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EFFECTS OF ANOXIA AND FLOODING ON ALCOHOL DEHYDROGENASE IN ROOTS OF *GLYCERIA MAXIMA* AND *PISUM SATIVUM*

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Key Word Index—*Glyceria maxima*; Gramineae; *Pisum sativum*; Leguminosae; roots; anoxia; flooding; alcohol dehydrogenase.

Abstract—Flood tolerant *Glyceria maxima* and intolerant *Pisum sativum* were compared in respect of the effects of anoxia and flooding on the maximum catalytic activities of alcohol dehydrogenase in their roots. Small (< 73%) increases in enzyme activity occurred when excised roots of both species were incubated in nitrogen for up to 2 days. Further incubation in nitrogen rapidly and permanently damaged the roots of both species. Enzyme activity in flooded roots of *Glyceria* was about double that in corresponding non-flooded roots. A marginally greater difference was found for roots of *Pisum*. It was concluded that the two species respond so similarly to the above treatments that variation in the extent of induction of alcohol dehydrogenase is unlikely to be a significant factor in determining their ability to tolerate flooding.

INTRODUCTION

Alcohol dehydrogenase (EC 1.1.1.1) in roots may play a key role in determining the ability of plants to tolerate flooding. Davies [1] has suggested that the onset of anoxia leads to accumulation of acid that lowers cytosolic pH, which then causes a switch from acids to ethanol as the major product of fermentation. Crawford [2] has argued that "it is the limitation of ethanol production that is the key to endurance of low-oxygen environments". The aim of the work reported in the present paper was to determine the effects of anoxia, and of flooding, on the maximum catalytic activity of alcohol dehydrogenase in the roots of the flood tolerant *Glyceria maxima* [3, 4] and of the non-flood tolerant *Pisum sativum* [5]. We have paid particular attention to the problems of measuring maximum catalytic activities of enzymes in higher plants [6].

RESULTS AND DISCUSSION

Effects of anoxia

The effects of anoxia on the activity of alcohol dehydrogenase in roots cannot be determined merely by placing the roots of an intact plant in nitrogen. This is because transport of oxygen from shoot to root could nullify any attempt to keep the root anoxic. Accordingly, we investigated the effects of anoxia by excising the roots and incubating them in water under nitrogen. As controls, replicate samples of excised roots were put on damp cotton wool and incubated in air. To determine the time over which measurements of the enzyme should be made, we investigated the effect of anoxia and excision on the roots' survival. We estimated survival time by measuring the ability of the excised roots to respire in air after periods of anoxia (Table 1). In the controls in air the roots of both species survived satisfactorily for 3–4 days, although the rate of respiration fell steadily. In contrast, when the excised roots were incubated in nitrogen, their respiration rate on return to air was found to have been drastically diminished. The effect of even 24 hr anoxia was marked. After 48 hr anoxia the respiration of both species was reduced by over 90%. The relative inability of roots, incubated in nitrogen for 48 hr or more, to respire on return to air does not appear to have been due to lack of substrate. Addition of 4 mM sucrose to such roots, after removal from nitrogen, did not significantly increase their respiration. These results strongly suggest that excised roots of both species are quickly killed by anoxia, and that any study of the effects of anoxia on alcohol dehydrogenase in excised roots should be confined to the first 24–48 hr anoxia. Our results also suggest that there is little difference between the excised roots of the two species in their ability to withstand anoxia. Indeed, if there is a difference, it is that the roots of the flood tolerant *Glyceria* are the more susceptible.

Table 1. Effects of excision and incubation in nitrogen on oxygen uptake of roots of *Glyceria maxima* and *Pisum sativum*

Days incubated	Oxygen uptake (μl/hr per g fr. wt) *Glyceria*		*Pisum*	
	Air	Nitrogen	Air	Nitrogen
0	267 ± 6	267 ± 6	387 ± 9	307 ± 8
1	205 ± 4	35 ± 6	174 ± 1	158 ± 2
2	274 ± 23	13 ± 2	142 ± 1	30 ± 6
3	158 ± 11	15 ± 6	113 ± 3	16 ± 2
4	125 ± 7	20 ± 2	—	30 ± 4

The excised roots were incubated at 25° in the dark under air or nitrogen. Oxygen consumption in air was determined immediately after excision and at intervals thereafter. Values are means ± s.e. of estimates from triplicate samples.

