Properties of Herbage in Relation to Equine Dysautonomia: Biochemical Composition and Antioxidant and Prooxidant Actions

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To investigate the etiology of equine dysautonomia (ED), a degenerative polyneuropathy affecting grazing horses, the biochemical composition and antioxidant/prooxidant activities of aqueous extracts of plants collected from ED pastures were determined. Plants collected immediately after an outbreak of ED had reduced antioxidant and weak prooxidant activities when compared with control plants (plants collected from ED pastures out of ED season and control plants from ED pastures that were grown under favorable conditions). ED plants also had significantly increased concentrations of fructose and low molecular weight phenolic compounds, significantly more of one amino acid zone (probably valine), significantly less tartaric acid, and a nonsignificant decrease in ascorbic acid content when compared with control plants from ED pastures that were grown under favorable conditions. These findings suggest that ED plants may be under oxidative stress, possibly due to chilling, drought, or fungal colonization. However, experimental drought and chilling of plants did not reproduce the biochemical alterations identified in ED plants. It is possible that the altered biochemical content of ingested plants may contribute, directly or indirectly, to the development of ED in grazing horses.

Keywords: *Equine dysautonomia; antioxidant; prooxidant*

INTRODUCTION

Equine dysautonomia (ED), or "grass sickness", is a polyneuropathy affecting the central, peripheral, and enteric nervous systems of grazing horses (Cottrell et al., 1999). ED has a localized geographical distribution, occurring in northern Britain, northern Europe, the Falklands, and Patagonia (Cottrell et al., 1999). Interestingly, there are no confirmed reports of ED in Ireland, despite the large grazing horse population. Although ED is associated with grazing, the etiology is not known. Proposed causes of ED include mycotoxins (Robb et al., 1997) and infection with *Clostridium botulinum* type C (Poxton et al., 1997). There is at present no evidence to support involvement of a toxic plant (Robb et al., 1997). ED shows a strong seasonal incidence, being most common in spring, and occurs predictably after periods of dry, cool (<10 °C), and frosty weather (Doxey et al., 1991). Grasses from ED fields are colonized by increased numbers of *Fusarium* spp. and *Epicoccum* spp. (Robb et al., 1997). We hypothesized that ED is associated with the ingestion of plants which are under metabolic stress due to adverse weather and/or fungal colonization. Plants subjected to these biotic stresses may undergo oxidative stress and have increased production of reactive oxygen species and lipid peroxides and reduced concentrations of antioxidants (Bartosz, 1994; Mahan, 1994; Prasad, 1996; Haraguchi, 1997; Sairam et al., 1998; Smirnoff, 1998). They may also have increased concentrations of glutamate, aspartate, malonate, nitrate, and secondary metabolites including phenolics. These metabolites could be toxic to grazing horses, especially when the antioxidant content of ingested plants is concomitantly reduced. The aim of this study was (a) to determine the biochemical composition of plants collected from ED pastures, with particular emphasis on the antioxidant and prooxidant properties, and (b) to compare the biochemical composition and fungal colonization of plants collected from ED pastures with those of plants subjected to experimental drought and chilling.

MATERIALS AND METHODS

Collection of Plants from ED Pastures. Plants (wet weight = 100 g) were collected from 12 fields in central Scotland on two occasions. First, ED samples were collected as soon as possible (always within 72 h) after a horse grazing that particular field had developed ED (nine acute and three subacute cases). These samples were collected between February and May. Second, control samples were collected from the

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same fields in September when ED is relatively uncommon. To ensure that samples were representative of the entire field, each sample comprised a mixture of at least 10 aliquots which were collected at points situated along an imaginary "W" pattern in the field. Herbage samples were collected by manual pulling from areas that had been grazed and not from the ungrazed "roughs". Samples included all plant types present and all parts of plants except the roots. Care was taken to avoid sampling of plant roots and adherent soil. All samples were free of rain and dew at the time of sampling. Samples were rapidly frozen within 20 min of collection by mixing with dry ice pellets and then stored in airtight bags at −70 °C prior to analysis.

Assessing the Effect of Experimental Drought and Chilling on Plant Metabolism and Fungal Colonization. In this experiment, four 0.5 m^2 turfs were collected from each of two permanent pastures in central Scotland that had a history of ED in previous years. The turfs had >15 cm deep soil to ensure that the roots and associated soil layer remained intact. The turfs were relatively species poor, containing perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*), rough meadowgrass (*Poa trivialis*), creeping buttercup (*Ranunculus repens*), Timothy grass (*Phleum pratense*), Yorkshire fog (*Holcus lanatus*), daisy (*Bellis perennis*), and annual meadowgrass (*Poa annua*). Turfs were placed in wooden boxes and transported to a greenhouse and allowed to acclimatize for 4 days (days −4 to 0) in favorable conditions (ambient day temperature = 20−24 °C, ambient night temperature = 7−10 °C, daily watering, daylight). After acclimatization, two unstressed control turfs were maintained under favorable conditions for the duration of the experiment (days 0−21), namely, under glass with adequate watering and protected from frost. The other two "stressed" turfs were given no water on days 0−21 and chilled to ∼3 °C overnight on days 1, 5, 7, 12, and 14. Plant samples were collected from all turfs on days 0, 4, 7, 11, 14, and 21. The collected sample included all plant aerial organs including nongrass species except buttercups and daisies, which were removed and discarded at each sample collection point to prevent overgrowth. Samples were frozen at −70 °C prior to analysis.

