Acta Bot. Neerl. 41(3), September 1992, p. 271–281

Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus* L.

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SUMMARY

Two clones of the grass *Holcus lanatus* from a metal-contaminated (Hallen Wood, Avonmouth, UK) and an uncontaminated site (Totley, Sheffield, UK) accumulated cadmium from Cd-amended hydroponic cultures, the Totley material to two-fold higher concentrations than the Hallen Wood. The Totley clone showed impaired growth at relatively low Cd concentrations; the reduction in parallel tolerance indices (TIs) to 50% occurred at an external Cd concentration of 53 μM compared with 94 μM in the Cd-tolerant Hallen Wood material. In both clones Cd was transported to the shoots; in the non-tolerant (Cd-sensitive) Totley tissues the two-fold greater Cd accumulation was accompanied by a two-fold rise in lipid peroxidation, indicative of membrane damage by reactive oxygen species in the shoot, though not in the roots. Evidence for the involvement of activated forms of oxygen was also seen in the highly significant correlations between Cd uptake into the shoot and the activities of superoxide dismutase ($r = 0{\cdot}95$) and guaiacol peroxidase ($r = 0{\cdot}96$), but confined to the sensitive Totley material. It was concluded that one potentially highly-damaging effect of Cd was to promote the generation of partly-reduced and highly-reactive forms of oxygen in the Cd-sensitive clone and that the site of activated oxygen formation was the shoot rather than the root.

Key-words: cadmium tolerance, cadmium toxicity, *Holcus lanatus*, molecular damage, oxygen radicals.

INTRODUCTION

The molecular mechanisms of cadmium toxicity are not known with any certainty. In many areas of biology, highly-reactive free radicals have been implicated directly and causally in the molecular damage associated with exposure to a wide range of pollutants, drugs and other toxins (Halliwell & Gutteridge 1989) including a range of transition metals, particularly copper and iron. Free radicals have also been implicated in determining tolerance of and susceptibility to a range of heavy metals in plants (De Vos *et al.* 1989, 1991; De Vos & Schat 1991). In animal systems, Al^{3+} and Pb^{2+} have been shown to increase the rate of lipid peroxidation of membranes, possibly by binding to negatively-charged groups so potentiating the membrane to direct oxidative attack (Quinlan *et al.* 1988). Similar processes may be involved in Cd toxicity by accelerating Fe-catalysed lipid peroxidation (Halliwell & Gutteridge 1989).

In this present work we have examined the effect of exposure to cadmium on two clones of the grass *Holcus lanatus* L., one from a metal-contaminated site 2·8 km downwind from a major European smelting complex (Hallen Wood clone), the other from an uncontaminated site 250 km north (Totley clone). In particular we have examined the effect of Cd^{2+} feeding on the photosynthetic tissue, the source of oxygen and, in stressed plants, the source of partly-reduced and highly-reactive oxygen radicals. Our objective has been to seek evidence for the role that activated oxygen might play in the mechanisms underlying cadmium toxicity in plants.

MATERIALS AND METHODS

Plant materials

Clones of a cadmium-tolerant (Hallen Wood, Avonmouth, UK) and a non-tolerant (Totley, Sheffield, UK) race of *H. lanatus* (for site details see Baker *et al.* 1986) were developed from the growth of single vegetative tillers in 21 vessels containing full-strength Hoagland's Solution, set up in a controlled-environment room (growth conditions: 20°/15°C, 16 h/8 h day/night regime, relative humidity 75 ± 5%). The clones used were not the same as in previously published work (Baker *et al.* 1986), but were derived from material collected from the field sites in 1987 and subsequently cultivated in potting compost. Experimental material was prepared by dividing plants into uniformly-sized tillers, pruning existing roots from them, and then allowing them to regenerate roots in fresh Hoagland's Solution. These tillers were then used for the experiments detailed below.

Measurement of cadmium tolerance

Tillers with similar maximum root lengths (< 2 cm) were selected for measurement of cadmium tolerance. Experimental units were set up containing batches of 10 replicate tillers. The maximum root lengths of all tillers were measured and then the basal nutrient solution replaced with a series of full-strength Hoagland's Solution cultures, amended with Cd^{2+}, supplied from a $2CdCl_25H_2O$ stock solution. The concentration range employed was 0–110 μM Cd. All root lengths were remeasured after a period of 7 days. The root elongation data were used to calculate mean parallel indices of Cd tolerance (Wilkins 1978; Baker 1987) for each clone at each Cd concentration employed.

