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Morphological and physiological responses of lowland purple nutsedge (*Cyperus rotundus* L.) to flooding

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Abstract

Background and aims

Purple nutsedge (*Cyperus rotundus* L.) is a major weed of upland crops and vegetables. Recently, a flood-tolerant ecotype evolved as a serious weed in lowland rice. This study attempted to establish the putative growth and physiological features that led to this shift in adaptation.

Methodology

Tubers of upland *C. rotundus* (ULCR) and lowland *C. rotundus* (LLCR) ecotypes were collected from their native habitats and maintained under the respective growth conditions in a greenhouse. Five experiments were conducted to assess the variation between the two ecotypes in germination, growth and tuber morphology when grown in their native or 'switched' conditions. Carbohydrate storage and mobilization, and variation in anaerobic respiration under hypoxia were compared.

Principal results

Tubers of LLCR were larger than those of ULCR, with higher carbohydrate content, and larger tubers developed with increasing floodwater depth. Stems of LLCR had larger diameter and proportionally larger air spaces than those of ULCR: a method of aerating submerged plant parts. The LLCR ecotype can also mobilize and use carbohydrate reserves under hypoxia, and it maintained relatively lower and steadier activity of alcohol dehydrogenase (ADH) as a measure of sustained anaerobic respiration. In contrast, ADH activity in ULCR increased faster upon a shift to hypoxia and then sharply decreased, suggesting depletion of available soluble sugar substrates. The LLCR ecotype also maintained lower lactate dehydrogenase activity under flooded conditions, which could reduce chances of cellular acidosis.

Conclusions

These adaptive traits in the LLCR ecotype were expressed constitutively, but some of them, such as tuber growth and aerenchyma development, are enhanced with stress severity. The LLCR ecotype attained numerous adaptive traits that could have evolved as a consequence of natural evolution or repeated management practices, and alternative strategies are necessary because flooding is no longer a feasible management option.

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Introduction

Purple nutsedge (*Cyperus rotundus* L.) is usually considered an upland weed and, until the 1970s, it was reported to occur only occasionally or at low densities in flooded rice fields in the Philippines (Pablico and Moody, 1985). Surveys there showed that *C. rotundus* densities in lowland rice increased to 15 plants m⁻² in 1998 and to over 50 plants m⁻² in 2005, making it one of the most important weeds in rainfed lowland rice (Islam *et al.*, 2005). *Cyperus rotundus* ecotypes appear to thrive in the flooded conditions of lowland rice grown in rotation with vegetables (Baltazar *et al.*, 2006). This has substantial economic implications, because in almost half of the rice-growing areas in the Philippines vegetables are also grown during the dry season and there are substantial rice yield losses due to weeds.

The occurrence of lowland and upland ecotypes of *C. rotundus* has also been reported elsewhere in the world (Holm *et al.*, 1977; Chavez and Moody, 1986; Wills, 1998). These ecotypes have been shown to have a similar number of chromosomes, although morphological and genetic variations can be observed (Cruz *et al.*, 2001). Lowland ecotypes are taller, with greater biomass, larger leaves and tubers two to three times the weight of upland ecotypes (Baltazar *et al.*, 1997; Peña-Fronteras *et al.*, 2009). Genetic differences between upland and lowland ecotypes were also reported among populations from North America, India and Brazil (Okoli *et al.*, 1997), as well as among populations from Luzon (Philippines), where rice-vegetable rotations are widespread (Casimero *et al.*, 1999).

In flooded or waterlogged soils, oxygen deficiency in plants adversely reduces cellular ATP production through aerobic respiration, resulting in reduced growth and crop yield (Fukao and Bailey-Serres, 2004). The survival and proliferation of *C. rotundus* in flooded rice fields suggest that this weed has mechanisms to survive in oxygen-deficient environments. Adaptation to oxygen deficiency (anoxia or hypoxia) in flooded soils involves a combination of morphological and metabolic processes, particularly involving enzymatic systems (Ratcliffe, 1995). Oxygen deficiency in root tissue, for example, inhibits aerobic respiration and starch mobilization, and induces anaerobic fermentation pathways (Crawford, 1992). Ratcliffe (1995) detected anaerobically induced pyruvate decarboxylase (PDC) genes in maize seedlings and characterized the regulation of these genes at the transcription, translation and post-translation levels in oxygen-deficient plants using the gene family of alcohol dehydrogenase (ADH). In flood-tolerant rice genotypes, an increase in the activities of

enzymes associated with anaerobic metabolism, such as PDC and ADH, was observed (Sarkar *et al.*, 2006; Ismail *et al.*, 2009). These studies suggest the importance of anaerobic respiration for survival under low-oxygen stress.

Three key enzymes involved in anaerobic respiration are PDC (2-oxoacid carboxylase, E.C.4.1.1.17), ADH (alcohol:NAD oxidoreductase, E.C.1.1.1.1) and lactate dehydrogenase [LDH; L-(+)-lactate:NAD⁺ oxidoreductase, E.C.1.1.1.27]. Pyruvate decarboxylase catalyses the decarboxylation of pyruvate to yield carbon dioxide and acetaldehyde, ADH catalyses the reduction of acetaldehyde to ethanol and the regeneration of NAD⁺, and LDH catalyses the formation of lactate and also the regeneration of NAD⁺. Because ethanol easily diffuses out of tissues, plants tend to use alcoholic fermentation as the main metabolic pathway rather than lactate fermentation under anaerobic conditions. Lactate fermentation leads to lactate accumulation, resulting in cytoplasmic acidosis and toxicity. Tolerance of cytoplasmic acidosis was suggested, however, as one of the determinants of flooding tolerance in plants (Roberts *et al.*, 1985). Thus, regulation of cytoplasmic pH is central to the survival of plants growing in flooded conditions (Drew, 1997). The activity of LDH in *C. rotundus* in response to flooding, however, is not known and could play a role in regulating cytoplasmic pH and enhancing flood tolerance.

Plants capable of breaking down and using starch reserves in hypoxic or submerged soils are generally more tolerant of anaerobic conditions, provided energy reserves are adequate. Fermentable carbohydrates are one of the biochemical requirements in cereals for sustaining active fermentative metabolism under anaerobic conditions (Guglielminetti *et al.*, 2001; Ismail *et al.*, 2009). Anatomical adaptations may also contribute to tolerance of flooding, and an increase in aerenchyma in roots and stems is associated with flooding tolerance in some plant species, presumably by facilitating oxygen diffusion to roots (Benz *et al.*, 2007).

A lowland ecotype of *C. rotundus* has been shown to have larger tubers, greater reserves of soluble sugars and higher amylase activity under anaerobic conditions than an upland ecotype (Peña-Fronteras *et al.*, 2009). Furthermore, activities of ADH and PDC were induced in the roots of upland and lowland ecotypes within the first 24 h of germination under hypoxia, although activities decreased in the lowland ecotype between 24 and 48 h. In contrast, ADH and PDC activities in upland *C. rotundus* (ULCR) continued to increase between 24 and 48 h.

