

AERENCHYMA DEVELOPMENT AND ELEVATED ALCOHOL DEHYDROGENASE ACTIVITY AS ALTERNATIVE RESPONSES TO HYPOXIC SOILS IN THE *PIRIQUETA CAROLINIANA* COMPLEX¹

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The ability of plants to make morphological or physiological adjustments in response to environmental cues allows them to survive and reproduce under a wide range of conditions. One stress that plants are often exposed to is soil oxygen depletion due to flooding. Plants can respond to hypoxic soils by producing oxygen-conducting aerenchymous tissue or through induction of enzymes in the ethanolic fermentation pathway. Here we use greenhouse experiments to examine flood responses in plants of the *Piriqueta caroliniana* (Turneraceae) complex, which occupy a range of moisture regimes. Morphotypes and hybrids in this complex exhibited contrasting responses to hypoxic conditions. Genotypes from flooded habitats developed aerenchyma and did not substantially elevate levels of alcohol dehydrogenase (ADH) activity, an enzyme associated with anaerobic respiration. Plants from drier sites, on the other hand, did not develop aerenchyma but had much higher levels of ADH activity. Plants with aerenchymous tissue had substantially higher rates of growth under sustained flooding. Results are consistent with the hypothesis that aerenchyma development is an effective strategy in habitats subject to persistent flooding, while elevating activity of enzymes for ethanolic fermentation is effective only under ephemeral flooding. The range of phenotypic responses observed illustrates contrasting adaptive strategies that can lead to habitat isolation and evolutionary divergence.

Key words: adaptive plasticity; alcohol dehydrogenase; flooding; Florida; hybrid breakdown; hydric soils; hypoxia; Turneraceae.

Understanding how plants adapt to the wide range of environmental conditions they encounter is a major goal of evolutionary ecology. Intrinsic to the process of adaptation is the acquisition of specific morphological and physiological features that allow plants to flourish under a diversity of environmental conditions (Schlichting and Pigliucci, 1999; Visser et al., 2003). Due to their sedentary nature, plants are particularly subject to selection via aspects of the abiotic environment, including climatic and edaphic conditions, which may interact to have dramatic effects on soil moisture availability (Schlichting and Pigliucci, 1999; Gurevitch et al., 2002). Extremes of soil moisture (xeric or hydric soils) represent some of the most challenging conditions that plants face and often limit distributions because specific sets of traits are required for plants to persist under these severe circumstances. While plant stress responses under drought have received considerable attention, flooding and hydric soil conditions can also present major constraints on the distribution of a species (Jackson and Colmer, 2005).

The primary stress induced by flooding is reduced soil oxygen availability. Roots, rhizomes, and other subterranean plant organs usually obtain oxygen for respiration from their immediate environments (Drew et al., 2000). However, under flooded conditions, transfer of oxygen to the roots is effectively blocked due to microbial respiration and displacement of soil air particles by water (Drew et al., 2000). Sustained hydric soil conditions can subject plants to hypoxic (low oxygen) or even

anoxic (no oxygen) environments, and failure to adapt to water-induced oxygen stress can result in plant mortality (Blom et al., 1994). When flooding occurs regularly, we would expect selection for genotypes able to respond with physiological or anatomical modifications that allow sustained growth and reproduction.

Alcohol dehydrogenase (ADH) is one of two proteins in the ethanolic fermentation pathway that is responsible for the reduction of acetaldehyde, which is toxic to plant tissues, to ethanol, resulting in continuous regeneration of NAD⁺ in the cytoplasm (Chung and Ferl, 1999). Hence, induction of ADH can enhance survival of plants under flooded conditions (Johnson et al., 1994). Changes in enzyme activity levels have been noted within a day under hypoxic conditions and may occur more quickly under anoxic conditions (Keeley and Franz, 1979). Previous studies examining associations between ADH activity and flood tolerance have produced contradictory results (Chan and Burton, 1992), with some studies finding negative correlations (McManmon and Crawford, 1971; Marshall et al., 1973; Francis et al., 1974; Brown et al., 1976) and others finding positive relationships (App and Meiss, 1958; Mendelssohn et al., 1981; Torres, 1981; Chow, 1984). A broad taxonomic survey of plants typically found in aquatic and terrestrial habitats suggests that flood-intolerant species tend to have increased ADH activity in response to hypoxic soils, while flood-tolerant species typically have reduced ADH activity (McManmon and Crawford, 1971).

An alternative mechanism for coping with low oxygen soil environments is the development of aerenchymous tissue, which effectively forms air channels in the cortical tissue of the roots (Arber, 1920; Sculthorpe, 1967; Armstrong, 1979; Konings and Verschuren, 1980; Crawford, 1982; Justin and Armstrong, 1987; Fig. 1). Aerenchyma enhances diffusion of atmospheric or photosynthetic oxygen from the shoot to the roots and rhizosphere, allowing aerobic respiration and growth

