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RADICAL FORMATION AND ACCUMULATION *IN VIVO*, IN DESICCATION TOLERANT AND INTOLERANT MOSSES

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Water loss in a desiccation-sensitive moss resulted in destruction of chlorophyll, loss of carotenoids and increased lipid peroxidation, indicating the presence of damaging forms of activated oxygen. These effects were exaggerated when the plants were desiccated at high light intensities. During water-deprivation there was a build up of a free radical, detected *in vivo*, with a close correlation between molecular damage and radical accumulation. In contrast, in a desiccation-tolerant moss there was almost no indication of molecular (oxidative) damage. However a stable radical similar in type and concentration to that found in the desiccation-sensitive species accumulated, particularly under high irradiances. The stable radical appears to be one of the end-products of a process initiated by environmental stress, desiccation and high irradiance: its association with molecular damage depending on the degree to which the species is tolerant of desiccation. Identification of the radical in intact tissue from EPR and ENDOR studies, suggests that this is not a short-lived peroxy-radical but instead is relatively stable and carbon-centred.

KEY WORDS: *Dicranella palustris*, *Tortula ruraliformis*, mosses, free radical, desiccation.

INTRODUCTION

Mosses are generally poikilohydric (i.e. lacking means of restricting water loss). For many species water loss can be highly damaging, often lethal. Moss species which show a high tolerance of desiccation are exceptional and have attracted investigators (see[1,2] for extensive reviews) though the mechanisms conferring desiccation tolerance are by no means resolved.

In higher plants, water stress is known to impair photosynthesis, the principal route for the dissipation of absorbed light energy (see[3] for review) but without significantly decreasing the flow of electrons through the photosystems.[4,5] This may lead to enhanced leakage of electrons to oxygen by routes previously outlined.[6] In unstressed environments, higher plants and mosses appear to be adequately protected from oxidative damage in the chloroplasts through superoxide dismutase, several peroxidases, tocopherols, carotenoids, ascorbate and glutathione.[7–10] Under stressed conditions these protective mechanisms may be breached, leading to oxidative damage.[11,12] Chloroplasts prepared from droughted wheat plants generate superoxide in amounts proportional to the extent of water loss, when irradiated with

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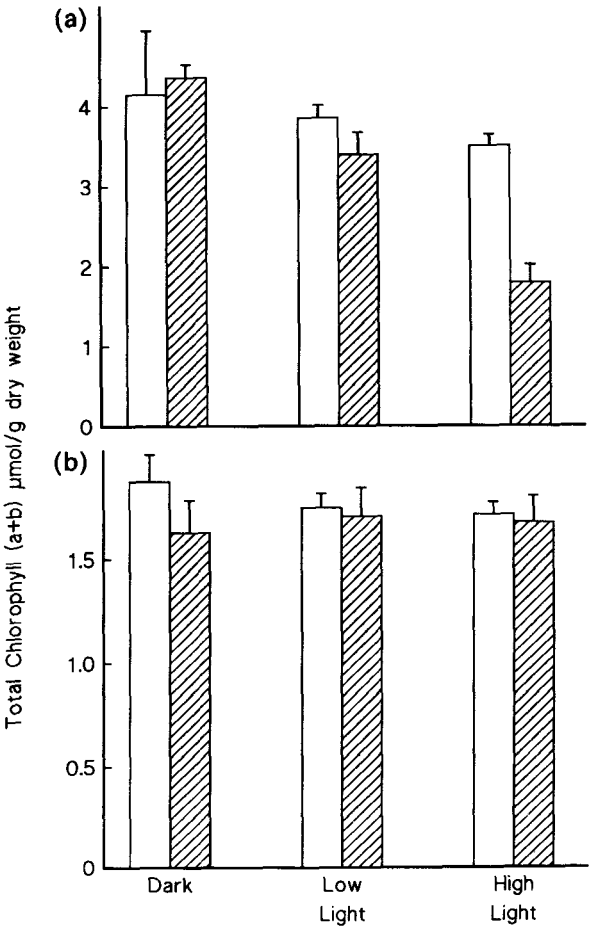

FIGURE 1 Total chlorophyll (*a* and *b*) µmol/g dry weight in (a) *D. palustris* and (b) *T. ruraliformis* in (□) non-desiccated controls and (■) after desiccation treatment under three different irradiances. Error bars = one standard error, where $n = 5$.

photosynthetically saturating light.[13] We were particularly interested to extend this work to mosses which lack the complex morphology and water-loss prevention mechanisms of higher plants. In the field, mosses will often be simultaneously exposed to bright sunlight and desiccating conditions. We report here the effect of desiccation under different light irradiances on two mosses from contrasting environments: *Dicranella palustris* from moisture-saturated or wet montane flushes and *Tortula ruraliformis* from dry, well-drained sand dunes.