To elucidate the traits associated with the shift in adaptation to flooded conditions, these studies built on

the work of Peña-Fronteras et al. (2009). We attempt to (i) assess putative anatomical changes that render the lowland *C. rotundus* (LLCR) ecotype more tolerant of flooded conditions; (ii) investigate whether the adaptation is constitutively expressed or induced by their environment, by ‘switching’ their native growth conditions and evaluating the effects of this change on growth of the two ecotypes; and (iii) determine the pattern of depletion of starch and maintenance of soluble sugar concentration as indicators of continued growth under anaerobic conditions, and the possible role that anaerobic respiration pathways could have by measuring the activities of two key enzymes, ADH and LDH, over an extended period under hypoxia.

Materials and methods

Tuber collection and maintenance

Tubers of LLCR plants were collected from fields where rice had been continuously cultivated in flooded soils, and tubers of ULCR plants were collected from fields used for continuous cultivation of vegetables in aerated soil. Tubers of each ecotype were allowed to sprout and plants were grown until maturity in 50-cm-diameter, 108-L-capacity plastic pots in a greenhouse. The LLCR ecotype was grown in soil continuously flooded to a depth of 7 cm with water, whereas ULCR was maintained in aerobic soil and watered as needed. Tubers from LLCR and ULCR plants were randomly selected and washed free of soil before use in subsequent experiments.

Experiment I: tuber biomass and germination

Tuber width, length, and fresh and dry weights were recorded from 100 tubers randomly selected from each of the LLCR and ULCR plants. The length and width of each tuber were measured with a micrometer (Model IP54, Fred Fowler Co., Newton, MA, USA), and tubers were oven-dried at 70 °C before weighing.

Ten tubers of both LLCR and ULCR were sown in sterilized clay loam soil at 1-cm depth in 12-cm-diameter plastic trays and sprouted in either (i) moist soil or (ii) soil flooded with 2 mm of standing water. Trays with tubers were placed in natural light and temperature conditions in the greenhouse, and sprouted tubers were counted daily for 30 days. Treatments were replicated four times.

Experiment II: seedling growth responses to three water regimes

Tubers of LLCR and ULCR were placed in moist soil to ensure uniform sprouting. Ten tubers with 1-cm-long shoots of both LLCR and ULCR were sown in sterilized

clay loam soil in 12-cm plastic trays under three water regimes for 14 days: 0 (moist aerated soil), flooded to 2-mm water depth and flooded to 20-mm water depth. The plants were grown under ambient light and temperature in the greenhouse. At 14 days, plants were harvested and separated into roots, shoots and tubers. The lengths and dry weights of roots and shoots, and the lengths, widths and dry weights of tubers were determined. Plant and tuber samples were oven-dried at 70 °C before determining their dry weights. Treatments were replicated three times.

Experiment III: measurement of aerenchyma (air space) in roots and shoots

Ten tubers of both ULCR and LLCR were sown in moist sterilized clay loam soil in plastic trays and allowed to sprout. At 5 days after sowing, five tubers from each of ULCR and LLCR, with shoots emerging but the first leaf still folded, were selected and then grown for 3 days in pots with 2-mm water depth and another five sprouted tubers were grown for 3 days in pots with moist aerated soil. Cross-sections of both roots and shoots of each ecotype were then made 1 cm from the base and examined under a microscope. Root and shoot surface area, and air spaces or aerenchymatous tissues in the cross-sectional area were measured (Image Software J 1.31c, National Institutes of Health, USA), and root and shoot diameters were calculated from surface area measurements. Data were collected as three replicates with two measurements per replicate.

Experiment IV: carbohydrate concentrations in tubers and enzyme activities in roots during germination

This experiment monitored changes in non-structural carbohydrate contents in tubers and activities of ADH and LDH in roots of the two ecotypes. Tubers of ULCR and LLCR were placed in moist sterilized clay loam soil in plastic trays to ensure uniform sprouting. When 1 cm of the hypocotyl had emerged, 10 uniformly sized sprouted tubers of LLCR and 20 uniformly sized sprouted tubers of ULCR were planted in 95 mm × 95 mm × 55 mm plastic trays filled with sterilized clay loam soil. Sprouted tubers of ULCR were grown in aerated moist soil (native) or in soil flooded with 2 mm of water (switched), and sprouted tubers of LLCR were grown in aerated moist soil to simulate their ‘switched’ habitat or in soil flooded with 2 mm of water as their native habitat. Because of their slower growth under flooded conditions, a larger number of ULCR sprouted tubers were sown to ensure sufficient root material for analyses. The experiment was conducted under ambient greenhouse conditions. After 5 days, sprouts with

emerging roots and shoots but with the first leaf still folded were subjected to hypoxia treatment by placing them in airtight Erlenmeyer flasks filled with 300 mL of distilled water, through which high-purity N₂ gas was flushed to reduce and maintain the oxygen concentration at about 0.5 mg L⁻¹ (Ellis and Setter, 1999). The oxygen concentration was monitored using a dissolved-oxygen meter (YSI 85; YSI Inc., Yellow Springs, OH, USA). The flasks were covered with aluminium foil to prevent sprouts from undergoing photosynthesis. Roots and parent tubers were excised from the sprouted plants at 0, 12 and 24 h, and then at 24-h intervals for a period of 144 h, and stored at -20 °C until used for enzyme assays and for measuring the concentrations of soluble sugars and starch, respectively. The carbohydrate content of tubers was calculated based on tuber dry weight. Roots were used in the enzyme activity assays because they are more likely to be exposed to hypoxia in waterlogged or flooded natural soils than shoots. Leaves of most herbaceous plants generally produced relatively small amounts of fermentation products (Kimmerer and MacDonald, 1987). Treatments were replicated three times.

Experiment V: activities of ADH and LDH following growth in aerated and flooded conditions

This experiment was conducted to assess the activities of ADH and LDH in roots of sprouted parent tubers of the two ecotypes grown continuously for 5 days under flooded (2 mm of water) or aerated conditions, or grown under aerated conditions for 5 days, followed by 48 h of hypoxia, in flasks containing water flushed with nitrogen gas. The protocol used in Experiment IV was also used in this trial, and treatments were replicated three times.

Enzyme extraction and activity assays in roots

Extraction of ADH and LDH from roots followed the procedure of Valdez (1995). About 200 mg of root tissue were ground in 1.2 mL of extraction buffer in a pre-cooled mortar placed on ice. The extraction buffer contained 100 mM *N*-Tris((hydroxymethyl)methyl-2-amino)ethanesulphonic acid (TES), 2 mM MgCl₂·6H₂O, 1.0 mM ethylenediaminetetraacetic acid disodium salt, 20 mM dithiothreitol and 0.25 % (w/v) Triton X-100. A measured volume of the extract was transferred to 1.5-mL Eppendorf tubes containing bovine serum albumin (BSA) to a final concentration of 1%. The remaining extract was used to determine protein concentration (Bradford, 1976), with BSA as the standard.

