Aspects of the prevention of light damage during drying and rehydration of the desiccation-tolerant grass *Eragrostis nindensis.*

Nicola Bergh

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Supervisor: Dr JM Farrant

ABSTRACT:

There are two main mechanisms of desiccation-tolerance in angiosperms. Both of these (poikilochlorophylly and homoiochlorophylly) involve adaptations to prevent light-induced damage as the plants dry and rehydrate. The poikiolchlorophyllous grass *E. nindensis* was investigated to determine physiological responses to light during drying, and mechanisms of tolerance of dehydration. The desiccation-sensitive *E. curvula* was investigated simultaneously as a control in order to compare responses of tolerant and sensitive relatives. Quantum efficiency of photosystem II was determined using chlorophyll fluorescence parameters and levels of photosynthetic pigments (chlorophylls and carotenoids) and of anthocyanins were measured. Electrolyte leakage of drying and rehydrating leaves was monitored to determine the extent of damage to membranes. Quantum efficiency and photosynthetic pigment contents were reduced in both plants dehydrated to <2% RWC; only *E. nindensis* recovered to initial levels. Both plants accumulated anthocyanins but these reached greater levels in *E. nindensis* and were found on the entire length of the leaf. On rehydration, *E. nindensis* lost the anthocyanins as it reconstituted chlorophylls. Neither species showed marked increases in electrolyte leakage but *E. curvula* did not recover on rewatering.

Abbreviations used:

EL - electrolyte leakage

HC - homoiochlorophyllous desiccation-tolerant plant programme

MC - moisture content

PK - poikolochlorophyllous desiccation-tolerant plant

RWC - relative water content (%)

INTRODUCTION

Desiccation-tolerance is a highly-specialised trait which is extremely rare in angiosperm vegetative tissues. Resurrection plants have evolved to exist in the unique niche of pockets of shallow sand, often on rocky outcrops where no other plants can survive the frequent and complete dehydration of the soil. They are generally slow-growing and unable to compete with faster-growing neighbours in deeper soils. Despite the rarity of this ability, it has a wide taxonomic distribution which has led Oliver (1996) to suggest that desiccation tolerance has evolved separately on at least twelve occasions. It would thus be expected that there may be several different mechanisms of desiccation-tolerance and indeed two very different strategies occur in vegetative tissues. In the first, the photosynthetic pigments and thylakoid membrane system are dismantled during drying and reconstituted on rehydration(Sherwin & Farrant 1996). This process is thought to be an adaptation to prevent interaction of light with drying and rehydrating photosynthetic machinery. In the second mechanism the structure of the photosynthetic machinery is largely retained during drying and rehydration (homoiochlorophylly). Such plants possess other adaptations to prevent light-induced damage. Tuba *et al* (1998) consider the two strategies to be ecological adaptations determined by the duration of drying/wetting cycles commonly experienced by the plants. Homoiochlorophyllous plants would thus have evolved to dehydrate and green up faster than poikilochlorophyllous plants.

Poikilochlorophylly is thought to be of major adaptive value in the prevention of light-chlorophyll interactions during stages of intermediate tissue moisture content because under these conditions light becomes extremely dangerous to leaves. Both HC and PK plants possess adaptations to prevent exposure of chlorophyll to light at these times because reduced water contents inhibit normal physiological activity but do not prevent deleterious reactions (Sherwin & Farrant 1998). Both protection and avoidance mechanisms may be important during these stages.

Photosynthesis is especially sensitive to water stress (Sherwin 1995) even more so than respiration (Seel, Hendry & Lee 1992 on mosses cited in Sherwin 1995). At RWC < 30% this is thought to be due mainly to membrane damage (Kaiser 1987 in Sherwin 1995). Membranes particularly sensitive to desiccation damage. The entire photosynthetic

apparatus is based in the thylakoid membranes, and proper functioning of photosystems, light-harvesting complexes and associated proteins depends on sound thylakoid structure. Thus light-induced damage to these systems must be avoided in drying plants. When chlorophyll pigments are excited by light at water contents which prevent full functioning of metabolic systems, oxygen free radicals are produced. These entities are extremely reactive and cause damage to many cellular molecules, including membranes. The extreme measure of the dismantling of the chloroplast membrane system is the way in which poikilochlorophyllous desiccation-tolerant plants prevent this damage. This also prevents photobleaching and degradation of unprotected chlorophyll in the dry state.

Homoiochlorophyllous plants employ other mechanisms to deal with the potential damages of light to dry leaves. These include reorientation and extensive folding of drying leaves to protect chlorophyll from light, accumulation of shielding and quenching pigments and upregulation of antioxidant compounds (Sherwin & Farrant 1998).

Many of the known angiosperm resurrection plants are indigenous to Southern Africa. Frequently-studied examples include species from the genera *Myrothamnus, Craterostigma* and *Xerophyta*. Desiccation tolerance has excited much interest due to the possible potential for learning about mechanisms which may increase drought-tolerance in crop plants. Desiccation-tolerance in grasses has inspired interest due to the potential use of such grasses as grazing in order to extend stock-farming into arid areas, or maintaining higher stocking rates in areas where water availability limits grazing. In 1974, Gaff & Ellis surveyed the summer-rainfall areas of Southern Africa for sedges and grasses tolerant of near-total dehydration. Of the 80 species examined, the 11 which could recover from the air-dry state appeared to be restricted to the subfamily Eragrostoideae sensu Tateoka (1957) and within this subfamily to the tribes Eragrostideae, Sporoboleae and Chloridae. These three tribes are considered closely related by Hutchinson (1979) (cited in Gaff & Ellis 1974) and have Kranz metabolism and presumably, the C4 photosynthetic pathway (Gaff & Ellis 1974). These authors speculate that these tribes possess a potential for desiccation tolerance which is realized in only a few genera, suggesting that the trait evolved relatively recently.

Most tolerant grasses were found to be homoiochlorophyllous and to fully regreen after 24 hours or less of rehydration. *Eragrostis nindensis* Ficalho & Hiern (=E. denudata) was one

Most tolerant grasses were found to be homoiochlorophyllous and to fully regreen after 24 hours or less of rehydration. *Eragrostis nindensis* Ficalho & Hiern (=E. denudata) was one of the few poikilochlorophyllous species and was reported to take 48 hours for regreening after full imbibition (Gaff & Ellis 1974).

Although poikilochlorophyllous species take far longer to recover from the dry state than homoiochlorophyllous species, their recovery rate is extremely rapid relative to sensitive species which need to grow from seed or from a dormant underground storage organ after drought. Desiccation-tolerant grasses are thus potentially extremely advantagous pasture species in aridland stock farming. *E. nindensis* was identified as having potential for introduction to semi-arid pastures by Gaff & Ellis in 1974 since it is fairly palatable and nutritious. Walter & Volk, 1954 cited in Gaff & Ellis (1974) found a high protein and phosphate content in the foliage. Also, the tussocks possess strong roots which allow the plucking of leaves without wrenching the plant from the soil, a requirement for tolerating grazing . Although *E. nindensis* has very slow growth rates, it has fairly low levels of potentially toxic secondary metabolites and is highly regarded as sheep fodder in Namibia (Sutaryono & Gaff 1992). *E. nindensis* was also one of the few desiccation-tolerant grasses (together with *Sporobolus lampranthus*) capable of growing on deeper soils as well as in the typical resurrection-plant xerosere and this allows them to achieve broad tussocks of considerable age growing between taller grass species on flats with deeper soils (Gaff & Ellis 1974).

E. nindensis is a perennial grass with a wide southern African distribution (Fig. 1). It is generally found on bare exposed areas and stony sandy soils (Gibbs Russel *et al* 1990). Gaff & Ellis (1974) found that like other desiccation-tolerant grasses, *E. nindensis* often grows in soil only 1 - 2 cm deep.

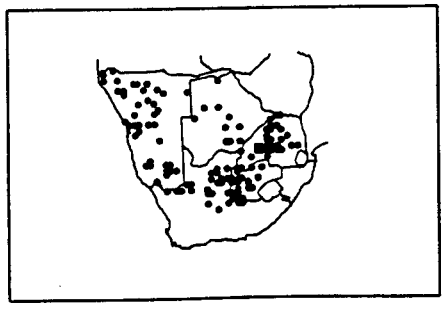

Fig. 1. Distribution of *E. nindensis* (from Gibbs Russell *et al* 1990)

The aim of this study was to investigate physiological changes occurring during drying and rehydration of *E. nindensis*. For comparison, the desiccation-sensitive congener *Eragrostis curvula* (Schrad.) Nees was also examined. This is a widespread and common Southern African grass, generally occurring in high rainfall areas (Gibbs Russel *et al* 1990) which was also screened for desiccation tolerance by Gaff & Ellis and found to be unable to recover from the dry state.