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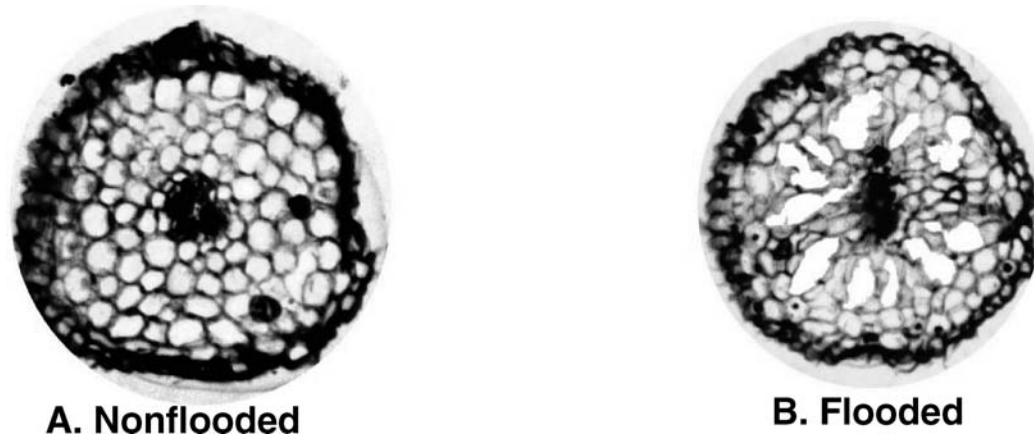


Fig. 1. Transverse sections taken 2 cm from the root tip of the same genotype of *Piriqueta caroliniana* (recombinant hybrid) after 2 mo of growth in (a) a nonflooded and (b) a flooded environment. Aerenchyma is evident in roots from the flood-treated plant as open air chambers and collapsed cells in the cortical tissue.

to be maintained under hypoxic conditions (Armstrong and Gaynard, 1976; Lambers and Smakman, 1978; Armstrong, 1979; Armstrong and Webb, 1985; Drew et al., 1985). Aerenchyma can also maximize oxidation of harmful elements and facilitate the removal of harmful gases from the root tissue and surrounding soil (Kawase and Whitmoyer, 1980; Shannon et al., 1996; Colmer, 2003). While species like teosinte (*Zea luxurians*) and rice (*Oryza sativa*) have constitutive aerenchyma development (Drew et al., 2000), other plants like corn (*Zea mays*) and cordgrass (*Spartina patens*) develop this tissue facultatively, with oxygen availability typically acting as the primary environmental factor triggering aerenchyma development (Visser et al., 2003).

A number of studies have examined aerenchyma production and ADH expression in response to hypoxic soil conditions (reviewed in Drew, 1997; Blom, 1999; Jackson and Armstrong, 1999; Drew et al., 2000), and other studies have investigated intra- and interspecific variation in plant growth in response to flooding (Fenster, 1997; Bell and Sultan, 1999; Nabben et al., 1999; Vignolio et al., 1999; van Eck et al., 2004). However, there are few analyses that link physiological and anatomical responses to the fitness of plants under flooded conditions (e.g., Naidoo et al., 1992; Ram, 2000; Grimoldi et al., 2005), particularly for intraspecific comparisons (Linhart and Baker, 1973; Keeley, 1979; Keeley and Franz, 1979; Chan and Burton, 1992; Lenssen et al., 2004). Moreover, none of these studies examined the effects of both ADH expression and aerenchyma production in the same species, so our understanding of flood tolerance strategies comes from interspecific surveys. Interspecific comparisons can be misleading because it is not possible to control for the confounding effects of suites of phenotypic traits that may be associated with each species, making it difficult to assign fitness differences to a particular trait.

In this study we use plants from the *Piriqueta caroliniana* (Walter) Urban complex (Turneraceae) and their hybrids to examine the alternative strategies of increased ADH activity and aerenchyma formation as adaptations to flooding and hypoxic soils. This study focuses on two interfertile morphotypes within this complex that occur in habitats with contrasting flooding regimes and on their advanced generation hybrid derivatives, which occupy a broad hybrid zone across central Florida (Martin and Cruzan, 1999; Maskas and Cruzan,

2000). The *caroliniana* (C) morphotype is found in dry habitats of northern Florida in sites characterized by high pine and turkey oak scrub associations (Martin and Cruzan, 1999; Picotte et al., 2006). The well-drained, quartz sand soils of these habitats are virtually never inundated (Myers and Ewel, 1990). Plants of the V (*viridis*) morphotype occupy the poorly drained, limestone-sand soils characteristic of the slash pine-palmetto flatwoods of southern Florida and are subject to inundation for extended periods of time during the wet (May–December) season (Abrahamson and Hartnett, 1990; Martin and Cruzan, 1999; J. J. Picotte, D. M. Rosenthal, J. M. Rhode, and M. B. Cruzan, unpublished manuscript). The hybrids in central Florida (H) were derived from interbreeding between the C and V morphotypes that was initiated at least 20 and perhaps more than 100 generations in the past (Maskas and Cruzan, 2000; Cruzan, 2005). These H genotypes occupy habitats in central Florida that are similar to the C morphotype (J. J. Picotte et al., unpublished manuscript).

