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# Adaptedness of Variants at an Alcohol Dehydrogenase Locus in *Bromus mollis* L. (Soft Bromegrass)

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*Abstract*

Alcohol dehydrogenase is highly polymorphic in many plant and animal species. Here we report evidence that the naturally occurring, electrophoretically detectable allozyme variants of the $Adh_{1B}$ locus in *B. mollis* can respond differentially to environmental stresses. It is argued that alcohol dehydrogenase activity is specifically involved in response to these stresses.

Crude extracts of predominantly selfed seeds sampled from plants of known $Adh_{1B}$ genotype were assayed for their ADH activity in the forward and backward reactions. The seeds from $Adh^{S}_{1B}\,Adh^{S}_{1B}$ plants produced extracts about 12% less active in both directions than seeds from their $Adh^{F}_{1B}\,Adh^{F}_{1B}$ counterparts. Such $Adh^{S}_{1B}\,Adh^{S}_{1B}$ plants were also shown to produce about 13% more dry matter when grown under continuous flooding in the greenhouse whereas no difference between genotypes was detected in control pots. The seeds of $Adh^{F}_{1B}\,Adh^{F}_{1B}$ plants showed an advantage over the alternative homozygotes in more rapid germination at 2°C, but no difference was found at 15°C. Thus the variants are differentially adapted, and this is likely to play a role in the maintenance of the polymorphism in natural populations.

## Introduction

There is considerable interest among population biologists in the role which enzyme polymorphism might play in increasing the adaptedness (as defined by Dobzhansky 1968) of individuals and of natural populations, i.e. their capacity to survive and reproduce in a given range of environments. In this connection, genetically defined variants of alcohol dehydrogenase (ADH; EC 1.1.1.1) have been the subject of much experimental effort, especially in *Drosophila melanogaster* and *Zea mays*. For example Gibson (1970), Schwartz (1971), Felder *et al.* (1973), Vigue and Johnson (1973) and Day *et al.* (1974) have characterized biochemical differences between electrophoretically detectable alleles. Kojima and Tobari (1969), Marshall *et al.* (1973), Bijlsma-Meeles and van Delden (1974) and Morgan (1975) have reported allelic responses to different kinds of selection pressures. In view of the increasing body of evidence which implicates differential levels of ADH activity with flooding tolerance in plants (Crawford 1967, 1972; Schwartz 1969; Marshall *et al.* 1973; Francis *et al.* 1974), we assayed the catalytic activity of electrophoretically defined ADH variants found in a natural population of soft bromegrass (*Bromus mollis* L). In conjunction, we tested the response of these ADH variants to environmental stresses which were assumed to be related to the functioning of the enzyme (differential flooding tolerance and germination rates at low temperature).

*B. mollis* is a predominantly self-pollinated annual, and in south-eastern Australia it has been notably successful in colonizing a diversity of microhabitats. The species is tetraploid, and genetic studies indicate that in seeds and flooded roots the enzymic

products of two homeologous alcohol dehydrogenase loci, $Adh_{1A}$ and $Adh_{1B}$, are detectable electrophoretically (Brown *et al.* 1974). In the population studied the $Adh_{1A}$ locus is monomorphic for the *F* allele and codes for a monomer which can polymerize with the product of the $Adh_{1B}$ locus. The $Adh_{1B}$ locus is polymorphic for two alleles which occur with about equal gene frequency.

## Materials and Methods

*Field Sampling*

Collections of seed from a natural population 15 km west of Holbrook, N.S.W., were made in November 1972 and November 1973. The 1972 collection, previously described (Brown *et al.* 1974), was made at five positions spaced about 100 m along a water course. At each position, two samples each of about 100 plants were collected. One sample came from a wet microhabitat adjacent to the creek, and the other came from a more xeric microhabitat on the bank 20 m away from the first. Later, the seed of each maternal plant was separately packaged. In 1973, another collection was made in these five and in three new positions. The genotype at the polymorphic alcohol dehydrogenase ($Adh_{1B}$) locus of each field-grown maternal plant was determined by electrophoresis of the sample of seed (Brown *et al.* 1974). The remaining seed provided the material for the three experiments reported here. Experiment 1 employed the 1972 material, and experiments 2 and 3 the 1973 material.

