Anoxia tolerance in the aquatic monocot Potamogeton pectinatus: absence of oxygen stimulates elongation in association with an unusually large Pasteur effect

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Abstract

Elongation by stems of overwintered tubers of Potamogeton pectinatus (L.) is strongly promoted over several days by oxygen-free conditions. Characteristics of the respiration underpinning this unusual response were examined. Anaerobic plants produced ethanol and CO₂ in approximately equimolar amounts, indicating that glycolysis coupled to alcoholic fermentation was the principal CO₂-producing respiratory pathway. Rates of CO₂ evolution by aerobic and anaerobic whole plants (shoot and tuber) were similar, suggesting a rate of glycolysis three times that of aerobic plants, i.e. a strong Pasteur effect. In the shoot alone, anaerobic CO₂ production was twice the aerobic rate indicating a 6-fold increase in the rate of glycolysis in this tissue. Anoxic stems contained more sucrose at a stronger concentration than slower-growing aerobic stems or anaerobic leaves, demonstrating that sugar supply to the site of most rapid growth exceeded demand in the absence of oxygen. Concentrations of potentially toxic acetaldehyde in the external medium were small (approximately 0.2 mol m⁻³) during anoxia and on return to aerated conditions. Lactic acid was undetectable under anaerobic conditions and in vivo ³¹P-NMR analysis of shoots revealed a cytoplasmic acidification of only ≤ 0.2 pH units. In contrast, shoots of Pisum sativum, an anoxia-intolerant species, showed much stronger cytoplasmic acidification when transferred to oxygen-free conditions.

Key words: Anaerobiosis, aquatic plants, cytoplasmic acidification, ethanolic fermentation, nuclear magnetic resonance (NMR) spectroscopy, Pasteur effect, wetland plants.

Introduction

Potamogeton pectinatus is an aquatic monocot that overwinters as small tubers in the beds of lakes and rivers where oxygen supply can be severely limited or extinguished. A notable feature is that elongation by the terminal shoots of tubers is strongly promoted by anaerobic conditions. The response is highly unusual since the sudden imposition of this stress kills growing cells of most other species in only a few hours (e.g. Arabidopsis shoots; Ellis et al., 1999). The growth-promoting effect of anoxia in P. pectinatus is located in the stem rather than the leaves and can be sustained for up to 2 weeks (Summers and Jackson, 1994). Furthermore, this anaerobic growth is supported by cell divisions and is susceptible to growth-regulating influences of hormones and gravity normally associated with aerobic cell expansion (Summers and Jackson, 1994, 1996). The resulting strong, directional, anaerobic elongation provides submerged P. pectinatus with the means to escape from the sediment of streams and lakes into better-lit, oxygenated conditions closer to the water surface.

Many wetland or riverine species are well-adapted to the low-oxygen conditions which naturally occur in their habitat, and some can tolerate several months of total anoxia (Barclay and Crawford, 1982). However, in most other species studied hitherto, anaerobic shoot elongation is much slower than in well-aerated conditions (e.g.
Schoenoplectus lacustris, Barclay and Crawford, 1982). Exceptions are the rice coleoptile where a relatively small and short-lived stimulation is sometimes observed (Alpi and Beevers, 1983; Perata et al., 1992) and Potamogeton distinctus, a species very similar to _P. pectinatus_ (Ishizawa et al., 1999).

Anoxia-tolerance can only provide temporary protection against anaerobic surroundings and plants must gain access to oxygen (and carbon dioxide) for continued survival. Whether anoxia tolerance is of long duration (as in _P. pectinatus_) or much shorter (as in most land plants), cells must employ specialized biochemical and metabolic means to survive during the temporary absence of oxygen. This is all the more evident for _P. pectinatus_ as it extends its stem abnormally quickly as part of the shoot growth shown by this species under oxygen-free conditions.

In the present work, an attempt was made to identity the major respiratory pathway of _P. pectinatus_ under anaerobic conditions. The available respiratory substrates and other metabolic features were also assessed, such as rate of glycolysis, lactate formation and cytoplasmic acidosis that underpin the unusually strong and sustained shoot growth shown by this species under oxygen-free conditions.

### Materials and methods

#### Plant material and growing conditions

Tubers of _Potamogeton pectinatus_ (L.) were collected from a shallow stretch of the River Evenlode, Oxfordshire, UK and stored in damp grit at 2 °C in the dark until required. All experiments were performed within 6 months of collection. Individual tubers of similar mass were weighted down by approximately 6 g of 5 mm diameter glass ballotini in 150 mm tall, 23 × 10⁻⁶ m³ test tubes and grown on in darkness. An anaerobic workstation (Forma Scientific, Inc., Ohio, USA) was used to provide continuous and verifiable oxygen-free conditions (Summers and Jackson, 1994). Control plants were grown similarly but in an aerobic workstation. Shoot extension was measured over the 5 d following the onset of elongation. This usually commenced 1 d after transferring the tubers from cold storage to the 20 °C conditions of the workstations. Anaerobic treatment involved maintaining the plants either in a humid anaerobic gas phase, or in water that had been de-oxygenated within the workstation for 24 h. Sparging submerged plants with air (5 × 10⁻³ m³ min⁻¹), or growing the shoot in humid air provided fully aerated conditions.

Seeds of _Pisum sativum_ L., cv. ‘Meteor’ were soaked in tap water overnight, sown in moist vermiculite and grown in the dark for 7 d at 20 °C. Internodal tissue from pea plants was used for comparison with _P. pectinatus_ in the ³¹P-NMR experiments.