The activity of ADH was assayed using the method of Valdez (1995). The reaction mixture for ADH assay (1 mL)

consisted of 51.8 mM TES at pH 7.0, 20 µL of crude extract, 0.17 mM NADH and 10.02 mM acetaldehyde. The mixture was allowed to stand for 2 min before the addition of acetaldehyde. Absorbance was read at 340 nm at 30 °C for 420 s using a spectrophotometer (Model DU 800; Beckman Coulter Inc., Fullerton, CA, USA). The activity of LDH was assayed using the method of Davies *et al.* (1974). The reaction mixture for LDH assay consisted of 0.15 M Tris-HCl (pH 8.0), 0.1 mM NADH, 2.5 mM 4-methylpyrazole hydrochloride, 200 µL of the crude extract and 5 mM sodium pyruvate. After addition of the crude extract, the mixture was allowed to stand for 2 min before sodium pyruvate was added. Absorbance was read at 340 nm at 30 °C for 120 s. One unit of enzyme activity is defined as 1 µmol of NADH oxidized per milligram of protein per minute.

Soluble carbohydrate concentrations in tubers Soluble sugar, starch and total carbohydrate concentrations were determined from five tubers per replication, from both LLCR and ULCR plants used in the enzyme assay. The procedure of Fales (1951) as modified by Peña-Fronteras *et al.* (2009) was used to determine soluble sugars. Briefly, tubers were washed with distilled water, peeled and cut into small pieces, oven-dried at 70 °C and ground to a fine powder. Approximately 200 mg of powdered sample were extracted twice with 80 % ethanol at 80 °C for 10 min. An aliquot was added to 5 mL of anthrone reagent. The absorbance of each sample was read at 620 nm and the concentration of soluble sugars was derived from a standard curve using glucose.

Starch concentration was determined by enzymatic hydrolysis following the method of Setter *et al.* (1989), and modified by using the anthrone method in the colorimetric determination (Yemm and Willis, 1954). The residue obtained after analysis of soluble sugars was oven-dried at 70 °C overnight. A sample of ~200 mg was added to acetate buffer pH 4.6 and placed in a boiling-water bath for 3 h, and then 2 mL of acetate buffer pH 4.6 and 1 mL of amyloglucosidase solution were added. The mixture was incubated in a water bath at 37 °C for 24 h. About 5 mL of anthrone reagent were added to a portion of the extract and the absorbance of each sample was read at 630 nm. The quantity of glucose released from the enzymatic hydrolysis was determined from a standard curve. Values for starch concentration were determined by multiplying those of sugars by a factor of 0.90. Total soluble carbohydrates were determined as the sum of soluble sugars and starch concentration.

Statistical analysis

Data from each experiment were analysed using ANOVA based on respective models for Experiments I–III using CROPSTAT 7.2 (IRRI, 2007). For comparison of morphological features in Experiment I (dry weight, length and width of tubers), a one-way ANOVA was undertaken. Assessments of tuber sprouting in relation to the water regime (Experiments I and II) were analysed as a split-plot design with water regime as the main plot and *C. rotundus* ecotype as the subplot. Measurement data on aerenchyma in stems and roots and cross-section measurements (Experiment III) were analysed within a randomized complete block design. For the time-course studies (Experiments IV and V) on sugar, starch, carbohydrate and enzyme activities, data were analysed as a split-split-plot design (water regime, ecotype and time) with ANOVA, as implemented in Proc Mixed procedures of the SAS 9.1.3 (SAS Institute, 2003). Treatment means were compared using the least significant difference (l.s.d.) at $P = 0.05$.

Results

Variation in morphological and anatomical features

Tuber biomass and sprouting The LLCR tubers were larger and had almost 4-fold the dry weight of the ULCR tubers (Table 1). Both ecotypes took 4–5 days to reach 100% sprouting in moist soil, but over 25 days to reach a maximum of 50% sprouting in flooded soil, reflecting a marked reduction in the rate of tuber sprouting in flooded soil (Fig. 1). The LLCR ecotype appeared to maintain slightly higher rates of tuber sprouting under both conditions, although differences were not significant.

Shoot and root growth responses to different water regimes Flooding adversely affected the shoot and root growth of both ecotypes. Shoot lengths as well as plant dry weights decreased with increased water depth to 20 mm. Whether grown in moist or in flooded soil, LLCR plants were over 30% taller and 50% heavier, and their tubers were three times larger than those of ULCR (Fig. 2). The tuber dry weight of LLCR increased significantly with increasing water depth (Fig. 2B).

Aerenchyma tissue in roots and shoots Both LLCR and ULCR appear to have similar root and shoot anatomy, but stems of LLCR had greater diameter than those of ULCR, independent of growth conditions (Figs 3 and 4). Roots of LLCR were also slightly larger than those of ULCR, although the differences were not significant.

Table 1 Length, width, and fresh and dry weights of ULCR and LLCR tubers collected from plants grown in the greenhouse. Values are means of 100 tubers

	Plant type		Standard error of the difference (SED)
	Upland	Lowland	
Dry weight (mg tuber^{-1})	196	743	23
Length (mm tuber^{-1})	14	20	0.42
Width (mm tuber^{-1})	7	12	0.15

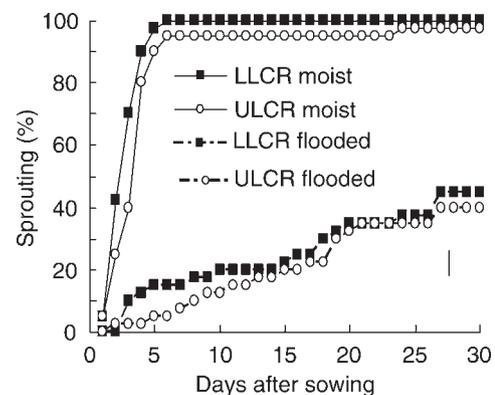


Fig. 1 Per cent of ULCR and LLCR ecotype tubers sprouting in moist soil and in soil flooded with 2 mm of water in Experiment I. Vertical bar indicates l.s.d. at $P = 0.05$.

The proportion of the cross-sectional area of stems occupied by aerenchyma tissue in LLCR was about twice that of ULCR grown under either aerobic or flooded conditions. Moreover, the area of aerenchyma tissue was greater in both ecotypes grown in flooded soil, but the increase was significant only in stems of the LLCR ecotype (Fig. 4). On the other hand, the percentage of root cross-sectional area occupied by aerenchyma tissue was similar in both LLCR and ULCR grown in aerobic soil, and this increased by about 15% in both ecotypes grown in flooded soil (Fig. 4).