EXPERIMENTAL

Dicranella palustris (Dicks.) Crundw. ex E.F. Warb. was collected from Rydal Fell, Cumbria (national grid reference NY 363084) and *Tortula ruraliformis* (Besch.)

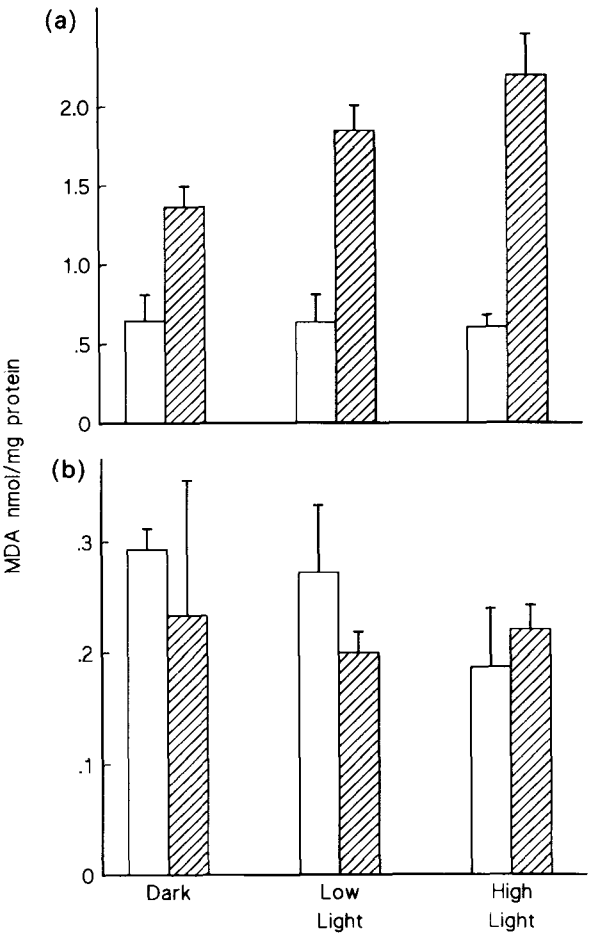

FIGURE 2 Lipid peroxidation (as thiobarbituric acid-reactive compounds) formed in (a) *D. palustris* and (b) *T. ruraliformia* in (□) non-desiccated controls and (■) after desiccation treatment under three different irradiances. Error bars = one standard error, where $n = 5$.

Grout. from Newborough Warren, Anglesey (SH 405363). The top 5 mm of healthy green tissue were cut, washed, blot-dried and sealed in perspex cabinets for 72 h at either 100% relative humidity (control) or 85% relative humidity (desiccated) and illuminated for 14 h daily at three levels of irradiance, nil (darkness), 80–100 μmol $m^{-2} s^{-1}$ (low light) or 1100–1200 μmol $m^{-2} s^{-1}$ (high light) (irradiance measured at the plant surface) using metal halide–tungsten lamps supplemented at the highest irradiance with a Björkman lamp (Hansatech Ltd., King's Lynn, Norfolk, U.K.) suspended over a water heat-trap. Day/night temperatures were maintained at 22 and 15°C.

Lipid peroxidation was determined as the concentration of thiobarbituric acid-reactive substances, equated with malonylaldehyde (MDA), as in[14] but with butylated