We varied the pH, and the concentration of each

component, of the reaction mixtures to obtain the optimum conditions for the assay of alcohol dehydrogenase. We did this, for both species, with freshly excised roots, and with excised roots that had been under nitrogen for 24 hr. We investigated whether significant losses of enzyme activity occurred during extraction of the roots. We did this in recovery and in recombination experiments. In the former we prepared duplicate samples of roots, extracted one in the standard way, and the other similarly except that a measured amount of pure alcohol dehydrogenase was added to the roots prior to homogenization. Comparison of the activities in the two extracts showed how much of the added enzyme had survived the complete process of homogenization and extraction. The amounts of pure enzyme added were always comparable to the endogenous activity of the sample of roots. Recoveries were determined for freshly excised roots and for excised roots that had been under nitrogen for 24 hr. For both species the averages of our estimates of recovery lay between 88% and 112% of the added activity. In recombination experiments we prepared a sample of freshly excised roots, a sample of roots that had been under nitrogen for 24 hr, and a mixed sample that contained equal weights of freshly excised and incubated roots. We did this for both species. The activities found in the mixtures ranged from 81–116% of the values predicted from the measurements made on the separate components of the mixtures. In view of the above, we suggest that our estimates of alcohol dehydrogenase in excised roots reflect the maximum catalytic activities of our samples.

Table 2 shows the effect of incubation under nitrogen on the maximum catalytic activity of alcohol dehydrogenase in the excised roots. The activities in comparable samples of *Glyceria* roots incubated in air remained relatively stable over 24 hr and ranged from 8.7–10.7 nkat per g fr. wt. In comparable samples of pea roots in air activity fell gradually over 42 hr from 52.8 to 20.8 nkat per g fr. wt. Activity in *Glyceria* roots under nitrogen rose slightly over 24 hr but was still comparable to that found in roots incubated in air. No significant change in activity was detected in pea roots under nitrogen. However, activity in such roots was slightly higher than that in corresponding roots in air because of the decline in the latter. This pattern of enzyme behaviour is not altered by expressing activity as nkat per mg protein. In a single experiment the following values were obtained: *Glyceria*, 1.6 (immediately after excision), 4.9 (24 hr in nitrogen), 4.9 (48 hr in nitrogen); pea, 3.9 (immediately after excision), 3.9 (24 hr in nitrogen), 2.9 (42 hr in nitrogen). We conclude that anoxia does not cause any marked increase in the maximum catalytic activity of alcohol dehydrogenase in excised roots of *Glyceria* and pea. We also conclude that there is no significant difference between the two species in the way in which the maximum catalytic activity of the enzyme in excised roots responds to anoxia. Per fresh weight the activity of the enzyme in pea roots was substantially higher than that in *Glyceria* roots: this difference largely disappears when activity is expressed per mg protein.

Table 2. Effect of incubation under nitrogen on activity of alcohol dehydrogenase in excised roots of *Glyceria maxima* and *Pisum sativum*

Hr after transfer to nitrogen	Enzyme activity (nkat per g fr. wt)	
	Glyceria	*Pisum*
0	7.1 ± 0.5	41.7 ± 7.7
2	8.6 ± 1.0	41.2 ± 7.2
12	10.1 ± 1.2	49.2 ± 12.7
18	10.4 ± 1.6	47.1 ± 11.5
24	12.3 ± 1.1	38.9 ± 13.9
42	—	52.5 ± 25.5
48	9.4 ± 4.2	—

The excised roots were incubated at 20° in the dark under air or nitrogen. Samples were assayed at the times indicated: values are means ± s.e. of estimates from five samples, each from separately grown batches of plants.

Effects of flooding

The response of excised roots to incubation in nitrogen will reveal inherent ability of root tissue to tolerate anoxia. However, such experiments do not necessarily show how roots respond to flooding. This is because excision can affect root metabolism by interfering with the supply of substrate and growth substances. More importantly excision, and incubation in nitrogen, eliminate the possibility of transport of oxygen to the flooded root via intercellular air spaces. We therefore determined the effects of flooding on the maximum catalytic activity of alcohol dehydrogenase in roots of *Glyceria* and pea that were still attached to the plants.