Biochemical Composition of Herbage. Biochemical analyses were performed on the 12 ED samples, 2 stressed turfs, and 2 unstressed turfs at all time points. To prepare aqueous plant extracts, 15 g of wet weight of frozen leaves (plants were weighed rapidly to minimize weight change due to condensation of moisture from the air) was added to 100 mL of distilled water and blended with a turbo-mix for 1 min at ∼20 °C. This procedure was performed to mimic chemical changes (e.g., precipitation, oxidation, polymerization) to the solutes occurring during mastication. The resultant suspension was filtered through muslin, a 10 mL aliquot being frozen at −70 °C for assessment of antioxidant and prooxidant activities. The remainder was frozen overnight at −40 °C, then thawed at room temperature and centrifuged at 20000*g* for 10 min at 20 °C. The supernatant was decanted into 10 mL aliquots and stored at −40 °C. Aliquots of the supernatant were analyzed as follows:

Analysis of Soluble Phenolics. The A_{260} of a 50-fold dilution was determined as an indication of total water-extractable phenolic material. For analysis of specific phenolics, 1 mL aliquots of each supernatant were passed through a C_{18}-substituted silica column (BondElut, 100 mg packing) that had been pretreated with 3 mL of methanol followed by 3 mL of water. After a rinse with 5 mL of H_2O to remove nonadsorbed solutes, retained aromatics were eluted with methanol (2 mL). Aliquots of the eluate were subjected to high-pressure liquid chromatography (HPLC) on a 25 × 0.46 cm column of C_{18}-substituted silica (Spherisorb 5ODS2). Elution was with a linear gradient extending from water/butan-1-ol/acetic acid (98.3:1.2:0.5 by volume) to acetonitrile/butan-1-ol/water (98.3:1.2:0.5) in 30 min. The eluent was monitored for absorbance at 260 nm.

Analysis of Soluble Sugars. A 50 μL aliquot of each supernatant was subjected to paper chromatography in ethyl acetate/pyridine/water (8:2:1, v/v/v) according to the descending method for 15 h. Authentic glucose, fructose, and sucrose were chromatographed as markers. Sugars were stained following the $AgNO_3$ method (Trevelyan, 1950), and the intensities of the stained spots were recorded on a semiquantitative visual scale (Fry, 1988).

Analysis of Free Amino Acids. A 50 μL aliquot of each supernatant was subjected to paper chromatography in butan-1-ol/acetic acid/water (12:3:5, v/v/v) according to the descending method for 48 h. The 20 common amino acids were run as markers. Amino acids were stained with ninhydrin, and the intensities of the stained spots were recorded on a semiquantitative visual scale.

Analysis of Organic Acids. A 1 mL sample, after the addition of 1 mg of butanoic acid as an internal standard, was passed through a 1 mL column of the strong cation exchanger, Dowex-50 (H^+ form), followed by a 1 mL column of the weak anion exchanger, Amberlite IRA95 (OH^- form). Anions adsorbed on the latter column were eluted with 1 mL of 0.5 M HCl and analyzed by HPLC on a 30 × 0.78 cm column of sulfonated polystyrene (Bio-Rad Aminex HPX-87H) with isocratic elution in 2.5 mM H_2SO_4. The column was kept at 40 °C, and carboxylic acids were detected in the eluate by absorbance at 210 nm.

Tannin Content. Total soluble tannins were estimated by measurement of the ability of the samples to precipitate bovine serum albumin that had been stained with Remazol brilliant blue (Waterman and Mole, 1994). Tannic acid was used as a standard.

Assessment of Antioxidant and Prooxidant Actions. The prooxidant and antioxidant properties of aqueous extracts of plants collected from the 12 ED pastures and 12 control pastures and from plants in the 2 stressed and 2 unstressed turfs collected at all time points were determined. Samples that were stored at −70 °C were used. Aqueous plant extracts were assessed for their abilities to modulate hydroxyl radical ($OH^•$)-dependent deoxyribose oxidation (Aruoma, 1994a). The $OH^•$ produced in the Fenton-type reaction in the mixture iron(III)−EDTA/ascorbate/H_2O_2 attacks deoxyribose to form products that on heating with thiobarbituric acid, at low pH, yield a pink chromogen that can be measured as absorbance changes at a wavelength of 532 nm. The samples were assessed for their abilities to inhibit the deoxyribose damage in the presence (a test of antioxidant activity) and absence (a test of prooxidant activity) of added ascorbate. Samples were assayed triplicate, with data varying by <15%. The ability of the plant extracts to inhibit peroxidation of ox brain phospholipid liposomes, which undergo rapid nonenzymatic peroxidation when incubated in the presence of $FeCl_3$ and ascorbic acid, was assessed essentially as described in Aruoma et al. (1993). A volume of 0.3 mL each of the grass samples was tested in a final assay volume of 1 mL. The antioxidant propyl gallate was used as a positive control.