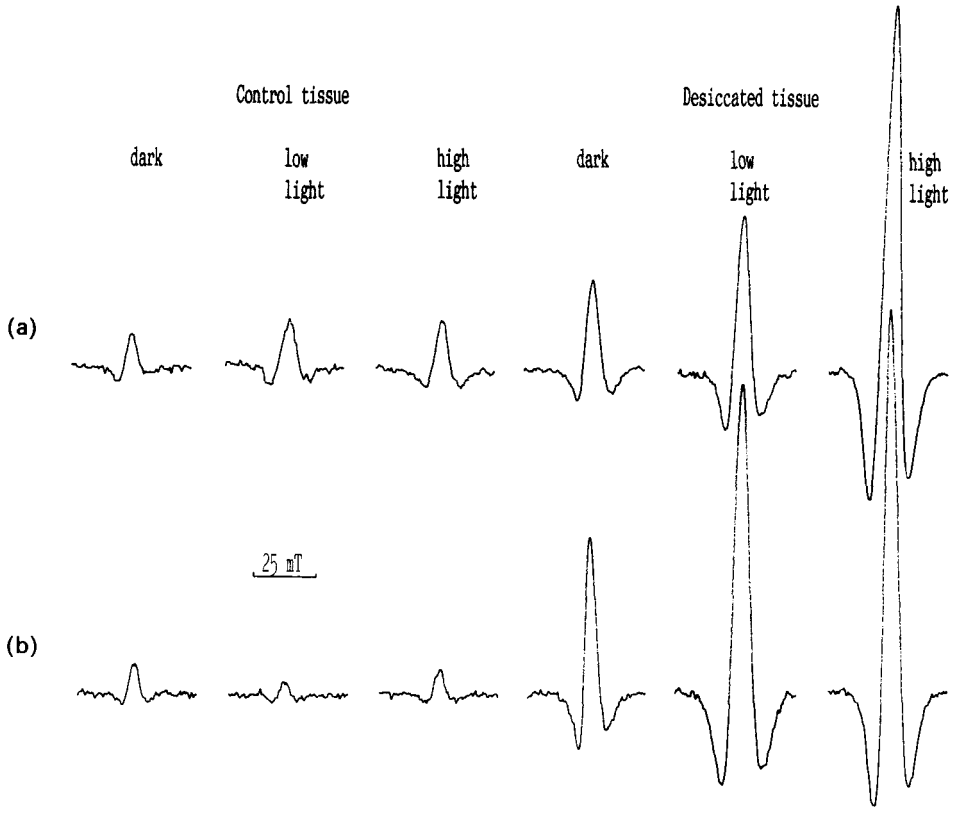

FIGURE 3 Second derivative of EPR spectra of approximately equal volumes of intact tissue of (a) *D. palustris* and (b) *T. ruraliformis* following desiccation under three different irradiances, together with non-desiccated controls.

hydroxy-toluene (0.05% w/v) routinely included as an anti-oxidant and quantified using 1,1,3,3, tetra-ethoxypropane as a standard. Protein content was determined by the method of.[15] Chlorophylls and carotenoids were estimated from ammoniacal acetone extracts, the chlorophylls from the extinction co-efficients of[16] and carotenoids from the formula in.[10] Electron paramagnetic resonance (EPR) and electron-nuclear double resonance (ENDOR) spectra were measured on a Brucker ER 200 D spectrometer at room temperature and 109 K respectively using intact plant material packed to a constant height and density.

RESULTS

Two markers of molecular damage were monitored: the destruction of chlorophyll and peroxidation of lipids. In *D. palustris*, in the absence of light there was little or no destruction of chlorophyll following desiccation (Figure 1a). On exposure to low or high light irradiance during desiccation, 12% and 50% respectively and of the total chlorophyll was destroyed. Under the highest irradiance the chlorophyll $a:b$ ratio

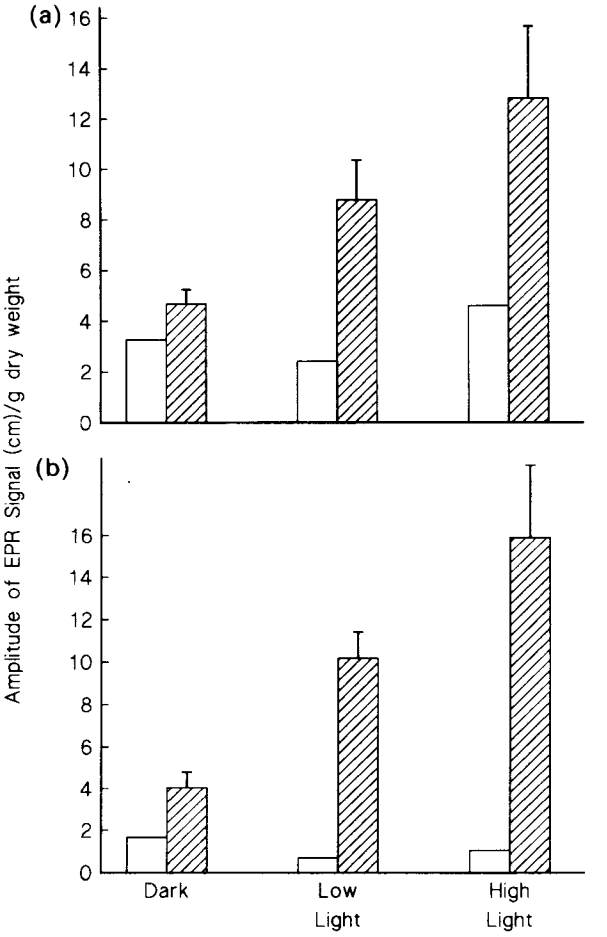

FIGURE 4 The mean amplitude of the EPR signals form intact tissue of (a) *D. palustris* and (b) *T. ruraliformis* in (□) non-desiccated controls and (■) after desiccation treatment under three irradiances, the signal amplitude being adjusted for the differences in dry weight of the tissues. Replication: control tissues 1 to 2, desiccated tissues 3.