Young *Glyceria* plants were grown in non-flooded sand for a week. Then some were flooded and the rest left non-flooded. At the time of flooding and at intervals up to 4 weeks thereafter representative plants were dug up and alcohol dehydrogenase in the apical 4–6 cm of the roots was measured. After the last sampling we measured the redox potential and pH of the media in which the plants had grown. Values of + 356 ± 12 (mean ± s.e. of nine estimates) and pH 6.9 were obtained for the non-flooded media. The corresponding values for the flooded media were + 2.7 ± 4.2 and pH 5.8. There is little doubt that the roots of the non-flooded plants were in an acrobic and those of the flooded plants in an anaerobic environment [7].

The pH and composition of the assay mixtures were optimized for extracts of roots from non-flooded plants, and from plants that had been flooded for 7, 21 and 28 days. Recoveries of alcohol dehydrogenase added before extraction of roots from 4-week flooded plants and from non-flooded control plants were 97% and 94%, respectively. Activity in a mixture of roots from 4-week flooded and corresponding non-flooded plants was equal to that predicted from measurements made on the components of the mixture. We think our estimates of the enzyme in roots of flooded and non-flooded *Glyceria* are reliable.

Flooding increased the activity of alcohol dehydrogenase in *Glyceria* roots (Table 3). A smaller increase occurred in the non-flooded roots. Thus the net effect of flooding was a modest increase, at the most a doubling, of activity. We detected no significant effect of flooding on the protein content of the roots, so the above pattern is observed whether enzyme activity is related to fr. wt or protein. In comparable studies McManmon and

Table 3. Effect of flooding on activity of alcohol dehydrogenase in roots of *Glyceria maxima*

Days from start of treatment	Enzyme activity (nkat per mg protein)		Protein content (mg per g fr. wt)	
	Non-flooded	Flooded	Non-flooded	Flooded
0	0.86 ± 0.12	0.86 ± 0.12	7.6 ± 0.4	7.6 ± 0.4
1	0.92 ± 0.09	1.05 ± 0.31	11.0 ± 0.8	10.6 ± 2.0
7	1.43 ± 0.23	2.10 ± 0.38	9.5 ± 1.0	9.8 ± 1.2
21	1.83 ± 0.27	3.13 ± 0.58	8.3 ± 1.0	7.7 ± 1.0
28	1.84 ± 0.27	3.45 ± 0.84	11.4 ± 1.7	8.5 ± 1.6

All values are means ± s.e. of estimates from three samples of roots.

Crawford [8] used the complete root systems of *Glyceria*. We also measured alcohol dehydrogenase in the complete root system of 4-week flooded *Glyceria* and the corresponding non-flooded controls. We obtained values of 4.92 and 0.78 nkat per mg protein, respectively. These values are comparable to those in Table 3 and add further support to our conclusion that flooding causes a small increase in alcohol dehydrogenase activity in *Glyceria* roots.

In the first experiments with peas we germinated and grew them for a week in non-flooded conditions, flooded them and measured alcohol dehydrogenase in extracts of the apical 4–6 cm of the roots at the start of flooding and 1 day later. Values (mean ± s.e. of 3 samples) of 14.5 ± 2.1 and 17.9 ± 5.7 nkat per mg protein, respectively, were obtained. After 5 days flooding the seedlings were dying and no activity could be detected in extracts of the roots, although recovery of added enzyme was close to 100%.

In the next set of experiments we grew peas in non-flooded conditions for 36 days, then flooded some and left the rest as controls. After 4 weeks flooding we measured the redox potential and pH of the media in which the peas had grown. Values of +231 ± 5, +490 ± 15 (each is the mean ± s.e. of nine estimates) and pH 6.5 and pH 6.4, respectively, were obtained for flooded media and the corresponding non-flooded media. Thus flooding led to an appreciable lack of oxygen. Alcohol dehydrogenase was measured at the start of flooding and 2, 10 and 25 days later. For these measurements the apical 4–6 cm of the young roots of two plants that had received the same treatment were excised, randomized and divided into three samples (fr. wt 0.15–0.4 g), each of which was assayed. There was considerable variation between replicate samples, but the results suggested that flooding caused alcohol dehydrogenase activity to increase to a maximum sometime in the first week and to decline thereafter. We attribute the variation to the extreme sensitivity of peas to flooding and to differences in the time it took individual plants to succumb.