Assessment of Plant Fungal Colonization. The number and species of fungi colonizing leaves from the two control and two stressed turfs were determined at each time point, as previously described (Robb et al., 1997). Briefly, at each sampling point, 50 representative plant leaves were cut with scissors from each of the turfs and soaked in 1% v/v sodium hypochlorite for 1 min to kill surface contaminant organisms. The 50 leaves were placed individually on the surface of five sterile culture plates containing potato dextrose agar, streptomycin (100 μg/mL), and penicillin (60 μg/mL) and incubated at room temperature in the dark for 7−10 days. The proportion of plant fragments with fungal growth, the number of fungal colonies per 50 leaves, and the identity of the fungi were determined.

Statistical Analysis. As the antioxidant/prooxidant and fungal colonization data were not normally distributed, within-group comparisons were made using the Wilcoxon rank sum test and between- and among-group comparisons were made using the Mann Whitney and Kruskall−Wallis tests, assuming a significance level of 5%. The normally distributed plant biochemical data were analyzed using the *t* test.

Table 1. Content of Water Extractable Phenolic Compounds and Organic Acids in Plant Samples[a]

treatment	A_{260} of 50-fold diluted extract (mean ± SE)	content of organic acids (mg/g of fresh weight) (mean ± SE)					
		oxalic	tartaric	ascorbic	unknown	succinic	acetic
stressed turf	0.418[b] ±0.017	7.627 ±0.547	1.664 ±0.339	1.335 ±0.264	18.201 ±3.976	5.972 ±0.668	0.754 ±0.114
unstressed turf	0.357 ±0.011	7.276 ±0.708	1.532 ±0.287	0.940 ±0.248	13.508 ±2.250	4.558 ±0.396	0.606 ±0.124
ED plants	0.526[c] ±0.032	6.050 ±0.642	0.587[d] ±0.262	0.709 ±0.272	13.483 ±2.867	3.563[e] ±0.543	0.519 ±0.113

[a] Data are for the ED plants ($n = 12$), and mean data for the 7−21-day samples (inclusive) of the stressed ($n = 16$) and unstressed control ($n = 16$) turfs. [b] Significantly different from unstressed sample ($p < 0.01$). [c] Significantly different from stressed sample ($p < 0.01$); significantly different from unstressed sample ($p < 0.01$). [d] Significantly different from stressed sample ($p \sim 0.02$); significantly different from unstressed sample ($p < 0.05$). [e] Significantly different from stressed sample ($p \sim 0.01$).

RESULTS

Biochemical Analysis. The absorbance values at 260 nm of a 50-fold dilution were taken as an indication of total water-extractable phenolic material. The major plant components extractable in water and contributing to A_{260} were expected to be low molecular weight phenolic compounds, with proteins, nucleotides, amino acids, and inorganics having a relatively minor contribution in comparison. The ED plants had significantly higher contents of extractable UV-absorbing solutes than the samples from the experimental turfs ($p < 0.01$) (Table 1). In addition, extracts of the stressed turfs had significantly raised A_{260} values compared with those of the unstressed control turfs ($p < 0.01$) (Table 1).

Fractionation of Soluble Phenolics. Despite the quantitative effects indicated by A_{260} measurements, HPLC did not reveal any consistent qualitative differences in the pattern of phenolic compounds between the ED plants and stressed or unstressed experimental turf samples. Representative HPLC profiles for the day 14 stressed and unstressed turf samples illustrate the variation among individual extracts and the lack of consistent trends (Figure 1). The variation indicates that appreciable chemical differences existed among individual "mouthfuls" consumed from the pasture. This is likely to be due to species-specific differences between the grasses and dicot herbs present in the pasture and also to genetic polymorphisms within a given plant species.

Analysis of Organic Acids. Quantitative analysis by HPLC also revealed few differences between the ED plants and the glasshouse turf samples (Table 1). However, the ED plants contained significantly less tartaric acid than the experimental turf samples (Table 1). The standard deviation in organic acid contents was high, indicating considerable variation among individual extracts, as noted for soluble phenolics.

Analysis of Soluble Sugars. All of the samples contained high levels of fructose and glucose; some contained detectable traces of sucrose and one to three unknown sugars, probably fructo-oligosaccharides. The ED plants contained significantly more fructose than the turf samples ($p < 0.001$) (Table 2). The experimental drought and chilling treatment had no effect on the monosaccharide content of the turf samples (Table 2).

Analysis of Free Amino Acids. Semiquantitative analysis of the water-extractable amino acids present in the turf samples indicated few differences between the treatments (Table 3). The ED plants contained significantly more of one amino acid zone (probably valine) than the turf samples ($p < 0.01$). The stressed turf samples accumulated a somewhat higher content of the basic amino acids than did the unstressed control turfs.