Like many other desiccation-tolerant plants, the habitat of *E. nindensis* contains no other vegetation to provide shade and the plants are thus subjected to high radiation levels during dehydration.

The photosynthetic machinery is the plant system most sensitive to moisture stress (Kruger 1995) and so serves as a good indicator of metabolic status. The present study thus investigated changes in functioning of photosystem II using measurements of chlorophyll fluorescence, and levels of photosynthetic pigments (chlorophylls and carotenoids) were determined at various stages during drying and rehydration.

When excited by light, chlorophyll molecules dissipate the excitation energy in three ways: heat, fluorescence or to the light reactions of photosynthesis. The emission of red fluorescence is an indication of the amount of energy being diverted away from photosynthesis by photosystem II and so is a sensitive measure of the physiological state of this part of the photosynthetic apparatus.

Dark-adapted leaves, when exposed to light levels low enough to prevent the occurrence of measurable photochemistry, emit a minimum or baseline fluorescence Fo. This is thought to represent the fluorescence emission when all reaction centres are open . Increases in Fo may indicate damage to PSII or prevention of transfer of excitation energy from antenna complexes to the reaction centre (Sherwin 1995).

The maximal fluorescence value Fm represents fluorescence emission when all the PSII reaction centres have been closed by the delivery of a strong pulse of actinic light. A high Fm is indicative of normal PSII functioning. Decreases in Fm generally indicate damage to

the photosynthetic electron transport chain such that excitation energy is lost as heat and via energization of the thylakoid membranes (Bilger & Schreiber 1986 in Sherwin 1995)

Damage to PSII can thus be detected by either an increase in Fo or a decrease in Fm. This is quantified by calculating the variable fluorescence Fv = Fm-Fo. The ratio Fv/Fm is proportional to the quantum yield of PSII and in healthy photosynthetic tissue is typically between 0.75 and 0.85 (Sherwin 1995).

This study investigated aspects of the prevention of free-radical damage and protection from UV radiation by measuring levels of anthocyanins and carotenoids in leaves during drying and regreening. Anthocyanins are flavonoids which have been implicated in protection from UV radiation damage - in other words to act as 'sunscreens' (Hopkins 1995) since they absorb strongly in the UV-B region of the spectrum. This is supported by the fact that anthocyanin synthesis is induced by UV radiation (Hendry 1993). The absorption spectrum of pelargonin, a widespread anthocyanin, shows a peak around 500nm, a wavelength at least theoretically suited to the quenching of some activated chlorophyll species. Anthocyanins are also hypothesised to act as free-radical scavengers or antioxidants (Larson 1988 cited in Farrant & Sherwin 1998).

Measurements of electrolyte leakage were use to assess structural integrity of cell membranes during drying and rehydration. This parameter gives an indication of changes in membrane configuration (Sherwin & Farrant 1996) which are indicative of damage. The ability to maintain membranes unchanged through the stresses which would greatly alter unprotected lipid bilayers is one of the characteristics of angiosperm desiccation tolerant plant species.

MATERIALS AND METHODS:

PLANT MATERIAL

Eragrostis nindensis and *E. curvula* (variety Ermelo) plants were grown from seed donated by Dr Roger Ellis of the Agricultural Research Council, Pretoria. *E. nindensis* seed was collected just west of Leeupan, Etosha. Plants were grown in a 1:1 mixture of potting soil and river sand and maintained in a greenhouse for approximately one year prior to the experiment. At the time of the experiment, the plants had not been subjected to any prior water stress and so had not been acclimated to tolerance.

The *E. curvula* specimens used to calculate full turgor MC were collected in Cape Town and only adult plants were available. Although the youngest tillers were used, these were still larger and more robust than the *E. curvula* tillers used for the dehydration. The use of a different ecotype at a different growth stage may have affected RWC values for *E. curvula*.

Experiment 1:

In an initial experiment, six *E. nindensis* plants were dried under laboratory conditions by witholding water. Once leaf relative water content (RWC) had dropped to between 5 and 10%, plants were left for a week and then rehydrated by watering well on the first day and then keeping soil moist for the duration of the rehydration. Measurements were then taken twice daily for the first three days and thereafter once a day until RWC reached pre-dehydration values.

Experiment 2:

In this experiment, 32 *E. nindensis* and 16 *E. curvula* plants were dried under controlled conditions. These consisted of a temperature range of 20 - 26°C, photoperiod of 12 hours and light intensity of 400 μmol photons. m^{-2}. s^{-1} . As in Experiment 1, dehydration was effected by witholding water. After twenty days of dehydration when leaf RWC averaged 20-40%, plants were transferred to a phytotron chamber where they were maintained at a temperature of 18 - 25°C and a photoperiod of 12 hours. Light intensity was approximately 800 μmol photons. m^{-2}. s^{-1}. After a further 10 days in this environment leaf water contents averaged 3.5% and plants were transferred to a germination chamber under fluorescent light

but with the same temperature and day-length settings, where trays of silica gel could be used to reduce humidity. After two weeks in this low-humidity treatment the plants were moved into the laboratory and rehydrated as before.

At regular intervals during drying and rehydration, plants were sampled for electrolyte leakage (EL), chlorophyll fluorescence (FLU) and pigment content. These measures were compared on the basis of RWC not time in order to account for differences in the drying rates of the two species. Moisture content and fluorescence values for *E. nindensis* represent measurements taken during both experiments; all other measurements were taken during Experiment 2 only.

1. MOISTURE CONTENT

Tillers were removed from plants as close to the base as possible. Old dead parts were discarded and the tiller or leaf weighed to determine fresh mass. Chlorophyll fluorescence and then EL measurements were taken on each leaf, after which leaves were dried in a 70°C oven for 24 hours. Leaves were allowed to cool over silica gel and then weighed to determine dry mass. Moisture content was calculated as follows:

MC = (fresh mass - dry mass) / dry mass ($g\ H_20.\ g^{-1}$ dry mass)

Relative water content (RWC) could then be calculated using the following formula:

RWC (%) = MC sample leaf / MC of leaf at full turgor

This is a measure of how moist a leaf is relative to its maximum possible moisture content, and allows comparisons between species. Full-turgor moisture content was obtained as follows: seventeen tillers were excised from well watered plants and placed in test tubes with enough water to cover the base. The tubes were then placed in the dark in a plastic cover for one to two days to allow maximal water uptake. Tillers were weighed as above to obtain full-turgor water content. The average of these values was used as full-turgor MC for each species.

2. PIGMENT CONTENT

Three replicates of approximately 0.02g of leaf material were used for each assay. Assays were performed at six intervals during the course of Experiment 2 as described in Table 1.

Table 1: Moisture contents of *Eragrostis nindensis* and *E. curvula* at the times of sampling for measurement of pigment contents.

code	description	RWC *E. nindensis*	RWC *E. curvula*
FH	Fully hydrated	80 - 90 %	80 - 90 %
PH	Partially hydrated	30 %	no measurements of chlorophylls or carotenoids; anthocyanin content at 75% RWC
DRY	Dehydrated	2%	2%
24HR	24 hours after first rewatering	2%	<10%
48HR	48 hours after first rewatering	37%	<20%
R	Rehydrated	~80%	no measurements: plants were obviously dead and starting to degrade.

A. CHLOROPHYLL AND CAROTENOID CONTENT

Leaf material was cut into fine pieces and placed in 10ml of 100% acetone. Extraction occurred overnight in the dark at 4°C. The absorbance (A) of the extract was read at wavelengths of 661.6 nm, 644.8 nm and 470 nm using a Beckman DU 650 spectrophotometer. Chlorophyll and carotenoid contents were calculated using the adjusted extinction coefficients as described by Lichtenthaler (1987).

Chlorophyll a $= 11.24\,(A_{661.6}) - 2.04\,(A_{644.8})$

Chlorophyll b $= 21.13\,(A_{644.8}) - 4.19\,(A_{661.6})$

Chlorophyll (a+b) $= 7.05\,(A_{661.6}) + 19.09\,(A_{644.8})$

Total carotenoids

(xanthophylls & carotenes) $= [1000(A_{470}) - 1.90\,(\text{chl a}) - 63.14\,(\text{chl b})] / 214$

The equations give pigment content in μg per ml of plant extract. Values were adjusted to mg pigment.ml^{-1}.g^{-1} estimated dry mass.

