



The aquatic resurrection plant *Chamaegigas intrepidus* – adaptation to multiple abiotic stresses and habitat isolation

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ABSTRACT: *Chamaegigas intrepidus* is a tiny poikilohydrous member of the Linderniaceae growing endemically in ephemeral rock pools on granite outcrops in Central Namibia. Habitat conditions are characterised by (1) frequent and fast desiccation and rehydration during the rainy summer season, (2) complete dehydration during the dry winter season lasting up to 11 months, (3) intensive solar irradiation and high temperatures during the dry season, (4) diurnal oscillations of pH in the pool water up to 6 units, (5) extreme nutrient deficiencies, especially nitrogen. Anatomical, biochemical and physiological adaptations to this complex of extreme environmental conditions are discussed such as contractive xylem, accumulation of carbohydrates, dehydrins and abscisic acid during desiccation, and the role of amino acids, ammonium, urea and urease for nitrogen nutrition. Furthermore, *Chamaegigas* populations on single inselbergs are genetically isolated, whereas gene flow between sub-populations from different pools on one inselberg is rather high. This pattern of gene flow is discussed in the context of the breeding system of *Ch. intrepidus*.

KEY WORDS: poikilohydrous cormophytes, limnology, desiccation, gene flow, Namibia

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INTRODUCTION

Although desiccation-tolerant (poikilohydrous) angiosperms comprise some 0.1% of all flowering plants only (POREMBSKI 2011), they have attracted the interest of plant scientists for more than a century. The first botanist to describe such species was the German taxonomist Kurt Dinter, who mentions four desiccation-tolerant angiosperms (*Chamaegigas intrepidus*, *Craterostigma plantagineum*, *Myrothamnus flabellifolia* and *Xerophyta viscosa*) in his flora of South-West Africa (DINTER 1909). The most spectacular species among them is the tiny *Chamaegigas intrepidus* Dinter, formerly *Lindernia intrepidus* (Dinter) Oberm., a member of the Linderniaceae (formerly Scrophulariaceae) family, growing endemically in Namibia.

Poikilohydrous plant species can equilibrate their leaf tissues with air humidity down to 0% and can be revived from this air-dried state (GAFF 1971). These ‘resurrection plants’ usually grow as pioneer species on sites with very harsh environmental conditions, especially limited seasonal water availability, e.g. on rock outcrops (POREMBSKI 2011). Most of them are found in (sub-)tropical Africa, Western Australia, India and South America, and only a small number occurs in Northern and Central America and in Europe. Desiccation tolerance has evolved within angiosperms independently several times, both among monocotyledonous and dicotyledonous plants, but not in gymnosperms.

Resurrection plants within the dicotyledons are mainly found within Gunnerales (Myrothamnaceae) and Lamiales (Gesneriaceae, Linderniaceae, Plantaginaceae).

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Both Gesneriaceae and Linderniaceae contain more than a dozen species. Some poikilohydrous members of the genus *Lindernia* and *Ch. intrepidus* colonize seasonally water-filled rock pools (POREMBSKI 2011).

Chamaegigas intrepidus grows in shallow ephemeral rock pools on granite outcrops in Central Namibia (Fig. 1). Nearly 100 years ago, Kurt Dinter described the extreme environmental conditions of the plant's natural habitat (DINTER 1918). Due to high temperature and a high evaporative demand of the atmosphere the bottom of the pool in which he discovered this resurrection plant remained permanently dry for at least half a year. During this time characterized by high solar irradiation, extreme temperatures and nearly completely dry air the plants survived as tiny rhizomes (diameter about 1 mm) and shrivelled leaves densely covering the bottom of the pool in a 1 cm thick layer of sand grains, dehydrated algae, dead daphnias, animal faeces and leaf litter. After the first summer rain falls had filled the small pools, a dense mat of small green *Chamaegigas* leaves covered the bottom of the pools within minutes. After two days Dinter discovered pink-coloured flowers in the midst of small rosette leaves floating on the water on top of a thin stem. A detailed

description on the history of discovery of *Ch. intrepidus* can be found in HEILMEIER & HARTUNG (2009).

Chamaegigas is adapted to the fluctuations of wet and dry conditions at its habitat through its ability for fast de- and rehydration. When water from the pools has been lost through evaporation, plants dry within less than two hours (GAFF & GIESS 1986). After rewatering, the vegetative organs regain full metabolic activity within two hours (HICKEL 1967). The extremely fast recovery after rehydration and the long survival in the desiccated state make *Chamaegigas* by far the most impressive of all poikilohydrous angiosperms. In the following habitat conditions and the plant's mechanisms of adaptation to its complex stressful environmental conditions at the anatomical, biochemical and physiological level will be described as well as implications of its isolated geographical distribution for generative reproduction and gene flow.

HABITUS, DISTRIBUTION AND HABITAT

Chamaegigas intrepidus is a small aquatic plant with two types of leaves: (i) 8 - 15 mm long lanceolate submerged leaves on a short main axis, (ii) two decussate pairs of sessile



Fig. 1. Temporarily water-filled rock pool at the end of the summer rainy period with *Chamaegigas intrepidus* on a granite outcrop on the farm Otjua (Omaruru District, Central Namibia)

floating leaves which form a small rosette on top of the thin stem which is attached to the main axis. The length of the stalk (1.5 to 10 cm) depends on the water level in the pools. Two flowers are produced in the rosette of the floating leaves (HEIL 1924). Their appearance is fully described in SMOOK (1969) and FISCHER (1992). The rhizome grows close to the bottom of the pools and bears a dense mat of fine roots.