*Experiment 1—Tolerance to Flooding*

The procedure was a modification of that described by Marshall *et al.* (1973). A 2-mm portion of the endosperm distal to the embryo was cut from each seed of maternal plants heterozygous for alcohol dehydrogenase, and used to determine the individual $Adh_{1B}$ genotype of that seed by standard electrophoretic procedures. Four seeds of each of the three *Adh* genotypes in each family were obtained and germinated in petri dishes. The seedlings were then transplanted into pots containing coarse sand in the glasshouse and watered twice daily, once with half-strength nutrient solution and later with tap water. Electrophoretic typing continued until a complete set of 12 plants for each family was obtained. There were 10 such sets of seedlings which were the progeny of 10 heterozygotes collected from the wet sites at positions 2, 3 and 4 (see Brown *et al.* 1974 for positions) and a similar number from the dry sites, yielding a total of 240 plants in the glasshouse. After 6 weeks, when all plants were well established, the four pots of each treatment were numbered and allocated randomly to two replications each of a control and a flooding treatment. Under the control regime the pots were watered twice daily as before. The remaining plants were placed in stainless steel tanks containing half-strength nutrient solution. The level of solution in the tanks was maintained near the sand surface, and the solution was completely renewed fortnightly. After 2 months the tillers and roots were thoroughly washed and their dry weights determined separately. Flowering did not occur, probably because no vernalization treatment was given.

*Experiment 2—Alcohol Dehydrogenase Activity*

Enzymic assays were made on seed extracts using a modification of the procedure of Bonnischen and Brink (1955). A separate series of assays was conducted on seed collected at each of the eight points along the creek. In each series, sets of six crude extracts were prepared simultaneously, corresponding to seed borne by $Adh^{F}_{1B}\,Adh^{F}_{1B}$, $Adh^{F}_{1B}\,Adh^{F}_{1B}$ and $Adh^{F}_{1B}\,Adh^{S}_{1B}$ and $Adh^{S}_{1B}\,Adh^{S}_{1B}$ plants in either the wet or the dry microhabitats. The order of processing extracts within each set was varied from day to day. The seed borne by $Adh^{F}_{1B}\,Adh^{F}_{1B}$ homozygotes is expected to consist mostly of $Adh^{F}_{1B}\,Adh^{F}_{1B}\,Adh^{F}_{1B}$ endosperm, except for the rare (5%) outcrosses ($Adh^{F}_{1B}\,Adh^{F}_{1B}\,Adh^{S}_{1B}$). Similarly, the seed on $Adh^{S}_{1B}\,Adh^{S}_{1B}$ plants is expected to contain mainly $Adh^{S}_{1B}\,Adh^{S}_{1B}\,Adh^{S}_{1B}$ endosperm. On the other hand, heterozygous maternal plants produced seed of the four different endosperm genotypes with equal frequencies. Fifteen seeds from each of six different maternal plants of the same *Adh* genotype and from the same site were pierced to facilitate imbibition and soaked overnight. The bulked sample of 90 imbibed seeds was frozen in liquid nitrogen and ground in 15 times its weight of 0·1 M tris–HCl buffer, pH 8·0, containing 5 mM dithiothreitol. After centrifugation at 27 000 *g* for 15 min at 2°C, the supernatant was filtered through 'Miracloth'. The filtrate was used to determine the specific ADH activity of the preparation (Dixon and Webb 1964) as nanomoles of NADH consumed or produced per minute per milligram of soluble protein (hereafter abbreviated to ADH activity) in the forward and

backward reaction. The assay solution in the forward direction comprised 2·0 ml of 0·1 M tris–HCl, pH 8·0, 0·5 ml of enzyme extract, 0·2 ml of 15 mM NADH, and 0·3 ml of 0·1 mM acetaldehyde. The assay for the backward reaction was identical except for the replacement of NADH by 0·5 ml of 6 mM NAD, replacement of acetaldehyde by 1·0 M ethanol, and the use of 0·2 ml of enzyme extract. In preliminary assays these saturating non-inhibitory amounts of substrate gave optimal and repeatable activities. The reaction was initiated by the addition of ethanol or acetaldehyde after determining the background activity with the cofactor in the absence of the alcohol or aldehyde substrate. Activity was measured by following the change in NADH concentration at 340 nm using a Varian recording spectrophotometer. Two measurements of both activities were made on each extract. The concentration of protein in the extracts was determined by the method of Lowry *et al.* (1951) using bovine serum albumen as standard.