B. ANTHOCYANIN CONTENT

Leaf material was cut into fine pieces, snap-frozen with liquid nitrogen and ground using a mortar and pestle. The ground material was lyophilised overnight and stored at -70 ° C until analysis at completion of the experiment. To 5mg of freeze-dried material was added 10 ml of acidified methanol consisting of methanol: water: concentrated HCl in the ratios 79:20:1. Extraction then occurred at 4°C for 48 hours. The solutions were then centrifuged and the supernatant removed made up to 12 ml with acidified methanol. The absorbance was read at 530 and 657 nm using the same spectrophotometer described above. Anthocyanin concentration was then calculated according to Sherwin & Farrant (1998):

Anthocyanin concentration $= [A_{530} - (0.33A_{657})] \ / \ 0.05$ [A.(g dry wt)$^{-1}$]

3. CHLOROPHYLL FLUORESCENCE

Chlorophyll fluorescence was measured using a modulated portable fluorometer (OS-500: Opti-Sciences, USA) in order to calculate the quantum efficiency of the leaves. Three segments from each sampled tiller were placed in a cuvette and dark-adapted for 10 min before reading. Modulation intensity and detector gain were set such that instantaneous fluorescence F_T of each leaf remained within constant limits. Initial fluorescence F_O was measured at a light intensity not sufficient to induce variable fluorescence. Maximal fluorescence F_M was measured using a saturation intensity of approximately 4 000 μmol. m^{-2}. s^{-1} with a duration of 0.9 sec. Variable fluorescence F_V was calculated from $F_M - F_O$ and this was used to determine quantum efficiency F_V/F_M.

4. ELECTROLYTE LEAKAGE:

After determination of Fv/Fm, leaf segments (generally about 5 to 12 segments per leaf) were placed in 18ml purified Milli-Q water and conductivity of the solution measured at five-minute intervals for 40 minutes using a portable conductivity meter (CRISON CDTM-523). The rate of leakage was calculated from the slope of the line generated in the initial linear phase of the time course of leakage and corrected for leaf dry mass to obtain a measure of conductivity in $\mu Siemens.cm^{-1}.min^{-1}.g^{-1}$ dry mass.

RESULTS:

1. MOISTURE CONTENT

Initially, both plants contained green, fully expanded leaves. *E. curvula* plants contained more dead (brown) leaves than *E. nindensis* at the start of the experiment. *E. nindensis* leaves had a higher absolute moisture content, although RWC values were similar for both species when fully hydrated.

E. nindensis:

Mean full-turgor MC = **3.56** ±0.50 g H_20 $.g^{-1}$ dry mass

E. curvula:

Mean full-turgor MC = **1.63** ±0.29 g H_20 $.g^{-1}$ dry mass

Fig. 2. *Eragrostis nindensis* plants during dehydration. Left = fully hydrated; centre = initial stages of drying (note leaf folding); right = dry.

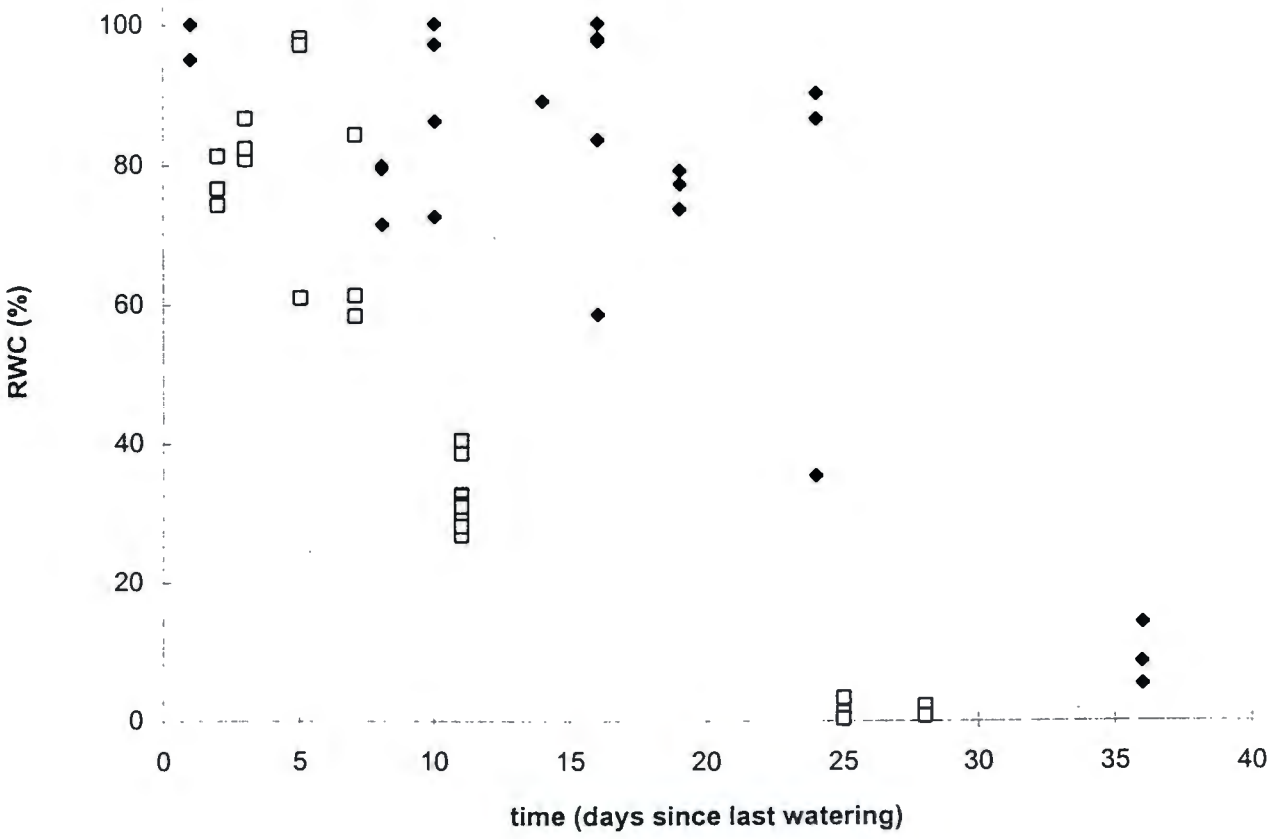

Figure 3. Moisture content of drying *E. nindensis* leaves

E. nindensis:

Figure 2 shows plants at various stages of drying. In both experiments, drying appeared to be slow with plants taking 20 - 30 days to reach RWC of below 10% (Fig. 3). *E. nindensis* responded to water deficit very early in the dehydration time course. The first visible signs of drying, a twisting along the length of the leaf, occurred within a week of witholding water when the leaves were still at a high RWC (70 - 80%). Between RWC of 70% and 40% leaves rolled up lengthwise, enclosing the adaxial leaf surface. As folding began, hairs along the blade margin became oriented inwards to form a loose meshwork across the opening to the adaxial surface (see insert to Fig. 4). The torsion also served to present the abaxial surface, which has a greater density of hairs, to the light.

Fig. 4. Prominence of leaf hairs in dry *E. nindensis* (RWC < 2%). Insert shows folded dehydrating leaf (RWC approx. 40 - 50%) showing orientation of hairs across the fold join.

Drying involved very striking colour changes which were due to changes in pigment content (see below). As water content continued to decline, leaves became more tightly rolled with one margin tucked inside the other. Older leaves folded around younger shoots as they dried; such young shoots were then completely enclosed in older leaf tissue and dried leaves were cylindrical in shape. In the dry state, the lowest recorded leaf RWC was 0.75% although mean dry RWC was 1.7%. Culm bases and roots were also extremely dry, containing less than 0.04g H_20. g^{-1} dry mass.

Figure 5. shows the time course of rehydration for *E. nindensis*. Rehydration rates were very variable between leaves on the same plant and even those on the same tiller; however, the first leaves took 44 - 50 hours to start greening although hydration began prior to this. Leaf rehydration was accompanied by unrolling and greening of the blade. Occasionally, expansion and unrolling occurred before greening. In tightly rolled leaf sheaths or younger leaves enclosed by older shoots, greening occurred in the tightly-rolled tissue and unrolling only occurred later. Greening progressed up the leaf from the base, preceded by the point of unrolling which represented a sharp transition between fully green, expanded leaf tissue and dry, rolled brown tissue (Fig 6.) The transition zone was yellow-orange in colour and was the only site where an intermediate stage between dry and rehydrated tissue could be seen.

Once approximately two-thirds of the leaf length had rehydrated, greening rate slowed and many green leaves retained dry tips.