Analysis of roots and shoots

Harvested plant dry-matter was oven-dried at 85°C and weighed, then dry-ashed overnight in 5 ml pyrex ignition tubes at 475°C in a muffle furnace. Ashed samples (> 10 mg original dry weight) were taken up in 5 ml 1·5 M nitric acid; samples weighing less than 10 mg were made up in 2 ml. Cadmium concentrations in the tissue digests were measured by atomic absorption spectrophotometry (Pye Unicam SP 1900).

Biochemical assays

Lipid peroxidation was determined as the concentration of thiobarbituric acid-reactive substances, equated with malonyldialdehyde (MDA) as used by Heath & Packer (1968), but with butylated hydroxy-toluene (90·05% w/v), routinely included as an anti-oxidant and quantified using 1,1,3,3, tetra-ethoxypropane as a standard. Superoxide dismutase

(EC 1.15.1.1.) activity was determined using the xanthine–xanthine oxidase–nitro blue tetrazolium system (Halliwell, 1975), quantified by the method of Giannopolitis & Reis (1977). Peroxidase (EC 1.11.1.7.) activity was monitored as the formation of tetraguaiacol using the method of Chance & Maehly (1975). Protein was determined according to the method of Bradford (1976).

RESULTS

Parallel tolerance indices (TIs) for tillers from the two clones showed that a reduction in the indices to 50% occurred at concentrations of 53 and 94 μM Cd respectively in the Totley and Hallen Wood clones (Fig. 1), illustrating the overall difference in Cd tolerance between the two clones.

Cadmium accumulated in roots and shoots of both clones, the concentration in the Totley material being generally greater than in Hallen tissues, though not to a statistically significant degree (Fig. 2). At external Cd concentrations greater than 20 μM the uptake was linear in an approximately 1:1 proportion between internal and external concentrations. However, the accumulation was some 10-fold greater in the root than shoot in both clones.

TBA-reactive products (a widely used measure of lipid peroxidation) accumulated in shoot tissue of the Totley clones, rising from 80 nmol (at 0 Cd) to 130 nmol g^{-1} fresh wt (110 μM Cd, Fig. 3a). In the Hallen shoot-tissue, the rise in TBA-reactive material was from 43 to 80 nmol g^{-1} fresh wt over the same range of Cd concentrations. The TBA-reactive products accumulating in the Totley shoots were 40–50 nmol g^{-1} fresh wt greater than in Hallen tissues at the higher Cd concentrations. At the points of 50% reduction in parallel tolerance index (Totley 53 μM, Hallen 94 μM Cd) the concentration of accumulated TBA-reactive products were respectively 110 and 70 nmol g^{-1} fresh wt in the shoots. In contrast, in the roots no difference in the concentration of TBA-reactive compounds in either clone could be detected against what appeared to be a high background of interfering pigments present in these tissues.

There was a significant increase in the activity of two enzymes closely associated with processing of activated forms of oxygen. Superoxide dismutase (SOD) activity in the shoots was highly variable particularly in material exposed to the highest concentrations of Cd (Fig. 4). In the Totley material the mean specific activity of SOD increased from 4·9 U mg^{-1} protein (at 0 Cd) to 17·5 U mg^{-1} protein at the higher concentrations of Cd. There was, however, no statistically-significant increase in SOD activity in Hallen tissues. The specific activity of SOD in the roots was generally many-fold lower than in the shoots with no clear indication of increased activity in one clone over the other.

The activity of peroxidase in the shoots also increased in the Totley material, some four-fold at the higher Cd concentrations (Fig. 5). Again there was no significant increase in activity in the Hallen tissues. Similar increases were noted in root material but these were not generally statistically significant.