All parts of the plant become totally dry when the rock pools dry out. Submerged and floating leaves get shrivelled. However, whereas mature floating leaves are considered desiccation-sensitive, immature floating leaves and submerged leaves are desiccation-tolerant (GAFF 1971). Thus *Ch. intrepidus* is exceptional in possessing two types of leaves with such a distinct contrast in drought tolerance on the same plant.

Chamaegigas grows endemically in Namibia (FISCHER 1992), exclusively in areas with granite outcrops (inselbergs) in the semi-desert and savanna transition zone (GIESS 1969, 1997). This arid to semi-arid region receives 160 to 570 mm precipitation per year, with rainfalls on only 20 to 70 days during summer (November to April), and a high variability from year to year. A few (5 to 12) rainy days alternate with a number (up to 60) of dry days during the wet season (HICKEL 1967). During dry days the shallow rock pools (maximum water level ca. 15 cm) usually dry out completely. In total, these ephemeral pools may be filled with water for some 40 to 85 days over the whole wet season. Thus the plant may experience 15 to 20 rehydration-dehydration cycles during a single rainy season (GAFF & GIESS 1986). Annual average temperature is 20 °C. However, air temperatures may rise up to 42 °C during the dry season, and sun-exposed rocks heat up to 50 °C at least (DINTER 1918). Average air humidity is 40%, but only 22% at the end of the dry season (September) (HICKEL 1967).

Due to the only thin layer of debris at the bottom of the rock pools, the water is very poor in nutrients. Furthermore, mineral nutrients will be leached out from the sediment after extensive rainfalls, when the shallow pools may overflow (GAFF & GIESS 1986). This can cause more severe nutrient deficiencies, especially during the time when there is plenty of water for sustained growth. On the other hand, animals deposit urine and dung on the rock surface, which are subsequently washed into the pools by rain water (HEIL 1924). Thus, especially during the early part of the wet season, there may be high amounts of urea and perhaps other dissolved organic nitrogen (DON) compounds present in the water, which are steadily diluted by leaching and plant uptake during the growth period.

Chamaegigas is physiologically active during the warm rainy season, whereas it survives the long (up to 11 months) dry season in the dehydrated state (HICKEL 1967). Due to its unique habitat, *Ch. intrepidus* suffers from a complex set of extremely harsh and interacting environmental

conditions, part of which are common for all resurrection plants (extremely severe edaphic drought and high solar irradiation concomitant with high air temperature and low air humidity during the dry season). The aquatic habitat, however, exposes the plant to additional stressors, namely recurrent flooding and drying cycles, low nutrient contents and drastic diurnal fluctuations of pH of the water during the wet season. Furthermore the geographical isolation of the inselbergs will limit gene flow affecting both genetic differentiation between populations and genetic diversity within populations.

We investigated this complex set of multiple stressors during several seasons in the natural habitat of *Ch. intrepidus* in Central Namibia (cf. Fig. 1 in HEILMEIER *et al.* 2005). The research on ecophysiological adaptations to abiotic environmental factors was performed on the farm Otjua, Omaruru District at a height of ca. 1400 m above sea level (for site description refer to HEILMEIER & HARTUNG 2001, 2011).

The resurrection plant *Chamaegigas*: Anatomical, physiological, biochemical and molecular adaptations to desiccation and high irradiance stress. Like any resurrection plant, *Ch. intrepidus* is exposed to severe dehydration, concomitant with intensive insolation. This combination of extreme environmental factors imposes three kinds of stress to the plants: (i) mechanical stress, (ii) destabilization and loss of integrity of supramolecular structures like membranes, (iii) oxidative stress (FARRANT 2000; VICRÉ *et al.* 2004a).

Anatomical adaptations of leaves and roots. Hydrated resurrection plants generally do not exhibit xeromorphic anatomical features. *Chamaegigas intrepidus*, being an aquatic resurrection plant, in particular does not possess any well-developed traits to protect it from water loss. Among all poikilohydrous cormophytes, *Ch. intrepidus* shows the fastest water loss, being air-dry even within one hour (GAFF 1977). This high rate of dehydration can only be tolerated by anatomical peculiarities at different levels from the cell to the whole organ. Compared to dehydrated leaves, the length of desiccated submerged *Chamaegigas* leaves is only 10 to 20% (HEIL 1924; HICKEL 1967). This drastic shrinkage is only possible due to the unique existence of contractive tracheids (SCHILLER *et al.* 1999). These xylem elements, which exist in the submerged leaves only, have thin longitudinal cell walls with broad spiral strengthenings and terminal plates with a small lumen. During the shrinking process these specialised xylem elements contract like an accordion. The shrinking process is accompanied by intensive wrinkling of the epidermis, which is caused by a tight connection between the plasma membrane and the cell wall resulting in contraction

of the whole cell during dehydration. Through this cytorrhysis plasmodesmatal connections between cells are maintained (HARTUNG *et al.* 1998). Shrinkage of leaves and cell wall folding in the resurrection plant *Craterostigma wilmsii* during dehydration is associated with modulation of mechanical properties of the wall (increased strength and flexibility), which may be caused by modification of xyloglucans and other changes in the polysaccharide content (VICRÉ *et al.* 2004b). Curling of older exterior leaves over interior buds may protect dried young tissue from photodamage (PROCTOR & PENCE 2002). Furthermore, accumulation of high amounts of anthocyanins causing the red colour of the floating *Chamaegigas* leaves protects from the high UV radiation and photo-oxidative damage (FARRANT 2000).