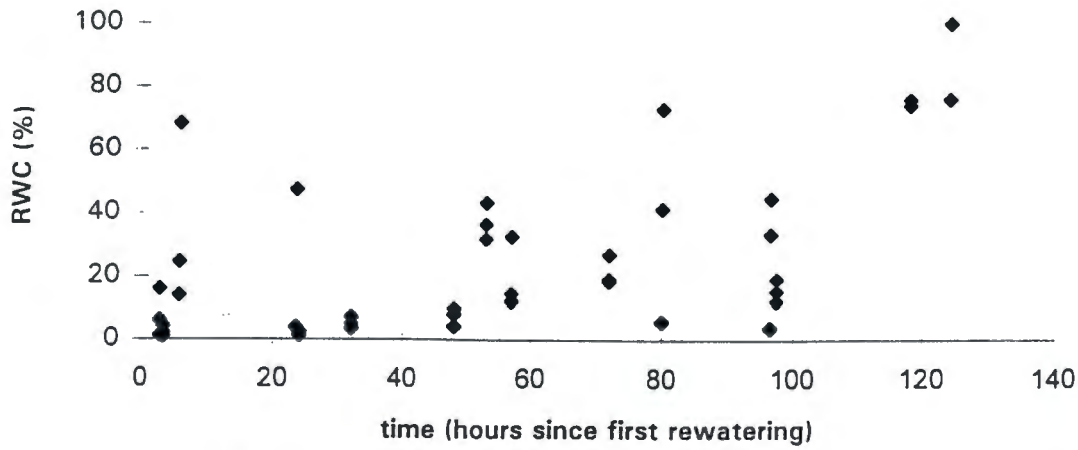

Fig 5. Moisture content of rehydrating leaves of *E. nindensis*

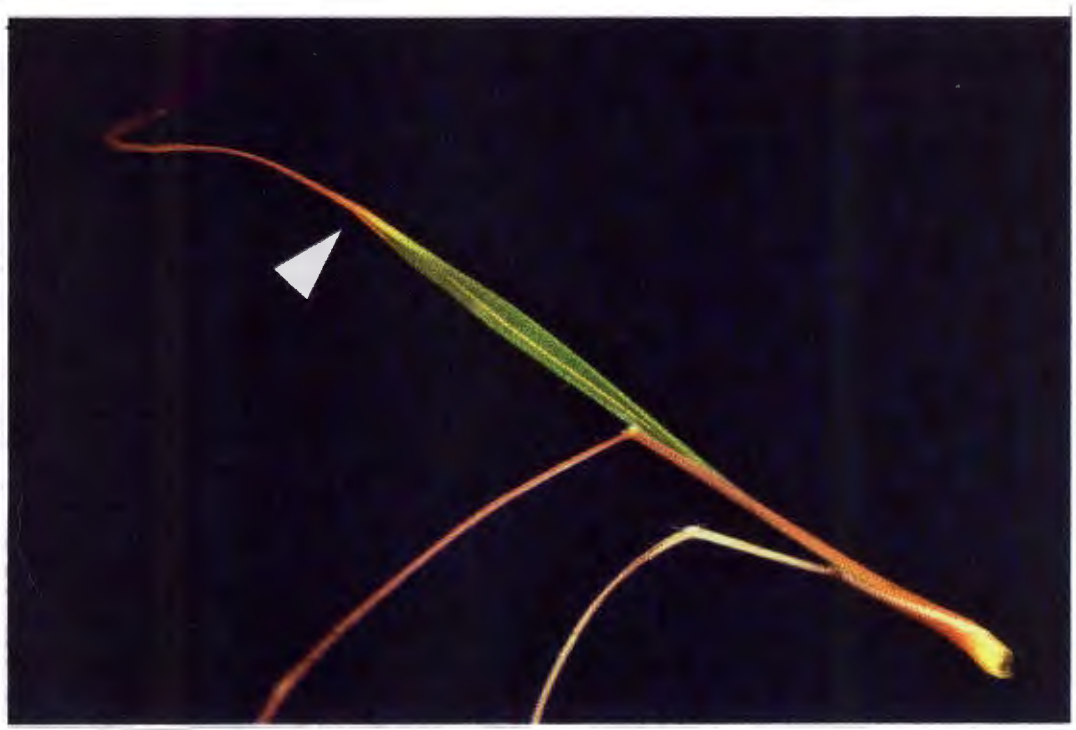

Fig. 6. Rehydrating *E. nindensis* leaf showing transition (arrowhead) between green and dry tissue. A young shoot which survived the dehydration enclosed within the older leaf is visible.

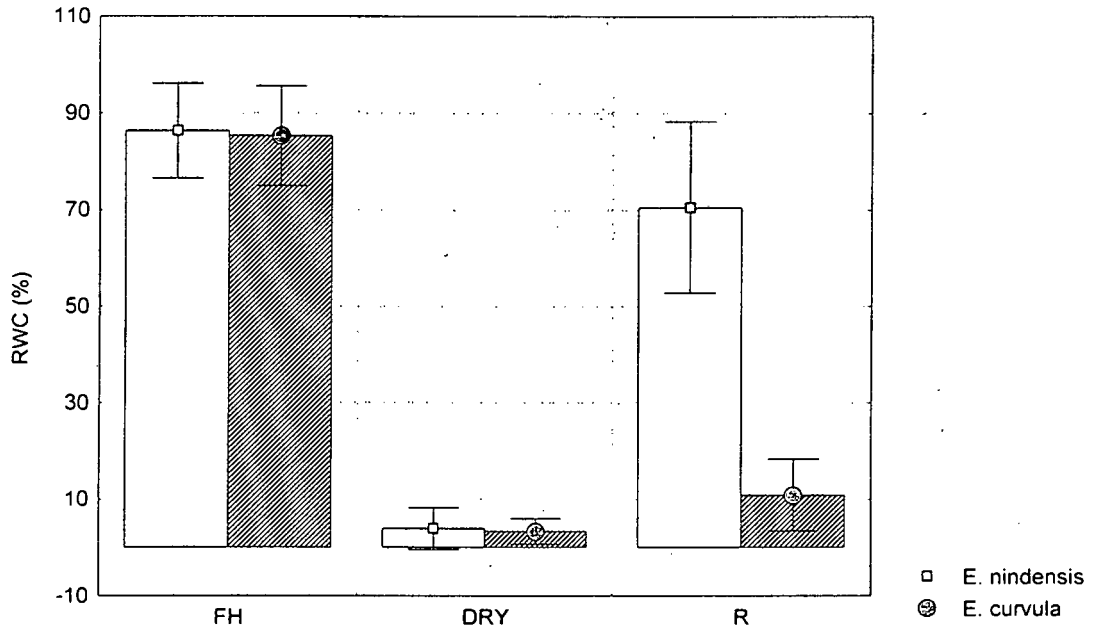

Figure 7. Moisture contents of E. nindensis and E. curvula during drying and rehydration.

FH = fully hydrated; R=rewatered.

Some plants took several days to start greening and not all tillers on a plant survived. Rehydrated plants all retained some dry leaves. All the *E. nindensis* plants used in the study showed some signs of regreening and most recovered fully, producing abundant green foliage within a week of rehydration.

E. curvula:

Leaves of this species maintained high moisture contents after *E. nindensis* had already begun to lose moisture. When the latter species averaged 30% RWC, *E. curvula* was still at 75% RWC. Leaf curling also occurred in this species. As RWC dropped below approximately 80%, many *E. curvula* plants were producing new shoots. At the termination of the dehydration, leaves had dried down to less than 2% RWC and roots and culm bases to less than 0.05g H_20. g^{-1} dry mass. Figure 7 shows moisture content of *E. curvula* in relation to *E. nindensis* at various stages of drying and after rehydration. *E. curvula* leaves did not rehydrate on watering. This is probably due to complete embolisation of root xylem vessels. Dry leaves excised and placed with the bases in water did rehydrate but did not recover other physiological activity. *E. curvula* did not recover from desiccation.

