Free Rad. Res. Comms., Vol. 8, No. 1, pp. 61–66
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PLANTS UNDER DROUGHT-STRESS GENERATE ACTIVATED OXYGEN

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(Received June 22, 1989, in revised form July 10, 1989).

Lysed chloroplasts prepared from droughted wheat plants generate $O_2^{\cdot-}$ on illumination as detected by electron spin resonance spectroscopy, the amplitude of the signal increasing with the severity of water deprivation. Following a similar time-course as radical formation and chlorophyll destruction, there was a significant increase in the accumulation of iron in the droughted shoots to reach an estimated concentration in the cell sap of about 2.5 mM. The evidence suggests that superoxide generated as a result of impaired electron transport in the chloroplasts reacts with the high concentration of accumulating iron resulting in the formation of hydroxyl radical, the probable cause of the primary pathologies observed in droughted plants.

KEY WORDS: Wheat, superoxide, hydroxyl radical, iron, drought.

INTRODUCTION

Much of the pathology of drought-induced damage in plants has been described in terms of gross morphology and physiology and recently reviewed.[1,2] At the subcellular level, drought damage causes the overall inhibition of protein synthesis, inactivation of several chloroplast enzymes, particularly those associated with CO_2 fixation,[3] impairment of electron transport,[4] increased membrane permeability[5] and increased activity of enzymes of the chloroplast H_2O_2 scavenging system.[6] Many of these descriptions are indistinguishable from those associated with natural, full-term senescence in plants.[7] In recent years there has been increasing evidence that forms of activated oxygen play a central role in the senescence of leaves,[8–10] in ripening fruits[11] and maturing flowers.[12] We present, here, evidence that damage as a result of drought also involved the formation of oxygen radicals and that much of the pathology that follows drought treatment is probably a consequence of the generation of superoxide in damaged chloroplasts.

EXPERIMENTAL

Wheat plants (*Triticum aestivum* L. cv. Sappo) were grown on Rorison's nutrient solution for 4 weeks after which drought treatments were exerted by suspending the roots in air, each succeeding day for 2 hr., 4 hr., 8 hr., then 12 hr. for the fourth and subsequent days of treatment. Control plants were placed in distilled H_2O during the drought treatment as in.[13]

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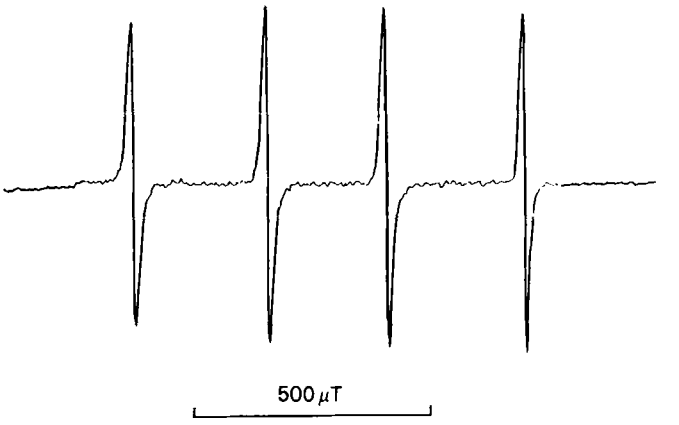

FIGURE 1

Chloroplasts were isolated in 100 mM K-phosphate buffer, at pH 7.5, containing 300 mM sucrose osmoticum as in[14] and ruptured by re-suspending for 10 min. in ice-cold 100 mM phosphate buffer, pH 7.5 before centrifugation at 7000 × g for 30 sec. and re-suspension in the same medium.

Electron spin resonance studies were carried out using a Varian E3 spectrometer at room temperature, the chloroplast membranes being diluted in phosphate buffer, pH 7.5, containing the spin trap Tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid) (final concentration 20 mM). Membranes were illumination by white light (irradiance 200W/m^2) and the e.s.r.spectra recorded after 2 min. of continuous illumination (or as described below). The amplitude of the Tiron semi-quinone signal was quantified using the illuminated riboflavin system of.[15] All e.s.r. determinations were carried out on membranes prepared within the preceeding 2 to 4 hours and stored in the dark on ice.

Iron determination was by atomic absorption spectroscopy. Enzymes and reagents were obtained from Sigma Chemicals, Poole and Boehringer Corporation, Lewes.