DISCUSSION

Of the two clones, that from Totley (the 'control', uncontaminated site) showed the expected greater sensitivity to Cd. The parallel tolerance indices (TIs) suggested an EC_{50} of 53 μM. In the Hallen material (from the polluted metalliferous site) the EC_{50} rose to 94 μM, confirming that it was significantly more tolerant of Cd than the Totley clone. The

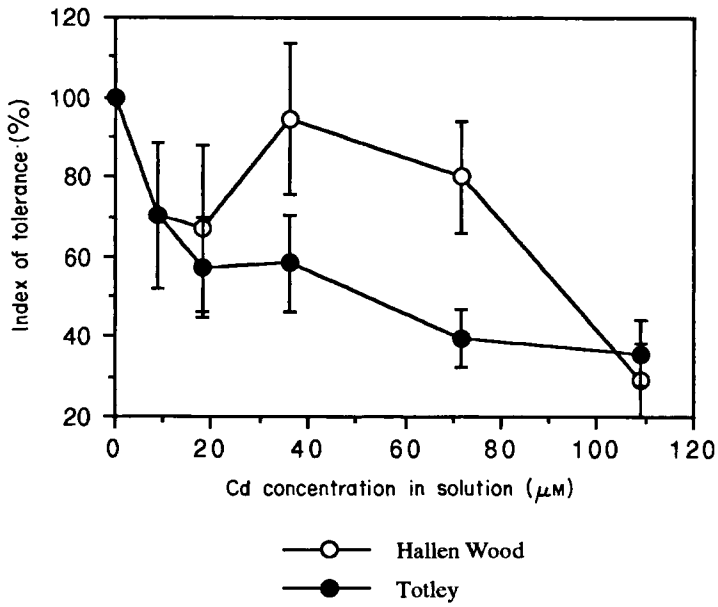

Fig. 1. Parallel indices of Cd tolerance for *H. lanatus* tillers from the Hallen and Totley clones grown in Hoagland's Solution over the concentration range 0–110 μM Cd. Values are means for batches of 10 replicate tillers + SE.

significant differences in TI detected between the two clones over the Cd concentration range 36–72 μM Cd were of a similar magnitude to those reported by other workers for this species (Coughtrey & Martin 1977; Baker *et al.* 1986) but the EC_{50} values are considerably higher. The latter authors found EC_{50} values of about 32 and 20 μM Cd for their Hallen Wood and Totley clones respectively. These differences most likely relate to the use of a complete, full-strength Hoagland Solution in the present work, whereas Baker *et al.* (1986) used a modified medium deficient in phosphate and sulphate in which root elongation responses to Cd would have been more sensitive. The two clones used here were also different, and, although they could be broadly classified as 'Cd tolerant' and 'Cd sensitive', their responses to cadmium were clearly less pronounced than previously characterized clones. When compared with TI data for cadmium in *Silene vulgaris* (Verkleij & Prast 1989) the EC_{50} values are also high, but in their studies Verkleij & Prast used a one-quarter strength Hoagland Solution as the basal medium in which Cd sensitivity would be increased.

The concentrations of Cd in both shoots and roots of the Totley clone were significantly greater than in the Hallen clone over most of the Cd-concentration range employed. Coughtrey & Martin (1978) studied Cd uptake by tolerant and non-tolerant clones of *H. lanatus* from a Hoagland Solution amended with either 9 or 18 μM Cd. They found slightly more Cd accumulating in the roots of their tolerant clone than in their sensitive one but the differences were statistically significant only at the lower Cd concentration employed. A similar (and significant) restriction of Cd transport to the shoots of their tolerant clone was apparent at both Cd concentrations. The Cd concentrations in both roots and shoots of Coughtrey & Martin's plants were generally much higher than in the present study probably reflecting the extended period of Cd treatment in their experiment (21 days cf.