In contrast to submerged leaves, neither floating leaves nor roots show such a dramatic longitudinal shrinking upon dehydration. Roots, however, drastically shrink in the lateral direction by 30 to 35% due to an exclusive shrinkage of the rhizodermis and the exodermis (HEILMEIER *et al.* 2002). The large single-layered cortex cells, however, do not collapse, not even shrink after complete desiccation, although they have extremely thin walls. Due to the utmost stability of the cortex cells longitudinal shrinkage of roots is minute (3% only). The thin walls of the large cortex cells must be extremely stiff to resist shrinkage. However, no remarkable staining that could point to an extraordinarily chemical composition of cell walls could be seen as it seems to be the case with the cell walls of leaves of *Craterostigma wilmsii* (see above). Thus the anatomical structure of the roots represents a special kind of adaptation of *Ch. intrepidus* to its extreme environment since the maintenance of the physical root structure stabilises the sediment of the pools during the long dry winter season by maintaining mechanical contact between the intertwined fine roots and the inorganic and organic particles of the sediment (HEILMEIER & HARTUNG 2001).

As demonstrated already by HEIL (1924) and HICKEL (1967), the root exodermis contains passage cells with outer cell wall thickenings. They are believed to be an adaptation of roots to drought and are not known for roots of homoiohydrous aquatic plants. HICKEL (1967) concludes that in desiccated roots, when the collapsed rhizodermis and exodermis form a layer that resembles a velamen radicum (HEILMEIER *et al.* 2002), the outer pad, which would close the passage cells, acts like a valve and slows down the loss of water.

Molecular, biochemical and physiological adaptations

Carbohydrates

During desiccation, mechanical stress is not only imposed at the tissue level, but also at the cellular level:

The progressive decrease in water content destabilizes membranes and other supra-molecular structures due to detrimental molecular interactions, altered biochemical activities and increased concentrations of cellular contents. In resurrection plants, membranes and cytoplasm are assumed to be protected by sugars, compatible solutes and various proteins (VICRÉ *et al.* 2004a; DINAKAR *et al.* 2012). Poikilohydrous plants exhibit high amounts of carbohydrates, predominantly sucrose, but also trehalose and raffinose (VICRÉ *et al.* 2004a; DINAKAR *et al.* 2012), and octulose in *Craterostigma plantagineum* where it is converted during desiccation to sucrose (BIANCHI *et al.* 1992). In *Chamaegigas*, besides sucrose (140-180 µmol g⁻¹dw), stachyose (approximately 180-250 µmol g⁻¹dw) is the most dominant carbohydrate. Raffinose is present, however, at a relatively low level (approx. 20 µmol g⁻¹dw). Glucose and fructose are substantially higher in leaves (180-200 µmol g⁻¹dw and 60-120 µmol g⁻¹dw respectively) than in roots (20-30 µmol g⁻¹dw). During dehydration the monosaccharide content of the leaves decreases, in contrast to an increase in stachyose and sucrose content. HEILMEIER & HARTUNG (2001) suggested that glucose and fructose are incorporated into sucrose and stachyose, as has been discussed by WHITTAKER *et al.* (2001) for *Sporobolus stapfianus* and *Xerophyta viscosa* as a consequence of increased leaf hexokinase activity. In the European resurrection plant *Haberlea rhodopensis* accumulation of sucrose and verbascose was accompanied by upregulation of genes which are related to carbohydrate metabolism (GECHEV *et al.* 2013). For *Ch. intrepidus*, the total content of soluble carbohydrates in air dry tissues of roots and submerged leaves ranged from 14 to 26% with stachyose (roots) and sucrose (leaves) as the dominant sugars. Similarly, in desiccated leaves of the three Gesneriaceae resurrection plants *Ramonda nathaliae*, *R. myconi* and *Haberlea rhodopensis* sucrose levels were markedly increased compared to control leaves, whereas raffinose pools were constitutively high, but stachyose was nearly absent, as found by MÜLLER *et al.* (1997). These authors interpret the constitutive presence of raffinose as a pre-adaptation facilitating vitrification. In *Ramonda serbica* the content of sucrose increased during desiccation as well, contributing to osmoregulation and membrane stabilization (ŽIVKOVIC *et al.* 2005).

The function of sugars such as sucrose, raffinose or stachyose or other galactosyl-sucrose oligosaccharides is the suppression (i) of crystallisation of protoplastic constituents, (ii) of the fusion between membrane systems and (iii) of protein denaturation (HOEKSTRA *et al.* 2001), and the promotion of glass formation at low water content (CROWE *et al.* 1998). Since at a glassy state a liquid has a high viscosity, chemical reactions are slowed down and interactions between cell components are prevented.

Therefore a glassy state is highly stable and ideal to survive desiccation (HOEKSTRA *et al.* 2001). Sucrose and trehalose serve as osmoprotectants for biomembranes and stabilize biomolecules, e.g. proteins. In highly desiccated cells, according to the 'water replacement hypothesis' these sugars can substitute water in the glassy cytoplasm by binding the proteins and lipids via hydrogen interactions (HOEKSTRA *et al.* 2001).

Dehydrins

A similar function as for sugars according to the 'water replacement hypothesis' is ascribed to LEA (late embryogenesis abundant) proteins, which are assumed to provide a 'hydration shell' to proteins and other macromolecules and thereby prevent damage due to water loss (HOEKSTRA *et al.* 2001; GECHEV *et al.* 2012). They may also synergistically interact with sugars such as trehalose and thus prevent aggregation of proteins during desiccation (MOORE *et al.* 2009). LEA proteins are rapidly synthesized during the late stage of embryogenesis or in response to dehydration in vegetative tissues of resurrection plants. The most studied group of LEA proteins are dehydrins ('LEA D11 family'; VICRÉ *et al.* 2004a).