We did further experiments similar to those just described except that the peas were grown for 42 days before being flooded, and the sampling procedure was altered. At each time, two plants as identical as was practicable were chosen, one flooded and one non-flooded. Four complete secondary root systems were excised from the flooded plant and amalgamated into a single sample for analysis. The same was done for the non-flooded plant. Thus at each time of sampling we compared one flooded plant with one unflooded plant. The assay for alcohol dehydrogenase was optimized for extracts of roots taken at the start of flooding and for extracts of flooded and non-flooded roots taken 7 days later. In a recombination experiment between roots flooded for 7.5 days and their corresponding non-flooded roots, the activity in the mixture was 108% of the predicted value. Recoveries of added enzyme were: at the start of flooding, 64%, 92%; after 7 days flooding, 92%, 98%. We suggest that our estimates of the enzyme in pea roots reflect the maximum catalytic activities of the roots. The results of these experiments (Table 4) demonstrate two points. First, the values for roots taken at the start of flooding confirm that there was considerable variation between plants that had received the same treatment. This was variation in the amount of active enzyme rather than variation in total protein. Second, despite this variation it is clear that for each comparison at a specific time, the activity of alcohol dehydrogenase was higher in the flooded than in the non-flooded roots. Thus flooding increased the maximum catalytic activity of alcohol dehydrogenase in pea roots but the increase was not large.

Conclusions

The maximum catalytic activity of alcohol dehydrogenase in the root systems of *Glyceria* and pea is substantial. This is expected from previous studies of the apical 6 mm of the roots [9, 10]. In neither species does anoxia or flooding cause any very marked change in the activity of the enzyme. Small increases occurred in both species. On flooding the increase in pea appears to be greater than that in *Glyceria*. However, the increased activity in pea roots was not sustained, as the plants died. We think it unlikely that this relatively minor difference between the two species is a significant cause of the intolerance of peas to flooding. This view is reinforced by the recent demonstration that pea cells can withstand concentrations of ethanol likely to exceed those that occur on flooding [11]. In view of our evidence that excised roots of *Glyceria* and pea are more or less equally susceptible to anoxia, we think it probable that the major distinction between the two species in respect of flood tolerance is the presence in *Glyceria* of aerenchyma that would prevent roots in flooded soil from becoming totally anoxic.

Our flooding experiments are closely comparable to those of McManmon and Crawford [8]. Nonetheless, we

Table 4. Effect of flooding on activity of alcohol dehydrogenase in roots of *Pisum sativum*

Days from start of treatment	Enzyme activity (nkat per mg protein)		Protein content (mg per g fr. wt)	
	Non-flooded	Flooded	Non-Flooded	Flooded
0	2.0, 6.0, 26.1	—	1.7, 1.7, 1.9	—
0.5	10.1	19.0	1.3	1.8
1.5	21.0	78.7	2.5	1.8
2.5	18.7	59.1	2.4	2.0
3.5	36.7	49.4	1.9	2.3
4.5	39.4	58.7	2.7	1.6
5.5	46.2	70.2	1.8	1.3
6.5	7.9	35.7	2.8	1.1
7.0	—	46.6	—	2.6
7.5	43.0	127.0	2.6	2.9

did not find the marked difference in response of alcohol dehydrogenase to flooding in the roots of *Glyceria* and pea that was reported by these authors. For the reasons given previously [9] we believe that ours are the more reliable estimates of alcohol dehydrogenase. For these reasons, and, more particularly, because of the data in the present paper, we think untenable the recent statement [12] that "studies have shown that in various tissues susceptible to anaerobic injury there is a marked induction of alcohol dehydrogenase with little effect being produced on the flood-tolerant species".