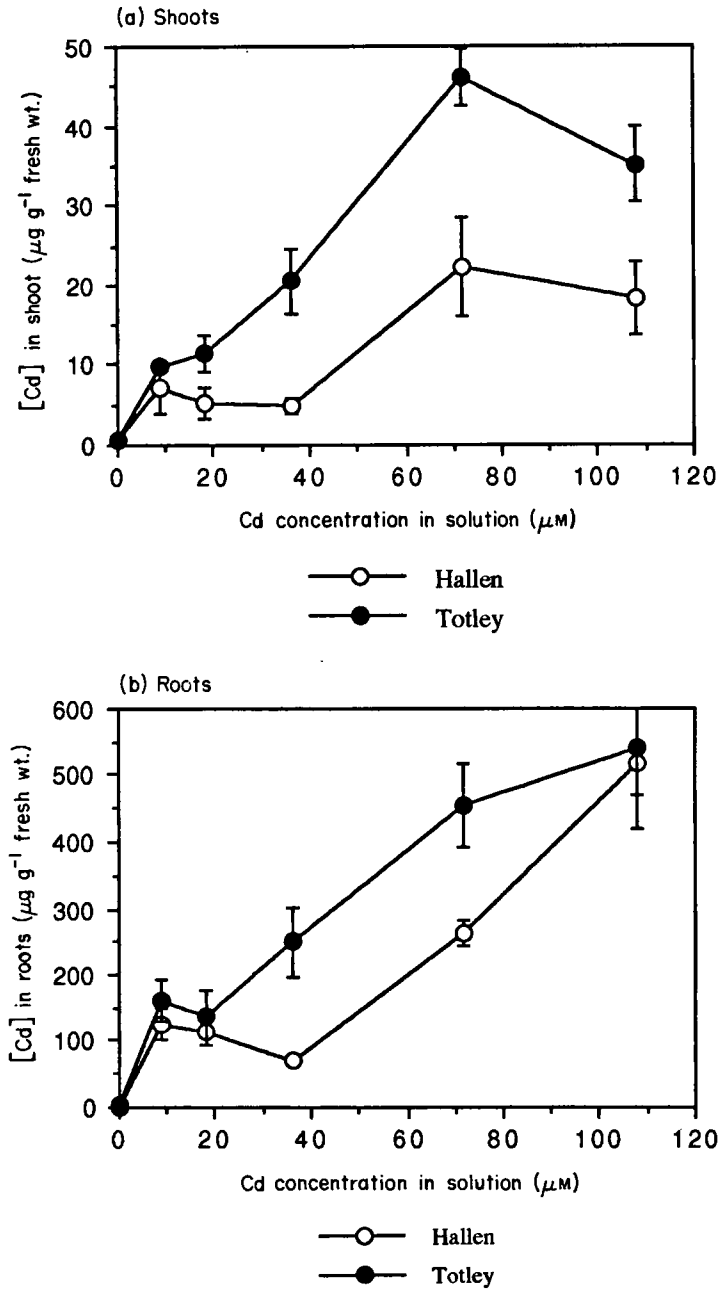

Fig. 2. The relationship between tissue Cd concentrations and external Cd concentration in (a) shoots and (b) roots of the Hallen and Totley clones of *H. lanatus* grown in Cd-amended Hoagland's Solution. Values are means of three replicates ± SE.

7 days). Verkleij & Prast (1989) showed similar patterns of metal uptake to those of Coughtrey & Martin in their hydroponic studies on Cd uptake by tolerant and non-tolerant *Silene vulgaris* populations. What emerges from all the works discussed is a