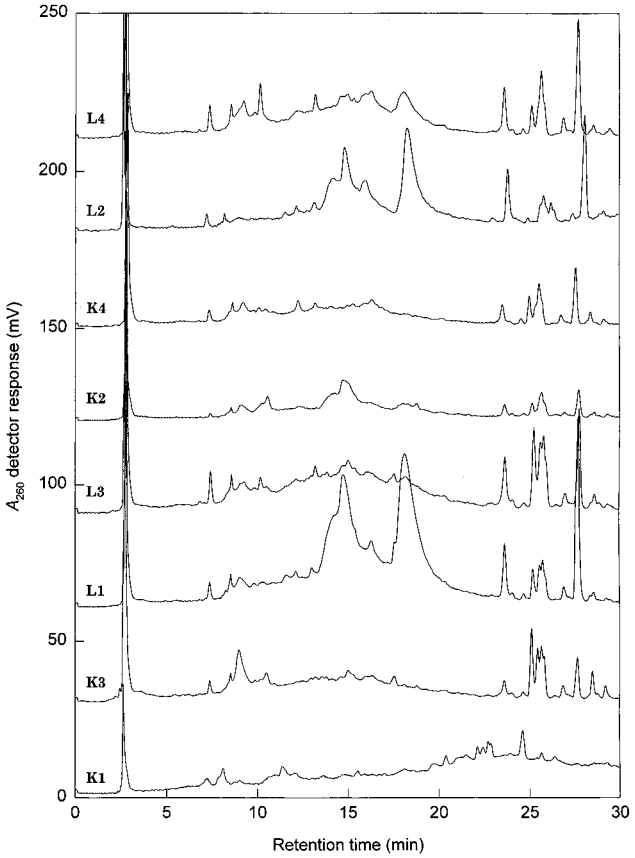

Figure 1. Representative HPLC traces of phenolic compounds water-extracted from turf samples. The extracts shown are from 14-day glass-house-grown turf. Samples K1, K3, L1, and L3 were stressed (by cold and drought); samples K2, K4, L2, and L4 were unstressed controls.

Table 2. Relative Content of Water Extractable Monosaccharides in Plant Samples[a]

treatment	relative fructose content (mean ± SE)	relative glucose content (mean ± SE)
stressed turf	2.63 ± 0.12	3.50 ± 0.13
unstressed turf	2.69 ± 0.12	3.44 ± 0.15
ED plants	3.58 ± 0.14[b]	3.83 ± 0.11

[a] Data are for all of the ED samples ($n = 12$), and mean data for the 7−21-day samples (inclusive) of the stressed ($n = 16$) and unstressed control ($n = 16$) turfs. Monosaccharide contents were scored on a visual scale: −, +, ++, +++, and ++++ indicate 0−4, respectively). [b] Significantly different from stressed sample ($p < 0.001$); significantly different from unstressed sample ($p < 0.001$).

Tannin Content. All of the plant extracts contained very small amounts of soluble tannins, which are therefore unlikely to play any appreciable role in disease aetiology.

Table 3. Relative Content of Water Extractable Amino Acids in Plant Samples[a]

	intensity of ninhydrin staining of amino acids present in					
treatment	zone 3 (Val, Trp)	zone 5 (Ala, Thr, Glu, Gly, Ser)	zone 6 (Asp)	zone 7 (Asn)	zone 8 (Arg, His, Lys)	zone 9 (cysteic acid)
stressed turf	1.63 ±0.20	1.38 ±0.17	2.94 ±0.11	2.63 ±0.17	1.81[b] ±0.13	1.00 ±0.15
unstressed turf	1.33 ±0.19	1.06 ±0.19	2.50 ±0.20	2.13 ±0.20	1.25 ±0.17	0.88 ±0.08
ED plants	2.82[c] ±0.24	1.91 ±0.23	2.82 ±0.24	2.00 ±0.48	1.91 ±0.23	1.27 ±0.22

[a] Data are for all of the ED plants ($n = 12$), and mean data for the 7−21-day samples (inclusive) of the stressed ($n = 16$) and unstressed control ($n = 16$) turfs. Amino acid contents in the six major zones of a paper chromatogram were scored on a visual scale: −, +, ++, +++, and ++++ indicate 0−4, respectively) (mean ± SE). [b] Significantly different from unstressed sample ($p < 0.02$). [c] Significantly different from stressed sample ($p < 0.01$); significantly different from unstressed sample ($p < 0.001$).