2. PIGMENT CONTENT

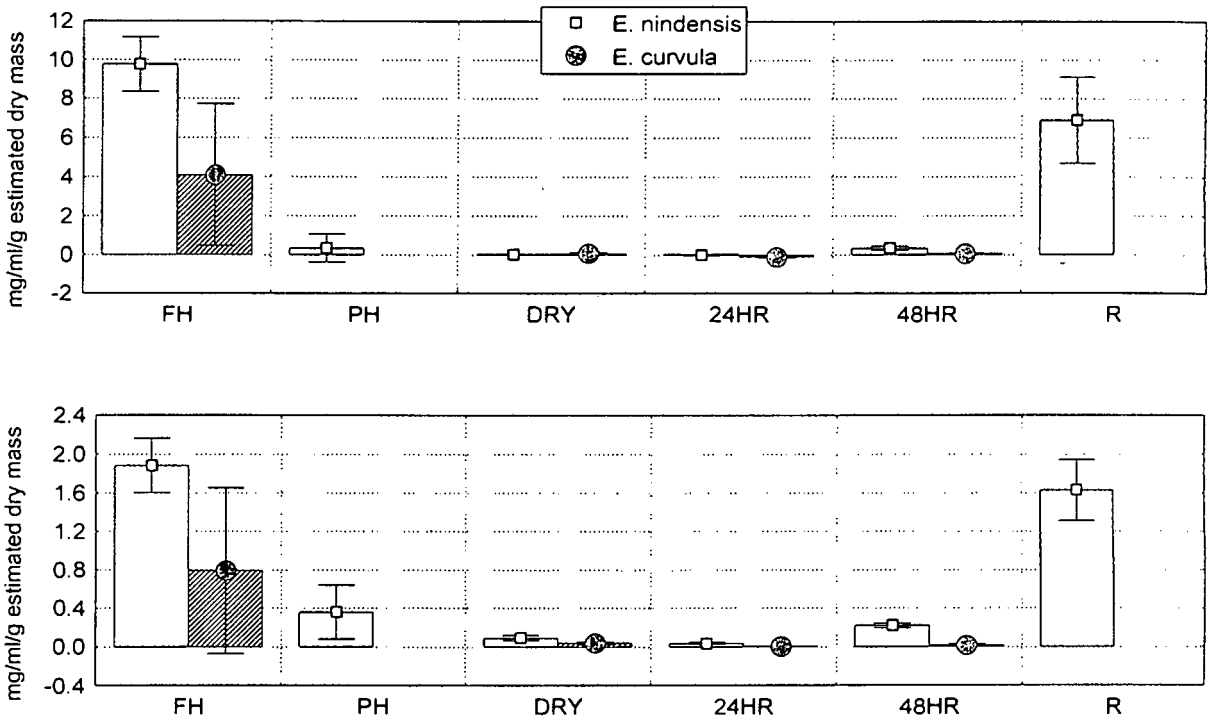

Figure 8. Photosynthetic pigment contents in leaves of *E. nindensis* and *E. curvula*. a - total chlorophyll (a + b) . b- total carotenoids (xanthophylls and carotenes). FH = fully hydrated; PH=partially hydrated; 24HR= 24 hours after first rewatering; 48HR= 48 hours after rewatering; R = rehydrated. For moisture contents at different stages see Table 1.

E. nindensis:

This species had a greater total chlorophyll content than the sensitive control (Figure 8a). As the leaves folded, the blades became less green, taking on a faded grey-green colour. This was indicative of loss of chlorophyll and occurred as RWC dropped to 40%. At 35% RWC, leaves began to change from grey-green to reddish purple (see Fig. 9) and by the time leaves had reached 30% RWC, chlorophyll content had dropped to 3.5% of fully hydrated leaves. This occurred very rapidly - within 10 days of witholding water - and coincided with a large increase in anthocyanin content (Fig. 9). Figure 10 shows a rehydrating *E. nindensis* leaf surrounded by dry leaves. Note the dark pigmentation on the dry leaves. Anthocyanin contents remained high below 30% RWC but were completely broken down once rehydrating leaves reached above approximately 40% RWC. By the

time plants were fully hydrated, anthocyanins were no longer detectable by the methods used in this study.

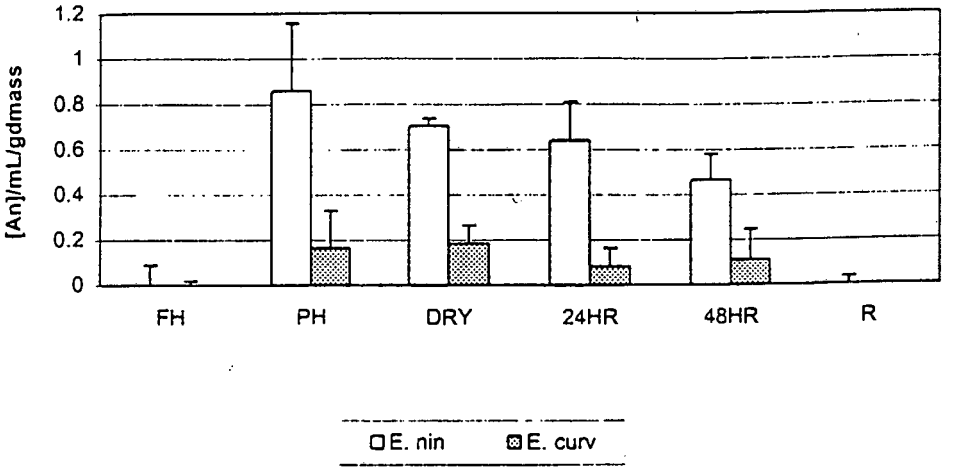

Fig 9: Anthocyanin contents of *E. nindensis* and *E. curvula* leaves

In the dry state, chlorophyll was not detectable in leaves of *E. nindensis* (Fig 8a) and only reached measurable levels 48hours after rewatering (RWC 37%, chlorophyll content 3.5% of maximum). In rehydrated leaves, chlorophyll levels were comparable to those in leaves prior to dehydration. The values for chlorophyll content in rehydrated leaves were lower than control values as many of these leaves retained a dry tip at the time of measurement.

Figure 8(b) shows changes in total carotenoid content in both species. Carotenoid breakdown occurred later than chlorophyll breakdown in drying *E. nindensis* and resynthesis preceded that of chlorophyll synthesis during rehydration. When the chlorophyll concentration had dropped to 3.5% of hydrated control, carotenoid levels were still at 24% of control. When the chlorophyll levels had returned to 3.5 %; carotenoid levels had already reached 12%. By the last assay, chlorophyll had only reached 70% of control values while carotenoid levels had reached 86%. Plants did not lose all carotenoids in the dry state and these pigments may have contributed to the orange-red or brown colouration of dry leaves.

Figure 10. Rehydration of darkly pigmented leaves of *E. nindensis* during Experiment 2. Most of the leaves are not showing signs of rehydration. A greening leaf is visible (centre).

E. curvula:

This species remained green for a long period and still had green leaves when *E. nindensis* had lost all its chlorophyll. In the initial stages of drying, *E. curvula* accumulated red pigment, presumably anthocyanin (Fig. 9) on the leaf sheaths only. This was in response to mild water stress as (RWC 75%). There was no further increase in anthocyanin level with dehydration but the pigmentation, once laid down, persisted throughout further stages of drying and after rewatering. In the dry state chlorophyll content had dropped to below 2% of that found in hydrated plants (Fig 8a). Chlorophyll content did not recover upon rehydration . Carotenoid content shows a similar drop during dehydration and some carotenoids are detectable in the dry state (Fig 8b). These did not increase on rehydration.

3. Chlorophyll Fluorescence

E. nindensis:

Figure 11 shows changes quantum efficiency of photosystem II (QE) with drying and rehydration. Fully hydrated plants had a QE of 0.8 (Fig. 11a); the graph clearly shows two outliers with lower than average QE. These leaves, despite having high RWC, were both very twisted which may indicate that chloroplast dismantling had begun. Quantum effeciency dropped as RWC passed 60%. When plants were at a RWC of 30% and chlorophyll content at 3.5% of control, QE was below 0.4. In the dry state when chlorophyll was absent from the leaves, measured QE averaged 0.3. Both measures are indicative of non-functioning photosystem II enzymes (Sherwin 1995).