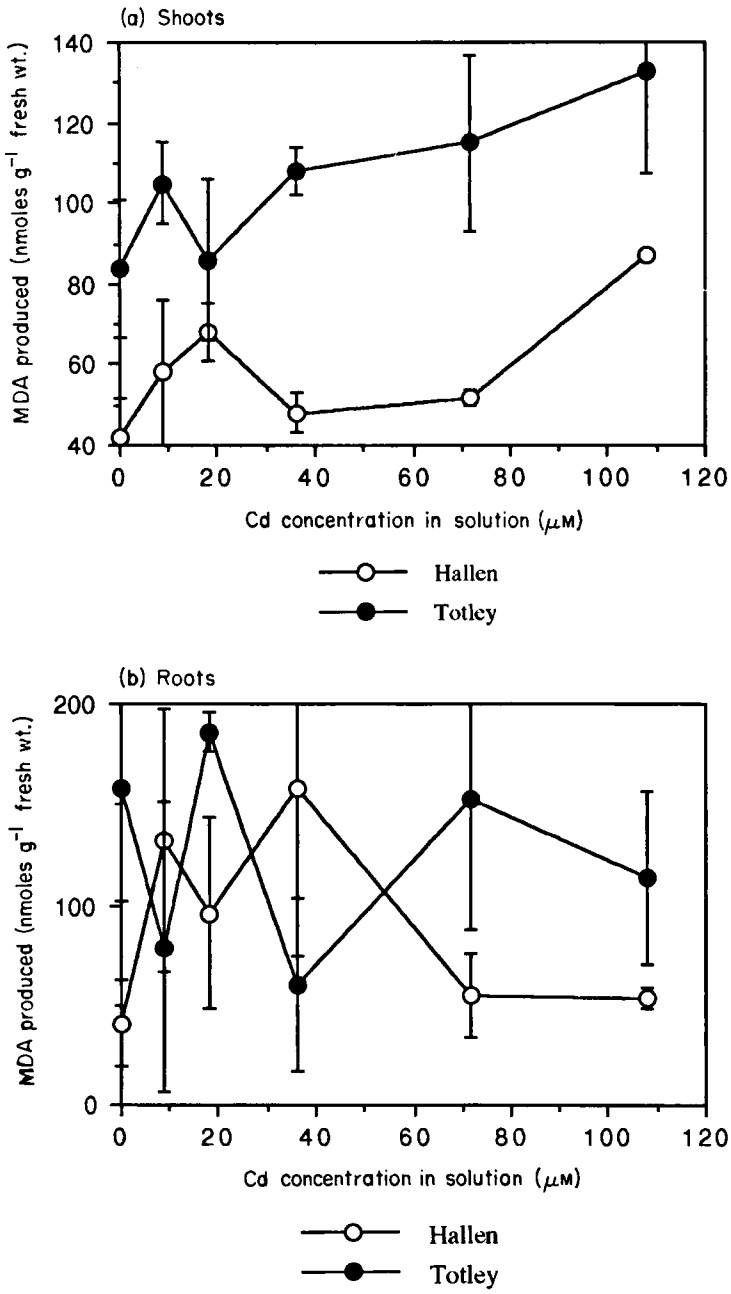

Fig. 3. The effect of Cd treatment on malonyldialdehyde (MDA) production in (a) shoots and (b) roots of cadmium-tolerant (Hallen) and non-tolerant (Totley) clones of *H. lanatus*. Values are means of three replicates ± SE.

reduced concentration of cadmium accumulating in the shoots of tolerant genotypes by comparison with Cd-sensitive plants; the root responses are more variable and may, to some extent, reflect differing patterns of Cd adsorption and exchange by roots in addition to any intrinsic differences in internal transport of the metal.

