give a good impression of the complex mechanisms involved, these are not species-specific. Many adaptations are similar in different species but a single syndrome cannot be identified when several species and their adaptations on the respective levels are compared. This correlates with the findings presented above. This also implies that evolutionary trends of desiccation tolerance via the analysis of the adaptive levels cannot be deduced on a small scale; trends may only be deduced on an extremely large scale, i.e. bryophytes vs. pteridophytes vs. angiosperms (cf. Ch. 1.3.).

Nevertheless, many points for the connectedness of adaptive levels can be made. As mentioned in Ch. 1.1., several more or less artificial categories to delineate different types of desiccation-tolerant plants exist. One of these attempts distinguishes fully and modified desiccation-tolerant plants (Toldi et al. 2009). This distinction proves to be useful in the context of this work because it is based on morpho-anatomical features. Fully desiccation-tolerant species dry out rapidly due to the lack of evaporatic control as they have no water-retaining structures but they are also marked by their ability to survive rapid drying; their desiccation tolerance is often times constitutive, i.e. many cellular components necessary for a successful rehydration are constitutively expressed, these species are always prepared for dehydration events and they mainly rely on repair strategies (Oliver et al. 1998). This form of desiccation tolerance is often times regarded as evolutionary primitive (Oliver et al. 2000). Most of these species are usually non-

vascular plants, mainly bryophytes, lichens and algae. However, a few desiccation-tolerant vascular species (mainly Hymenophyllaceae) probably belong to this group as well due to their lack of water-retarding traits.

Modified desiccation-tolerant species on the other hand are only able to survive slow drying. This mainly refers to vascular plants which usually exhibit morpho-anatomical structures that retard water loss, e.g. sclerenchyma. It does not only retard water loss and therefore prolongs the time for the induction of relevant protection mechanisms but it also retards water uptake upon rehydration (Blomstedt et al. 1998; Gaff & Churchill 1976). This is not necessarily unfavorable because a too rapid rehydration can be harmful as well. This type of desiccation tolerance is usually referred to as "induced", an evolutionary derived form reserved for vascular plants. These species are not always prepared for complete desiccation but need more time to induce cellular protection systems. Modified desiccation-tolerant plants usually rely on protective strategies because they have more time to induce relevant mechanisms. Both classifications attribute a crucial role to morpho-anatomical traits because they are an important factor in the control of water relations, i.e. the rate of water loss and therefore the existence of specific morpho-anatomical structures are directly correlated.

In the following few paragraphs examples for constitutive and induced desiccation tolerance will be given. In *Tortula ruralis*, a desiccation-tolerant bryophyte, several clues for constitutive desiccation tolerance were identified. Dehydrins, a special group of late embryogenesis abundant proteins (LEAs), were found in the hydrated state (Oliver et al. 1998). LEAs are common proteins in plants and they are usually associated with abiotic stresses such as drought or cold (Tunnacliffe & Wise 2007). They are however rarely found in the hydrated state and are thought to be involved in cellular protection mechanisms. Another example is the high amount of sucrose in *T. ruralis* in the hydrated state (10% dw) (Oliver et al. 1998). Since sugars function as osmoprotectants they can be regarded as another protective mechanism. Again, the peculiarity compared to desiccation-tolerant vascular plants is the permanent storage of amounts of sugar in the hydrated state.

A third and last example is the occurrence of mRNA particles (mRNPs). mRNA transcripts of rehydrins, a group of proteins important during rehydration (no LEAs), in the hydrated state are quickly transformed into mRNPs upon dehydration and can easily

be transformed from mRNPs to mRNAs upon rehydration which speeds up the rehydration process in general (Oliver et al. 1998; Wood & Oliver 1999). Obviously, a recovery program upon rehydration is performed (cf. Oliver et al. 2000). This is of course only a very limited selection of constitutive desiccation tolerance; for further information the reader may be referred to excellent reviews (e.g. Oliver et al. 2005). In modified desiccation-tolerant species constitutive patterns are extremely rare. Changes regarding the cellular protein and osmoprotectant composition, translational control are usually only induced upon dehydration (e.g. Blomstedt et al. 1998; Gaff et al. 2009; Ingle et al. 2007).

However, even among desiccation-tolerant angiosperms transition states can be encountered. In *C. wilmsii* for instance large amounts of the sugar 2-octulose were identified. This is unusual for two reasons: large amounts of sugar are usually not stored in hydrated desiccation-tolerant vascular plants; this is however very common in fully desiccation-tolerant species. The second peculiarity lies in the sugar itself. Most desiccation-tolerant vascular species use sucrose as an osmoprotectant whereas 2-octulose seems to be confined to this species. Obviously, the large amount of sugar in the hydrated state suggests that *C. wilmsii* uses a constitutive mechanism like many non-vascular plants, at least for the sugar metabolism (Farrant et al. 1999). Therefore, the strict delineation of constitutive and induced desiccation tolerance resp. fully and modified species should not be too dogmatic.

After this account on fully and modified desiccation-tolerant species other examples which demonstrate links between several adaptive levels will be given. First, the connection between morpho-anatomical traits on an ultrastructural level and the physiological strategy is addressed. As mentioned briefly in Ch. 1.3. homoiochlorophyllous and poikilochlorophyllous desiccation-tolerant species exist. The former group is comprised of some monocots, all dicots and cryptogams. These species retain their chlorophyll upon dehydration (Toldi et al. 2009). As a consequence they are able to photosynthesize relatively quickly upon rehydration. Species of the latter group on the other hand dismantle their photosynthetic apparatus completely (Bewley 1979; Hambler 1961; Toldi et al. 2009). These species naturally need more time for a complete recovery. This strategy appears to be limited to monocots. On an ultrastructural level the chloroplasts of poikilochlorophyllous species are similar to