The *Piriqueta* complex's broad ecological range makes it an excellent system for investigating adaptive responses to contrasting environmental conditions. In the experiments described later, we use parental morphotypes and their recombinant hybrids from crosses to examine aerenchyma production and ADH expression in response to hypoxic soil conditions. Using recombinant hybrid genotypes among morphotypes and their naturally occurring hybrids allowed us to examine the effects of individual phenotypic traits in a broad range of genetic backgrounds. We specifically asked: (1) Do genotypes vary in their ability to form aerenchyma in their roots? (2) Do genotypes differ in their level of root ADH activity in response to short-term hypoxic conditions? (3) What are the fitness consequences of the presence of aerenchyma for plants growing under persistently flooded and nonflooded conditions? Based on habitat differences among morphotypes, we predicted that V genotypes, which typically occupy flooded habitats in southern Florida, would have the ability to develop aerenchyma in their roots, while plants from C and H populations would not form aerenchyma, but may have elevated ADH expression in response to hypoxic conditions. We also predicted that the presence of aerenchyma would contribute more to survival and growth under persistently hypoxic conditions than would elevated ADH expression.

MATERIALS AND METHODS

General methods: greenhouse crosses—Plants were propagated from field-collected seeds from seven populations of each of the parental morphotypes (C and V) and their hybrid derivatives (H) to produce hybrid genotypes for these experiments. Plants from field-collected seed were crossed to produce six sets of reciprocal F₁ hybrids (CH, HC, CV, VC, VH, and HV), which were backcrossed to their parental genotypes to produce first generation backcross hybrids (BC₁). The majority of hybrid genotypes used in the experiments consisted of recombinant hybrids, which were produced by haphazardly crossing BC₁ genotypes within each of the six original F₁ lineages for two consecutive generations to produce second and third generation recombinant hybrids (RH₂ and RH₃). Approximately 600 genotypes from parental populations (C, H, and V) and from the BC₁, RH₂, and RH₃ generations were propagated by vegetative cuttings, and a subsample of these plants was used for the experiments.

General methods: histological analysis of aerenchyma—Root tissue samples from the experiments described were collected for histological analysis to assess the presence or absence of aerenchyma under contrasting environmental conditions. The first 2 cm of each plant's primary root tip was collected for quantification of aerenchyma. Methods used for dehydration and tissue embedding procedures were modified from Sass (1951). Root tip samples were stored in 1.5-mL microcentrifuge tubes containing formalin-acetic acid-alcohol (FAA) until the samples were dehydrated in a series of six solutions consisting of increasing ratios of 95% ethanol to tertiary butanol (TBA) and dH₂O. Infiltration with Paraplast (Structure Probe, West Chester, Pennsylvania, USA) was performed over 4 days at 60°C, and mounted tissue samples were cut into 20 µm slices with a Leica (Wetzlar, Germany) RM 2035 microtome. Mounted sections were dehydrated and stained in 95% ethanol plus fast green FCF (0.1% w/v) for 30 s and digitally photographed using a Leica microscope with a Q-imaging Retiga 1300 camera (Burnaby, British Columbia, Canada). The presence or absence of aerenchyma in the cortical tissue was determined by examining these digital images.

General methods: root protein extraction—To examine variation in ADH activity among genotypes in the experiments, we extracted proteins from root tips of plants exposed to aerobic and hypoxic or nearly anoxic conditions. Root samples were collected from the first 2 cm of the root tip unless stated otherwise. Fresh roots were ground with Teflon pestles in ice cold extraction buffer (100 µL/0.01 g wet mass) containing 0.01 M MgCl₂, 0.01 M KCl, 0.2 M sucrose, 0.1 g/250 mL EDTA, 5.0 g/250 mL polyvinyl-pyrrolidone (PVP), 0.1 M Tris-HCl buffer (pH 8.5). Each sample was centrifuged at 4°C at 16 000 *g* for 5 min. Supernatant was collected and used immediately for activity assays. The remaining supernatant was divided equally into two separate microcentrifuge tubes and stored in -80°C for future protein quantification.

General methods: ADH activity—In the experiments, we used spectrophotometric measurements of the reduction of NAD to NADH in the presence of ethanol substrate to quantify differences in ADH activity among genotypes in flooded and nonflooded environments by applying a modified version of methods described by (Chan and Burton, 1992). Tissue

supernatant (100 µL) was added to ADH activity assay mixtures containing 600 µL of 0.05 M Tris-HCl buffer (pH 8.5), 200 µL of 1.7 mM NAD in dH₂O, and 200 µL 95% ethanol. Activity was assayed in a temperature controlled (30°C) Shimadzu (Kyoto, Japan) UV-1700 spectrophotometer using the UV Probe 2.01 software provided. Measurements were taken at 340 nm, and ADH activity was standardized against total protein concentration of the tissue. Protein concentration was measured at 562 nm using bovine serum albumin (BSA) standard and reagents provided in BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, Illinois, USA).

Experiment 1: aerenchyma development in flooded and nonflooded environments—We exposed recombinant hybrid genotypes to flooded and nonflooded conditions to determine whether individual genotypes had constitutive or facultative (plastic) expression of aerenchyma in response to flooding and to assess the effect of aerenchyma production on plant growth. Between four and six cuttings were propagated from BC₁ (*N* = 55) and RH₂ (*N* = 126) genotypes, for a total of 724 cuttings representing 181 genotypes. Cutting tips were dipped in rooting hormone, placed in peat pellets in the greenhouse, and watered as needed to promote root and vegetative growth for approximately 1 mo. When the majority of plants had established roots and developed three to four internodes, cuttings were transferred into six separate round shallow plastic tubs (three flooded and three nonflooded; 120 cm diameter × 30 cm deep) in a randomized incomplete block design.