declined from about 2.29 ± 0.10 to 1.73 ± 0.03 indicating selective and severe destruction of chlorophyll *a*. The concentration of total carotenoids was also reduced, by over 50% at the highest irradiance (data not shown). In *T. ruraliformis* desiccation treatment had no effect on chlorophyll concentrations, nor carotenoids, with or without illumination (Figure 1b).

Compared to non-desiccated controls, there was a significant rise in MDA in desiccated *D. palustris*, increasing with the level of irradiance from 2.3-fold (dark), to 2.9-fold (low light), to 3.6-fold (high light regime) (Figure 2a). There was no significant increase in MDA in *T. ruraliformia* during desiccation (Figure 2b).

Intact tissue of *D. palustris* produced a distinct EPR signal, the amplitude increasing 2 to 3-fold above control values in material which had been both desiccated and

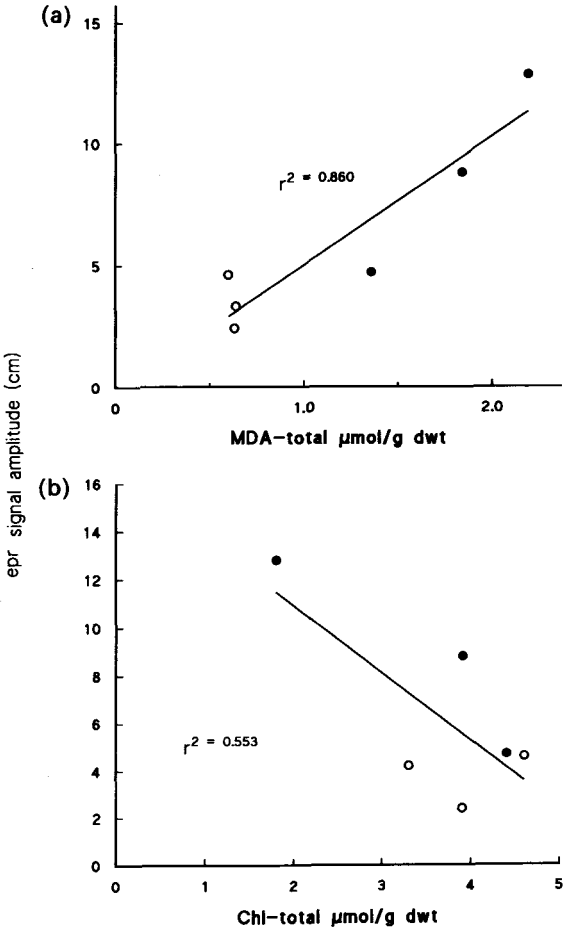

FIGURE 5 The relation between mean EPR signal amplitude (as in Figure 4) and (a) lipid peroxidation or (b) total chlorophyll concentration of *D. palustris*, showing Spearman's co-efficient and line of regression.

illuminated (Figure 3a). Surprisingly, broadly similar results were obtained from *T. ruraliformis* (Figure 3b). The data when quantified and adjusted for the small differences in dry weight of the samples are shown in Figure 4a and b. In non-desiccated tissue, the signal amplitude from *D. palustris* was 2 to 3-fold greater than in *T. ruraliformis*. Desiccation however resulted in similar values in both species with the signal amplitude increasing with increasing irradiance. There was a strong correlation between the amplitude of the EPR signal and the destruction of chlorophyll (r = 0.553) (Figure 5a) and between signal amplitude and lipid peroxidation (r = 0.860) (Figure 5b) in *D. palustris*. No such correlation exists in *T. ruraliformis* (data not shown).