Fully hydrated submerged leaves of *Chamaegigas* synthesise proteins which show similarities with the ABA (abscisic acid) inducible desiccation related proteins (dehydrins) of *Craterostigma plantagineum* (BARTELS *et al.* 1990). Even after four weeks of optimal hydration the dehydrin amount is high. In fully hydrated roots, however, dehydrins are not detectable. When hydrated roots were treated with ABA, dehydrins were formed, as it was the case when roots desiccated (SCHILLER *et al.* 1997).

Abscisic acid

The results on the induction of the expression of dehydrin related genes confer strong arguments on the role of abscisic acid during acquisition of desiccation tolerance in *Chamaegigas*, acting as a signalling molecule in the transduction path between perception of water loss and gene expression (HARTUNG *et al.* 1998; VICRÉ *et al.* 2004a). In *Ch. intrepidus*, ABA concentration increases 20- to 30-fold during dehydration in submerged leaves and roots, whereas in floating leaves the increase is always less than fivefold (SCHILLER *et al.* 1997). The increase in ABA concentration is linearly correlated with the decrease in osmotic potential, without any threshold, which indicates a rather sensitive response of ABA biosynthesis to dehydration. Due to a slow degradation rate, *Ch. intrepidus* maintains high ABA concentrations in hydrated tissues.

Degradation of ABA is much slower in *Chamaegigas* tissues than in those of mesophytic plants such as

Valerianella locusta. After 24 h pre-incubation with labelled (¹⁴C) ABA 3% of the label were recovered in the degradation product phaseic acid (PA) in *Ch. intrepidus*, but 31% in *Valerianella locusta* (SCHILLER 1998). Leaves of many other plants metabolise ABA even more rapidly (90-100% within one day).

The content of ABA conjugates, predominantly the glucose ester of ABA (ABA-GE), is low in *Ch. intrepidus*, without distinct fluctuations of ABA-GE. Roots contained less ABA-GE than submerged leaves (SCHILLER *et al.* 1997). Since conjugated ABA compounds are deposited in the vacuoles where no further degradation is possible, they have previously been regarded as an indicator of stress history of plant tissues. However, this is not the case in *Chamaegigas*, which can release excess ABA to the surrounding medium (see below). Therefore, deposition of ABA conjugates into the vacuoles is not necessary.

Photosynthesis

Due to the exposed nature and the elevation (up to 1500 m a.s.l.) of the rock outcrops, photosynthetic photon flux density (PPFD) regularly exceeds 2000 µmol m⁻² s⁻¹ during summer (HEILMEIER & HARTUNG 2001) and even basal submerged leaves which are covered by a dense layer of floating leaves may experience PPFD > 300 µmol m⁻² s⁻¹ (WOITKE *et al.* 2004). Especially when the pools dry out, basal leaves are exposed to full sun. A major mechanism of these leaves to protect against high irradiance load is the folding and curling during the shrinking process as described above.

Especially when becoming dehydrated, leaves can be severely damaged by the high light intensity prevailing on the exposed outcrops (DINAKAR *et al.* 2012). Measurements of chlorophyll fluorescence of both submerged and floating leaves showed a continuous decrease of PSII efficiency with leaf drying in both leaf types (WOITKE *et al.* 2004), as has also been found for leaves of *Haberlea rhodopensis* (GEORGIEVA *et al.* 2007) and *Ramonda serbica* and *R. nathaliae* (AUGUSTI *et al.* 2001; GASHI *et al.* 2013). As AUGUSTI *et al.* (2001) and DEGL'INNOCENTI *et al.* (2008) discuss for *R. serbica*, this decrease in PSII efficiency is mostly attributable to non-radiative energy dissipation via zeaxanthin, especially during early stages of dehydration. A controlled reduction of photosynthesis has been interpreted as a protection against photo-oxidation (FARRANT 2000). At very low levels of water content, when activity of phenolic peroxidases is rather low (SGHERRI *et al.* 2004), reduced ascorbate and glutathione were found to prevent photo-oxidative damage in *R. serbica* (AUGUSTI *et al.* 2001), whereas non-photochemical quenching was greatly reduced (DEGL'INNOCENTI *et al.* 2008).

After rehydration, even the floating leaves, which

had been previously assumed to be desiccation-sensitive (HICKEL 1967; GIESS 1969; GAFF 1977; GAFF & GIESS 1986), regained their full photosynthetic capacity as inferred from PSII efficiency within 18 hours. Even after repeated dehydration and rehydration there was no damage due to photoinhibition in either leaf type (WOITKE *et al.* 2004). However, whether desiccation tolerance exists also over longer periods in floating leaves, as it is the case for submerged leaves, remains doubtful.

Adaptations of the aquatic resurrection plant *Chamaegigas* to its specific limnic habitat: pH fluctuations and nutrient shortage

Conductivity and nutrient contents of the pool water.