*Experiment 3—Germination*

From both the wet and dry microhabitats at each of six of the positions sampled in 1973, 10 seeds borne by four plants of each of the two *Adh* homozygotes were set apart—960 seeds in total. Seeds were assayed only from the two homozygotes because (i) this enabled larger samples of these two treatments, (ii) a biopsy of each seed to determine its genotype (as in experiment 1) would affect its germination properties, and (iii) sets of five seeds derived from heterozygous plants would theoretically show greater variability (seed to seed) if a difference did not exist. The 10 seeds were weighed and five seeds were placed on moistened filter paper in petri dishes that had been autoclaved. These dishes were placed in a cold room at 2°C. The remaining five seeds were similarly placed in petri dishes which were then placed in a cabinet maintained at 15°C. In none of the treatments were the seeds pierced. The day of germination of each seed was recorded when the radical reached 2 mm in length.

The data from each of the three experiments were analysed as a split plot experiment. In general, higher-order treatment interactions were not significant and the appropriate pooled error mean square was used to test the significance of treatment effects.

## Results

*Experiment 1*

The dry weights accumulated by the control and the continuously flooded plants under glasshouse conditions are summarized in Table 1. Under flooding, there was greater dry weight of leaves and a marked reduction in the weight of roots compared with control conditions. The analysis of variance of the six attributes in Table 1 showed the major source of variation to be that between family means, within habitats. This indicated that the families were highly heterogeneous, and that inter-family genetic variance was high. There was also a difference between the performance of the progeny of heterozygotes sampled from the xeric as opposed to the mesic microhabitats. The xeric material tended to yield more under both experimental conditions. The difference was statistically significant for leaf (and whole plant) weight when tested against the families mean square. This difference was not in the anticipated direction; it was expected that xeric material would be at a relative disadvantage in the flooded treatment. The actual result might have arisen either from the fortuitous combination of highly heterogeneous material or from the relatively warm conditions in the glasshouse where the flooding tanks were slow to cool at night (maximum temperatures ranged up to 33°C).

Nevertheless, there was a significant difference between *Adh* genotypes in their accumulation of dry weight under flooded but not under control conditions. Plants with the $Adh^{S}_{1B}$ $Adh^{S}_{1B}$ genotype grew 13% more than their selfed full sibs. This magnitude of difference was found in families originating from both the wet and dry microhabitats.

**Table 1. Dry weight (g) of leaves and roots of the three $Adh_{1B}$ genotypes grown under control and flooded conditions, and treatment means and analysis of variance**

| | Leaves | | Roots | | Total | |
|---|---|---|---|---|---|---|
| | Control | Flooded | Control | Flooded | Control | Flooded |
| *Adh* genotype | | | | | | |
| *FF* | 3·15 | 4·51 | 2·07 | 1·06 | 5·22 | 5·57 |
| *FS* | 3·04 | 4·33 | 2·12 | 1·06 | 5·16 | 5·39 |
| *SS* | 3·16 | 5·07 | 2·06 | 1·18 | 5·22 | 6·25 |
| S.e. of difference[A] | 0·11 | 0·33 | 0·10 | 0·06* | 0·18 | 0·36* |
| Microhabitat | | | | | | |
| Dry | 3·10 | 5·17 | 2·19 | 1·15 | 5·28 | 6·32 |
| Wet | 3·14 | 4·10 | 1·98 | 1·05 | 5·11 | 5·16 |
| S.e. of difference[A] | 0·10 | 0·43* | 0·18 | 0·18 | 0·84 | 0·48* |
| Source of variation | d.f. | Mean squares | | | | |
| Microhabitat | 1 | 0·048 | 34·03* | 1·323 | 0·270 | 0·87 | 40·37* |
| Genotype | 2 | 0·190 | 5·91 | 0·039 | 0·200* | 0·06 | 8·23* |
| Families with microhabitats | 18 | 0·320 | 5·51** | 1·002** | 0·199** | 2·13** | 7·01** |
| Treatment interactions | 38 | 0·282 | 2·06 | 0·227 | 0·044 | 0·74 | 2·35 |
| Residual | 59 | 0·209 | 2·27 | 0·178 | 0·077 | 0·57 | 2·77 |

[A] The standard error of the difference between treatment means.
* $P < 0·05$. ** $P < 0·01$.