chloroplasts of many other plants. During dehydration however, these so-called "desiccoplasts" dismantle their complete photosynthetic apparatus, i.e. chlorophyll (a + b), most carotenoids, thylakoid membranes, and osmiophilic plastoglobuli. The pigment loss in chloroplasts in leaves of *Xerophyta scabrida* (Velloziaceae) was documented. After 48 hours of desiccation 87% of the chlorophyll a and b content was lost whereas only 57% of the carotenoids were lost (Tuba & Lichtenthaler 2011). Leaves then turn yellow. The desiccoplasts have an oval shape in the dry state and generally resemble gerontoplasts which are typical of desiccation-sensitive plants before they shed their leaves in autumn. At this point, the chloroplasts do not function properly anymore due to the successive loss and breakdown of photosynthetic pigments and thylakoid membranes (Tuba & Lichtenthaler 2011). The major difference to a geronotplast however is that desiccoplasts regain their full function after 2-3 days upon rehydration. In desiccoplasts first re-greening and photochemical activity occur after approx. 24 hours, which is followed by the rebuilding of thylakoid membranes and the complete reassembly of the photosynthetic apparatus (Tuba et al. 1994; Tuba et al. 1993). The speed of resynthesis is relatively quick which can probably be attributed to the fact that the majority of needed components is stored in the plastoglobuli since their number gradually declines upon rehydration.

The second example comes from an interesting case study of the fern *Mohria caffrorum*. This species is a part-time desiccation-tolerant plant with a sensitive period during the rainy season (Farrant et al. 2009). This is highly exceptional but it hints to the ecological significance of desiccation tolerance as an adaptation to habitat-specific environmental conditions. Aside from this, morpho-anatomical structures change in accordance with the changing degree of desiccation tolerance. During the dry (i.e. desiccation-tolerant) period fronds curl easily as a protection against excess light and therefore damage of the photosynthetic apparatus. Scales on the abaxial (exposed) side of fronds are present which probably mask chlorophyll, hereby reducing the damage of excess light and therefore elevated reactive oxygen species (ROS) production. This connection between scales and a low ROS production could be demonstrated (Farrant et al. 2009). The total leaf area of desiccation-tolerant individuals was also reduced compared to those from the rainy season. These characteristics are highly relevant because fronds from the desiccation-sensitive period did not curl but simply wilted and

scales were absent. This clearly indicates the adaptive significance of morpho-anatomical structures in desiccation-tolerant plants and their connectedness to other adaptive levels.

Another interesting example which demonstrates, in this particular case, the effects of physiological changes on morpho-anatomical structures is *Polypodium vulgare* (Polypodiaceae). Here, a pretreatment with abscisic acid (ABA), a phytohormone associated with desiccation tolerance, altered the ultrastructure of cell walls and the plasmamembrane (Zenkteler & Bagniewska-Zadworna 2005). It has long been known that a pretreatment with ABA enhances or sometimes even induces desiccation tolerance in many species but the direct links to morpho-anatomical changes had not been revealed before. In conclusion, several links between the four adaptive levels can be identified. However, systemic approaches to desiccation tolerance are very rare and should be encouraged in the future to unravel the full connectedness of all four adaptive levels.

5. First non-destructive leaf growth rate determination of desiccation-tolerant, mat-forming monocots

5.1. Abstract

Desiccation-tolerant plants are adapted to habitats characterized by harsh environmental conditions such as infrequent rainfall and high temperatures. Consequently their growth rate is assumed to be very low. Among desiccation-tolerant vascular plants this has only been destructively quantified in two studies. The absolute growth rate of *Afrotrilepis pilosa* leaves (Cyperaceae), a dominant mat-forming, desiccation-tolerant species on inselbergs in West Africa, was determined along a climatic (precipitation) gradient in Ivory Coast over a period of three months (June-September, rainy season interrupted by a short dry season). A non-destructive leaf-marking method was applied for terrestrial plants for the first time and its suitability and possible modifications are discussed. Absolute growth rates of leaves differed along the climatic gradient but were not statistically significant. The lowest mean absolute growth rate was found among leaves in drier surroundings (1.74 cm), the highest in the humid south west (6.91 cm), which is low compared to European pasture grasses. A leaf turnover of at least 6 leaves p.a. can be assumed.

5.2. Introduction

Rock outcrops such as inselbergs are a center of diversity for desiccation-tolerant plants. Some monocotyledonous desiccation-tolerant species form extensive mats (up to 40-60 m^2) on the occasionally very steep slopes of inselbergs. In South America desiccation-tolerant Velloziaceae are mat formers whereas the Cyperaceae *Afrotrilepis pilosa* is the dominant mat former on West African inselbergs. These mats are an important habitat because substrate is accumulated due to the decomposition of organic material which creates a habitat for animals and retains water which would otherwise be lost as run-off. Although desiccation tolerance allows plants to colonize azonal habitats such as inselbergs they have a very low growth rate, especially compared to homoiohydrous plants which require a fairly constant water supply. This is commonly known as the desiccation tolerance productivity trade-off hypothesis (Alpert 2000, 2006; Oliver et al. 2000). The net primary production of some lichens and bryophytes have been studied in the past (for a brief overview cf. Kappen & Valladares 1999) but the absolute growth

rates of leaves of desiccation-tolerant vascular species have rarely been quantified. It is for instance known that some Velloziaceae may attain a height of up to 4 m and an age of 550 years (Alves 1994). A study of *A. pilosa* destructively estimated the growth of one individual to 0.5 cm p.a., i.e. an individual of 1 m height could easily have an age of 200 years (Bonardi 1966; Chevalier 1933). However, all quoted studies used destructive methods. Due to the low productivity and establishment success of many desiccation-tolerant vascular plants non-destructive measurement methods should be applied. Therefore *A. pilosa* as a dominant desiccation-tolerant species on West African inselbergs was chosen for this pilot study to non-destructively determine the growth rate of leaves and *A. pilosa* mats along a climatic (precipitation) gradient for the first time and compare these results to the destructive method used by Bonardi (1966).