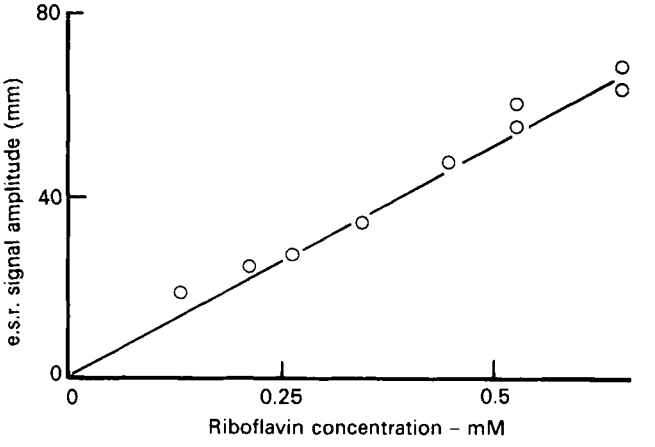

FIGURE 2

RESULTS

Chloroplasts, prepared from plants subjected to 4 days of drought treatment (with 26 cumulative hr. of water deprivation), when lysed and illuminated, produce an e.s.r. signal as in Figure 1. The amplitude of the signal was quantified against solutions of riboflavin, of known concentration, similarly illuminated in the presence of Tiron. During continuous illumination the amplitude of the signal from riboflavin increases to reach a maximum after 180 sec. Decreasing the level of irradiance by 50% had no effect on the rate of Tiron semi-quinone formation. Accordingly, a calibration curve of signal amplitude against concentration of riboflavin was established. The relationship was linear to a concentration of riboflavin of about 1 μM with a correlation of $r = 0.991$ (Figure 2). Lysed chloroplasts in 20 mM Tiron, pH 7.5, on illumination showed a more rapid rise in signal amplitude than with riboflavin to reach a stable maximum after about 60 sec. continuous illumination. On darkening, the signal decayed with a half-time of about 30 sec. (about 100 sec. in the riboflavin standards).

Chloroplast membranes from control plants were diluted to give a range of chlorophyll concentrations and, on illumination (for 120 sec.), the relationship between e.s.r. signal amplitude and membrane concentration, measured by chlorophyll content, was established (Figure 3). At high concentrations of chlorophyll ($> 0.6\,\mu$M) the maximum amplitude was reached rapidly (within 30 sec.) but also quickly decayed, possibly as a result of O_2 depletion. At concentrations between approximatley 0.025 and 0.40 μM, maximum amplitude was achieved within 120 sec. and was stable for at least several minutes. At pigment concentrations below 0.01 μM the maximum amplitude was not reached in less than 7 min. A chlorophyll concentration of 0.22 μM was used in subsequent experiments.

The effect of chloroplast membranes of several compounds, implicated in oxygen radical chemistry, was examined. Using membranes from plants given 4 days of drought treatment, bovine superoxide dismutase at 200 U/ml reduced the e.s.r. signal

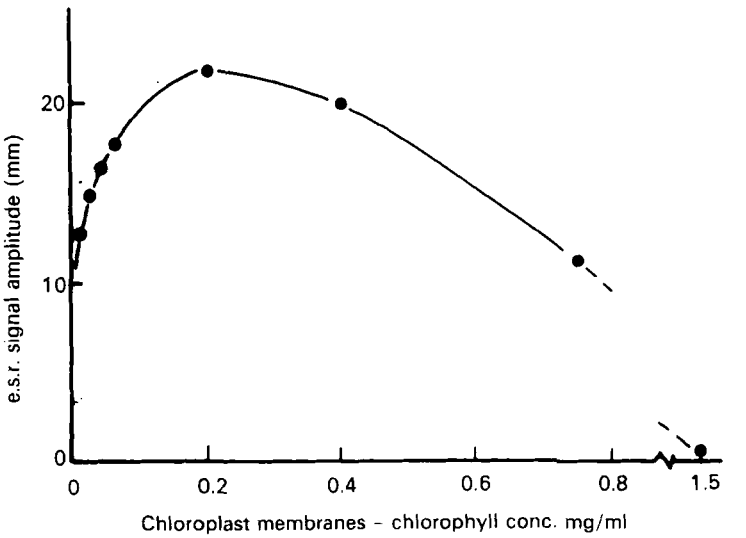

FIGURE 3

amplitude by 40%. Added catalase at 200 U/ml had no effect. Reduced glutathione at 10 mM decreased the amplitude by 85%. With superoxide dismutase and glutathione there was no reduction in the half-life of the signal on darkening indicating that these additions react with superoxide rather than directly with the Tiron radical. The signal generated by preparations of membranes with Tiron was absolutely dependent on light.