Water level in water-filled pools with *Chamaegigas* ranged from 4 to 13 cm (median 7.5 cm). Average daily water loss due to evaporation is some 12 mm in flooded pools and increases to 23 mm in drying pools. In pools drying out loss of water caused the dissolved compounds to be concentrated as measured by the electrical conductivity. Average conductivity in flooded pools was 36 $\mu\text{S cm}^{-1}$. A decrease in water level by a factor of two increased conductivity on average up to two-fold, in some cases up to four-fold. After refilling by rain, conductivity decreased to the low values before the pools started drying out (HEILMEIER & HARTUNG 2001). This indicates that towards the end of the wet season, when these measurements were taken, only minute amounts of nutrients are washed into the pools from the surrounding rock surface.

The low conductivity values of the pool water are related to small concentrations of dissolved ions and indicate a poor buffer capacity in the pool water. The most abundant metallic cation in the water of pools with *Ch. intrepidus* was sodium (average concentration 190 mmol m^{-3}), followed by calcium (70 mmol m^{-3}), potassium and magnesium (30 mmol m^{-3} each). Median concentrations of chloride and sulfate were about half that of Na^+ and Ca^{2+} respectively. Phosphate concentrations were below detection limits in most of the cases (HEILMEIER & HARTUNG 2001). These values are by a factor 2 to 40 lower than in temporary waters in the Namib Desert (KOK & GROBBELAAR 1980, 1985).

Mineral nutrient contents in the sediment of the rock pools at the end of the dry season are in most cases higher than those given by GAFF & GIESS (1986) for *Chamaegigas* rock pools. This is especially true for potassium, magnesium (ca. 5 g kg^{-1} dw each) and phosphorus (ca. 0.6 g kg^{-1} dw), whereas the content of total nitrogen (3.5 g kg^{-1} dw) is somewhat lower than the value from GAFF & GIESS (1986).

Among nitrogenous compounds both, inorganic and organic N species, were considered as possible nitrogen sources for *Chamaegigas*. Whereas nitrate was below detection limit in most cases, ammonium was much more

abundant, with maximum concentrations in drying pools of 0.6 mM (HEILMEIER & HARTUNG 2001). During drying out of the pools, median concentration increased by a factor of 2.5, whereas maximum concentration increased 8.5-fold. Amino acids were also present in the low micromolar concentration range in flooded pools (SCHILLER *et al.* 1998a). In flooded pools the most abundant amino acids were serine, glycine and asparagine, which were found in nearly all pools with *Ch. intrepidus*. Less common, although also with high concentrations when present, were glutamine and alanine. During drying, the relative proportions of amino acids changed, with asparagine, serine and glycine found in nearly all samples. However, their median and maximum concentrations increased less (1.5- to 2.5-fold and 6- to 12-fold respectively) compared to all amino acids (3- respectively 20-fold). Four amino acids could be detected in almost dry pools only, namely glutamine, threonine, arginine and tyrosine. These additional amino acids were present in high concentrations, except for glutamine. Thus, during drying out of the pools, the spectrum of amino acids became more diversified both in frequency and concentrations of individual amino acids.

The most abundant N compound both in flooded and drying pools was urea. The general time course of urea concentration during drying out of the pools was similar to NH_4^+ . Evaporation of the water caused a dramatic increase for both compounds, which was however more pronounced in NH_4^+ (HEILMEIER *et al.* 2001).

Diurnal oscillations of pool water temperature, pH and CO_2 concentration

Water temperature showed similar diurnal oscillations as air temperature, however, with a smaller amplitude. Maximum values reached ca. 35 °C during the day, minimum temperatures at night were comparable to nocturnal air temperature (HEILMEIER & HARTUNG 2001). In parallel to diurnal fluctuations of water temperature we found oscillations in pH of the pool water, with maximum values in the afternoon and minimum values at night. In flooded pools maximum pH values recorded in 1998 were close to 10 in the afternoon, minimum near 5 in the morning. Still higher pH values in the afternoon (pH 12) were measured in 1995 (SCHILLER *et al.* 1997). Average diurnal pH amplitude was about 2.5 in both years. Thus, these diurnal pH fluctuations are somewhat larger than those found by SCHOLNICK (1994) for ephemeral desert pools in Utah (USA).

Diurnal oscillations of pH were mainly a consequence of changing solubility of CO_2 due to fluctuations of water temperature. During the daytime, when water temperature was above 30 °C, CO_2 was completely lost from the pools by diffusion. This raised the pH to strong alkaline values up to pH 10. Most of the inorganic carbon was present in

the form of HCO_3^- , with peak concentrations about 100 times higher than maximum CO_2 concentrations during the night. After sunset, when water temperature steadily decreased, CO_2 concentration increased due to respiration throughout the whole night, which caused a rapid decrease in pH down to slightly acidic values. However, as soon as water temperature increased in the morning, CO_2 concentration rapidly dropped to zero (HEILMEIER & HARTUNG 2001). Thus for submerged *Chamaegigas* plants CO_2 is not available for photosynthesis during most part of the daytime.

Adaptations of Chamaegigas intrepidus to diurnal oscillations of pool water pH and related effects

As shown above, *Ch. intrepidus* has to cope with substantial diurnal fluctuations in the pool water pH. Therefore we investigated the effect of external pH and dehydration on intracellular pH of *Chamaegigas* roots and leaves via ^{31}P NMR spectroscopy (SCHILLER et al. 1998b). High external pH (10.0) caused only a negligible alkalinisation of the root cytoplasm, and drastic dehydration led to a small alkalinisation of leaf vacuoles at pH 10. These results imply an unusually effective pH regulation which is consistent with the adaptation of *Chamaegigas* to numerous adverse environmental factors. According to NMR analysis dehydration had no effect on the pools of inorganic phosphate and phosphocholine. Since membrane damage is usually accompanied by an increase of inorganic phosphate and phosphocholines, this indicates that membranes are most effectively protected from damages due to low water potentials.