5.3. Material and Methods

To determine the absolute growth rate of *A. pilosa* leaves non-destructive leaf-marking methods frequently used in seagrass research were modified and applied to terrestrial plants (Short et al. 2001; Zieman 1974). A similar method has only been used once for the analysis of pasture grasses (Tallowin et al. 1995). During the rainy season in June 2010 four different inselbergs were visited in Ivory Coast (IB 1–4) (Fig. 28). IB 1-3 are located on an axis of decreasing precipitation towards the north whereas IB 4 is located in the humid south west. On each inselberg 5-7 *A. pilosa* mats (23 total) were randomly chosen and their size was measured. Up to 3 leaves (old and young) of one individual were marked with a permanent marker or nail polish at the lamina base (point 0) and in the middle of the lamina to confirm that leaves exclusively grow from a basal meristem (Fig. 29). Aluminum tags were attached to each marked individual. Altogether 113 individuals and 261 leaves were sampled. After the short dry season (September 2010) growth was measured from the lamina base to the marking and between markings during the rainy season. The newly produced biomass of leaves (increment below the marking) was clipped, air-dried and weighed after two weeks. For statistical analysis JMP 5.0.1.2. (SAS Institute) was applied. To increase the accuracy of results, only green leaves were included in the statistical analysis (n=75 for absolute growth rate determination, n=57 for dry weight determination).

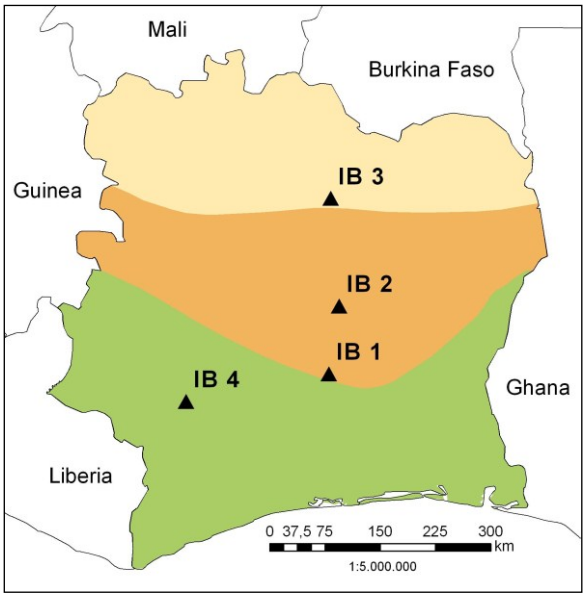

Figure 28 Location of IB 1-4 in Ivory Coast. Precipitation is high in the humid south and decreases towards the north. Color code: green: rainforest zone (humid and wet), orange and beige: savanna (drier towards the north).

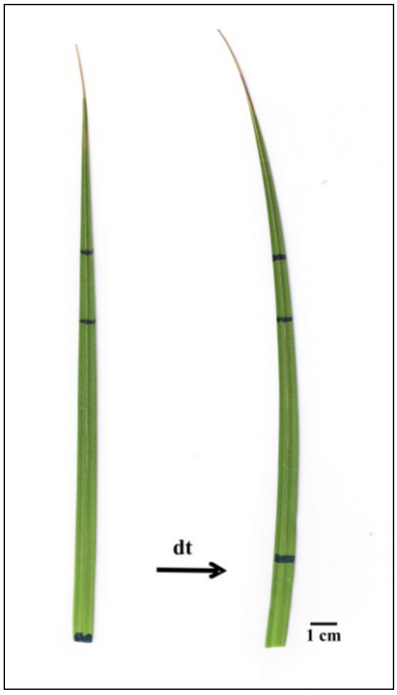

Figure 29 Marked lamina of *Afrotrilepis pilosa* (initial marking (left), after approx. 3 months (right)).

5.4. Results

All sampled mats, 90% of all individuals but only 50% of all marked leaves were re-located in September 2010. The mat size did not change from June to September 2010. Leaf growth was only detected at the lamina base (i.e. from a basal meristem), not in the middle of the lamina. The highest mean growth was found on IB 4 (6.91 cm) with 37 cm being the maximum for one leaf, the lowest absolute growth was found on IB 2 (1.74 cm) which is located in drier surroundings. Leaves on IB 1 and IB 3 had similar mean absolute growth rates (Fig. 30). The Tukey-Kramer test did not indicate significant differences between the four inselbergs. The correlation between mat size and leaf growth was not significant, either. The analysis of the mean dry weight indicated a significant higher value for IB 4 compared to IB 1-3 (Fig. 31).