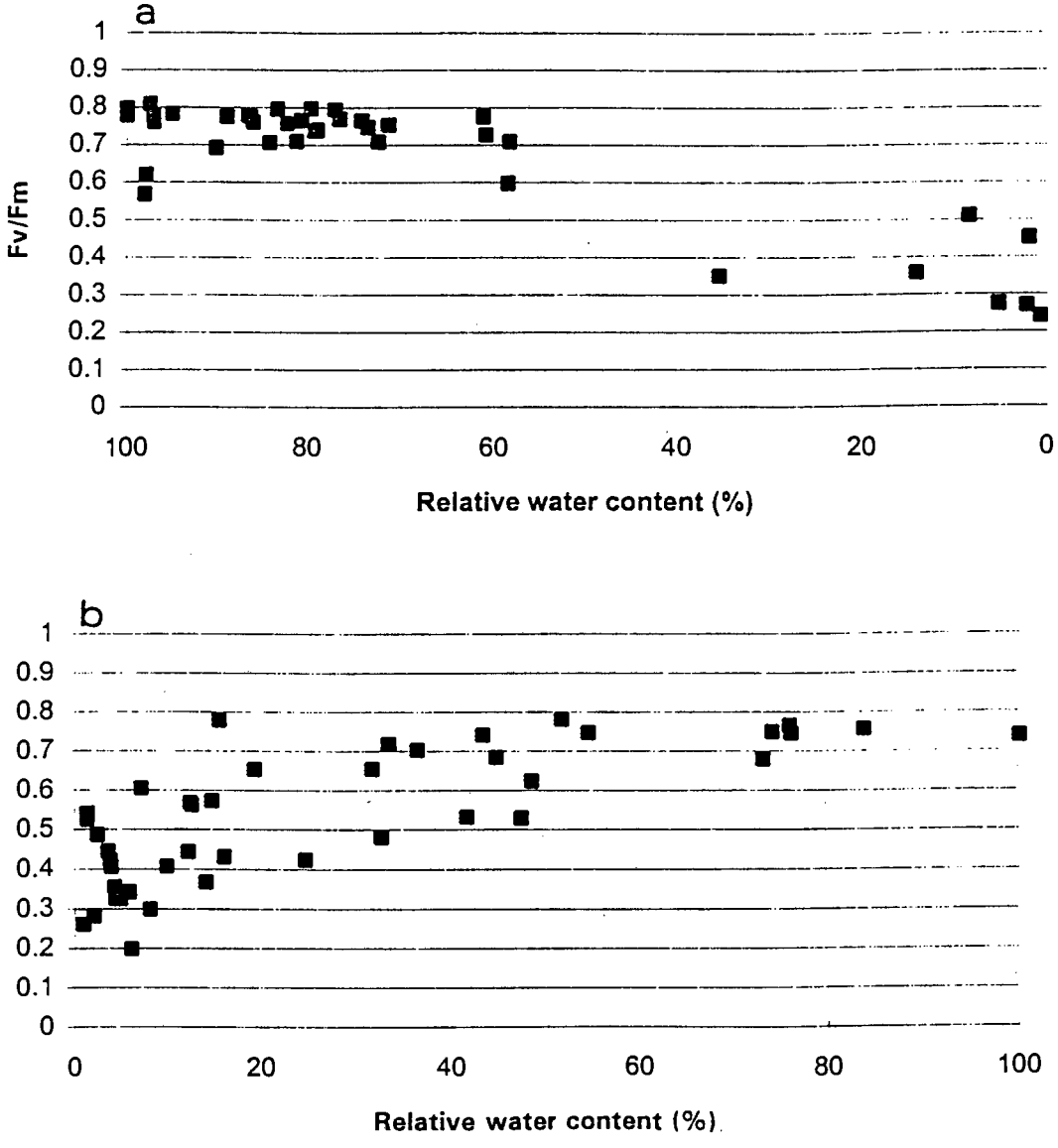

Fig 11. Quantum efficiency of dehydrating (a) and rehydrating(b) *Eragrostis nindensis* leaves

During rehydration QE increased indicating a reactivation of photosystem II activity. There was initially a large scatter of values (Fig 11b) probably due to different recovery rates of chlorophyll and photosynthetic enzymes. At RWC of 37% when 3.5% of the chlorophyll had been resynthesised, QE was at 0.6. Quantum efficiency returned to initial values at RWC between 70 and 80%. This occurred after 120 hours of rehydration.

E. curvula:

Figure 12 compares Fv/Fm values of *E. curvula* and *E. nindensis* in the hydrated, dry and rehydrated states. In the dry state, *E. curvula* QE levels were as low as those of the tolerant species. On rewatering, QE values rose slightly but never returned to initial levels.

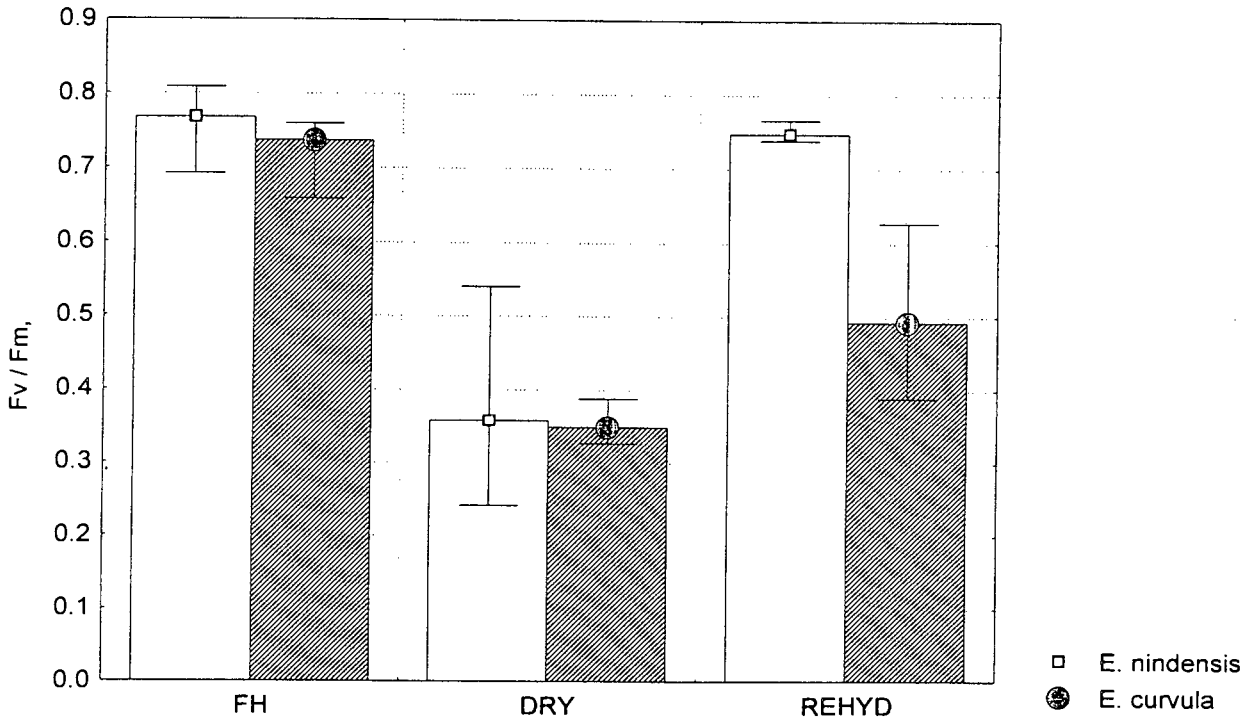

Figure 12. Quantum efficiency of E. nindensis and E. curvula leaves during drying and rewatering. FH = fully hydrated (80-90%RWC); DRY=<2%RWC; REHYD=rewatered (*E. nindensis* at 70% RWC; *E. curvula* at 20% RWC)

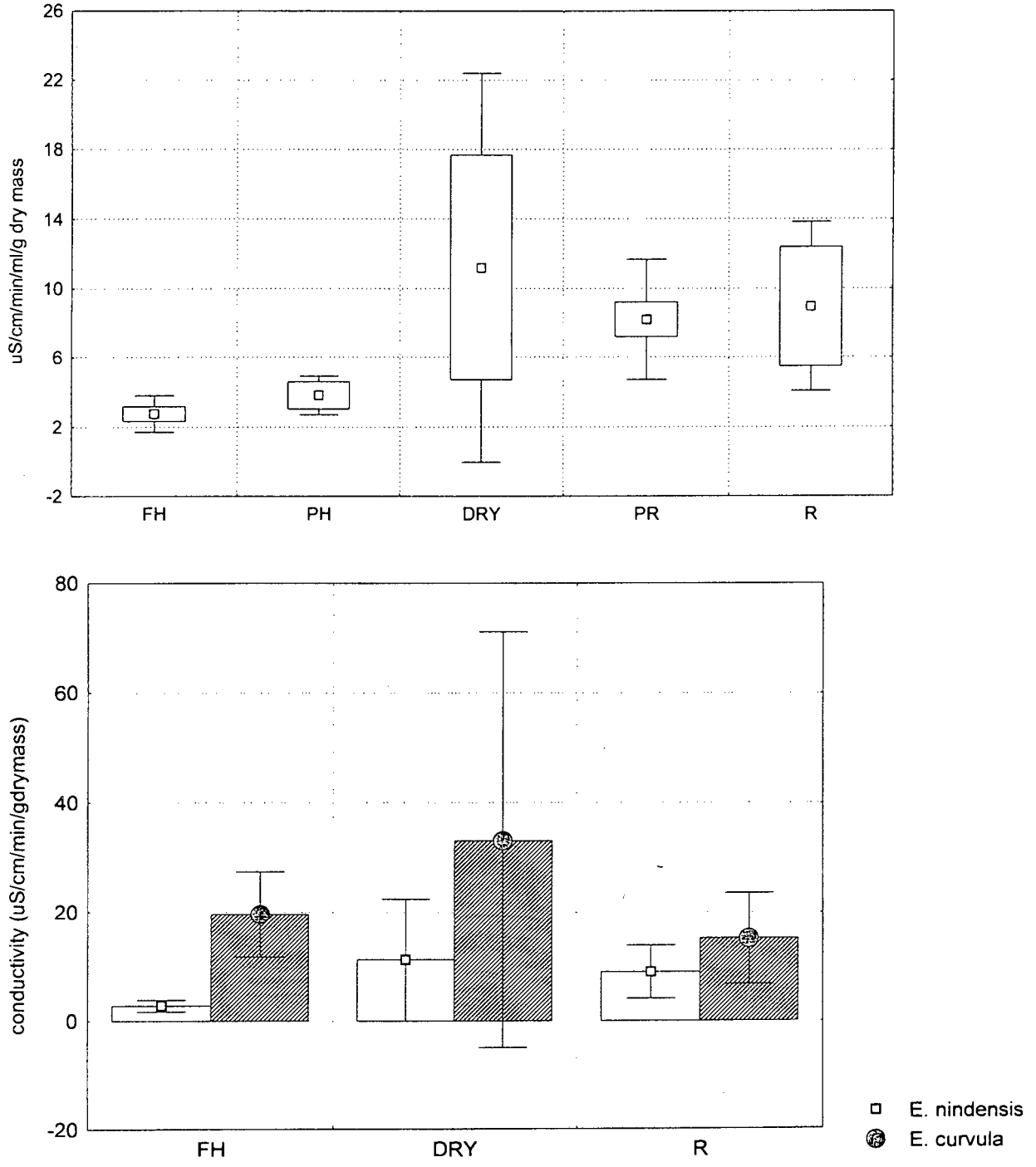

Figure 13. Electrolyte leakage of leaves during drying and rehydration.