Due to the drastic diurnal pH fluctuations, reaching alkaline pH values up to 12, we expected serious losses of ABA to the pool water considering that ABA distributes within *Ch. intrepidus* according to the anion trap concept (HARTUNG & SLOVIK 1991; SLOVIK et al. 1995). A significant loss of ABA during the afternoon hours might be a serious problem, especially because during this time pools usually dry up. *Chamaegigas intrepidus*, however, seems to be well adapted to these conditions. Rosettes preloaded with ^{14}C ABA lost significantly less ABA to the medium than the rosette of the mesophytic terrestrial plant *Valerianella locusta* when transferred to an alkaline medium (SCHILLER et al. 1997).

As shown above, during daytime the pool water loses nearly all its CO_2 which has been accumulated during the night time. Therefore, photosynthesis is either severely limited by CO_2 , or *Chamaegigas* has to use alternative carbon sources like hydrogen carbonate. By measuring the diurnal course of O_2 concentration in the pool water, HEILMEIER & HARTUNG (2001) proved that *Ch. intrepidus* performs photosynthesis with submerged leaves even when the medium is completely devoid of CO_2 , indicating

use of HCO_3^- . Using advanced techniques of chlorophyll fluorescence, WORTKE et al. (2004) could demonstrate a significant role of the floating leaves for total plant carbon gain. Since stomata of floating leaves are assumed to stay open continuously, CO_2 will be a major source for photosynthesis for the plant as long as floating leaves are active.

Adaptations of Chamaegigas intrepidus to nitrogen deficiency of the pool water

As shown above *Chamaegigas* has to cope with extreme conditions of nitrogen deficiency. While nitrate is virtually absent in the rock pools, urea, ammonium ions and the amino acids glycine and serine occur in low concentrations (micromolar range). Urea cannot be utilised by *Ch. intrepidus* directly. In uptake experiments with ^{14}C - and ^{15}N -labelled urea no incorporation and utilisation of urea could be observed, even after long incubation periods (up to 5 days), when the root systems were cleaned carefully (HEILMEIER et al. 2000). When the natural sediment or jack bean urease was present in the medium, urea-N was accumulated in tissues of *Ch. intrepidus*. Furthermore ^{15}N NMR spectra performed with *Chamaegigas* roots after incubation with ^{15}N -urea did not show any ^{15}N signals such as ammonium, glutamine and glutamate in the absence of urease. However, when ammonium was released by the action of urease ^{15}N could be utilised by *Ch. intrepidus*. Urease therefore plays an essential role for the acquisition of urea-N. It is important to note in this context that urease can survive the harsh conditions in the desiccated rock pools during the Namibian winter at temperatures up to 60 °C, complete dryness and high UV irradiation. Thus, this remarkable resistant enzyme seems to be a key factor for survival of *Chamaegigas* (and also of other plants that depend on urea-N deposited from animals on the rock outcrops and flushed into the pools during the early parts of the rainy season).

Ammonium, which originates from urea especially in drying pools, may become extremely deficient, especially in highly alkaline pool water where it can be lost as ammonia to the atmosphere. Then *Chamaegigas* switches to utilisation of glycine, which is most abundant among amino acids in the pool water, as shown after incubation with ^{15}N glycine, both under laboratory and field conditions (SCHILLER et al. 1998a). ^{14}C -Glycine can be taken up by *Chamaegigas* roots with a high affinity glycine uptake system ($K_m = 16 \mu\text{M}$).

When *Chamaegigas* rosettes or isolated root systems were incubated with ^{15}N glycine ^{15}N NMR spectra showed a glycine, serine, glutamine and glutamate signal (HARTUNG & RATCLIFFE 2002). This agrees with the action of glycine decarboxylase (GDC) and mitochondrial serine hydroxy methyl transferase (SHMT; Cl-metabolism, MOUILLON et al. 1999) in the *Chamaegigas* roots, which seem to

metabolise glycine much more rapidly than, e.g., maize roots (HARTUNG & RATCLIFFE 2002). This could be a part of an adaptation to the natural habitat with glycine as one of the dominating N sources, especially when other forms of nitrogen are nearly absent from the pool water.

Glycine has been shown earlier to be the dominant N source for arctic and alpine plants (CHAPIN *et al.* 1993; RAAB *et al.* 1996) and a number of sedges from different ecosystems (RAAB *et al.* 1999). SCHMIDT & STEWART (1999) have demonstrated glycine as the main soil derived N source for a wide range of wild plants in Australian communities. *Chamaegigas* is the first example of an aquatic vascular plant showing that amino acids such as glycine and serine can be an important N source.

Adaptations of *Chamaegigas* to habitat isolation: Breeding system and gene flow. As an endemic plant *Chamaegigas* grows in an extremely small area of Central Namibia. Compared to plants of a wide distribution, such populations often suffer from genetic impoverishment, i.e. a small number of polymorphic gene loci and a loss of alleles. Inbreeding within small populations and genetic drift may cause a reduced degree of heterozygosity and a fixation of harmful alleles. Additionally the isolated occurrence of *Chamaegigas intrepidus* on inselbergs of approximately 25 km distance may reduce a genetic exchange via seed dispersal and pollen transfer drastically. Thus restricted genetic diversity should severely limit the potential for adaptation in *Chamaegigas* to its stressful environmental conditions.