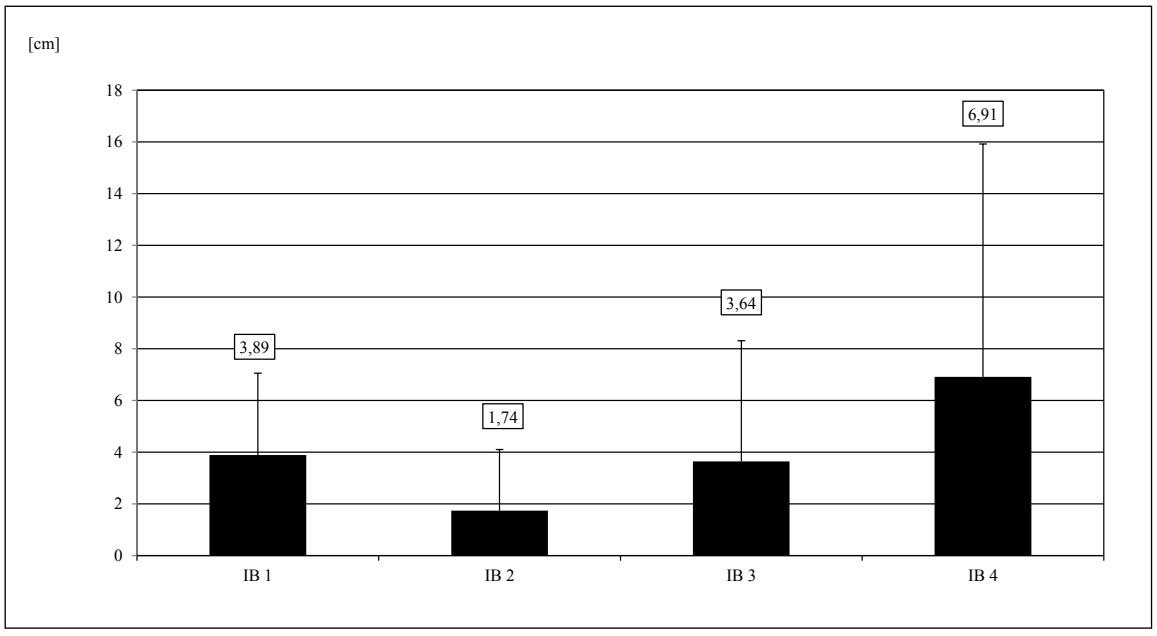

Figure 30 Mean absolute growth rate of all individuals on IB 1-4 (differences among inselbergs are not significant).

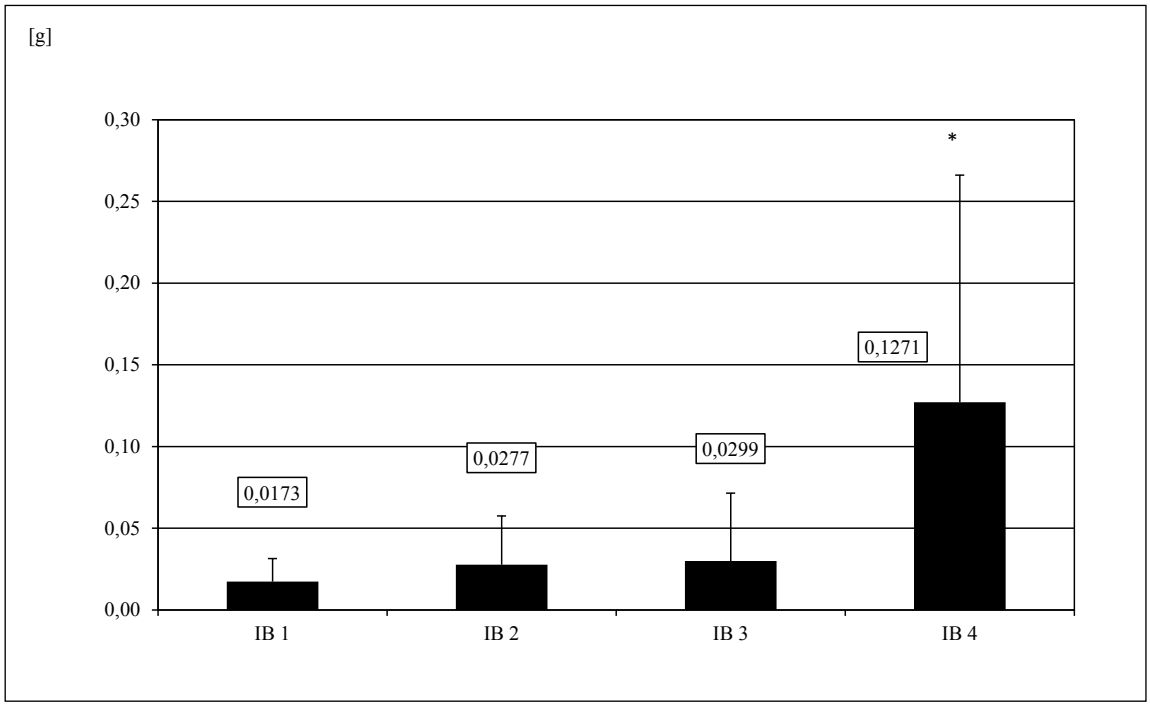

Figure 31 Mean dry weight of newly produced biomass (IB 4 significantly different).

5.5. Discussion

5.5.1. Absolute growth rate

No significant differences between the absolute growth rate and climatic zone (i.e. amount of precipitation) or mat size and individual leaf growth could be validated. The high mean growth of leaves on IB 4 however indicates a much higher absolute growth rate in the humid south west compared to inselbergs located in drier surroundings. The apparently more favorable environmental conditions on IB 4 are reflected in the leaf morphology as well. Leaves of *A. pilosa* in the south west were more vigorous, longer and wider (up to 70 cm, 1.5 cm wide) compared to those on the other three inselbergs (approx. 30-50 cm, c. 0.5-1 cm wide). This morphological difference explains the statistical difference between IB 1-3 and IB 4: since leaves on IB 4 are much larger compared to those on IB 1-3 their weight is naturally higher.

Outer (older) leaves marked in June were almost exclusively dead in September. Although no information about the age and life history of single leaves are available the low growth rates suggest that outer leaves died soon after the end of the rainy season in

June 2010. Apparently all leaves undergo a certain number of wetting and drying cycles before they die and energy is completely allocated to younger leaves. These outer leaves which have a higher photosynthetic leaf area are important to gain a positive carbon balance in the rainy season but once the leaf area and the cost for rehydration become too high they are discarded and replaced by inner (younger) leaves.