(a) ***E. nindensis*** **(b)** ***E. nindensis*** **and** ***E. curvula*****. FH=fully hydrated; R=rewatered. For MC values see Fig 12.**

4. Electrolyte leakage

E. nindensis:

Leakage of fully hydrated control plants low (Fig 13a.) This measure increased slightly during dehydration but generally remained low. In the dry state there was a large range in recorded leakage values. This decreased during rehydration but leakage rates were higher in plants which had undergone drying and rehydration than in pre-dehydrated plants, possibly due to the fact that plants had not been previously dehydrated.

E. curvula:

Figure 13b compares leakage of *E. curvula* to that of *E nindensis*. Fully hydrated leaves of *E. curvula* had greater rates of electrolyte leakage, and this was true at all stages in the experiment. Dry *E. curvula* had maximal leakage rates and the values were lowest in rewatered leaves from this species. This result is surprising but the decrease is not large and may be attributable to differences between individual leaves.

DISCUSSION:

Eragrostis nindensis is desiccation-tolerant, as its vegetative tissue recovers fully from dehydration to less than 2% RWC (absolute moisture content of 0.07g $H_20.g^{-1}$ dry mass in both roots, leaves and culm bases). RWC's below 70% are outside the range experienced by sensitive tissues, which are killed by a RWC lower than 30% (and many cannot survive below much higher levels). Relative water content of 30% has been shown to be the critical point at which membrane damage becomes severe (Sherwin 1995).

The drying rates for these plants were extremely slow compared to rates in the wild: Gaff (1977) reports drying times in the field for leaves of several desiccation-tolerant species ranging between 1h and 4d. Potted plants allowed to dry by witholding water typically take far longer to desiccate - in one study, the time taken to reach moisture contents of below about 0.5 g $H_20.g^{-1}$ dry mass in potted plants varied between 3 and 30 days (Sherwin 1995). In the present study, drying rates were slow enough for induction of tolerance to occur during initial stages of drying, presuming such induction is required.

Drying rate in *E. nindensis* is initially faster than *E. curvula*. It is not clear whether this is due to physical effects such as different soil drying rates or to physiological differences between the two species. In either case, changes occur in *E. nindensis* at higher RWC than *E. curvula*. Some desiccation tolerant plants (eg *Myrothamnus flabellifolia*) do not close stomata in response to water stress and lose thus they simply lose moisture in equilibration with the environment (Sherwin *et al* 1998). Some authors hypothesize that plants possess mechanisms to slow the rate of drying in order to have enough time to activate protective mechanisms (Dalla Vecchia *et al* 1998). Although *E. nindensis* does not appear to prevent water loss, leaf folding may play a role in slowing the drying rate.

Rolling of leaves protects the adaxial (upper) leaf surfaces especially in the initial stages of drying when folding of hairs across the opening to the adaxial surface may

also contribute to increased light reflectance. The abaxial surface is protected to some extent by its greater density of hairs. Rolling of the leaf also considerably reduces the surface area exposed to light. Thus the function of the first detectable response to drying appears to be related to reducing exposure of leaves to light.

Quantum efficiency began to drop simultaneously with the first signs of folding in *E. nindensis* leaves. The decrease in Fv/Fm is likely to be a result of either conversion of chlorophyll to its precursor molecules or orderly dismantling of the thylakoid membranes, or both. Damage to chloroplasts is unlikely to be a major reason for the reduction in QE due to the following reasons: (1) Since *E. curvula* does not recover from dehydration, it is reasonable to speculate that loss of chlorophyll and reduction in QE in this species is due to photobleaching, oxidative damage to membranes and molecules, and accumulation of other dehydration-related damages. Thus the retention of chlorophyll in *E. curvula* at MC's which in *E. nindensis* correspond to much lower chlorophyll levels indicate slower disappearance of photosynthetic pigments in this species. This is likely to represent the time course for chlorophyll degradation due to damage. The more rapid rate of chlorophyll loss in *E. nindensis* is thus likely have a different cause and to represent the time scale of chloroplast dismantling. (2) The fact that *E. nindensis* recovers from near-complete desiccation within several days of watering indicates that minimal damage has been sustained by its tissues.

This is the feature that makes desiccation-tolerant plants so remarkable. How does *E. nindensis* prevent light from damaging its subcellular components while in the vulnerable semi-dry state? Folding of the leaves together with the reorientation of hairs will reduce the number of photons striking chlorophyll molecules in the initial stages of drying. Leaf folding is a common feature of resurrection plants (Gaff 1977). The leaves of *E. nindensis* cannot, however, fold completely like some HC dicotyledonous plants which are able to curl leaves into very small surfaces (Sherwin & Farrant 1998 & 1996; Kruger 1997). Thus accumulation of masking pigments would be an important adaptation to protect thylakoid membranes at this stage. The increase in anthocyanins during dehydration is likely to play a large role in screening light from the chloroplasts. Anthocyanins have been implicated in both absorption of incident light (visible and UV wavelengths) and in free-radical scavenging (Sherwin & Farrant 1998) so it is likely that these pigments have a dual role in preventing light

damage. The subsequent rapid breakdown of chlorophyll completely prevents the production of free radicals by light in the chloroplasts. Preserving some carotenoids in drying leaves allows for quenching of chlorophyll that does become activated by light. The residual carotenoids will also contribute towards the defusing of superoxide free radicals produced in drying chloroplasts.

It is likely that the plant will also increase activities of other anti-oxidant compounds as has been shown in several other desiccation-tolerant species (Sherwin & Farrant 1998). These authors showed increased levels of the anti-oxidant enzymes glutathione reductase, ascorbate peroxidase and superoxide dismutase increased during dehydration of both a PK and an HC species. The desiccation-tolerant grass *Sporobolus stapfianus* (which belongs to the same subfamily as *E. nindensis*) has also shown to increase glutathione reductase activity during desiccation (Sgherri et al 1994 in Sherwin & Farrrant 1998).

The process of breakdown of the photosynthetic apparatus has been visualised via electron microscopy in studies of the poikilochlorophyllous resurrection sedge *Xerophyta viscosa*, which begins to break down its chlorophyll at approximately 30% RWC. Two species in this genus have been shown to lose virtually all chlorophyll in the dry state - *X. viscosa* (Sherwin & Farrant 1998) and *X. humilis* (Cooper 1997). In the dry state, the thylakoid membranes from chloroplasts of *X. viscosa* lost their ordered structure and became vesiculated. These changes disappeared on rehydration (Sherwin & Farrant 1996) and are likely to represent the state in which thylakoid components are stored in the dry cells.

The decline in quantum efficiency during drying and rehydration may be interpreted to be due to disruption in transfer of excitation energy from PSII to the photosynthetic electron transport chain, due to disruption of thylakoid membrane structure. Reduction in Fv does not neccessarily indicate damage to PSII but could also be a result of protective regulatory processes that serve to dissipate the excess excitation energy(Sherwin 1995). Such processes may include orderly dismantling of the photosystem as well as accumulation of accessory pigments to quench excitation energy. Anthocyanins and carotenoids may be involved in this quenching (Sherwin & Farrant 1998).