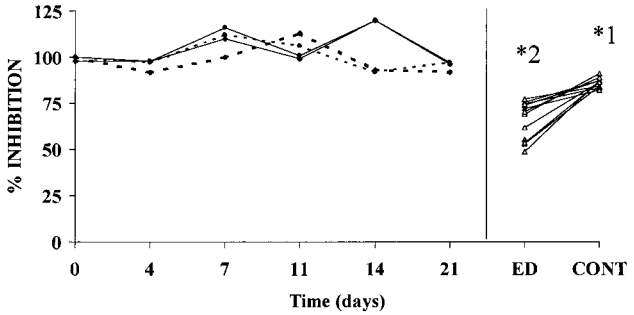

Figure 2. Antioxidant activity of plant extracts from control ($n = 2$, dotted lines) and stressed ($n = 2$, solid lines) turfs and of extracts of plants from equine dysautonomia pastures (ED, $n = 12$) and control pastures (CONT, $n = 12$), as determined by the percent inhibition of hydroxyl radical dependent oxidation of deoxyribose. *1 = significantly higher ($p < 0.01$) than ED plants; *2 = significantly lower ($p < 0.01$) than pooled data for the 7−21-day samples (inclusive) of the unstressed control ($n = 8$) turfs.

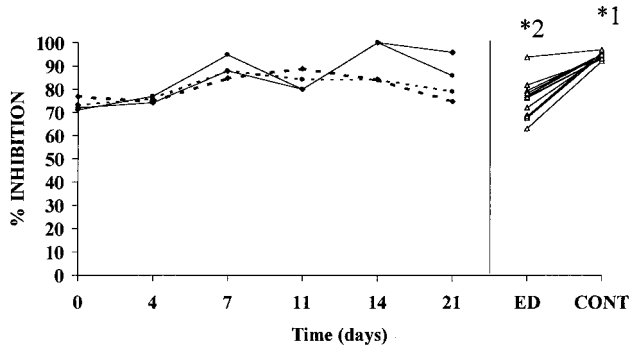

Figure 3. Antioxidant activity of plant extracts from control ($n = 2$, dotted lines) and stressed ($n = 2$, solid lines) turfs and of extracts of plants from equine dysautonomia pastures (ED, $n = 12$) and control pastures (CONT, $n = 12$), as determined by the percent inhibition of ox brain phospholipid liposome peroxidation. *1 = significantly higher ($p < 0.01$) than ED plants; *2 = significantly lower ($p < 0.01$) than pooled data for the 7−21-day samples (inclusive) of the unstressed control ($n = 8$) turfs.

Prooxidant and Antioxidant Properties. All plant extracts acted as antioxidants in both assay systems (Figures 2 and 3). Compared with plant extracts from the 12 control fields and compared with the mean ($n = 8$) data for the 7−21 day samples (inclusive) of the unstressed control turfs, ED plant extracts had significantly ($p < 0.01$) reduced antioxidant activity in the $OH^{\bullet}$-mediated deoxyribose damage assay (Figure 2) and in the ox brain phospholipid liposome peroxidation assay (Figure 3). The antioxidant activity of the control and stressed turf extracts did not change significantly during the experiment (Figures 2 and 3). Except for 7 of the 12 control plant extracts, all plant extracts acted as weak prooxidants, being capable of increasing the $OH^{\bullet}$-mediated deoxyribose damage, presumably by their ability to reduce Fe^{3+} ions in the Fe-EDTA complex (Figure 4). Their prooxidant activity in this assay was, however, weak compared with that of 100 μmol/L ascorbic acid, which stimulated oxidative damage by 532%. Compared with extracts from the 12 control fields and compared with the mean ($n = 8$) data for the 7−21 day samples (inclusive) of the unstressed control turfs, ED plant extracts had significantly ($p < 0.01$) increased prooxidant activity in the $OH^{\bullet}$-mediated deoxyribose damage assay (Figure 4). When compared with unstressed control turfs, the stressed turfs had significantly ($p < 0.05$) increased prooxidant activity in the $OH^{\bullet}$-dependent damage during days 7−14 (Figure 4).

Mycology. Culture of plant fragments from the unstressed control turfs and stressed turfs yielded a number of fungal species including *Acremonium* spp., *Alternaria* spp., *Aureobasidium* spp., *Cladosporium* spp., *Epicoccum* spp., *Fusarium* spp., *Geotrichia* spp., *Mucor* spp., *Mucoralis* spp., *Penicillium* spp., *Sordaria* spp., *Trichoderma* spp., and yeasts. *Cladosporium* spp. predominated in the stressed and unstressed groups at all time points. Throughout the experiment, there was no apparent alteration in the proportions of fungal species colonizing plants in either group. Between- and within-group comparisons showed that the total number