Carbohydrate contents and enzyme activities

Carbohydrate concentrations in tubers Total carbohydrate, soluble sugar and starch concentrations in unsprouted tubers, as a percentage of tuber dry weight, were higher in ULCR than in LLCR (data not shown). As LLCR tubers are more than three times larger than ULCR tubers, however, the total

carbohydrates as soluble sugar and starch contents per tuber before sprouting were much higher in LLCR than in ULCR (Figs 5 and 6). After sprouting for 5 days in native conditions and then incubating under hypoxia for 6 days, total carbohydrate, soluble sugar and starch

contents remained consistently higher in LLCR than in ULCR (Fig. 5). Soluble sugars in tubers of LLCR increased after sprouting in flooded soil, remained unchanged for the first 12 h of growth and then gradually decreased up to 48 h, followed by a progressive increase until the end of the trial. In general, starch content decreased with time in LLCR tubers. In tubers of ULCR, however, starch and soluble sugars remained more or less unchanged throughout the period of incubation under hypoxia.

When grown under switched conditions (2-mm flood for ULCR and moist soil for LLCR), the trends in total non-structural carbohydrates and starch contents in tubers were similar to those in tubers grown in their native conditions (Fig. 6). ULCR tubers showed no marked changes in soluble sugar and starch contents, whereas LLCR tubers showed increases in soluble sugar and decreases in starch contents within the first 12–24 h under hypoxia.

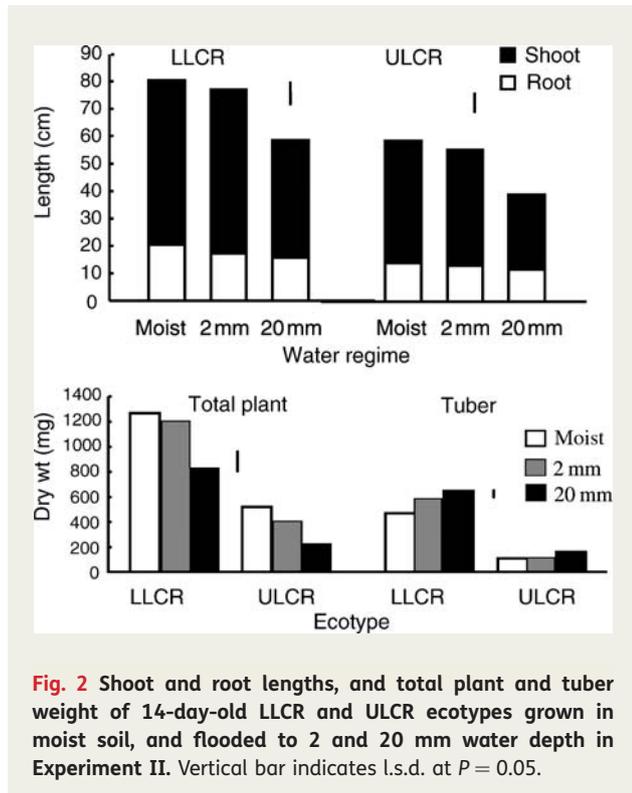


Fig. 2 Shoot and root lengths, and total plant and tuber weight of 14-day-old LLCR and ULCR ecotypes grown in moist soil, and flooded to 2 and 20 mm water depth in Experiment II. Vertical bar indicates l.s.d. at $P = 0.05$.

Induction of ADH and LDH activities in roots under aerobic and hypoxic conditions The activity of ADH in roots of 5-day-old plants of both ULCR and LLCR was low when roots were grown under aerobic conditions, but this activity increased in flooded soil, and the increase was substantially greater in roots of ULCR (Fig. 7A). Switching the conditions for sprouted tubers from aerobic to hypoxic considerably increased the activity of ADH in both ecotypes; however, the increase was more dramatic in roots of ULCR. Lactate dehydrogenase activity was similar in roots of both

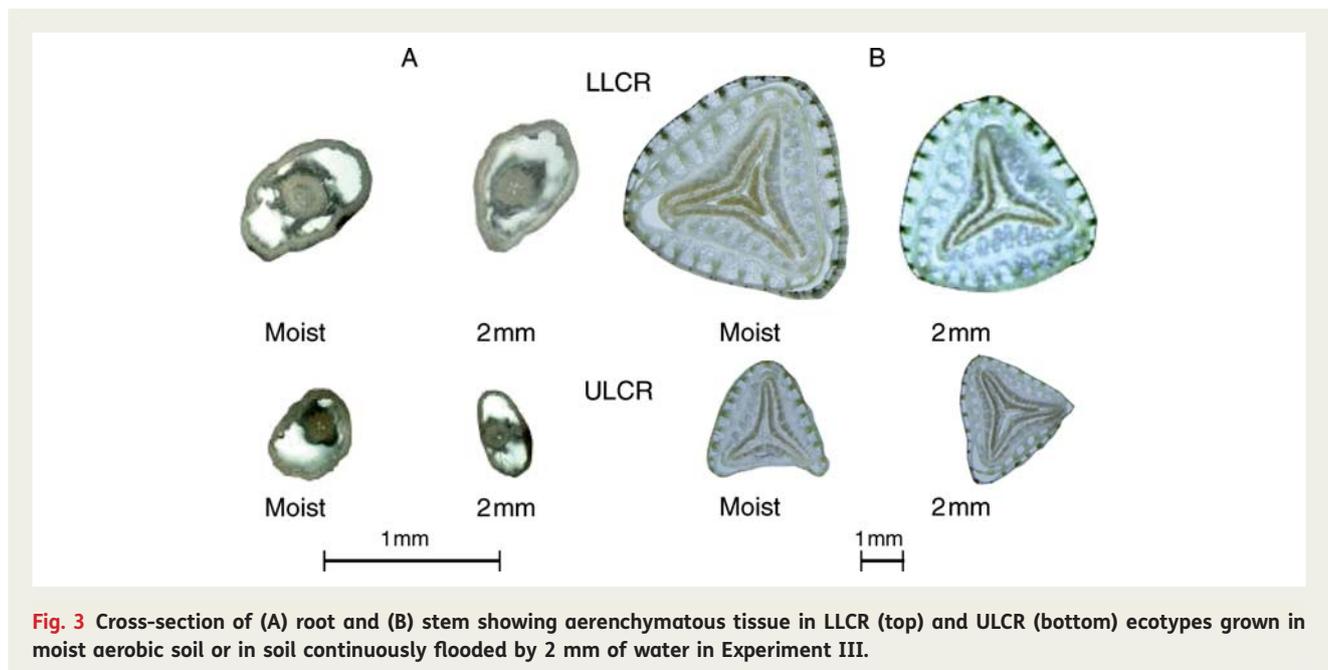


Fig. 3 Cross-section of (A) root and (B) stem showing aerenchymatous tissue in LLCR (top) and ULCR (bottom) ecotypes grown in moist aerobic soil or in soil continuously flooded by 2 mm of water in Experiment III.