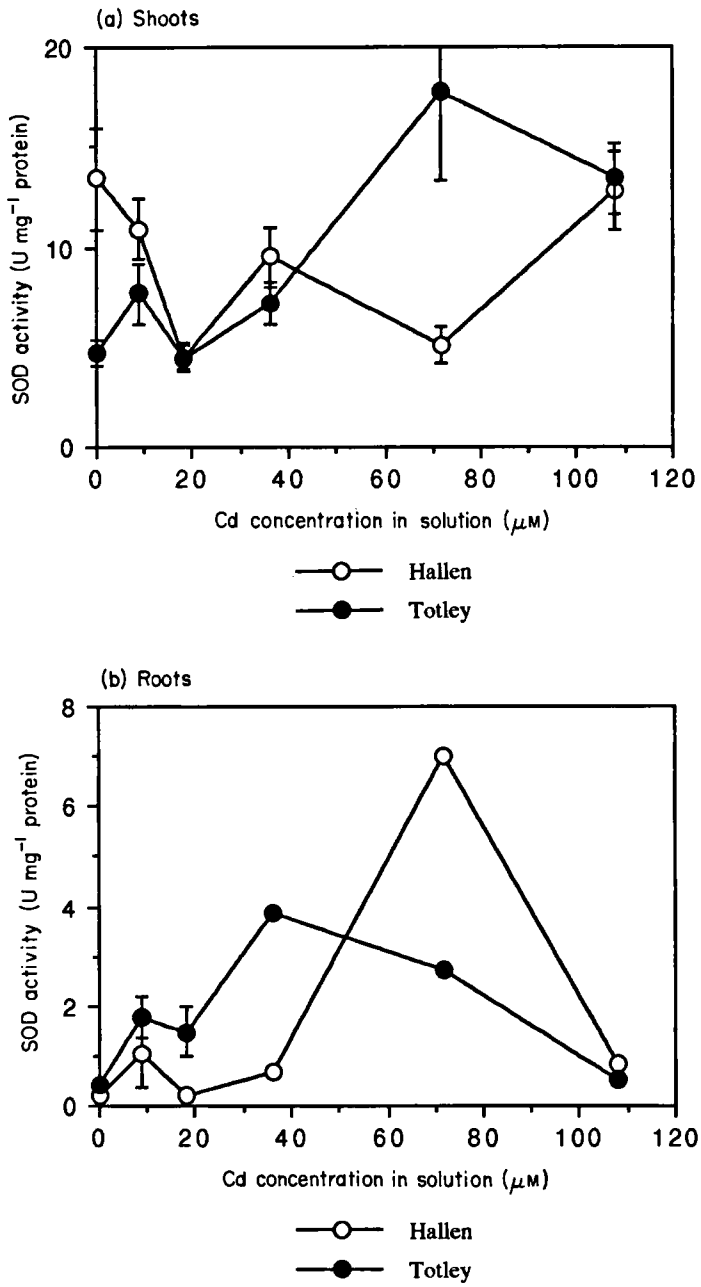

Fig. 4. The effect of Cd treatment on the activity of superoxide dismutase (SOD) in (a) shoots and (b) roots of cadmium-tolerant (Hallen) and non-tolerant (Totley) clones of *H. lanatus*. Values are means of three replicates ± SE.

One consequence of exposure to Cd was the reduction in growth in the root material. In mature plants, growth beyond that provided by finite organic reserves is dependent on the operation of autotrophic processes, a principal function of the shoot tissue. Evidence