**Table 2. ADH activity in the forward and backward direction of bulked seed extracts of different $Adh_{1B}$ genotypes, and treatment means and analysis of variance**

ADH activity is expressed as nanomoles of NADH consumed or produced per milligram of protein per minute

| | | Weight of soaked seeds (mg) | Protein per seed (mg) | ADH activity | |
|---|---|---|---|---|---|
| | | | | Forward | Backward |
| *Adh* genotype | | | | | |
| *FF* | | 5·2 | 0·24 | 100 | 20·9 |
| *FS* | | 5·3 | 0·24 | 100 | 19·9 |
| *SS* | | 5·7 | 0·25 | 88 | 18·3 |
| S.e. of difference | | 0·33 | 0·012 | 4·5* | 0·91* |
| Source of variation | d.f. | Mean squares | | | |
| Microhabitat | 1 | 0·15 | 0·0001 | 90 | 43·4 |
| Site error | 7 | 0·34 | 0·0016 | 194 | 19·9 |
| Genotypes | 2 | 1·09 | 0·0008 | 633* | 28·4* |
| Microhabitat × genotype | 2 | 0·37 | 0·0020 | 39 | 1·8 |
| Within site error | 28 | 0·90 | 0·0011 | 163 | 6·7 |

* $P < 0·05$.

*Experiment 2*

Extracts of seed sampled from plants of known $Adh_{1B}$ genotype differed in their ADH activity in both the forward (reduction of acetaldehyde) and backward (oxidation of ethanol) directions (Table 2). The $Adh^{S}_{1B}\,Adh^{S}_{1B}$ plants gave seeds with ADH activity about 11% less than that in seeds from plants of the other two genotypes. The difference, although small, is consistent in that it is statistically significant over 16 sets of comparisons which together involve a total of 96 maternal plants of each genotype. The standard errors for the mean activity were 3·2 for the forward reaction and 0·65 in the backward reaction. There were no systematic differences in seed weight and protein content which might operate to bias these results.

**Table 3. Germination of seeds derived from the two *Adh* homozygotes, and treatment means and analysis of variance**

| | | Weight of dry seed (mg) | No. germinated (of 5 seeds) | | Mean days to germination | |
|---|---|---|---|---|---|---|
| | | | 2°C | 15°C | 2°C | 15°C |
| *Adh* genotype | | | | | | |
| *FF* | | 2·86 | 4·8 | 4·5 | 28·0 | 6·6 |
| *SS* | | 2·80 | 4·7 | 4·5 | 32·5 | 7·0 |
| S.e. of difference | | 0·10 | 0·10 | 0·17 | 1·9* | 0·4 |
| Source of variation | d.f. | | Mean squares | | | |
| Microhabitat | 1 | 0·53 | 0·09 | 2·04 | 28 | 12·4 |
| Site error | 5 | 1·30 | 0·42 | 1·56 | 146 | 6·0 |
| Genotype | 1 | 0·08 | 0·26 | 0·0 | 486* | 3·2 |
| Microhabitat × genotype | 1 | 0·27 | 0·01 | 0·0 | 150 | 0·1 |
| Within site error | 82 | 0·23 | 0·24 | 0·69 | 90 | 4·5 |

* $P < 0·05$.

*Experiment 3*

In the germination experiment, the homozygous genotypes showed no differences in seed dry weight or germination percentage (Table 3). However, a statistically significant difference was detected in the speed of germination at 2°C. The seeds from $Adh^{S}_{1B}\,Adh^{S}_{1B}$ homozygotes were slower to germinate under this treatment. The benefit to the *FF* homozygotes in terms of rapidity of germination was about 14%. Because the distribution of both the percentage and the rapidity of germination was highly skewed, the analyses of variance were repeated using the angular and log transformation respectively. These transformations yielded similar results; in fact, the significance of the genotypic difference in rapidity of germination at 2°C was even more striking.

## Discussion

The evidence from the above experiments indicates that compared with the *F* allele, the *S* allele at the $Adh_{1B}$ locus of *B. mollis* (i) codes for alcohol dehydrogenase with lower activity in crude seed extracts, (ii) conditions a higher tolerance to flooding, but (iii) is associated with slower germination. A discussion of these three properties

follows in turn, with conclusions concerning their possible role in maintenance of the polymorphism.

The occurrence of allozymic variants of alcohol dehydrogenase associated with altered catalytic activity is well known in maize (Schwartz 1971; Felder *et al.* 1973; Marshall *et al.* 1973; Freeling 1976). This altered activity apparently arises from different specific activities per milligram of ADH protein, and/or from different relative amounts of each ADH allozyme. A distinction between these two mechanisms obviously cannot be drawn from measurements in crude preparations. However, regardless of its genetic basis, we are here concerned with the existence of a net differential ADH activity, and what is its possible physiological significance.