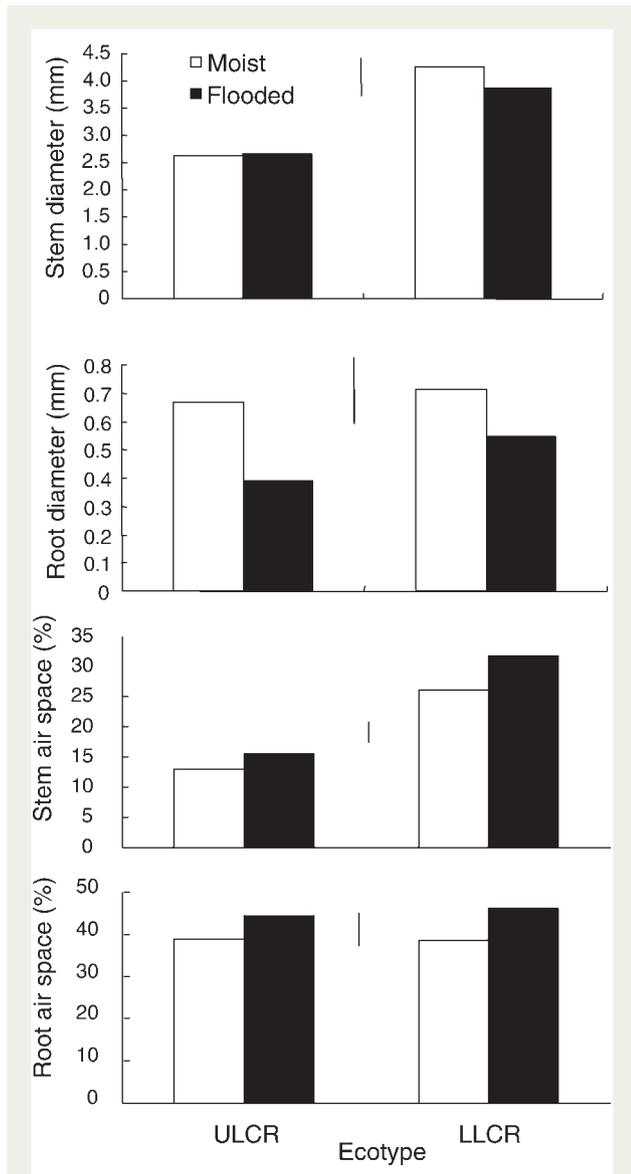


Fig. 4 Diameter and per cent aerenchymatous tissue of stem and root of ULCR and LLCR ecotypes grown in moist and flooded soil in Experiment III. Vertical bar indicates l.s.d. at $P = 0.05$.

ecotypes grown in aerated soils, but increased significantly only in roots of the ULCR ecotype when grown in flooded soil or when switched from aerobic to hypoxic conditions (Fig. 7B).

Patterns of ADH and LDH activities in roots growing under hypoxia The activity of ADH in roots of 5-day-old sprouted tubers of both ULCR and LLCR grown in their native conditions was similarly low (time zero, Fig. 8A), but increased significantly in roots of both ecotypes when switched to controlled hypoxia. Alcohol

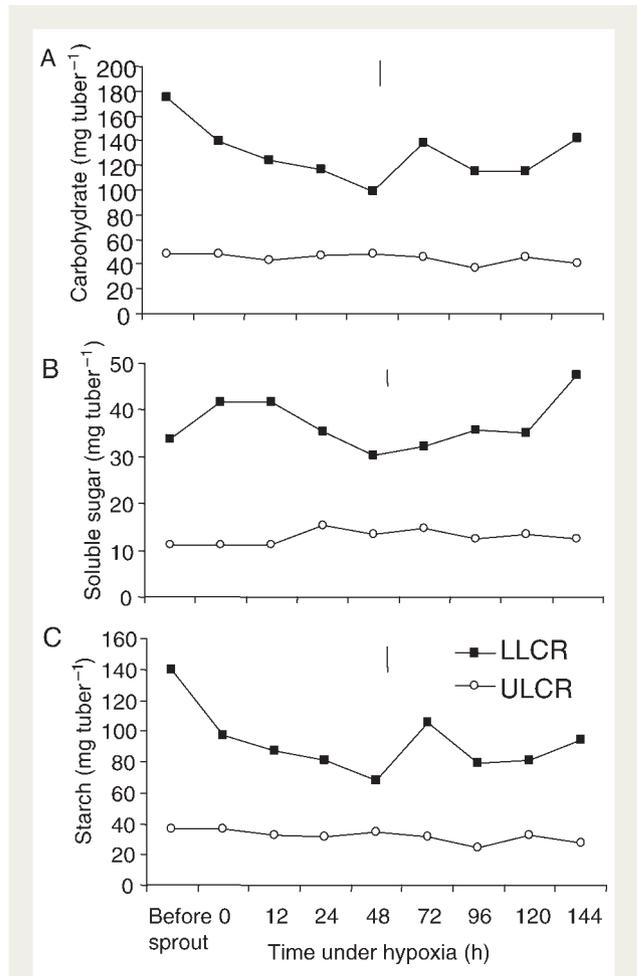


Fig. 5 Total carbohydrate (A), soluble sugar (B) and starch content (C) in fresh tubers before and after sprouting in their native conditions for 5 days (time 0) and then incubated under hypoxia for 144 h in tubers of *C. rotundus* ecotypes in Experiment IV. Vertical bar indicates l.s.d. at $P = 0.05$.

dehydrogenase activity in ULCR increased sharply between 24 and 72 h, followed by a rapid decrease from 72 to 144 h (Fig. 8A). Alcohol dehydrogenase activity in LLCR also increased within the same period, but to a lesser extent, and then decreased more gradually from 72 to 144 h (Fig. 8A). This enhanced ADH activity in roots of the LLCR suggests that the conditions in the flasks were more hypoxic than in flooded soil. Under 'switched' conditions, ADH activity increased in roots of ULCR but decreased slightly in roots of LLCR (point zero, Fig. 8B). However, after 12 h of incubation in flasks, the trend in activity became similar to that observed under hypoxia following growth in native conditions (Fig. 8B).

The activity of LDH in roots of both ecotypes was only about 1/10th that of ADH (Fig. 9A and B). When grown