However, the absolute growth rate of leaves of *A. pilosa* is higher than initially assumed but can still be classified as low, especially compared to European pasture grasses. Tallowin et al. (1995) measured the growth of four Poaceae (*Lolium perenne*, *Agrostis stolonifera*, *Holcus lanatus*, *Poa trivialis*) over periods of 14-21 days during the summer months. *L. perenne* had the highest daily lamina extension rates (ranging from ~ 3.3-6.5 mm), *P. trivialis* the lowest (ranging from ~ 1.4-4 mm). If these values are extrapolated for 3 months the absolute lamina growth rates of these temperate grasses are much higher than those of *A. pilosa* (with very few exceptions on IB 4). Alves (1994) reported a yearly leaf production of 8-10 leaves in some Velloziaceae, Bonardi (1966) estimated a leaf production of 6 leaves for *A. pilosa*. The latter is certainly a realistic, if not slightly pessimistic, assessment. These values and the annual increment of 0.5 cm found by Bonardi (1966) are, at least in some regions, comparable to values determined using a non-destructive method. Nevertheless, the data presented above are results from a one-year study and should therefore be interpreted carefully. The study should be repeated to include more data into the statistical analysis.

5.5.2. Leaf-marking method

The data demonstrate that the non-destructive leaf-marking method can be applied to *A. pilosa*. The method should be utilized for growth rate determinations of other monocotyledonous desiccation-tolerant mat-forming species on inselbergs in other parts of the world as well (e.g. Velloziaceae in South America) to gain deeper insights into the ecology of these plants.

The fact that some leaves were in fact clipped during this study has certainly not escaped the readers' attention. This procedure is however not necessary if the determination of the dry weight of samples is not desired. In fact, this method allows the subsequent marking of each marked lamina, i.e. the absolute growth rate of one leaf can be monitored over several time periods. It is nevertheless important to sample many

leaves and individuals because a considerable number of samples may be lost. Data derived from this method may not be as exact as from destructive methods but among species which are endangered, rare or where the in vivo growth rate is desired this method should be considered. In this pilot study old and young leaves were marked alike to test the impact of chemicals on leaves. Future studies should focus on inner (younger) leaves to allow an easier data interpretation. The impact of chemicals contained in permanent markers did not have a negative effect on leaves; among leaves treated with nail polish some samples turned brown which certainly impaired the water and solute transport (and therefore growth). Since this did not lead to a die-back of leaves and was confined to very few samples they were included in the analysis as well.

5.5.3. Absolute vs. relative growth rate

In this study the absolute growth rate of *A. pilosa* leaves was determined. The absolute growth rate describes the growth increment over time, i.e. mg increase/day. However, many publications usually refer to the relative growth rate, i.e. the increase in biomass per unit time per unit of biomass already present (g/g/day or mg/g/day) (Poorter 1989). If this is desired the method has to be modified. This would mainly involve destructive methods. In this case a certain number of undisturbed mats and 5-10 individuals per mat should be chosen. Ideally 3 inner leaves per individual are marked at the base with a permanent marker. At the first harvest point a few leaves (depending on the number of harvest points) are clipped, the total width (basal) and length of leaves and the length of growth increment (from base to mark) are measured. In the lab the dry weight of the whole leaf and the growth increment section are determined (at least 24 hours, 70 °C). This procedure is repeated at each harvest point. This modification should be tested because it allows a comparison to other species which would then be helpful to verify of falsify the desiccation tolerance productivity trade-off hypothesis. However, this procedure was not chosen for two reasons. First, a constant presence over at least one year (ideally more) near the plots is required which could not be realized. Second, the goal of this study was to find a non-destructive measurement method. This was accomplished but a functional synthesis of the marking and the destructive method should be realized in cooperation with researchers on site.

6. Outlook

Although the preceding chapters might suggest that a large body of research on plant desiccation tolerance does already exist additional research on the morpho-anatomical level is important to gain further insights into the mechanisms of desiccation tolerance of other species and plant groups as these differ from species to species to some extent. This is of course also true for the other three levels. Much work has now been done on leaves of desiccation-tolerant vascular plants but future research should, in addition to the studies quoted in Ch. 4, also focus on morpho-anatomical characteristics and adaptations of roots since only very few studies were devoted to roots and their adaptive value (Heilmeier et al. 2002; Porembski & Barthlott 1995). Roots as water and nutrient-absorbing organs probably play a key role in the water household of desiccation-tolerant vascular plants. Since temporal water scarcity characterizes habitats of many desiccation-tolerant vascular plants efficient water uptake mechanisms are likely to exist. Aside from more morpho-anatomical studies of roots physiological aspects should be examined. This should include the mechanisms of xylem refilling and the elimination of embolisms throughout the whole plant. The latter should be considered first and foremost because this has fierce implications for the biotechnological engineering of more drought-tolerant crops (cf. below).

The number of desiccation-tolerant pteridophytes is relatively high. This is in stark contrast to the research efforts devoted to this group. Obviously, these results also underline the need for more in-depth research concerning pteridophytes and their actual desiccation tolerance (not many species have been tested). First of all, extensive and thorough tests of several species which are thought to be desiccation-tolerant are required (sporo- and gametophyte). This is largely determined by their suspicious habitat (e.g. inselbergs, tree canopies) or taxonomic relationship. Once this is done comparative studies of their morpho-anatomical traits should be conducted. Micro-computed tomography could be used for these studies. A better knowledge of morpho-anatomical characteristics of desiccation-tolerant pteridophytes would allow an even more profound comparison of desiccation-tolerant species and their respective traits across plant groups (monocots and dicots, angiosperms and pteridophytes). Naturally, research on other adaptive levels is certainly needed as well and their possible links between morpho-anatomical traits, habitat and degree of desiccation tolerance should be

determined. This is especially interesting from an evolutionary point of view because it has often times been hypothesized that pteridophytes pose an evolutionary transition state in their desiccation tolerance which has to be tested on all adaptive levels (Oliver et al. 2000). Therefore species from families harboring desiccation-tolerant genera such as Schizaeaceae, Pteridaceae, Polypodiaceae and Aspleniaceae need to be considered first before families with less desiccation-tolerant species are examined. Research results from other desiccation-tolerant pteridophytes but also angiosperms can be used as a source of inspiration. Morpho-anatomical research of desiccation-tolerant lycophytes is even scarcer than that of ferns. Desiccation tolerance of corms of the genus *Isoetes* has been mentioned once but has not been studied extensively (Gaff & Latz 1978). A few basic studies about *Selaginella lepidophylla* (Selaginellaceae) and its adaptations to desiccation tolerance exist (Brighigna et al. 2002; Iturriaga et al. 2006; Lebkuecher & Eickmeier 1993) but this is certainly not sufficient and should be targeted in the near future. This would advance our knowledge of desiccation tolerance immensely.