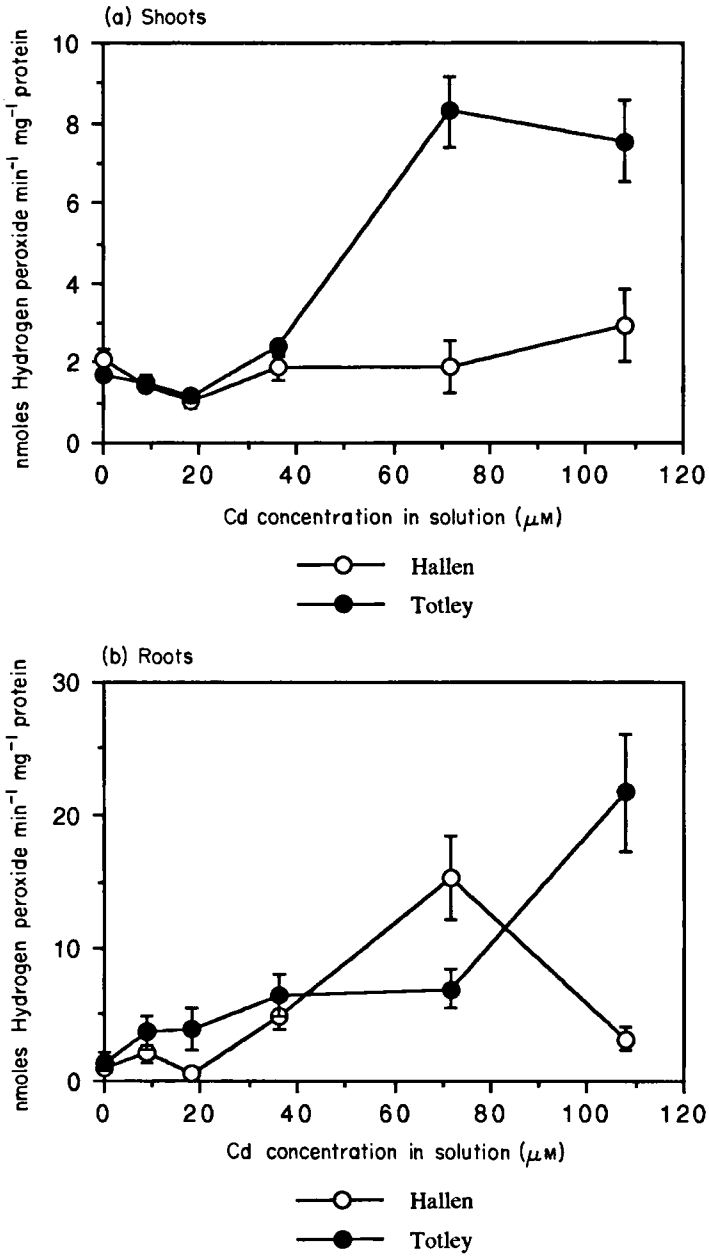

Fig. 5. The effect of Cd treatment on peroxidase activity in (a) shoots and (b) roots of cadmium-tolerant (Hallen) and non-tolerant (Totley) clones of *H. lanatus*. Values are means of three replicates ± SE.

of Cd-mediated subcellular damage to the shoot was provided by the accumulation of TBA-reactive products, widely equated in the literature (Gutteridge & Halliwell 1990) with peroxidation of membrane lipids and increased electrolyte leakage from plant cells (R.K. Wallace & P.C. Thorpe, unpublished data). In the case of plants, lipid

peroxidation is particularly associated with the chloroplast membranes (Price *et al.* 1989), the major source of reactive species known to initiate and propagate the abstraction of H and subsequent peroxidation of lipids. At 53 μM external Cd, shoots of the Totley material had accumulated 110 nmol of TBA-reactive products compared to 48 nmol in the Hallen tissues. A difference of 40–50 nmol g^{-1} fresh wt of TBA-reactive products between the two populations was apparent up to the highest Cd treatment (108 μM Cd) but there was no difference in their overall response to Cd treatment. Increased lipid peroxidation will impair autotrophic processes and, over time, must inevitably bring about a decrease in growth, one of the most obvious symptoms of Cd toxicity in plants. The accumulation of TBA-reactive compounds was a first indicator of the involvement of free radicals in subcellular damage to the shoot. This was further confirmed by examination of the activities of two enzymes directly involved in the processing of activated forms of oxygen. The activities of superoxide dismutase and peroxidase, two of the principal lines of defence against the reactive properties of respectively superoxide and its dismuted product hydrogen peroxide, showed a three- and four-fold rise in activity on exposure to Cd in the shoots of the Totley clones. Significantly perhaps, there was no change in the activity of these protective enzymes in the Hallen clones. The signal initiating the rise in activity of these enzymes is not known with certainty but is, in all probability, closely linked to the increased generation or accumulation of the two substrates O_2^- and H_2O_2. The relationship between the activities of SOD and peroxidase and with the concentration of Cd accumulating in the shoots is shown in Figure 6. In the Cd-tolerant Hallen shoots the correlation between SOD and peroxidase activities was not significant ($r=0{\cdot}63$, $P>0{\cdot}05$), neither were their correlations with Cd uptake ($r=0{\cdot}26$, $P>0{\cdot}05$ and $r=0{\cdot}46$, $P>0{\cdot}05$, respectively). In sharp contrast, in the sensitive Totley shoots there was a highly significant correlation between the two enzyme activities ($r=0{\cdot}96$, $P<0{\cdot}001$) and between Cd uptake and the activities of SOD ($r=0{\cdot}95$, $P<0{\cdot}01$) and peroxidase ($r=0{\cdot}96$, $P<0{\cdot}001$).

One of the toxic effects of many heavy metal ions has long been thought to involve membrane damage in the roots. Recent work with both *Silene vulgaris* (De Vos *et al.* 1989, 1991) and *Mimulus guttatus* (Strange & Macnair 1991) has shown the initial effect of copper is to damage the cell membranes. The resulting leakiness of metal-damaged membranes can be used to distinguish tolerant and non-tolerant genotypes by measuring K^+ efflux (Wainwright & Woolhouse 1977). Differences in the responses of tolerant and non-tolerant genotypes of *M. guttatus* can be detected rapidly and the leakiness of the membranes results in non-tolerant individuals showing a greater short-term uptake of copper which can be expected to lead to greater cellular damage and accelerated death of cells, both in the root and shoot. In the longer-term, a greater accumulation of the metal would be predicted in the shoots of the susceptible genotype, as was found in the case of *H. lanatus* in the present work. Such observations suggest that the mechanism of tolerance (to copper at least) resides in some way in the membrane, either by structural alteration, or by the production of some protective compound or system constitutively. Further work is required to confirm and differentiate these hypotheses.