In the case of *B. mollis*, endosperm of homozygous genotype $Adh^{F}_{1A}\,Adh^{F}_{1A}\,Adh^{F}_{1A}$; $Adh^{S}_{1B}\,Adh^{S}_{1B}\,Adh^{S}_{1B}$ exhibits three active alcohol dehydrogenase isozymes *FF*, *FS*, and *SS*. Thus seeds borne by plants of genotype $Adh^{S}_{1B}\,Adh^{S}_{1B}$ contain all the multimers found in the heterozygote $Adh^{F}_{1B}\,Adh^{S}_{1B}$ but in different ratios (Brown *et al.* 1974). Bulked seed extracts from $Adh^{F}_{1B}\,Adh^{F}_{1B}$ plants contain mostly *FF* dimers. They also contain trace amounts of *FS* and *SS* dimers because about 5% of the seeds are genetically detectable outcrosses. Thus the presence in the allopolyploid of the monomorphic homeologous locus ($Adh_{1A}$) and the capacity for heteropolymerization shown by its product act to buffer the expression of genetic variability. Conversely, the relatively small difference in ADH activity detected in extracts of bulked seeds indicates that the activity difference between pure *FF* and *SS* isozymes might be substantial.

A relationship between allozymes with lowered catalytic activity for alcohl dehydrogenase and a higher tolerance to waterlogging has been found in a polymorphic inbred line of maize (Marshall *et al.* 1973). The present results indicate that the maize model can have a counterpart in natural populations of plants. The lowered tolerance of the $Adh^{F}_{1B}$ homozygotes is presumably due to the higher levels of ethanol accumulated in the roots of this genotype under anaerobic conditions (Crawford 1967). Thus the difference in performance between $Adh_{1B}$ genotypes of *B. mollis* under flooded conditions does argue that this locus could respond to selection in nature.

Since experiment 1 demonstrated that there could be circumstances under which the $Adh^{S}_{1B}$ allele would be at an advantage, a situation was sought under which the reverse selection pressure might apply. For example, in *Drosophila pseudoobscura*, Marinkovic and Ayala (1975) show how an allozyme genotype with superior performance at a certain stage of the life cycle may have low fitness at another stage. Alcohol dehydrogenase is known to occur constitutively with high activity in seeds and pollen in many plants. The enzyme functions to regenerate $NAD^{+}$ from NADH and thereby facilitate further glycolysis and a high level of anaerobic production of free energy. Thus any impairment to the reaction rate might be expressed as a retardation of the germination process. Under conditions of low temperature, the duration of the phase for which the seed is dependent on anaerobic respiration would be prolonged and thereby any difference would be magnified. The results of Table 3 confirm this prediction. This finding for *seeds* contrasts with our previously published data on effective pollen gene frequencies and Mendelian segregation ratios, which suggest that $Adh^{S}_{1B}$ *pollen* is not at a disadvantage compared with $Adh^{S}_{1B}$ pollen, as is also the case in maize (Schwartz 1969).

The present data show, therefore, that the variants are differentially adapted, but that the magnitude of these differences is small. This is consistent with the inherent

conservatism of expression of variability for this polymorphism in *B. mollis*. It is impossible to translate these measurements of relative adaptness into relative selective coefficients or Darwinian fitness values. The apparently very small magnitude of effects on growth could be magnified under competitive conditions, and thus amount to a severe selective pressure. Conversely the remarkable phenotypic plasticity of this species (Jain *et al.* 1970) could render this observed difference negligible. In our intensive survey of one population, we did not find evidence of microgeographic differentiation for allele frequencies according to soil moisture conditions (Brown *et al.* 1974). Likewise, the small increase in germination speed could be a very important selective component in the life cycle of a spring annual plant. In this life cycle, the seeds are dormant in summer, imbibe the autumn rainfall when cold ground temperatures are encountered but must be active to respond at the first sign of the spring season.

In conclusion, the laboratory experiments described indicate that the naturally occurring variants of alcohol dehydrogenase in *B. mollis* can respond differentially to certain selective regimes. The $Adh^{F}_{1B}$ allele is more adapted to rapid germination in cold temperatures whereas the $Adh^{S}_{1B}$ allele is more adapted to waterlogged conditions. Such variation in the direction of selection could play a role in maintaining the alcohol dehydrogenase polymorphism in natural populations (Haldane and Jayakar 1963).

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