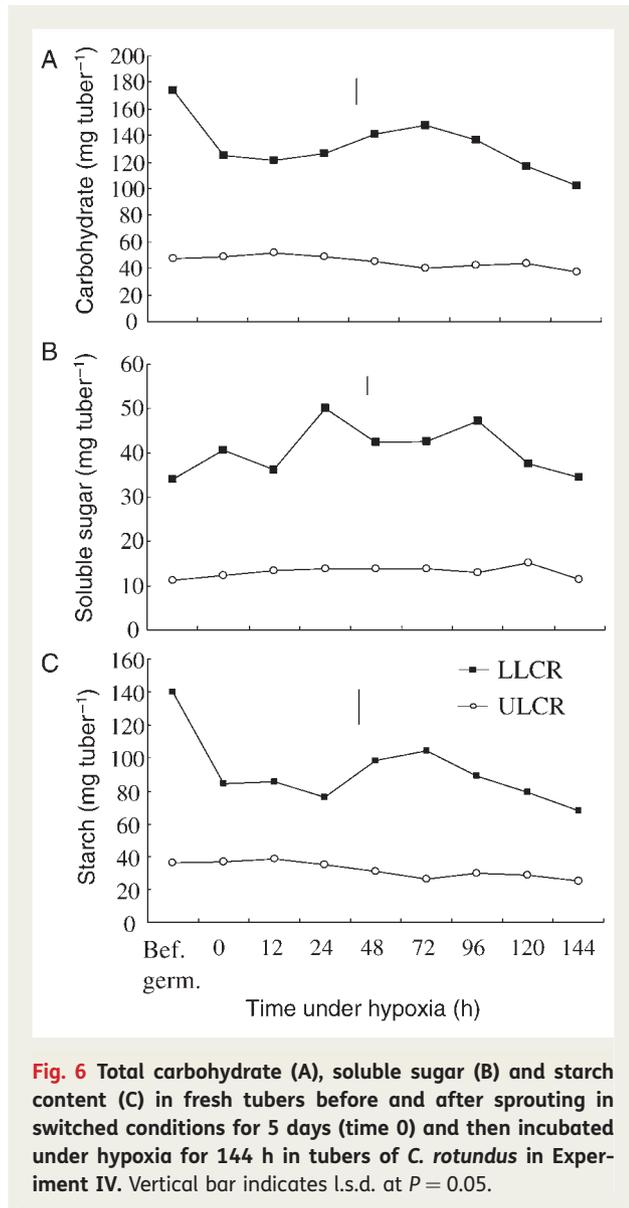


Fig. 6 Total carbohydrate (A), soluble sugar (B) and starch content (C) in fresh tubers before and after sprouting in switched conditions for 5 days (time 0) and then incubated under hypoxia for 144 h in tubers of *C. rotundus* in Experiment IV. Vertical bar indicates l.s.d. at $P = 0.05$.

under native conditions before controlled hypoxia treatment, LDH activity in both ecotypes increased significantly within the first 48 h of hypoxia and then decreased progressively until 144 h (Fig. 9A). In those grown under ‘switched’ conditions, however, LDH activity in ULCR roots at the start of the hypoxic treatment was significantly higher than in LLCR roots (Fig. 9B). This increased further for the first 24 h under hypoxia and then decreased progressively with time; it remained higher, however, than that of LLCR during most of the duration of the trial. The activity of LDH in LLCR roots under hypoxia was low and remained relatively steady for the duration of the experiment (Fig. 9B). These data support those indicating that LDH activity increased substantially under flooding or hypoxia only in ULCR roots (Fig. 7B).

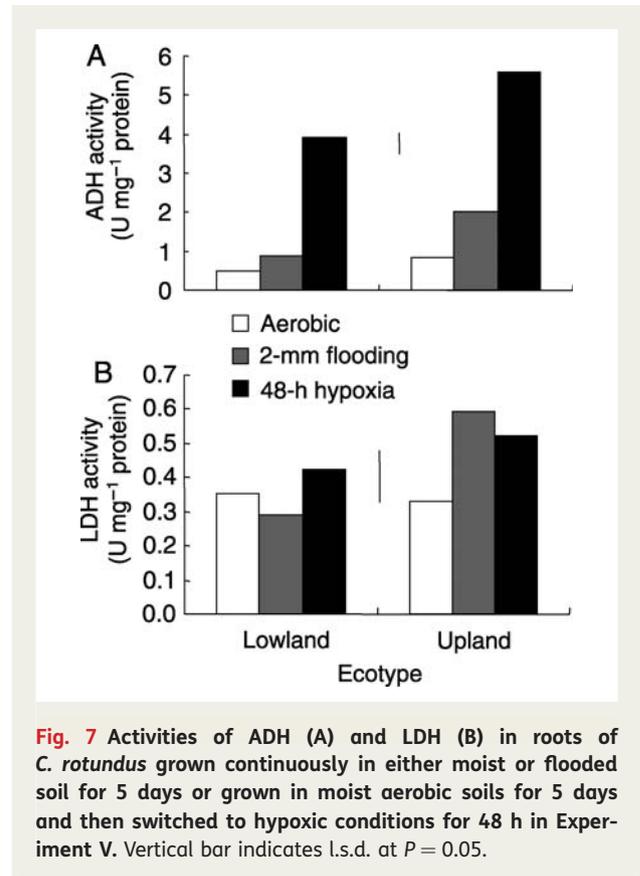


Fig. 7 Activities of ADH (A) and LDH (B) in roots of *C. rotundus* grown continuously in either moist or flooded soil for 5 days or grown in moist aerobic soils for 5 days and then switched to hypoxic conditions for 48 h in Experiment V. Vertical bar indicates l.s.d. at $P = 0.05$.

Discussion

Our studies indicate that adaptation of the LLCR ecotype to flooding is associated with a set of morphological, anatomical and physiological features that distinguish it from ULCR.

Growth, morphology and anatomy

Variation in tolerance of flooded conditions in these two ecotypes may involve processes triggered after tuber sprouting. Sprouting of LLCR tubers in the field probably starts during the 10–15 days of land preparation before rice transplanting, and when the soil is still sufficiently aerobic. If this is the case, sprouting of tubers might be suppressed if farmers maintained anaerobic soil conditions during land preparation before transplanting or direct seeding.

Faster growth of LLCR will ensure a competitive advantage to establish it as a weed in flooded rice fields. The LLCR ecotype produced tubers that are more than three times larger than those of ULCR, and with an increasing tendency to invest more in tuber growth with increasing severity of flooding (Fig. 2B). These data suggest the importance of the initial investments in tuber growth as well as the ability to maintain faster

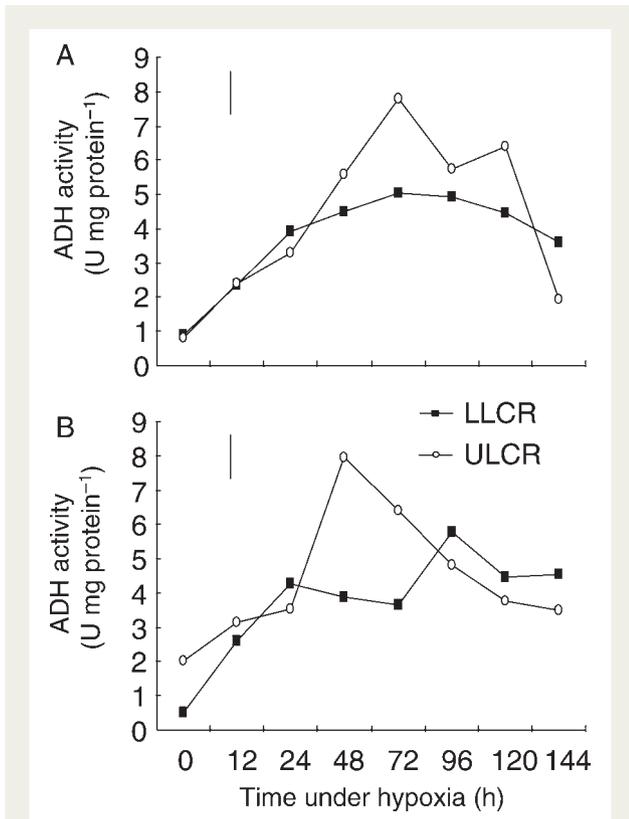


Fig. 8 Activities of ADH in roots of LLCR and ULCR ecotypes grown in their (A) native and (B) switched conditions for 5 days and then subjected to hypoxic treatment for 144 h in Experiment IV. Vertical bar indicates l.s.d. at $P = 0.05$.