Chloroplasts were prepared from plants after drought treatment for 3, 4 or 5 days together with controls, and diluted in Tiron-buffer to a constant concentration of chlorophyll of 0.22 μM. On illumination there was an increase in the signal amplitude positively correlated to the duration of water-deprivation treatment (Figure 4). After 3 days of treatment (total 14 hr. water deprivation) the amplitude was not significantly different from control ($P > 0.5$), after 4 days (= 26 hr. water deprivation) to some 70% above controls, quantified as riboflavin equivalents.

Using leaf tissue similarly deprived of water, the internal cellular concentration of iron was determined and calculated on the assumption that the metal was dissolved in the cell sap and not deposited on walls or membranes. After 4 days of drought treatment the concentration of iron increased 2.3-fold over controls (Table 1). This increase in the uptake of iron, from the nutrient solutions, continued to the 7th day of drought treatment when the internal iron concentration reached 2.55 mM. Expressed as iron concentration per g dry weight, the droughted tissues accumulated some 3-fold more iron than controls after 7 days (Table 1). Coinciding with this

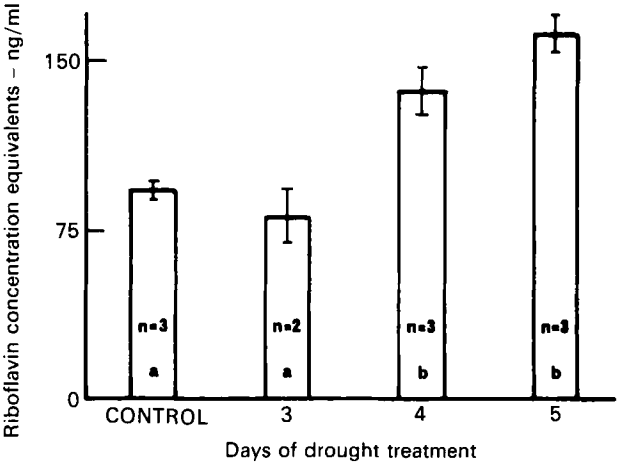

FIGURE 4

increase, there was a loss of chlorophyll. By day 7 of drought treatment, the pigment concentration had declined to 56% of control (per g dry weight of tissue) (data not shown).

DISCUSSION

Electron spin resonance studies of chloroplasts prepared from droughted wheat show that superoxide is generated during illumination. The source of electrons will most

TABLE I
Concentration of iron in the shoots of plants subjected to drought treatment. The cell sap was determined as the difference between tissue fresh and dry weight. Result are the mean of 5 determinations. The values for the drought treatments for day 4 and 7 were significantly different at the 5% level or better.

Days of Drought Treatment	mmol Fe/l cell sap		μmol Fe/g dry wt.	
	Control	Drought	Control	Drought
0	0.487	-	2.42	-
2	0.445	0.599	3.04	3.34
4	0.516	1.169	3.96	4.92
7	0.406	2.553	2.73	8.21

likely be chlorophyll.[16] The chanelling of electrons to oxygen rather than through the chloroplast electron transport chain to $NADP^+$ may be considered as a key part of the pathology of drought. Impairment of the electron transport chain appears to become significant only after the 3rd day of treatment (totalling 14 hr. of water deprivation). After this time the e.s.r. signal amplitude increases, which, because of it's sensitivity to added superoxide dismutase, indicated enhanced generation of superoxide. Further drought-treatment results in additional superoxide formation. Using riboflavin as a standard has allowed these increases to be quantified. However it is probable that the extraction methods used here to isolate chloroplasts will have resulted in some damage to the electron transport system in both drought-treated and control material. This would explain the unexpectedly high amount of superoxide generated in the controls.

Associated with the damage to the chloroplast electron transport system and with superoxide formation, drought-treatment also results in a significant uptake of iron, from the nutrient solution, into the leaf tissue. Much of the increase occurred in the 4th to 7th day of treatment when the plants had access to the external supply of iron for only 12 hours daily. Only those plants which had previously been deprived of water showed a significant increase in iron uptake. The 5-fold increase in iron concentration, in the cell sap, to 2.55 mM over 7 days is likely to result in iron participating directly in the formation of the highly reactive hydroxyl radical by well documented mechanisms (e.g.[17]). In turn this or similar[18] highly oxidizing species are likely to react with chlorophyll leading to the destruction of the pigment by described mechanisms.[19,20] As pigment destruction continues, so the overall capacity of the chloroplast to generate oxygen radicals through the photosynthetic apparatus would diminish although this stage was not detected in our e.s.r. studies.

Acknowledgements

AHP acknowledges the support of an SERC studentship, GAFH acknowledges assistance from NERC and from the Nuffield Foundation.

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Accepted by Prof. B. Halliwell