Pools were filled with 10 cm of river sand. One 11.4 kg bag of mulch was added to each pool and mixed thoroughly with the sand to encourage microbial activity and development of hypoxic soils. Pools used for the flooded treatment had holes drilled into their sides approximately 1 cm above the level of the sand to allow excessive water to drain and avoid completely submerging the vegetative portion of the plants. Pools used for the nonflooded treatment had holes drilled in their bottoms to allow the soil to thoroughly drain after each watering. Flooded pools were set on a sprinkler timer to water them twice daily, while nonflooded pools were watered every 3–5 d. Measurements of plant height, leaf number, leaf area, and aboveground biomass (estimated as the product of plant height and number of leaves; Rhode and Cruzan, 2005) were recorded immediately following transplantation and every 2 wk for the duration of the experiment. At the end of 8 wk, roots were collected for aerenchyma quantification and were placed in FAA-filled microcentrifuge tubes.

Experiment 2: root region variation in ADH activity—Roots of RH₃ genotypes from greenhouse crosses (*N* = 60) were exposed to hypoxic conditions to assess differences in ADH activity along their lengths and to confirm that sampling only root tips would be representative of the majority of the root system. We used recombinant hybrids in this experiment to obtain a wide range of ADH expression levels. Plants were subjected to flooded conditions for 14 d, then collected and rinsed with tap water to remove the soil. Roots were then dissected into thirds, and samples from the root tip, one third of the way up, and two thirds of the way up the root were collected for enzyme assays. Root segments were placed in ice cold extraction buffer and ground using Teflon pestles, and enzyme activity assays immediately followed this procedure.

Experiment 3: ADH activity in response to short-term anoxia—We determined whether parental morphotypes (three each of C and V) and their recombinant hybrids (six of the RH₃ genotypes) differed with respect to ADH expression when exposed to extremely hypoxic conditions. Plants were grown in a 50 : 50 soil : sand substrate under normoxic conditions prior to treatment. Selected plants were placed individually into separate sealable plastic bags filled with tap water after having their roots rinsed to remove excess soil. The vegetative portion of the plant remained protruding through the top of the bag, and bags were sealed around the stem of the plant. Bags were connected to tubing, which was used to bubble nitrogen gas (N₂) through the water, creating nearly anoxic conditions (<1% dissolved oxygen) around the root environments of each plant.

An O₂ sensor (YSI 5300, Yellow Springs, Ohio, USA) was used to measure O₂ levels throughout the experiment's duration. Water was removed from each container via a syringe and then placed into a 25 mL beaker. Dissolved oxygen content was measured within 1 min after the sensor was placed into the beaker right below the surface of the water. The O₂ levels of each sample were monitored for 2 min to ensure that they remained constant following the introduction of anoxic water to the beakers.

Root tissue was collected to examine differences in ADH enzyme activity after 0, 1, 2, 4, 6, 10, 12, and 16 h. At each sample time, primary root tips (2–3 cm in length) were removed from each cutting, immediately placed in ice-cold extraction buffer, and ground using Teflon pestles. Homogenate was centrifuged in a temperature-controlled centrifuge (4°C) at 16000 rcf for 5 min. Supernatant was collected and used immediately for measurement of ADH activity. Protein concentration was determined using a BCA Protein Assay Reagent Kit as described earlier.

Experiment 4: ADH activity in response to long-term hypoxia—Plants from field-collected seeds from 15 populations of parental morphotypes and their advanced-generation hybrid derivatives (five each of C, V, and H) distributed throughout Florida and southern Georgia were used to examine differences in ADH activity when exposed to hypoxic conditions over an extended time. The primary goal of this experiment was to determine whether plants differed with respect to ADH expression under reduced oxygen levels that were similar to conditions they might encounter in the field. Seeds were germinated in 7 × 7 cm pots under greenhouse conditions and grown for approximately 2 mo to establish roots. After 2 mo, potted plants were placed in plastic trays filled with water to the soil level. A total of 48 plants was used in this experiment: 12 C (representing nine genotypes), 12 V (12 genotypes), 12 H (12 genotypes), and 12 RH₃ (12 genotypes). Plants were monitored every 2 d to assure that water levels did not drop below the level of the soil over the 20 d period.

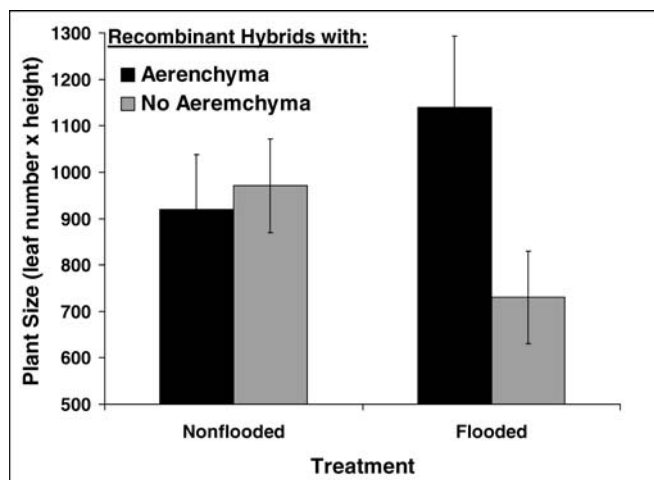


Fig. 2. The effects of aerenchyma on plant size (stem height \times number of leaves; means \pm SE), for backcross and recombinant hybrids from crosses among genotypes in the *Piriqueta caroliniana* complex grown under flooded and nonflooded conditions for 2 mo.