The relationship between seed and vegetative desiccation tolerance is another matter requiring attention (Leprince & Buitink 2010). Many mechanisms of vegetative desiccation tolerance resemble those of orthodox seeds. Current research indicates that mechanisms from seeds but also novel strategies evolved in vegetative tissues can be distinguished (Illing et al. 2005). Additionally, further knowledge about strategies in pollen and spores will be relevant. Once a better understanding of this complex issue has been gained more reliable conclusions about the evolution of desiccation tolerance in land plants may be drawn. Aside from evolutionary aspects results from studies concerning salt tolerance should be considered because many mechanisms seem to be comparable to those in desiccation-tolerant plants (Bartels & Sunkar 2005). Similarly, tolerance of very high or low temperatures seems to be linked to desiccation tolerance because many desiccated species are apparently able to endure extreme temperatures in the desiccated state (cf. references in Alpert 2000).

On the molecular level, the response to dehydration and desiccation in plants is multigenic. Despite the large body of research that has been devoted to different aspects of desiccation tolerance single-gene experiments to confer desiccation-tolerance in sensitive plants have not been satisfying (Bartels & Sunkar 2005; Holmström et al.

1996; Kishor et al. 1995; Peters et al. 2007). One major disadvantage is that the accumulated knowledge from a variety of disciplines is rarely combined to exploit its full potential and to understand the complexity of the underlying mechanisms of desiccation tolerance. Therefore, calls for a systems-based approach, especially on the physiological, biochemical and molecular level, have increased (Leprince & Buitink 2010; Moore et al. 2009). The establishment of databases to pool results from expressed sequence tags (EST) and genomic results will be another major step towards a holistic understanding of desiccation tolerance (Moore et al. 2009). This will be helpful to eliminate at least some black boxes associated with desiccation tolerance in plants and to facilitate the practical application of desiccation tolerance, i.e. the biotechnological engineering of more drought-tolerant crops. Until now more than 100 genes related to desiccation tolerance have been identified but only very few have been transferred to desiccation-sensitive plants. This will be a major challenge for the coming years (Moore et al. 2009).

Aside from much-needed research on these four adaptive levels ecological aspects of desiccation-tolerant (vascular) plants should be considered. This field has been even more neglected than morpho-anatomical adaptations. First steps have been taken in this doctoral thesis but much more endeavors should be devoted to this because it adds to our understanding of the ecology of desiccation tolerance in plants.

However, all these future endeavors will only be realizable if the habitat and species of desiccation-tolerant (vascular) plants and therefore the wide variety of adaptive strategies are conserved. Currently, forest canopies, habitat to a large number of desiccation-tolerant pteridophytes, are lost due to large-scale logging. The same applies to granitic and gneissic inselbergs around the globe, a diversity hot spot for desiccation-tolerant plants (Porembski 2011). Quarrying as a consequence of space and resource limitations has reduced the share of inselbergs immensely over the last few years. Additionally, human-lit fires are regionally very common. Although some desiccation-tolerant species (especially *M. squamosus*, *A. pilosa* and several Velloziaceae) are well-protected by their layers of leaf sheaths, the establishment of younger individuals is highly threatened. Therefore, scientific as well as conservation efforts will be of utter importance to enhance our understanding of desiccation tolerance.

7. Summary

Adaptations to desiccation tolerance in plants occur on the morpho-anatomical, physiological, biochemical and molecular level. The majority of research in the context of plant desiccation tolerance is currently centered on the latter three adaptive levels. The potential biotechnological application to economically important crops is one of the drivers behind these research efforts. As a consequence morpho-anatomical traits of desiccation-tolerant plants are rarely examined in detail although they are crucial for a complete understanding of plant desiccation tolerance.

Therefore the central research questions of this doctoral thesis were if, how and to what extent desiccation tolerance is represented on a morpho-anatomical level in vascular plants. For the first time their adaptive role during de- and rehydration was examined over a wide taxonomic range. This was mainly restricted to angiosperms and some pteridophytes since an integration of plant groups which lack water-retaining structures such as bryophytes would have biased the results. Finally, a comparative analysis within and across the chosen desiccation-tolerant species was carried out to determine whether a universal morpho-anatomical syndrome for desiccation-tolerant vascular plants exists.

In addition to light and scanning electron microscopy micro-computed tomography (μCT) was used for the first time to visualize the 3-D structure of desiccation-tolerant plants. A prerequisite for this research was the establishment of a decent anhydrous fixation method. From a wide variety of classic and unusual fixatives only vaseline conserved the water status of the sample properly. Since it also attenuates the X-ray beam significantly which decreases the image quality only dry material was examined in a μCT whereas turgescent material was examined under a light microscope.

Using these methods, the comparative morphology and anatomy of several species were examined under the aspect of desiccation tolerance for the first time. Several morpho-anatomical traits and their respective functions during de- and rehydration could be identified. These encompass the protective function of leaf involution (via bulliform cells above the midrib in many monocots), an accordion-like leaf contraction in some dicots (*Myrothamnus moschatus* and *Myrothamnus flabellifolius*) or leaf rolling (many other dicots). A stabilizing function can be attributed to sclerenchyma, especially among monocots which exhibit a much higher degree of xeromorphism compared to many desiccation-tolerant dicots. A parallel leaf venation, typical of monocots, seems to be of

adaptive value as well. In monocots it can be regarded as a phylogenetic legacy with high adaptive value. Desiccation-tolerant dicots often times have an almost parallel leaf venation as well which underlines its adaptive value. It seems to facilitate the leaf involution resp. rolling and protects the leaf from potentially damaging excess solar radiation.