However, molecular genetic investigation by means of AFLP-marker (amplified fragment length polymorphism) with hierarchical structuring of the populations (individuals nested within pools, pools nested within sites) has shown a surprisingly high genetic diversity within the plants of one site (inselberg), even within one pool (77% of total variation, DURKA *et al.* 2004). Genetic differentiation between sites (17% of total variation) was higher than genetic variation among pools within sites (6%). Thus *Chamaegigas* on the different inselbergs is highly differentiated genetically. Genetic differentiation is not correlated with geographic distance. Assumption of an island model and isolation by distance yields the average effective number of migrants per generation among sites to be 1.2, among pools 3.0. These spatial patterns of genetic diversity are in accordance with the breeding system and dispersal mode.

Although the pollen/ovule ratio (99) is rather low compared to xenogamous plant species (e. g. isolated populations of the narrowly endemic *Ranunculus weyleri*: >5000; CURSACH & RITA 2012), pollination experiments indicated that *Chamaegigas* is a predominantly outcrossing species and a large number of pollen could be found both

on the wild bee and beetle species visiting the flowers (DURKA *et al.* 2004). Two bee species (*Liotrigona bottegoi* and *Apis mellifera*) and mainly two beetle species of the genus *Condyllops* (*C. erongoensis* and a newly identified species) have been identified as main pollinators (WOITKE *et al.* 2006). The zygomorphous and slightly protandric flowers show a typical insect pollination syndrome: they are distinctly coloured, intensively scenting and provide a rich floral reward (abundant pollen grains), but they do not produce any nectar. Rather, dense layers of trichomes (400 to 1600 per mm²) can be found on the lower lip, similar to well-known oil-flowers (e.g. in Plantaginaceae species: MARTINS *et al.* 2013). However, there are no indications that *Chamaegigas* lives in an oil flower/oil-collecting bee symbiosis (WOITKE *et al.* 2006).

In conclusion this endemic species does not show any genetic impoverishment. The high genetic variability may be a result of high UV radiation at high altitude (up to 1500 m a.s.l.), which should result in many mutations, and a high gene flow both within inselbergs by pollinating insects and among inselbergs due to seed dispersal by wind or animals (HEILMEIER *et al.* 2005). This may provide the genetic basis for a successful adaptation of *Chamaegigas* to its extreme habitat conditions.

CONCLUSIONS

Already Kurt Dinter, when seeing *Chamaegigas intrepidus* for the first time, was highly impressed by this tiny plant, surviving the extreme conditions at its natural habitat, and gave the name ‘fearless dwarf giant’ to it. Even today this species is considered to represent the most spectacular resurrection plant (GAFF & GIESS 1986; HARTUNG *et al.* 1998) as it (i) shows the most rapid recovery after rewetting among all resurrection angiosperms, and (ii) possesses two types of mature leaves with contrasting drought tolerance on the same plant. Therefore, this species has been considered to be well adapted to its natural habitat, shallow, only temporarily water-filled rock pools. However, as shown here, desiccation is only one aspect of a large number of abiotic stress conditions, which also include high temperatures and solar radiation, especially in the ultraviolet range, drastic diurnal oscillations in external pH, low partial pressures of carbon dioxide during the day when plants are flooded, and low nutrient contents of the pool water. Thus, both acquisition of resources like carbon and nitrogen when they are scarce and protection of cellular and molecular structures from damage due to dehydration, alkaline, heat and UV stress are essential for the survival of this tiny plant at its extreme habitat. Moreover, the isolation of habitats of this endemic plant, occurring in a very restricted geographical area, evokes the danger of genetic impoverishment for *Ch. intrepidus*.