The EPR spectrum gives no immediate indication of the origin and structure of the free radical. To identify the radical further, the samples were cooled to 109 K. The

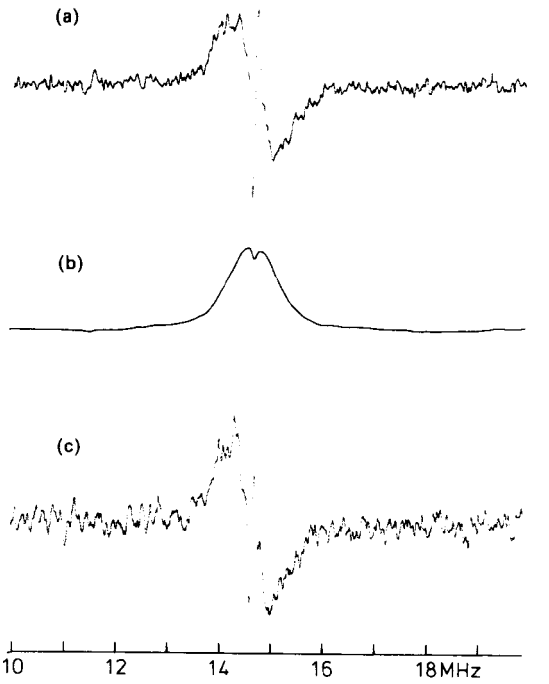

FIGURE 6 The ENDOR spectra of intact tissue of *D. palustris* at (a) 20 dB microwave power attenuation (b) the double integration of the above signal and (c) at 25 dB.

ENDOR response at the free proton frequency showed a rather featureless signal, characteristic of protons relatively far removed from the unpaired electron, with which they experience only a weak di-polar interaction. At 20 dB attenuation of the microwave power, the spectrum showed a sharp notch at the free proton frequency (Figure 6a); the double integration (Figure 6b) showing how this affects the appearance of the absorption spectrum. The depth of the notch was significantly reduced on increasing microwave power to 25 dB (Figure 6c). No signals were detected in the ^{14}H region from 0.5 to 10 MHz.

DISCUSSION

On the basis of sensitive markers of molecular damage associated with the formation of oxygen radicals,[12] the evidence is that *D. palustris* is a species sensitive to, and intolerant of, desiccation while *T. ruraliformis* is relatively resistant to drought. This conclusion correlates well with the known field performance and habitats of the two species and agrees with results obtained from other sensitive bryophytes.[17,18] The results indicate that the combination of desiccation and high irradiance was destructive in *D. palustris* and was closely and quantitatively associated with the build-up of a stable free radical. We interpret this to mean that by withholding water, particularly under high irradiance, reactive forms of activated oxygen were generated as previously reported in droughted wheat leaves.[13] These in turn initiated destruction of chlorophyll, peroxidation of lipids and build-up of a stable free radical.

The evidence from *T. ruraliformis*, however, demonstrates that accumulation of the stable free radical is independent of pigment destruction and lipid peroxidation, at least in this species. Because all evidence suggests that *T. ruraliformis* is tolerant of desiccation stress, the build-up of the stable radical in this moss is not an indicator that desiccation-induced damage has occurred at the molecular level. It is more likely that the effect of environmental stress (desiccation combined with high irradiance) resulted, as in wheat and other species, in the formation of activated forms of oxygen which, through a series of non-damaging radical transfer reactions, were recorded here as the accumulation of the stable radical. The identical environmental stress in *D. palustris* also gave rise to the stable radical but in this species the initiating forms of activated oxygen caused destruction of lipids and pigments. Whether or not damage will occur is presumably a function of the various molecular defences against activated oxygen which appear to determine, in part, tolerance (and intolerance) of desiccation.

Some indication of the origin and structure of the free radical would be helpful. At ambient temperature, EPR showed a symmetrical single line similar to that observed in germinating desiccated maize roots.[8] There was no change in shape when the temperature was cooled to 109 K. At this temperature any motion occurring at room temperature, and responsible for averaging anisotropic magnetic interactions, would be frozen out. The observation of a symmetrical line at low temperature indicates that the radical species has small *g*-anisotropy. This would be consistent with it being a carbon-centred radical. The dependence of the spectrum obtained at 109 K on microwave power, known from the earliest days of ENDOR spectroscopy[19] is characteristic of distant nuclei and reflects the relaxation characteristics of the system. As with EPR, the ENDOR observations do not lead readily to chemical identification of the radical formed here. The shape of the matrix proton ENDOR spectrum reflects the spatial distribution of the protons and which it may be possible to fit by modelling.[20,21] Our interim conclusion is that beyond being consistent with a carbon-centred radical, chemical identification will require further information beyond the finger-printing provided by magnetic resonance measurements.

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