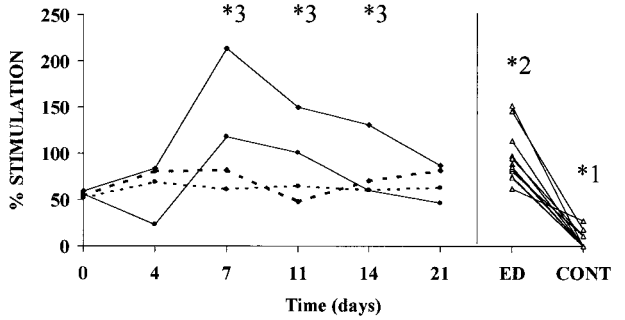

Figure 4. Prooxidant activity of plant extracts from control ($n = 2$, dotted lines) and stressed ($n = 2$, solid lines) turfs and of extracts of plants from equine dysautonomia pastures (ED, $n = 12$) and control pastures (CONT, $n = 12$), as determined by the percent stimulation of hydroxyl radical dependent oxidation of deoxyribose. *1 = significantly lower ($p < 0.01$) than ED plants; *2 = significantly higher ($p < 0.01$) than pooled data for the 7−21-day samples (inclusive) of the unstressed control ($n = 8$) turfs; *3 = prooxidant activity for the 7−14-day samples (inclusive) of the stressed ($n = 6$) turfs significantly higher ($p < 0.05$) than that for unstressed control turfs.

Table 4. Fungal Colonization of Stressed and Unstressed Plants[a]

	% plants with fungi		total no. of fungi	
day of expt	stressed turf	control turf	stressed turf	control turf
4	80 (42−100)	51 (40−84)	44 (36−72)	36 (22−48)
7	66 (48−82)	56 (50−62)	37 (26−65)	33 (29−36)
11	89 (70−92)	75 (66−94)	68 (15−138)	73 (36−127)
14	81 (75−100)	54 (48−92)	65 (26−75)	48 (42−51)
21	75 (61−100)	54 (48−92)	53 (45−84)	34 (28−135)

[a] There were no significant differences in the proportion of plant segments with fungal colonization or in the total number of fungi per 50 plant segments for unstressed and stressed turfs at any time point.

of fungi per 50 plant fragments and the percentage of fragments with fungal growth did not change significantly during the experiment (Table 4).

DISCUSSION

Plants collected from pastures immediately after an outbreak of ED had significant alterations in biochemical composition and antioxidant/prooxidant activity. When compared with control plants (plants collected from ED pastures out of ED season and control plants from ED pastures that were grown under favorable conditions), ED pasture samples had significantly reduced antioxidant activity and increased prooxidant activity. They also had significantly increased concentrations of fructose and extractable UV-absorbing (A_{260}) solutes (mainly low molecular weight phenolic compounds), significantly more of one amino acid zone (probably valine), significantly less tartaric acid, and a nonsignificant decrease in ascorbic acid content compared with controls (control plants from ED pastures that were grown under favorable conditions). Further analysis of the low molecular weight phenolic compounds by HPLC revealed considerable qualitative variation between individual extracts and a lack of consistent trends.

The reported alterations in biochemical composition and antioxidant/prooxidant activity of ED plants may contribute directly or indirectly to the etiology of ED in grazing horses. However, we cannot discount the possibility that these apparent alterations may be unrelated to ED and simply reflect the marked temporal variability in plant metabolism. In this respect, although the sampling technique was standardized, the relative quantities of each plant species within each sample, the stage of plant growth, and the weather conditions preceding sampling likely differed. However, such temporal changes in plant composition may be important in the etiology of ED, and indeed they may explain the strong seasonal and weather incidence of ED (Doxey et al., 1991). Two different control samples were used to compare data from ED plants. The first control was plants collected from the same ED fields, but out of ED season. Unfortunately, only antioxidant/prooxidant data were available for these samples. Many of the factors affecting plant composition, including pasture type (e.g., plant species, permanent pasture versus rotational, soil type, and geographical location) and pasture management (e.g., grazing density and fertilizer treatment), were unlikely to have changed significantly between these two sampling periods. Thus, the observed differences were considered to represent temporal variations induced by changes in weather, season, and unidentified biotic stresses. The second control, for both the antioxidant/prooxidant and the biochemical data, was plants from ED pastures that had been grown in a greenhouse under favorable conditions. This enabled us to test the hypothesis that ED plants may be under biotic stress.

The alteration in antioxidant/prooxidant activity, the increased concentrations of low molecular weight phenolic compounds, and the, albeit nonsignificant, reduction in ascorbate content suggested that the ED plants were under oxidative stress. Plants under oxidative stress have increased production of reactive oxygen species, lipid peroxides, and malondialdehyde, resulting in a reduced content of ascorbate, thiols, α-tocopherol, β-carotene, superoxide dismutase, reduced glutathione, glutathione reductase, and dehydroascorbate reductase (Mahan, 1994; Prasad, 1996; Bartosz, 1997; Haraguchi, 1997; Sairam et al., 1998; Smirnoff, 1998). The adaptive metabolic response to oxidative stress includes increased production of low molecular weight secondary products with antioxidant activity such as numerous phenolic compounds, carotenoids, nitrogenous, and sulfur-containing materials (Larson, 1995; Smirnoff, 1998). It is possible that the alterations in the concentrations of low molecular weight phenolic compounds, monosaccharides, and organic acids contributed to the alteration in antioxidant/prooxidant activity of ED plant extracts. Alternatively, the altered antioxidant/prooxidant activity of ED plants may be explained by the fact that many plant-derived antioxidants can act as prooxidants under certain circumstances (Laughton et al., 1989; Aruoma et al., 1993; Smith et al., 1992; Bowry and Stocker, 1993; Aruoma, 1994b, 1996; Kontush et al., 1996; Cao et al., 1997; Palozza et al., 1997; Sakagami and Satoh, 1997; Yamanaka et al., 1997; Fry, 1998; Palozza, 1998), with their antioxidant and prooxidant activities being dependent on their redox potential, their ability to chelate metal ions, the type of oxidizable substrate in use, and the biological environment in which they act (Schwartz, 1996; Roedig-Penman and Gordon, 1997; Palozza, 1998, Aruoma et al., 1998). The shift toward prooxidant activity observed in ED plants may occur when a particular antioxidant is present in very high concentrations, when there are reduced concentrations of the other synergistic antioxidants that normally protect the antioxidant activity of the molecule, or in the presence of transition metals such as the ferric ion. The significance of the alterations in fructose, tartaric acid, and amino acids is unclear.