P. caroliniana hybrid complex had significant effects on growth under hypoxic flooded conditions, but had little effect in well-oxygenated environments (Fig. 2). Presence of aerenchyma did not explain a significant proportion of the variation in the vegetative growth under nonflooded conditions ($F = 0.98$, $P = 0.3248$, $df = 1/107$). In contrast, plants grown in flooded conditions that possessed aerenchymous root tissue had higher levels of vegetative growth than their non-aerenchyma producing counterparts ($F = 5.34$, $P = 0.0231$, $df = 1/91$). Interactions between aerenchyma production and flood treatment for vegetative growth were due to the large effects of the presence of aerenchyma in flooded conditions in contrast with weak differences in vegetative growth due to the presence of aerenchyma in aerated soils ($F = 7.27$, $P = 0.0076$, $df = 1/200$; Fig. 2).

For plants growing in the flooded treatment, there were significant effects of aerenchyma presence and genetic composition on aboveground biomass. Under flooded conditions, the presence of aerenchyma had strong effects on the vegetative growth of individuals across all cytoplasmic genotypes (Fig. 3), but plants with H cytoplasmic genotypes were consistently larger than those with either cytoplasmic genomes inherited from the V or C morphotypes ($F = 3.76$, $P = 0.0269$, $df = 2, 91$). The composition of the nuclear genome also had significant effects on plant performance under flooded conditions. Although the proportion of the nuclear genome derived from the C and V morphotypes did not significantly affect plant growth in the flooded treatment, there were strong effects of nuclear genome composition based on the proportion derived from the H morphotype ($F = 3.24$, $P = 0.0257$, $df = 3/91$). In the flooded treatment, post hoc analyses using Tukey tests indicated that plants with 75% of the nuclear genome derived from H parents had significantly higher levels of vegetative growth (i.e., compared to 0, 25, and 50%; $P < 0.05$). Across both flooded and nonflooded treatments, the nuclear contribution from the C and H morphotypes did not have a significant effect on growth. However, recombinant genotypes tended to have higher growth rates as the proportion of V contribution to their genomes was reduced ($P = 0.0268$),

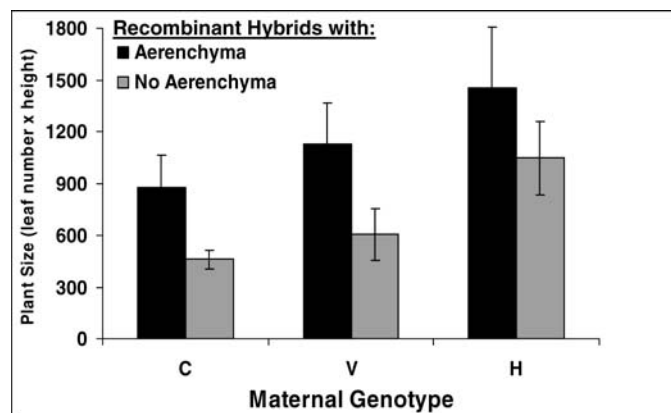


Fig. 3. The effects of the cytoplasmic genotype on plant size (stem height \times number of leaves) of plants in the *Piriqueta caroliniana* complex containing aerenchyma or no aerenchyma under flooded conditions (mean \pm SE). Inheritance of cytoplasmic genomes from *caroliniana* (C) or *viridis* (V) morphotypes, or from advanced generation hybrid derivatives (H) was inferred from crossing pedigrees.

but post hoc analysis using Tukey tests indicated that only those individuals containing either low (25%) or high (75%) proportions were significantly different ($P < 0.05$).

Experiment 2: root region variation in ADH activity—When tissue samples were collected along the length of roots, only slight differences in enzyme activity were detected among the three regions under flooded conditions ($F = 0.32$, $P = 0.7200$, $df = 2/177$). This result indicates that sampling root tissue from just the 2-cm root tip should be representative of ADH activity throughout most of the root system.

Experiment 3: ADH activity in response to short-term anoxia—When parental morphotypes and their recombinant hybrids were subjected to near anoxic conditions ($<1\%$ dissolved oxygen), they had differences in the levels of ADH activity (Fig. 4). The largest differences were observed after 12 h of exposure, when the C morphotype had the highest levels of enzyme activity compared to the V and RH₃ genotypes. Plants of the V morphotypes had a gradual increase in enzyme activity throughout the length of the experiment (16 h), but activity remained significantly lower than the C morphotype (Fig. 4). After 12 h, ADH activity in C genotypes decreased, while the activity levels in the V morphotype had only a slight increase (Fig. 4).