Although adaptive value could be ascribed to several morpho-anatomical traits the existence of a unifying morpho-anatomical syndrome in desiccation-tolerant vascular plants could not be confirmed since the variety of morpho-anatomical traits is very high on a small scale (i.e. within vascular plant families and groups such as monocots and dicots) and a large scale (angiosperms and pteridophytes) as well. Obviously, morpho-anatomical traits are not the only relevant adaptive factors for the survival of long periods of drought. The molecular, physiological and biochemical level have to be included as well. Current research results suggest that a universal strategy does not seem to exist on any of these levels either. The uncovering of links between all four levels is and will therefore be a major challenge for the coming years.

In addition to extensive morpho-anatomical studies an ecological study was conducted in Côte d'Ivoire. *Afrotrilepis pilosa*, a desiccation-tolerant Cyperaceae, forms dense mats on West African inselbergs. This species, as desiccation-tolerant vascular plants in general, is thought to grow extremely slowly due to harsh environmental conditions and desiccation tolerance itself (desiccation tolerance productivity trade-off hypothesis). A non-destructive leaf marking method was established to test this hypothesis. The absolute growth rate of individuals along a precipitation gradient was higher than expected but low compared to some temperate grasses. However, the relative growth rate needs to be determined as well as a combination of this non-destructive marking method and destructive harvesting methods.

8. Zusammenfassung

Anpassungen an pflanzliche Austrocknungstoleranz sind auf der morphologisch-anatomischen, physiologischen, biochemischen und molekularen Ebene zu finden. Der Großteil der aktuellen Forschungsbemühungen auf diesem Gebiet bezieht sich jedoch weitestgehend auf die letzten drei Ebenen. Mögliche biotechnologische Anwendungen der Mechanismen pflanzlicher Austrocknungstoleranz und deren Übertragung auf ökonomisch bedeutende Nutzpflanzen stellen die Motivation dieser Untersuchungen dar. Aus diesem Grund werden morphologisch-anatomische Anpassungen von austrocknungstoleranten Pflanzen nur (noch) selten detailliert untersucht, obwohl sie von entscheidender Bedeutung für ein umfassendes und komplettes Verständnis pflanzlicher Austrocknungstoleranz sind.

Ein zentrales Anliegen dieser Doktorarbeit war daher die Beantwortung der Fragen, ob, wie und in welchem Maße Austrocknungstoleranz auf der morphologisch-anatomischen Ebene repräsentiert ist. Die adaptive Funktion während des De- und Rehydrierungszyklus wurde untersucht. Diese Untersuchungen beschränkten sich vorwiegend auf Angiospermen und einige Pteridophyten, da eine Einbeziehung von Pflanzengruppen ohne transpirationsreduzierende Strukturen (z.B. Moose) die Resultate a priori verzerrt hätte. Abschließend wurde eine vergleichende Analyse innerhalb und zwischen den ausgewählten austrocknungstoleranten Arten durchgeführt, um festzustellen, ob ein universelles morphologisch-anatomisches Syndrom innerhalb austrocknungstoleranter Gefäßpflanzen existiert.

Neben der Licht- und Elektronenmikroskopie wurde Mikro-Computertomographie (μCT) genutzt, um erstmalig die 3-D-Struktur von austrocknungstoleranten Pflanzen zu visualisieren. Eine Voraussetzung für diese Untersuchungen war die Etablierung einer wasserfreien Fixierungsmethode. Aus einer großen Zahl an Fixiermitteln war es nur mit Vaseline möglich, den Wassergehalt der Probe konstant zu konservieren. Da die Ummantelung mit Vaseline jedoch gleichzeitig den Röntgenstrahl stark attenuiert und die Bildqualität herabsetzt, wurde nur trockenes Material im μCT untersucht werden. Turgeszentes Material wurde unter einem Lichtmikroskop untersucht.

Mit Hilfe dieser Methoden wurde die vergleichende Anatomie und Morphologie mehrerer Arten erstmalig unter dem Aspekt der Austrocknungstoleranz untersucht. Es konnten mehrere morphologisch-anatomische Merkmale und ihre spezifische Funktion

während des De- und Rehydrierungszyklus bestimmt werden. Dies umfasst die protektive Funktion der Blattfaltung (über bulliforme Zellen oberhalb der Mittelrippe bei vielen Monokotylen), eine Akkordeon-ähnliche Blattkontraktion einiger Dikotyler (*Myrothamnus moschatus* und *Myrothamnus flabellifolius*) und das Einrollen des Blattes (viele andere Dikotyle). Dem Sklerenchym kann eine stabilisierende Funktion zugeschrieben werden, insbesondere innerhalb der Monokotylen, die im Vergleich zu austrocknungstoleranten Dikotylen einen höheren Grad an Xeromorphie aufweisen. Eine parallele Blattnervatur, die fast nur innerhalb der Monokotylen zu finden ist, scheint ebenfalls von adaptivem Wert zu sein. Für Monokotyle kann eine Parallelnervatur als phylogenetisches Erbe mit hohem adaptivem Wert eingestuft werden. Austrocknungstolerante Dikotyle besitzen ebenfalls häufig eine (beinahe) Parallelnervatur, wodurch der adaptive Charakter dieser Struktur bestätigt wird. Auch hier scheint eine Parallelnervatur die Blattfaltung bzw. das Einrollen des Blattes zu begünstigen und das Blatt vor potentiell schädigender Sonneneinstrahlung im ausgetrockneten Zustand zu schützen.