Recovery of *E. nindensis* QE to pre-dehydration levels indicates full recovery of all PSII-associated photosynthetic apparatus. A study by Dace et al (1998) showed that in the PK Xerophyta humilis, such resynthesis was dependant on de novo translation but not transcription of nuclear genes. They hypothesise that drying initiates the production of mRNA's for chlorophyll biosynthesis, and that these are stably stored in the dried leaves until rehydration. Thus reconstitution of thylakoid membranes does not require activation of the DNA and the genome can remain condensed in its most highly protected form until RWC recovers. Such a mechanism may be operating *in E. nindensis*. Recovery of photosynthesis appears to lag behind recovery of moisture content. This was found to be true in other poikilochlorophyllous desiccation-tolerant plants (Tuba *et al* 1994) as the entire photosynthetic system had to be recreated before photosynthetic activity could begin again. Thus it would be expected that the sequence of events would be : first, recovery of turgor; second, recovery of cellular metabolism including protein synthesis; and third, reconstitution of the photosynthetic apparatus. The last parameter to recover appears to be chlorophyll content as this had not returned to initial levels at the time of last sampling, despite full recovery of QE and the healthy, dark green appearance of rehydrated leaves. Full recovery of photosynthetic function appears to require new genomic transcription (Dace et al 1998)

Chlorophyll lost in *E. curvula* due to photobleaching and free radical damage in water-stressed tissues was not recovered on rehydration. This species could not prevent damage due to desiccation and light and could not repair damage after water loss. The folding of the leaves in *E.curvula* may have been entirely mechanical due to loss of turgor as leaf cells dehydrated.

Envelopment of buds by folding leaves is also common to many resurrection plants (Gaff 1977). This also serves to protects younger shoots in both species. The tightly folded tillers are also likely to be better protected against mechanical damage while tissue is dry and brittle.

Gaff & Ellis (1974) cite evidence reveiwed in Levitt 1972 that younger, meristematic tissue is more tolerant of water stress than mature tissue. The new shoots produced by *E. curvula*, presumably in response to moisture stress, may have been a last attempt to replace older, damaged tissue with new shoots more capable of surviving the stress. This response is known in roots of xerophytes where drying soil stimulates production of new roots in order to mine deeper soil for receding moisture (Drew 1979) such that the plant can survive until the next rain event.

Role of carotenoids: these are important accessory photosynthetic pigments having two main functions: to intercept light at wavelengths inaccessible to chlorophylls, and to 'quench' activated chlorophyll and so prevent the formation of oxygen free radicals. Carotenoids are also antioxidants and protect the chlorophylls from destruction by light. This is called photobleaching and is a result of oxidative bleaching of chlorophyll by activated oxygen. The double bonds of carotenoids quench the activated oxygen molecule (Hendry 1993). This last function of the carotenoids will be especially important during dehydration. The lag in carotenoid breakdown is thus hypothesised to be a deliberate mechanism to protect drying leaves. Once completely dry, leaves can no longer produce free radicals and the inert metabolic state is stable until rehydration. The more rapid resynthesis of carotenoids on rehydration is likely to be important: the plant needs to have carotenoids present before it resynthesised chlorophyll because the carotenoids must be in place to accept excitation energy that might otherwise be damaging to rehydrating chloroplasts. This however does not appear to be true for all desiccation-tolerant plants: in *Myrothamnus flabellifolia*, recovery of carotenoid levels was slower (72hours) than that of chlorophylls (24hours) (Sherwin & Farrant 1996) . *M. flabellifolius* is a homoiochlorophyllous plant and so is likely to utilise other protective mechanisms during rehydration (eg greater levels of anti-oxidant enzymes). The retention of some carotenoids in dry *E. nindensis* as opposed to dry *E. curvula* is likely to be important in the quenching of activated oxygen in near-dry states.

Anthocyanin accumulation in response to both visible and UV light is common in both sensitive (Hopkins 1995) and desiccation-tolerant plants (Gaff 1977); sensitive plants often protect apical buds with red or orange anthocyanins. These buds contain

meristematic, actively dividing cells so in this case the anthocyanins are probably importatnt in preventing UV damage to DNA (Dace *et al* 1998). Accumulation of anthocyanins on the leaf sheaths of *E. curvula* is probably due to this response since purple leaf sheaths occur in field grown *E. curvula* (pers. obs). In grasses, the leaf sheaths protect the meristems and the newly-differentiated leaf tissue so accumulation of anthocyanins on the leaf sheaths is probably a response similar to that of anthocyanin accumulation in leaves surrounding apical buds in dicotyledons. The leaf sheaths of *E. curvula* are also the only parts of the leaf with any hairs present; this may contribute to light reflectance away from these parts of the tiller.

E. nindensis leaves accumulate anthocyanin along their whole length and the greatest amounts are laid down between the fully hydrated state and a moisture content of about 30% . At 31% the chlorophyll has already been substantially broken down. Thus it appears that anthocyanin accumulates simultaneously with chlorophyll breakdown which supports the role of anthocyanins in masking chlorophyll from light and preventing peroxidation damage (Sherwin & Farrant 1998). Gaff (1977) reports accumulation of anthocyanins in many desiccation-tolerant species during dehydration. He also states that "an intense purple-black colour in the dry leaves of most grass and some *Xerophyta* species is a reliable criterion for discriminating the viable dry leaves from the non-viable senescent leaves. The red colouration of dry viable leaves of *E. nindnesis* is not completely reliable, however." The results of this study agree with Gaff's observations as not all deeply-pigmented leaves recovered.

On rehydration, anthocyanin levels dropped slowly such that 48 hours after rehydration, leaves still retained approximately 50% of the anthocyanin level present in the dry state. The retention of anthocyanins during rehydration in *E. nindensis* is probably a mechanism for light-protection and free-radical scavenging while chlorophyll is being reconstituted. Sherwin & Farrant (1998) found similar retention of anthocyanins in rehydrating leaves of *Xerophyta viscosa*. The partially-synthesised photosynthetic apparatus is likely to be subject to the same light stresses as dehydrating leaves and anthocyanins as well as carotenoids will thus be important protective components during at this time.

The integrity of the cell membrane is indicated by electrolyte leakage rates. Fully hydrated *E. nindensis* leaves show very low leakage rates which do not increase substantially during dehydration. This is expected since in general, desiccation-tolerant plants exhibit constitutively low rates of leakage (Kruger 1995) which are indicative of the activation or upregulation of mechanisms to maintain membrane integrity during drying and subsequent rehydration. One of these mechanisms is the accumulation of compatible solutes which serve to maintain turgor but more importantly replace the hydration shell neccessary to maintain the configuration of membranes and other cellular structures. Ghasempour et al (1998) found that sucrose levels increased almost threefold and that raffinose and stachyose accumulated in air-dry leaves of *E. nindensis*. Levels of the extremely effective compatible solute trehalose also increased.

In the dry state the increased variation in leakage rates indicates different levels of membrane damage between sampled leaves. This may be explained by the fact that not all leaves recovered from desiccation and those with larger leakage rates may already have died. Since the conductivity assay is destructive, it is impossible to say which leaves would have rehydrated so it may be that a greater range of electrolyte leakage rates is normal in dehydrated tissue of this species. On rehydration, the variation in leakage rates between leaves decreases. Leakage rates however remain slightly higher than initial rates which may indicate that recently-dehydrated membranes are fragile for some time after rehydration. The increase is however not large and may not be significant: all studies to date report that leakage rates return to baseline levels on rehydration if they increased at all during the dry state (Sherwin & Farrant 1996; Cooper 1997; Kruger 1998).

The greatly increased range of leakage values for *E. curvula* in the dry state indicate that membranes have become disrupted and cells are not able to withstand the immersion in water required for the assay. This species does not possess mechanisms of membrane protection as cells did not recover on rehydration.

Eragrostis nindensis thus is able to dismantle its photosynthetic apparatus and break down chlorophyll during drying, while protecting the subcellular environment from oxidative damage and putting in place all those mechanisms which will ensure

membrane protection and the viability of cellular compartments, structures and macromolecules in the dry state. Light protection-mechanisms such as folding and anthocyanin accumulation are part of such protection. At the same time it is able to avoid the metabolic disruptions imposed by low water contents. In the dry state, it is able to persist for extended periods of time (on the order of months, Gaff 1977) and upon rehydration it refills embolised root xylem and rehydrates its tissues while preventing damage due to uncontrolled influx of water. During this time, synthesis of photosynthetic pigments in the correct order (ie carotenoids before chlorophylls) and in the correct proportions, and re-assembly of the thylakoid membrane systems must occur, while any damage incurred during drying must be repaired. During both drying and rehydration, antioxidant and quenching compounds must be produced in sufficient amounts in the correct cellular compartments to prevent extensive damage due to oxygen free radicals. Mechanical stresses imposed during the considerable shrinkage and expansion of drying and rehydration must be mediated. Physiological processess such as protein synthesis and gene transcription are resumed, respiration and photosynthesis are restarted and carbon gain begins anew.

None of these mechanisms are present in *E. curvula* in sufficient quantity to prevent damage during rehydration or effect recovery on rehydration.

The co-ordination of all these processes in order to induce tolerance to extreme desiccation in *E. nindensis* is a remarkable feat. Although the slow growth rates imposed on desiccation-tolerant tissue may limit the applicability of this species as a pasture grass, it will still be a useful fodder species which will provide grazing for stock as soon as a shower breaks a drought. For a stock farmer, this quick response to rain may mean the difference between maintaining and losing a herd after a prolonged drought.

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