Repeated-measure ANOVA and a priori contrasts indicated that the C morphotypes had higher levels of ADH activity than V and RH₃ genotypes combined (Fig. 4). Initial results for a repeated-measure ANOVA among the genotype groups for ADH activity was not significant ($P = 0.1069$). However, when the V and RH₃ genotypes were combined, the a priori contrast was significant ($P = 0.0437$), indicating higher levels of enzyme activity in the C morphotypes compared to the V and RH₃ genotypes across the 16-h period of this experiment. Levels of ADH activity in the V morphotype was not significantly different ($P = 0.6243$) when contrasted with the other two genotypes, and ADH activity was not different for the RH₃ compared to the C and V morphotypes ($P = 0.0882$; Fig. 4).

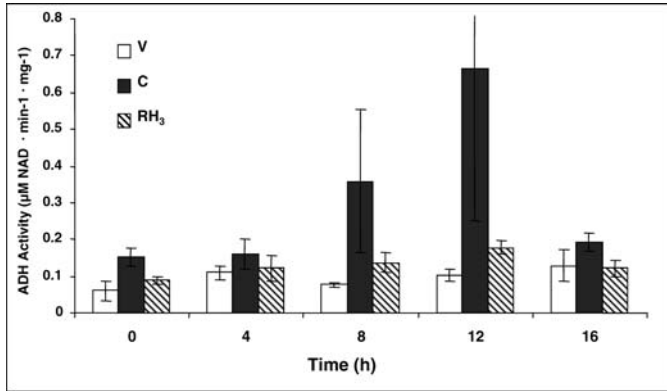


Fig. 4. Changes in alcohol dehydrogenase (ADH) activity (mean \pm SE; $\mu\text{M NAD reduced}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$) in the roots of different morphotypes (C and V) and third-generation recombinant hybrids (RH₃) of the *Piriqueta caroliniana* complex during exposure to nearly anoxic conditions for 20 h.

Experiment 4: ADH activity in response to long-term hypoxia—Over the course of this 20-d experiment, oxygen levels decreased from normoxic conditions to around 6% dissolved oxygen. When plants were subjected to decreasing levels of dissolved oxygen, they tended to increase levels of ADH activity in their roots to different degrees (Fig. 5). At the beginning of the experiment under normoxic conditions, ADH activity was low in root tissue across morphotypes and hybrids. As the level of oxygen in the soil decreased (2nd y-axis, Fig. 5), enzyme activity increased in the parental morphotypes as well as the RH₃ hybrids. After 4 d of the hypoxic conditions, increases in ADH activity were notable for plants of the C morphotype. For the last two sample periods, the level of ADH expression for C morphotype dropped because the roots of most of these plants became necrotic.

Repeated measures ANOVA indicated that there were differences in ADH enzyme activity among the parental morphotypes and RH₃ genotypes ($F = 8.05$, $P < 0.001$, $df = 2/35$; Fig. 5). A priori contrasts confirmed that the plants of the C morphotype expressed significantly higher enzyme activity compared to the other morphotypes and hybrids pooled together ($P < 0.0001$) and that the V morphotype expressed significantly lower levels of enzyme activity compared to C, H, and RH₃ plants combined ($P = 0.0007$). Enzyme activity in RH₃ and H genotypes were intermediate and not significantly different from the other plants ($P = 0.120$ for a priori contrasts). Root tip sections collected at the end of the experiment did not have any evidence of aerenchyma ($N = 48$).

DISCUSSION

Morphotypes and hybrid genotypes in the *P. caroliniana* complex had contrasting responses to hypoxic soil environments. Plants of the *caroliniana* (C) morphotype and the recombinant hybrid genotypes with a larger genomic contribution from C morphotypes elevated their ADH activity in response to low oxygen soils. On the other hand, plants of the *viridis* (V) morphotypes and those hybrids with larger genomic contributions from V morphotypes formed aerenchymous tissue but did not have increased ADH activity levels. Because

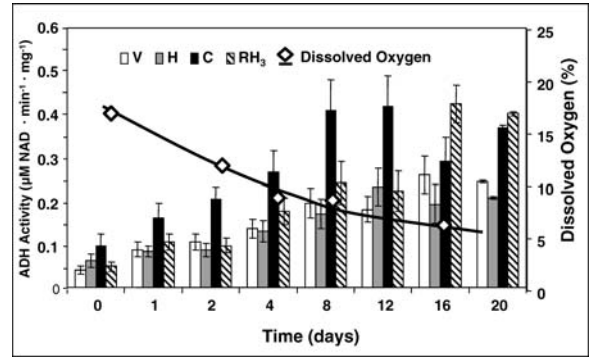


Fig. 5. Changes in soil oxygen content (open diamonds; line was fit by eye) and alcohol dehydrogenase (ADH) activity (mean \pm SE; $\mu\text{M NAD reduced}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$) in the roots of two morphotypes (C and V), their advanced-generation hybrid derivatives (H), and third-generation recombinant hybrids (RH₃) of the *Piriqueta caroliniana* complex exposed to extended hypoxic conditions (20 d).

treatments used in this study were designed to span the range of soil oxygen levels faced by *Piriqueta* in their natural habitats, we would expect to see similar responses under field conditions. Our observations of growth responses of these parental and hybrid genotypes suggest that there are two alternate strategies for coping with flooding. These results support the hypothesis that physiological adjustments represent a response that may be optimal for ephemeral flood conditions, while the formation of aerenchyma tissue represents a strategy for sustained growth under persistent flooding. Results from this study suggest that specific physiological and morphological responses expressed in plant roots have large effects on fitness in flooded environments and that the relative advantage of each strategy will depend on the duration of hypoxic soil conditions.