Obwohl einigen morphologisch-anatomischen Strukturen adaptiver Wert zugeschrieben werden kann, konnte die Existenz eines gemeinsamen morphologisch-anatomischen Syndroms innerhalb der austrocknungstoleranten Gefäßpflanzen nicht bestätigt werden, da die Variabilität vieler Merkmale selbst in einem kleinen (innerhalb der Gefäßpflanzen und deren Gruppen (Monokotyle, Dikotyle)) und großen Maßstab (Angiospermen, Pteridophyten) sehr groß ist. Offensichtlich sind morphologisch-anatomische Anpassungen nicht die einzig relevanten adaptiven Faktoren für das Überleben längerer Trockenperioden. Die molekulare, physiologische und biochemische Ebene müssen hier ebenfalls mit einbezogen werden. Die bisherigen Ergebnisse legen nahe, dass auf keiner adaptiven Ebene eine universelle Strategie existiert. Daher wird es zukünftig eine entscheidende Herausforderung sein, die Verbindungen der verschiedenen Ebenen freizulegen.

Neben ausgiebigen morphologisch-anatomischen Untersuchungen wurde im Rahmen dieser Arbeit eine ökologische Studie in der Elfenbeinküste durchgeführt. *Afrotrilepis pilosa*, eine austrocknungstolerante Cyperaceae, bildet dichte Matten auf westafrikanischen Inselbergen. Es wird angenommen, dass diese Art, wie austrocknungstolerante Gefäßpflanzen im Allgemeinen, aufgrund der extremen

Umweltbedingungen und der Fähigkeit zur Austrocknungstoleranz sehr langsam wächst (Austrocknungstoleranz-Produktivitätsabwägungs-Hypothese). Im Rahmen dieser Studie wurde eine nicht-destruktive Blattmarkierungsmethode entwickelt, um diese Hypothese zu testen. Die absolute Wachstumsrate entlang eines Niederschlagsgradienten war höher als die Hypothese erwarten ließ, jedoch gering im Vergleich zu einigen temperaten Gräsern. Allerdings sollte zur Bestätigung dieser Resultate noch die relative Wachstumsrate bestimmt werden. Dies kann als Kombination der nicht-destruktiven Blattmarkierungstechnik und destruktiver Erntemethoden erfolgen.

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List of Figures

List of Tables

Appendix

List of publications

Korte, N., Porembski, S. (2010). Suitability of different cover crop mixtures and seedlings for a new tree row management in an organic orchard. Gesunde Pflanzen 62:45-52.

Korte, N., Porembski, S. (2011). Anatomical analysis of turgescent and semi-dry resurrection plants: the effect of sample preparation on the sample, resolution, and image quality of X-ray micro-computed tomography (µCT). Microscopy Research and Technique 74: 364-369.

Korte, N., Porembski S. (2011). Wiederauferstehungspflanzen - Überleben an Extremstandorten. Natur Forschung Museum 141: 14-23.

Korte, N., Porembski, S. (2012). A morpho-anatomical characterization of *Myrothamnus moschatus* (Myrothamnaceae) under the aspect of desiccation tolerance. Plant Biology 14: 537-541.

Statement on the candidates' contribution

Chapter 2:

Based on the following publication:

Korte, N., Porembski, S. (2011). Anatomical analysis of turgescent and semi-dry resurrection plants: the effect of sample preparation on the sample, resolution, and image quality of X-ray micro-computed tomography (µCT). Microscopy Research and Technique 74: 364-369.

- practical (lab) work planned and conducted: testing of different fixation/preparation methods, scans at µCT
- development of experimental design
- writing and editing of the manuscript

Chapter 3:

Based on the following publication:

Korte, N., Porembski, S. (in press). A morpho-anatomical characterization of *Myrothamnus moschatus* (Myrothamnaceae) under the aspect of desiccation tolerance. Plant Biology.

- practical (lab) work planned and conducted: light microscopy, µCT
- development of experimental design
- writing and editing of the manuscript

Chapter 4:

Based on the following publication:

Korte, N., Porembski, S. (submitted). Leaf anatomical traits of desiccation-tolerant vascular plants: a comparative analysis.

- practical (lab) work planned and conducted: light microscopy, µCT, schematic prototypes, statistical analysis
- development of experimental design
- writing and editing of the manuscript

Chapter 5:

Based on the following manuscript (in preparation):

Korte, N., Konaté, S., Porembski, S.. First non-destructive leaf growth rate determination of desiccation-tolerant, mat-forming monocots.

- field work: experimental design, practical field work in Côte d'Ivoire
- data analysis
- writing and editing of the manuscript

List of contributions to conferences

“Botanical and first zoological studies of desiccation-tolerant, mat-forming monocots on inselbergs in Ivory Coast”, **poster presentation** at the conference “Status and Future of Tropical Biodiversity" of the Society for Tropical Ecology (Gesellschaft für Tropenökologie e.V. – gtö), 21-24 February 2011, Frankfurt, Germany.

“A fresh look on desiccation-tolerant vascular plants from an anatomical perspective”, **oral presentation** at the XVIII International Botanical Congress (IBC) 2011, 23 - 30 July 2011, Melbourne, Australia

Grants and scholarships

June 2011: Kongress- und Vortragsreisenprogramm (DAAD); used to cover additional travel expenses, registration fee for the IBC 2011

March 2011: grant within the context of the Professorinnenprogramm (BMBF) of the University of Rostock; used to cover travel expenses for the IBC 2011

June/September 2010: Kurzstipendium für DoktorandInnen (DAAD); used to cover travel expenses for two field work trips to Côte d'Ivoire

March and July 2010 (in cooperation with Prof. Porembski): two grants in the program "Initiierung und Intensivierung bilateraler Kooperationen" (Deutsche Forschungsgemeinschaft: PO 604/13-1); used to cover travel expenses for Prof. Porembski, N. Korte and the Ivorian student Hervé Kouadio during two field trips to Côte d'Ivoire: ecology and zoological studies of *Afrotrilepis pilosa* mats (Cyperaceae)

Selbständigkeitserklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, den _____

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