growth in flooded soil conditions. Earlier studies reported similar findings (Casimero et al., 1999; Peña-Fronteras et al., 2009); however, the interesting tendency of LLCR to produce larger tubers with increasing flooding stress was not reported previously, and reflects the plasticity of this ecotype to respond to particular flooding conditions even at this early growth stage. Given that the tubers are the main means of dispersal in this species, this ability of LLCR to divert more carbohydrates to developing tubers in flooded soils could provide a distinct advantage for subsequent establishment in flooded soils. The greater growth of LLCR, even under moist soil conditions, suggests that this ecotype may succeed ULCR in lowland and upland conditions unless the ULCR has other growth advantages (e.g. variable tuber dormancy or drought tolerance) that allow it to compete and survive effectively in aerobic soils.

During sprouting and early growth, sprouts are dependent on stored carbohydrates and soluble sugars in the tubers, and the ability to mobilize these for growth. With the lower efficiency of anaerobic compared with aerobic respiration, the former pathway is largely

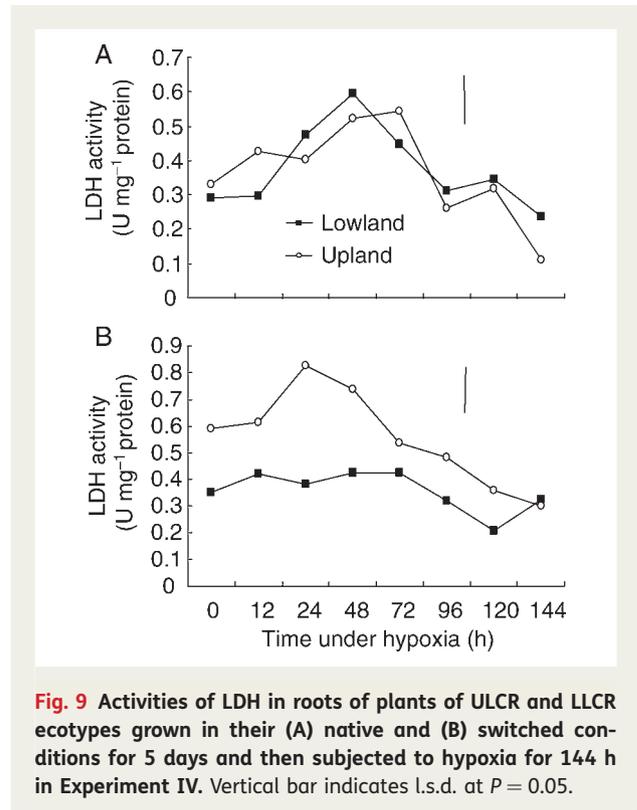


Fig. 9 Activities of LDH in roots of plants of ULCR and LLCR ecotypes grown in their (A) native and (B) switched conditions for 5 days and then subjected to hypoxia for 144 h in Experiment IV. Vertical bar indicates l.s.d. at $P = 0.05$.

reliant on an adequate supply of substrates and their efficient use. The larger tubers enable LLCR to store greater amounts of carbohydrates and soluble sugars, and this is probably an important adaptive feature to enable establishment in flooded soils. Crawford (1978) observed that plants tolerant of waterlogged soil have larger rhizomes and tubers than flood-sensitive plants. Furthermore, seeds of *Echinochloa crus-galli* var. *oryzicola*, which germinates and grows in flooded rice fields, are three to five times larger than seeds of *E. crus-galli* var. *praticola*, which germinates and grows only in upland soil (Yamasue, 2001).

A distinct and possibly important adaptive feature in flooded conditions is the larger diameter of stems of LLCR (Fig. 3), coupled with the increased proportion of air spaces or aerenchyma (Fig. 4). Aerenchyma contains large air spaces (Evans, 2003) and has long been associated with the enhanced performance of plants grown in oxygen-deficient environments (Visser et al., 2000). Large air spaces in the stems of LLCR may improve flooding tolerance by facilitating oxygen diffusion to the roots and submerged parts of the shoot.

Carbohydrate reserves and their utilization

Carbohydrate content and the pattern of its use during flooding differed between the two ecotypes. The LLCR

ecotype accumulated much more carbohydrate per tuber and maintained its ability to use these stored reserves during early growth in flooded soil. This is apparent from the progressive decrease in starch content, with maintenance or even an increase in soluble sugars during the first 6 days of growth under hypoxia (Figs 5 and 6). In contrast, both sugar and starch contents per tuber in ULCR were much lower, and remained more or less unchanged over the 6 days of hypoxia following tuber sprouting. Retaining the ability to break down and use these stored reserves under low-oxygen stress is also fundamental for survival, as most of the enzymes involved in starch catabolism became inactive when oxygen was not adequate (Perata *et al.*, 1992; Ismail *et al.*, 2009). Maintenance of high carbohydrate reserves and retaining the capacity to use them under low-oxygen stress could contribute considerably to LLCR's ability to flourish under lowland conditions.

To survive prolonged hypoxia, plants must have an adequate supply of fermentable substrates to fuel the anaerobic fermentation pathway (Gibbs and Greenway, 2003). This is particularly critical in early growth stages, before photosynthesis begins, and while the plant depends solely on stored reserves. The ability of LLCR to mobilize carbohydrate reserves in the tubers suggests the induction of amylolytic enzymes under oxygen-deficit conditions (Peña-Fronteras *et al.*, 2009). Peña-Fronteras *et al.* observed higher amylase activity and soluble sugar content in LLCR than in ULCR subjected to hypoxia for 48 h. Degradation of starch reserves under hypoxia was reported in flood-tolerant crops like rice, but not in upland crops that are intolerant of flooding such as wheat and barley. This may be due to the inactivity or absence of degrading enzymes in these crops when oxygen is deficient (Guglielminetti *et al.*, 2001; Ismail *et al.*, 2009). Starch breakdown in rice seeds during germination is governed by the activities of α -amylase, β -amylase, debranching enzyme and α -glucosidase (Guglielminetti *et al.*, 1995), and an α -amylase gene (*RAmy3D*) was reported to have higher expression under low-oxygen stress in rice genotypes tolerant of hypoxia during germination (Ismail *et al.*, 2009). Perata *et al.* (1992) observed that starch breakdown in flood-intolerant plants is arrested when oxygen is deficient. For ULCR, amylolytic enzyme activity may have been inhibited under hypoxic conditions.