The range of physiological and morphological responses observed in this complex illustrates the contrasting strategies for adaptation to hypoxic conditions that have been observed across different species in other taxonomic groups (Crawford, 1967). While each of these two strategies have been documented in different species (Vartapetian et al., 2003), this is the first analysis of the relative advantages of both responses within a single species complex. In *Piriqueta*, recombinant hybrids that had a larger proportion of their genome derived from the V morphotype were more likely to form aerenchyma, and the presence of aerenchymous tissue in the roots of these plants significantly improved their vegetative performance under hypoxic soil conditions. When these plants were exposed to hypoxic conditions, their ADH activity remained very low. However, under hypoxic conditions, plants with a larger proportion of their genome derived from the C morphotype increased their enzyme activity dramatically. These contrasting responses presumably reflect adaptation to the differences in flooding propensity of the two morphotypes' natural habitats.

The formation of aerenchyma occurs in many plants either as a response to environmental stressors (facultative) or as a part of normal development (constitutive; Evans, 2003). Approximately one-third of our samples possessed aerenchyma after being flooded for 2 mo. Because samples were collected only at the experiment's conclusion, it would normally be difficult to tell whether aerenchyma formation had been facultative or constitutive. However, because our experiments replicated the

same genotypes in flooded and nonflooded treatments, we were able to estimate that 30% of genotypes tested had constitutive aerenchyma, while for 36% of genotypes development of aerenchyma was facultative (i.e., was induced in only one environment). It is important to note that, because we only exposed these plants to two environments rather than the range of environments they might encounter, this may be an overestimate of the proportion of plants with constitutive aerenchyma production.

The observed facultative and constitutive responses for the development of aerenchyma among hybrid genotypes in the *P. caroliniana* complex may have consequences for their fitness under variable environments. We would expect that plants having facultative responses would be able to maintain high fitness under a broader range of soil oxygen levels. Plants with constitutive expression of aerenchyma may be at a disadvantage under drier conditions because producing aerenchymous tissue uses resources. On the other hand, some studies have suggested that aerenchyma is not necessarily disadvantageous under dry conditions. For example, the formation of aerenchyma has been noted in many nonwetland plants (Justin and Armstrong, 1987), and Baruch and Merida (1995) found that the proportion of aerenchyma in *Hyparrhenia rufa* increased under drought conditions. In the current study, the presence of aerenchyma did not appear to have strong negative effects on growth in nonflooded environments. The consequences of an inability to develop aerenchyma in response to inundation were quite severe, however, with C genotypes subjected to 20 d of hypoxia suffering extensive root necrosis. Given these results, the constitutive production of aerenchyma may be advantageous in environments subjected to periodic flooding, even if these alternated with drier periods.

It is interesting to note that while the majority of individuals had phenotypic responses to flooded conditions that were apparently adaptive, a smaller number of recombinant hybrid genotypes (15%) only produced aerenchyma under normoxic soil conditions, but not when exposed to flooded soils. As noted, producing aerenchyma in nonflooded soil does not necessarily reduce fitness, but it is clear that the lack of aerenchyma in hypoxic soils leads to high rates of mortality. Given that these genotypes were capable of producing aerenchyma but did not express this trait under hypoxic conditions suggests that this lack of response is due to dysfunctional combinations of genetic elements derived from the two parental species (i.e., hybrid breakdown: Dobzhansky, 1936; Muller, 1942; Orr, 1995; Turelli and Orr, 2000). Similar examples of environmentally induced hybrid breakdown have been observed for leaf morphological responses to drought in this species complex (J. J. Picotte et al., unpublished manuscript), and such examples of hybrid breakdown may be more common for hybrids derived from crosses between species adapted to contrasting environmental conditions.

One unexpected result from this study was the effect of cytoplasmic genotype on the fitness of recombinant hybrid genotypes. These results are consistent with previous studies, which noted that cytoplasmic genetic effects can have important ecological consequences for the fitness of individuals within a population (Rhode and Cruzan, 2005). While we might have expected that individuals possessing the cytoplasmic V genotype may have had an advantage under flooded conditions, this was not evident in our data. On the other hand, recombinant hybrids with cytoplasmic genotypes derived from H morphotypes had higher growth rates in the flooded

environment whether or not they had aerenchyma. These results are consistent with previous observations, which suggest that the hybrid derivative genotypes from central Florida are more vigorous than either the C or V morphotype (Rhode and Cruzan, 2005).

Genotypes that are unable to produce aerenchyma under flooded conditions must shift to a short-term strategy to compensate for decreasing levels of oxygen. The induction of ADH and activation of ethanolic fermentation pathways is one mechanism used by plants to cope with short-term flooding and to survive under anaerobic conditions (Drew, 1997; Tadege et al., 1999). There are disadvantages to increasing ADH activity as a response to hypoxic conditions, however, because it may have high energy costs, and products of the fermentation pathway contribute to cell death (Liao and Lin, 2001). In the present study, after approximately 12 d of hypoxia, plants that had the highest levels of ADH activity appeared noticeably unhealthy, with signs of necrosis in the root tips as well as chlorotic leaves. The rapidity with which the negative effects of elevated ADH activity were evident suggests that anaerobic responses may be advantageous only in environments that are subjected to short-term flooding.