EXPERIMENTAL

Plants. Glyceria maxima (Hartm.) Holmberg was obtained and grown as in ref. [9]: *Pisum sativum* L. cv Kelvedon Wonder was grown up to 5 days as in ref. [10]. For experiments with excised roots the apical 4–6 cm (Table 1) or 6 cm (Table 2) of young healthy *Glyceria* roots, and the apical 4 cm of the roots of 5-day-old peas were excised. Samples of 35–65 roots were put in 250 ml H_2O in 500 ml Erlenmeyer flasks that were gassed vigorously with O_2-free N_2 for 2 min and then sealed. For aerobic control samples the same number of roots were put on damp cotton wool floating in 250 ml H_2O in 500 ml Erlenmeyer flasks that were lightly plugged with cotton wool. Both sets of flasks were incubated in the dark without shaking. For the flooding experiments, young single shoots of *Glyceria* were taken from plants in water culture and transplanted into sand in plastic bowls (27 × 33 × 12 cm): there were six bowls and each contained 40 plants. The sand came to within 4 cm of the top of the bowl and was moistened with the soln used for water culture, the level of this soln was kept at 2.5 cm from the bottom of the bowl. Plants were grown like this for 7 days. Then in three of the bowls the level of the culture soln was raised to and kept at 1 cm above the level of the sand (flooded): the other bowls were left as they were (non-flooded). At intervals thereafter three plants from each treatment were taken from separate bowls and the apical 4–6 cm of the roots excised; those from the same treatment were randomized and samples of 15 roots (0.2–0.5 g fr. wt) were assayed. Peas were flooded similarly except that the concn of the culture soln was doubled. In the first experiments the peas were sown into moist sand, grown for a week in non-flooded conditions and then flooded; each sample for analysis contained 10 roots (apical 4–6 cm). In the other experiments the peas were grown in water culture for 5 days, transferred to sand culture and grown under non-flooded conditions for 36–42 days before some were flooded. *Glyceria* was grown in a greenhouse with natural illumination supplemented with artificial light (30 W/m^2 at base of shoot, photoperiod 18 hr) at 15–30°. Peas were grown in artificial light (50 W/m^2 at sand, photoperiod 18 hr) at 18°.

Enzyme assays. Samples (0.2–0.7 g fr. wt.) of *Glyceria* roots were homogenized as in ref. [9] with insoluble polyvinylpyrolidone (0.7 g/g fr. wt) in 50 mM glycylglycine buffer, pH 7.4, 10 mM 2-mercaptoethanol. Pea roots were extracted similarly except that the polyvinylpyrolidone was omitted from the homogenates of excised roots. Examination of the homogenates with a microscope revealed very few unbroken cells. The homogenates were centrifuged at 80 000 *g* for 30 min and the supernatants were assayed. Extracts were prepared at 4°, kept at 1° and assayed for alcohol dehydrogenase at 25° as in ref. [9]. For excised pea roots the reaction mixture contained in 3.0 ml: 93.3 mM glycylglycine, pH 8.8, 1.0 mM NAD, 100 mM EtOH. Optimization led to the following differences in other samples: *Glyceria*; excised, 2.0 mM NAD; flooded: day 0, 2.0 mM NAD; day 7, 140 mM glycylglycine, pH 9.3; day 21, 93.3 mM glycylglycine, pH 10, 2.67 mM NAD; day 28 and complete root system, 117 mM glycylglycine (pH 10.2), 2.67 mM NAD; pea, flooded, first experiment day 1 and 5, 93.3 mM glycylglycine, pH 9.3, 2.0 mM NAD; second experiment, 93.3 mM glycylglycine, pH 9.3, 100 mM EtOH; third experiment, 46.7 mM glycylglycine, pH 9.3, 2.0 mM NAD. Protein was pptd with 5% (w/v) trichloroacetic acid and measured as in ref. [10]. O_2 uptake was measured manometrically by Warburg's direct method at 25° with samples of 16 *Glyceria* roots or 10 pea roots in 2.0 ml 0.02 M KH_2PO_4 (pH 5.4). Redox potentials were measured with a Pt half-cell connected to a millivoltmeter; the reference was a standard calomel electrode.

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