The metabolic alterations noted in ED plants may be induced by the biotic stresses that have been associated with outbreaks of ED, namely, drought, chilling, and fungal colonization (Doxey et al., 1991; Robb et al., 1997). These biotic stresses have been shown to induce oxidative stress in susceptible nontolerant plants (Mahan, 1994; Prasad, 1996; Bartosz, 1997; Haraguchi, 1997; Sairam et al., 1998; Smirnoff, 1998). To compare the metabolic effects of drought and chilling on plants with the alterations in metabolism identified in ED plants, plants collected from ED fields not immediately associated with an outbreak were subjected to experimental drought and chilling. The experimental stress protocol did induce alterations in plant biochemical composition, but those alterations differed from those occurring in ED plants. Whereas the experimental stress increased plant prooxidant activity, in contrast to the ED plants, the antioxidant activity was un-

affected. Furthermore, the numbers and species of fungi colonizing the plants were not significantly affected by the experimental stress, whereas plants from ED pastures had significant alterations in fungal burden (Robb et al., 1997). Thus, the experimental drought and chilling protocol is not a good model for inducing the biochemical changes occurring in ED plants.

It was considered to be unlikely that any of the metabolites that were detected in elevated concentrations in ED plants could, per se, induce the neuronal degeneration that characterizes ED. However, because plants are considered to be a valuable source of essential antioxidants that afford protection against a number of diseases (Aruoma, 1994b; Johnson et al., 1994; Cook and Samman, 1996; Halliwell, 1996; Pezzuto, 1997), it is possible that the alteration in antioxidant/prooxidant activity may contribute to the development of ED in grazing horses. Given that the extracts of plants from ED pastures were weak prooxidants when compared with 100 μmol/L ascorbic acid, it is unlikely that their prooxidant activity per se could induce the neuronal degeneration that characterizes ED. However, plants with altered antioxidant/prooxidant activity, which are under metabolic stress, could potentially contribute to the development of ED if (a) the stressed plants also produce a neurotoxic metabolite that was not detected in this study, (b) the metabolic stress is a consequence of an interaction with a plant pathogen, such as a fungus, which is, per se, capable of producing a neurotoxin, or (c) the altered antioxidant/prooxidant activity of ingested plants alters the equine intestinal milieu, allowing overgrowth of neurotoxigenic bacteria, such as *Clostridium botulinum*, which has been implicated as a possible cause of ED (Poxton et al., 1997).

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LITERATURE CITED

Aruoma, O. I. Deoxyribose assay for detecting hydroxyl radicals. *Methods Enzymol.* **1994a**, *233*, 57−66.

Aruoma, O. I. Nutrition and health-aspects of free-radicals and antioxidants. *Food Chem. Toxicol.* **1994b**, *32*, 671−683.

Aruoma, O. I. Assessment of the potential prooxidant and antioxidant actions. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1617−1625.

Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem.* **1993**, *41*, 1880−1885.

Aruoma, O. I.; Deiana, M.; Jenner, A.; Halliwell, B.; Kaur, H.; Banni, S.; Corongiu, F. P.; Dessi, A. M.; Aeschbach, R. Effect of hydroxytyrosol on oxidative DNA damage and on low-density lipoprotein oxidation *in vitro J. Agric. Food Chem.* **1998**, *46*, 5181−5187.

Bartosz, G. Oxidative stress in plants. *Acta Physiol. Planta* **1997**, *19*, 47−64.

Bowry, V. W.; Stocker, R. Tocopherol-mediated peroxidation—the prooxidant effect of vitamin-E on the radical-initiated oxidation of human low-density-lipoprotein. *J. Am. Chem. Soc.* **1993**, *115*, 6029−6044.

Cao, G. H.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behaviour of flavonoids: Structure−activity relationships. *Free Radical Biol. Med.* **1997**, *22*, 749−760.

Cook, N. C.; Samman, S. Flavonoids—Chemistry, metabolism, cardioprotective effects and dietary sources. *Nutr. Biochem.* **1996**, *7*, 66−76.