The evidence from the present study highlights an important toxic effect of cadmium following its translocation to the photosynthetic shoots. There it brought about increased lipid peroxidation particularly in the Cd-sensitive material. The strong correlation between Cd uptake into the shoots and the enhanced activities of two enzymes involved in processing reactive species of oxygen was entirely confined to the Cd-sensitive clone. The interesting question remains as to why this did not occur in the tolerant (Hallen Wood)

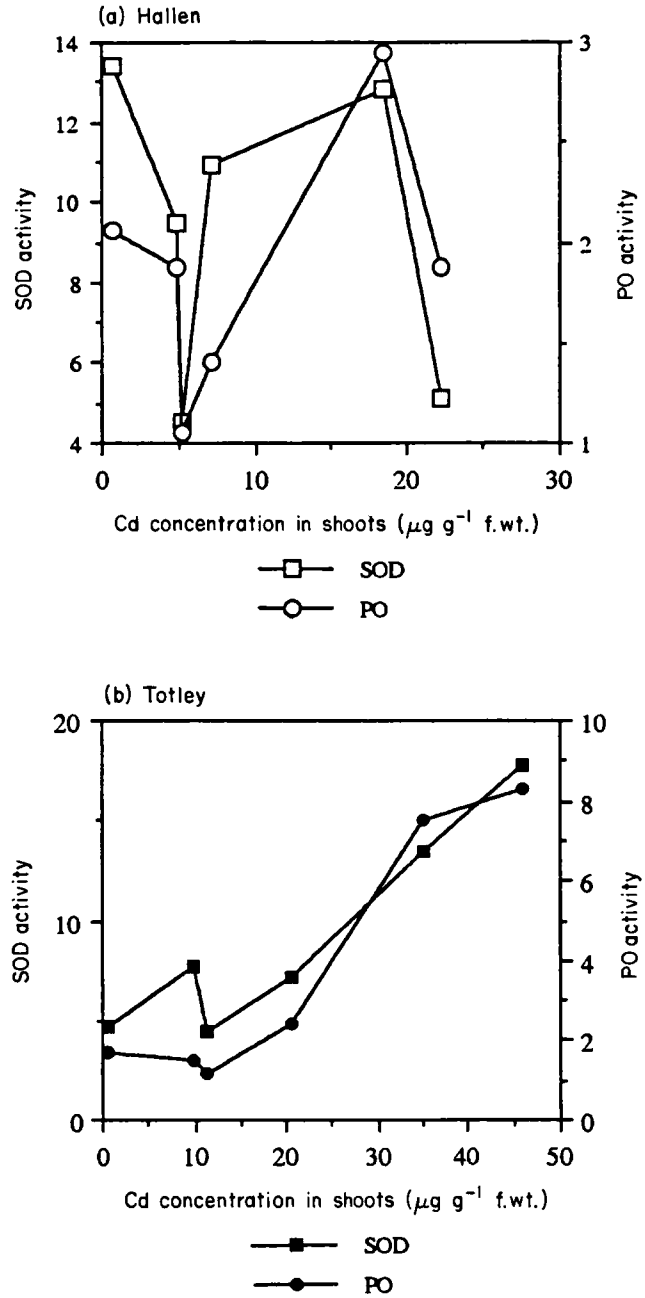

Fig. 6. The relationships between superoxide dismutase (SOD) and peroxidase (PO) activites and Cd concentration in the shoots of (a) Hallen and (b) Totley clones of *H. lanatus*.

tissues. The conclusion is that the toxicity of cadmium in sensitive plant material is closely associated with membrane damage following the generation of activated forms of oxygen arising in the photosynthetic tissues.

ACKNOWLEDGEMENTS

We thank the Natural Environmental Research Council (NERC) for research funding and the Science & Engineering Research Council (SERC) for provision of a Research Studentship for one of us (CFE).

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