The higher levels of ADH activity in individuals that inhabit flooded environments in the absence of soil oxygen are indicative of a short-term mechanism that can have deleterious effects on root tissue. Under anaerobic conditions there is an increase in enzymatic activity brought about by the ethanolic fermentation and consequently an increase in the concentration of ethanol. As a result of this increase in ethanol concentrations, Kiyosawa (1975) noted that there was a deleterious "fluidizing" effect on cell membranes. The continued production of ethanol beyond the tolerance of the plant can lead to the destruction of the mitochondrial membranes as well as the lipid cytoplasmic membranes (Crawford, 1977). After approximately 12 d of hypoxic conditions, the drought-associated plants in our experiment expressed significantly higher levels of ADH activity and displayed visible signs of stress. The costs of producing these enzymes and the inability to develop aerenchyma under extended hypoxic conditions appeared to have a significant negative impact on the health of the drought-associated plants.

While it would appear that ADH enzymes and the associated fermentation pathway may play an important role for short-term flood responses, the role of ADH in flood tolerance has been debated for decades (Ram, 2000). Several authors have argued that there are negative relationships between ADH activity and flood tolerance (McManmon and Crawford, 1971; Marshall et al., 1973; Francis et al., 1974; Brown et al., 1976), while others have argued that there should be a positive relationship between ADH activity and flood tolerance (Francis et al., 1974; Lin and Lin, 1992; Liao and Lin, 1995). The present study on *Piriqueta* supports the former hypothesis; higher levels of ADH activity were observed in the northern, more drought-associated-1.0-1.0(c)-478.1m ouc flooyed

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ADH under flooded conditions allowed individuals to survive anaerobic conditions for approximately 2 wk, at which point the inability to switch to a long-term strategy, i.e., aerenchyma production, led to high rates of mortality. In contrast, the southern and hybrid morphotypes appeared to be healthy throughout the duration of the experiments, as evident by the minimal signs of degradation to the root tissue as well as a steady increase in the vegetative growth of those individuals. It is also important to note that these genotypes did not display signs of root necrosis and leaf chlorosis as did plants of the C morphotype. While ADH enzyme levels were significantly lower in the V morphotypes in contrast with the hybrid derivatives and the C morphotypes, the production of aerenchyma was not evident after 2 wk of flooding. It is possible that there may have been aerenchyma development in the upper portion of the root, which we did not sample. However, the lack of aerenchyma in the roots of these plants during the first 20 d of exposure to hypoxic conditions is somewhat enigmatic because they maintained active growth during this time. In other species that have been analyzed, ADH is important for the removal of the byproducts of anaerobic respiration, so it is probable that plants of the V morphotype possess an alternative mechanism to avoid toxicity from the accumulation of acetaldehyde during the first few weeks of exposure to hypoxic soil conditions.

It is evident that flooding induces certain phenotypic responses that can be physiological or morphological in nature, and these responses may be adaptive for particular habitats depending on the typical maximum duration of hypoxic soil conditions. In the present study it is interesting to note that the H parental genotype often had the highest fitness across flooded and nonflooded environments. Because these genotypes are apparently recombinant hybrid derivatives of the C and V parental morphotypes (Martin and Cruzan, 1999; Maskas and Cruzan, 2000; Cruzan, 2005), it is possible that they may possess combinations of traits that allow them to sustain growth and high fitness under a diversity of environmental conditions. The range of physiological and morphological responses expressed by these naturally occurring hybrids suggests that they possess an ability to colonize a wider range of habitats. The overall higher fitness of H genotypes noted in this and previous studies (Rhode and Cruzan, 2005) and their potential for a broader range of phenotypic responses may have been instrumental in their spread across central Florida and the apparent displacement of the C and V parental morphotypes.

This study provides insights into how plants can maintain high fitness under fluctuating water levels through morphological and physiological responses. Plants in this species complex either utilize anaerobic respiration and changes in enzyme activity to eliminate toxic byproducts or they undergo more permanent anatomical changes to allow oxygen to diffuse to root tissues. The relative advantages of these two strategies depend on the duration of hypoxic soil conditions. Physiological adjustments may be beneficial if flooding is ephemeral, but this strategy leads to high levels of plant mortality if soils remain waterlogged for more than a few weeks. More permanent changes in root and stem anatomy, on the other hand, are necessary for continued growth in flooded environments, and plants that have aerenchyma do not appear to suffer any severe disadvantage under drier conditions. However, it is important to note that we did not examine the growth of plants under drought, and it is possible that the presence of air

chambers within roots may be disadvantageous when water is more severely limiting. These contrasting strategies represent the extremes of phenotypic plasticity reaction times: a short-term and rapid change that is advantageous for ephemeral environmental fluctuations, contrasted with a slower response that is advantageous under more sustained conditions. Continued investigations that utilize genotypes possessing these different responses will provide insights into the evolutionary advantages of contrasting phenotypic plasticity response times in environments with varying frequencies of temporal variation.

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