Cottrell, D. F.; McGorum, B. C.; Pearson, G. T. The neurology and enterology of equine grass sickness: a review of basic mechanisms. *Neurogastroenterol. Motil.* **1999**, *11*, 79−92.

Doxey, D. L.; Gilmour, J. S.; Milne, E. M. The relationship between meteorological features and equine grass sickness (dysautonomia). *Equine Vet. J.* **1991**, *23*, 370−373.

Fry, S. C. *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*; Longman: Harlow, Essex, U.K., 1988.

Fry, S. C. Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochem. J.* **1998**, *332*, 507−515.

Halliwell, B. Antioxidants in human health and disease. *Annu. Rev. Nutr.* **1996**, *16*, 33−50.

Haraguchi, H. Protection against oxidative stresses by plant secondary metabolites. *Plant Physiol. (Rockville)* **1997**, *114*, 232−233.

Johnson, I. T.; Williamson, G.; Musk, S. R. R. Anticarcinogenic factors in plant foods. A new class of nutrients. *Nutr. Res. Rev.* **1994**, *7*, 1−30.

Kontush, A.; Finckh, B.; Karten, B.; Kohlschutter, A.; Beisiegel, U. Antioxidant and prooxidant activity of α-tocopherol in human plasma and low-density lipoprotein. *J. Lipid Res.* **1996**, *37*, 1436−1448.

Larson, R. A. Plant defences against oxidative stress. *Arch. Insect Biochem. Physiol.* **1995**, *29*, 175−186.

Laughton, M. J.; Halliwell, B.; Evans, P. J.; Hoult, J. R. S. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin—effects on lipid-peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem. Pharmacol.* **1989**, *38*, 2859−2865.

Mahan, J. R. Thermal dependence of glutathione reductase; thermal limitations on antioxidant protection in plants. *Crop Sci.* **1994**, *34*, 1550−1556.

Palozza, P. Prooxidant actions of carotenoids in biologic systems. *Nutr. Rev.* **1998**, *56*, 257−265.

Palozza, P.; Luberto, C.; Calviello, G. Antioxidant and prooxidant role of beta-carotene in murine normal and tumor thymocytes: Effects of oxygen partial pressure. *Free Radical Biol. Med.* **1997**, *22*, 1065−1073.

Pezzuto, J. M. Plant-derived anticancer agents. *Biochem. Pharmacol.* **1997**, *53*, 121−133.

Poxton, I. R.; Hunter, L. C.; Brown, R.; Lough, H. G.; Miller, J. K. Clostridia and equine grass sickness. *Vet. Med. Microbiol.* **1997**, *8*, S49−S51.

Prasad, T. K. Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: Changes in antioxidant system, oxidation of proteins and lipids, and protease activities. *Plant J.* **1996**, *10*, 1017−1026.

Robb, J.; Doxey, D. L.; Milne, E. M.; Whitwell, K.; Robeles, C.; Uzal, F. The isolation of potentially toxigenic fungi from the environment of horses with grass sickness and mal seco. In *Grass Sickness, Equine Motor Neurone Disease and Related Disorders*; Hahn, C., Gerber, V., Herholtz, Mayhew, I. G., Eds.; Equine Veterinary Journal Ltd.: Newmarket, U.K., 1997; pp 52−54.

RoedigPenman, A.; Gordon, M. H. Antioxidant properties of catechins and green tea extracts in model food emulsions. *J. Agric. Food Chem.* **1997**, *45*, 4267−4270.

Sairam, R. K.; Shukla, D. S.; Saxena, D. C. Stress induced injury and antioxidant enzymes in relation to drought tolerance in wheat genotypes. *Biol. Planta.* **1998**, *40*, 357−364.

Sakagami, H.; Satoh, K. Prooxidant action of two antioxidants: Ascorbic acid and gallic acid. *Anticancer Res.* **1997**, *17*, 221−224.

Schwartz, J. L. The dual roles of nutrients as antioxidants and prooxidants: Their effects on tumor cell growth. *J. Nutr.* **1996**, *126*, 1221S−1227S.

Smirnoff, N. Plant resistance to environmental stress. *Curr. Opin. Biotechnol.* **1998**, *9*, 214−219.

Smith, C.; Halliwell, B.; Aruoma, O. I. Protection by albumin against the prooxidant actions of phenolic dietary-components. *Food Chem. Toxicol.* **1992**, *30*, 483−489.

Trevelyan, W. E.; Procter, D. P.; Harrison, J. S. Detection of sugars on paper chromatograms. *Nature* **1950**, *166*, 444−445.

Waterman, P. G.; Mole, S. *Analysis of Phenolic Plant Metabolites*; Blackwell: Oxford, U.K., 1994.

Yamanaka, N.; Oda, O.; Nagao, S. Prooxidant activity of caffeic acid, dietary nonflavonoid phenolic acid, on Cu^{2+}-induced low-density lipoprotein oxidation. *FEBS Lett.* **1997**, *405*, 186−190.

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