Activity of the enzymes ADH and LDH

In an earlier study (Peña-Fronteras *et al.*, 2009), the significance of induction of the anaerobic respiration pathway in adaptation to flooded soils was established; however, the measurements were made only up to 48 h. In the current study, we examined the activities of

ADH (the major limiting enzyme during the alcohol fermentation pathway), and of LDH (which alternatively converts pyruvate to lactic acid), over 6 days to assess the roles of the two pathways in tolerance of hypoxia in these *C. rotundus* ecotypes. We observed the induction of ADH in both ecotypes when grown in flooded soils, but with greater activity in ULCR. Increased activity of ADH in both ecotypes following flooding or hypoxia suggests a shift in their metabolic activities from aerobic respiration to alcohol fermentation under low-oxygen stress (Agarwal and Grover, 2006). Furthermore, we observed a greater increase in enzyme activity in plants grown in aerobic soil before the shift to hypoxia than in plants already growing in flooded soil before transfer to the hypoxic media, particularly in ULCR (Fig. 7A). The substantial increase in ADH upon shifting to hypoxic conditions is probably adaptive, but this might result in faster depletion of the already limited soluble sugars or fermentative reserves in ULCR. On the other hand, LDH showed a significant increase only in roots of the ULCR when grown in flooded soils or switched to hypoxic conditions (Fig. 7B). This might result in an increase in lactic acid production, leading to cellular acidosis. Low oxygen concentration has been reported to increase ADH and LDH activities by up to 5-fold in the roots of the marsh plants *Glyceria maxima* and *Senecio aquaticus*, and in rice roots (Smith and Ap Rees, 1979; Rivoal *et al.*, 1991). This increase in ADH and LDH activities is probably critical to maintain sufficient ATP through the anaerobic respiration pathways for seedling growth (Chen and Qualls, 2003).

In addition to LLCR's ability to mobilize starch into soluble sugars, the capacity to regulate ADH activity is a notable physiological adaptation that enables anaerobic fermentation to be sustained over a longer period, and possibly allows emergence of shoots from flooded soils. These findings concur with those of Peña-Fronteras *et al.* (2009). Furthermore, the current study shows that enzyme activity in both ecotypes peaked at 48–72 h, although ADH activity in LLCR was maintained at a much lower level than in ULCR. This could conserve sugar reserves in LLCR tubers while sustaining optimum anaerobic respiration during prolonged flooding. The lower sugar content of ULCR and the limited ability to mobilize starch into soluble sugars under hypoxia (Ismail *et al.*, 2009; Peña-Fronteras *et al.*, 2009) mean that available sugar reserves in the tubers could be rapidly depleted, resulting in a rapid decline in ADH activity after 72 h of hypoxia. This could involve a feedback response caused by a depletion of soluble sugars and inability to sustain anaerobic respiration.

The activity of ADH was considerably greater (8- to 11-fold) than that of LDH under hypoxia, indicating

that alcohol fermentation is probably the dominant pathway under anaerobic conditions in this weed. Alcohol dehydrogenase catalyses the conversion of acetaldehyde, which is toxic to plants, to ethanol, and regenerates NAD^+ to maintain glycolysis and substrate-level phosphorylation under anaerobic conditions (Davies, 1980). Ethanol accumulation can be prevented as it is soluble in the lipid bilayers and readily diffuses to the surrounding solution where it is diluted or metabolized by microorganisms (Drew, 1997). Lactate dehydrogenase catalyses the formation of lactate from pyruvate, which can result in cellular acidosis. Down-regulation or inhibition of LDH activity under hypoxia could, therefore, prevent cellular acidosis. The role of LDH in flooding tolerance may be explained by the Davies–Roberts pH stat hypothesis (Davies et al., 1974; Roberts et al., 1985). The LLCR ecotype appears more able to regulate lactic acid formation when subjected to low-oxygen conditions than ULCR (Figs 7 and 9B), which could partially account for differences in flooding tolerance.

Adaptive features of LLCR to flooding: are they constitutive or stress induced? The ability of LLCR to thrive in flooded conditions could be due to traits that are either expressed constitutively or induced by flooding stress, or a combination of these. Traits that differentiate the two ecotypes are expressed regardless of the growth conditions, although the extent of the expression in some of them is affected by the severity of the stress. For example, LLCR is taller and produces more biomass, larger tubers, more non-structural carbohydrate reserves, larger stems and higher proportions of air spaces in stems and roots. This ecotype also retains the ability to break down and use stored carbohydrates in the tubers (Figs 5 and 6) for shoot and root growth when grown in flooded soils. The activity of LDH in LLCR under flooded/hypoxic conditions appears moderated compared with ULCR (Figs 7 and 9), which may prevent the build-up of lactic acid and avoid its toxic effects. Apparently, the expression of some of these adaptive traits is enhanced in LLCR with low-oxygen stress, such as its tendency to divert more carbohydrates and produce progressively larger tubers with increasing depth of floodwater (Fig. 2), and the increased proportion of air spaces, particularly in stems, to facilitate oxygen diffusion to the submerged plant parts (Figs 3 and 4).

Conclusions and forward look

The presence of flood-tolerant ecotypes of *C. rotundus* means that in such cases flooding is no longer a viable management option against this weed once it has

sprouted. The adaptation of *C. rotundus* to flooded conditions seems to involve numerous shifts in growth and metabolism, including: (i) bigger tubers with higher carbohydrate and soluble sugar content, indicating a tendency to invest more in tuber growth in flooded conditions; (ii) larger stems and more air spaces or aerenchyma to facilitate oxygen diffusion to submerged plant parts; (iii) capacity to mobilize carbohydrate reserves and use them to generate energy through anaerobic respiration; (iv) capacity to optimize the use of carbohydrate reserves by controlling the activities of key enzymes such as ADH; and (v) down-regulation of LDH activity, possibly to prevent lactate accumulation and avoid cellular acidosis.

Because adaptive mechanisms involve changes in genetic composition or in gene frequencies within populations (Clements et al., 1983), it is possible that flooding tolerance in LLCR involves inherent changes in these characteristics. In flood-tolerant *E. crus-galli*, for example, germination under anaerobic conditions was suggested to be regulated by several genes (Fukao et al., 2003). If the flooding adaptation mechanism in *C. rotundus* is genetically controlled by a few major quantitative trait loci or genes, further elucidation of these mechanisms could lead to the identification of genes responsible for its flooding tolerance, which, in turn, could contribute to the development of submergence tolerance in crops and also possibly designing better control measures for this weed. Information gained from studying the tolerance mechanisms of various abiotic stresses in weeds could contribute to our understanding of the physiological and genetic basis of plant adaptation to adverse environments, as well as in designing effective weed management strategies, both of which may be used to enhance crop yield (Cooper and Hammer, 1996).

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Contributions by the authors

R.G.F. undertook the experimental work and laboratory analyses; all authors contributed to the planning of the research and to the manuscript.

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Conflict of interest statement

None declared.

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