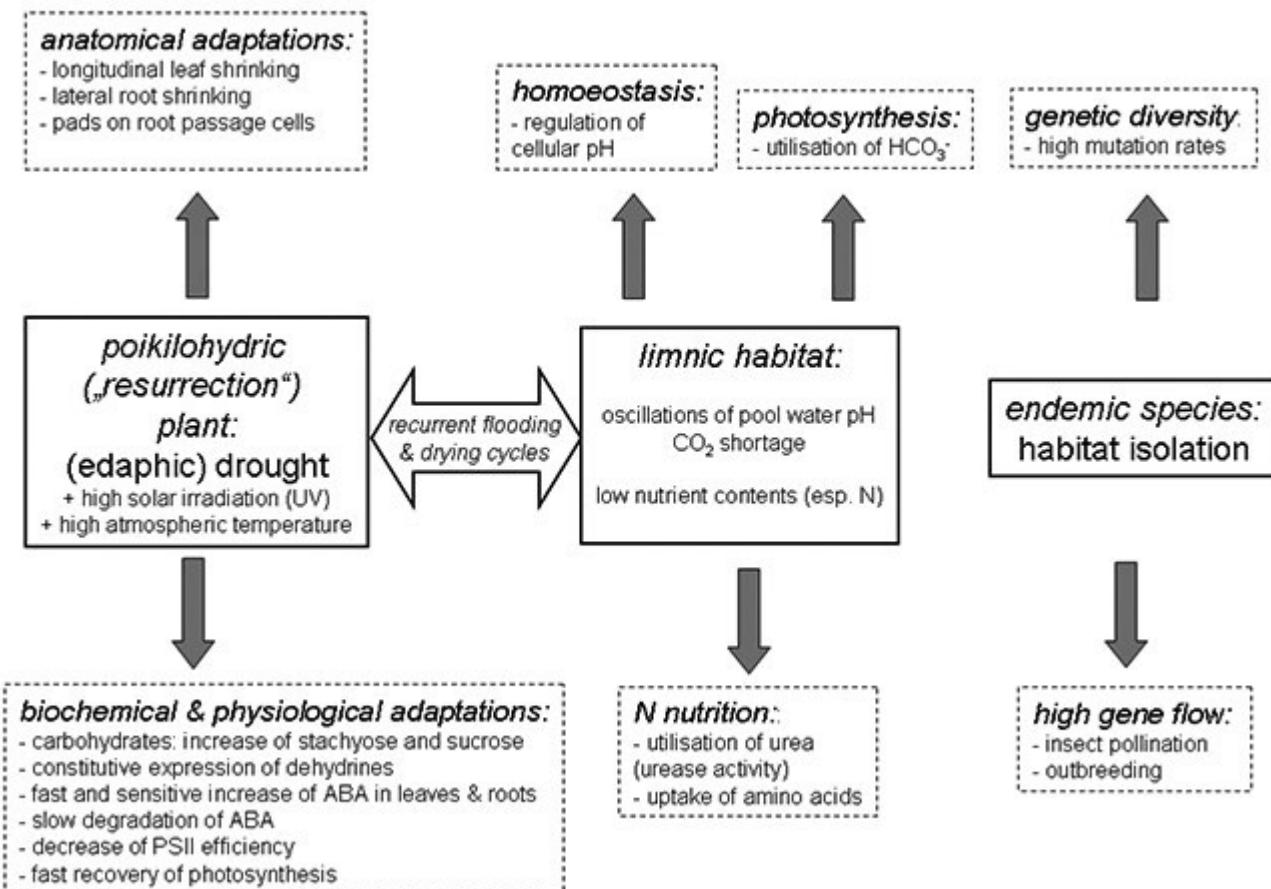


Fig. 2. The complex abiotic and habitat conditions (rectangles with full lines) and mechanisms of adaptation (rectangles with broken lines) for the aquatic resurrection plant *Chamaegigas intrepidus*

Nevertheless, this species is remarkably well adapted to this complex set of extreme and multiple stress factors (Fig. 2). These adaptation mechanisms are to a large extent constitutive, like synthesis of raffinose or dehydrins in submerged leaves also in the absence of desiccation. Similarly, constitutive expression of dehydrins has been found for *Ramonda nathaliae* by JOVANOVIĆ *et al.* (2011), and for *Haberlea rhodopensis* by GECHEV *et al.* (2012). In addition, the fast increase of ABA in submerged leaves and roots to dehydration is the most sensitive response of this kind among all resurrection spermatophytes examined so far. This orchestration of responses allows the plant to adapt to fast drying, within a few hours – in contrast to other resurrection plants like *Craterostigma plantagineum* which survive desiccation of its vegetative tissues only if drying is slow. Similarly, recovery from complete desiccation, which occurs within two hours is also the fastest among all resurrection angiosperms (GAFF 1977).

However, these stress responses do not come without cost. Just like the endemic European resurrection plant *Ramonda nathaliae* which grows on serpentine soils and thus suffers from a most unfavourable Ca/Mg ratio and high

levels of toxic trace elements (Ni, Cr, Co, Mn) in the soil, yielding in reduced size of leaves and rosettes (RAKIĆ *et al.* 2013), *Ch. intrepidus* has extremely small growth rates, with physiological activity being restricted to only very short and intermittent favourable periods of conditions, when rock pools are water-filled. The complex of various stress factors of mostly extended duration and high intensity requires a trade-off between investing resources in growth versus desiccation tolerance (ALPERT 2006). Niche partitioning between the less poikilohydrous *Limosella grandiflora* in deep parts of pools, which usually do not dry out, and the slow-growing poikilohydrous *Chamaegigas intrepidus*, which is restricted to the frequently desiccating shallow parts (HEILMEIER *et al.* 2005) allows the ‘fearless dwarf giant’ to survive as a perennial aquatic resurrection plant – without competition, but with a miracle of adaptation mechanisms to its unique environment.

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REZIME

Vodena vaskrsnica *Chamaegigas intrepidus* – adaptacije na višestruki abiotički stres i izolacija

Hermann HEILMEIER i Wolfram HARTUNG

C*hamaegigas intrepidus* je mala poikilohidrična biljka iz familije Linderniaceae. Raste kao endemična u povremenim barama na granitnom stenu u Centralnoj Namibiji.

Uslovi staništa rezultiraju: (1) čestim i brzim dehidratacijama, ali i rehidriranjem za vreme kišne letnje sezone, 2) potpunom dehidratacijom za vreme suve zimskog perioda, suša može da potraje i do 11 meseci, (3) biljke su izložene intenzivnoj sunčevoj radijaciji i visokim temperaturama tokom sušne sezone, (4) dnevnim oscilacijama u pH vode i do 6 jedinica, (5) ekstremnim deficijencijama nutrijenata, posebno azota. Adaptacije na anatomskom, biohemiskom i fiziološkom nivou zajedno sa kompleksom eksteremnih ekoloških uslova su diskutovani (kontraktivni ksilem, akumulacija ugljenih hidrata, dehidrini i ABA za vreme desikacije) kao i uloga aminokiselina, amonijaka, uree, i ureaze u ishrani azotom. Populacije *Chamaegigas*-a su genetički izolovane na jedinstvenom bregu, a protok gena među subpopulacijama je zanimljivo visok. U radu se diskutuje obrazac protoka gena u vezi sa načinom razmnožavanja i ukrštanja vrste *Chamaegigas intrepidus*.

Ključne reči: poikilohidrične kormofite, limnologija, desikacija, protok gena, Namibija