#### Gas chromatographic determination of sugars

Plant material was frozen, ground in liquid nitrogen and then homogenized in 5 × 10⁻⁶ m⁻³ 80% (v/v) ethanol. After centrifuging at 2500 g for 5 min, 1 × 10⁻⁹ m³ of supernatant and 300 × 10⁻⁹ m³ of 0.06% (w/v) internal standard (phenyl β-D-glucopyranoside) were taken to dryness in vacuo using a Speedvac Concentrator (Savant Instruments Inc., Hicksville, New York, USA). A two-stage derivatization procedure was adopted (Toba and Adachi, 1977) in which conversion of the reducing sugars to their oximes was followed by silylation of the hydroxyl groups to form trimethylsilylated (TMS) ethers. 20 × 10⁻⁹ m³ of hydroxylamine hydrochloride (BDH Ltd., Poole Dorset, UK) in pyridine (2.5 × 10⁻³ g m⁻³) was added to the residue and the mixture heated at 75 °C for 30 min. The sample was cooled to room temperature and 20 × 10⁻⁹ m³ of N-trimethylsilyl imidazole (Pierce, Rockford, Illinois, USA). However, with ethanolic fermentation problems can arise with acetaldehyde generation. Because of its effects on protein cross linkages acetaldehyde is much more toxic than ethanol (Perata and Alpi, 1991; Pfister-Sieber and Brändle, 1994). Moreover, it may be especially damaging to flooded or submerged plants when generated by the oxidation of accumulated ethanol after anoxic tissues are returned to aerated conditions (Monk et al., 1987).

While the efficiency of aerobic respiration, anaerobic fermentation is often strong in animal cells (Hochachka, 1991), in plants, the increase is usually only 3-fold at the most (Tsuji, 1968). After only a few hours anoxia, death of previously growing tissues occurs in intolerant species; a shortage of ATP presumably being a major contributing factor. This may also be one of the reasons why growth in anaerobic conditions is slowed in more tolerant wetland species. Other factors are also implicated in anoxia injury. These include cytoplasmic acidosis (Roberts et al., 1984a, b, 1985), the accumulation of toxic metabolites such as acetaldehyde and shortage of respirable substrates. Acidosis can arise, in part, from unregulated lactate fermentation, making it a potentially more harmful alternative to ethanolic fermentation.

During anoxia, energy (ATP) and chemical oxidizing power (e.g. NAD⁺) must be generated via pathways that do not use oxygen as an acceptor of reductant. Such pathways include lactate- and ethanol-fermentation. The accumulation of compounds such as arginine, asparagine, alanine, glutamine, γ-aminobutyric acid (GABA), malate, and glycerol has also been observed in anoxic tissues (Crawford, 1978; Henzi and Brändle, 1993). However, no major differences in energy-generating anaerobic pathways between tolerant and intolerant species have been discovered so far (Monk et al., 1984). It appears that glycolysis linked to ethanol fermentation (and to a lesser extent, lactate fermentation) is the principal means of supporting the re-oxidation and recycling of NADH needed to maintain glycolysis, ATP production and substrate level phosphorylation. Thus, it seems that anoxia-tolerance per se is a question of metabolic regulation rather than possession of novel, alternative energy-generating pathways (Henzi and Brändle, 1993).

In comparison with aerobic respiration, anaerobic fermentation is very inefficient. Only two molecules of ATP per molecule of glucose oxidized are generated, releasing only 5% of the ATP generated by aerobic respiration. To offset some of the approximately 95% loss in energy production, the rate of sugar oxidation through glycolysis and ensuing fermentation can increase (Paster effect). While the effect is often strong in animal cells (Hochachka, 1991), in plants, the increase is usually only 2–3-fold at the most (Tsuji, 1968). After only a few hours anoxia, death of previously growing tissues occurs in intolerant species; a shortage of ATP presumably being a major contributing factor. This may also be one of the reasons why growth in anaerobic conditions is slowed in more tolerant wetland species. Other factors are also implicated in anoxia injury. These include cytoplasmic acidosis (Roberts et al., 1984a, b, 1985), the accumulation of toxic metabolites such as acetaldehyde and shortage of respirable substrates. Acidosis can arise, in part, from unregulated lactate fermentation, making it a potentially more harmful alternative to ethanolic fermentation. However, with ethanolic fermentation problems can arise with acetaldehyde generation. Because of its effects on protein cross linkages acetaldehyde is much more toxic than ethanol (Perata and Alpi, 1991; Pfister-Sieber and Brändle, 1994). Moreover, it may be especially damaging to flooded or submerged plants when generated by the oxidation of accumulated ethanol after anoxic tissues are returned to aerated conditions (Monk et al., 1987).

In the present work, an attempt was made to identify the major respiratory pathway of _P. pectinatus_ under anaerobic conditions. The available respiratory substrates and other metabolic features were also assessed, such as rate of glycolysis, lactate formation and cytoplasmic acidosis that underpin the unusually strong and sustained shoot growth shown by this species under oxygen-free conditions.
Carbon dioxide production
Carbon dioxide production rates were estimated by placing individual plants (tuber with attached shoot) or excised shoots in 20 × 10⁻⁶ m³ test-tubes and excised tubers in 5 × 10⁻⁶ m³ Erlemeyer flasks. Vessels contained approximately 0.5 × 10⁻⁶ m³ distilled water and were sealed with ‘Subaseal’ puncture caps (Roslav, Macclesfield, UK) within the aerobic or anaerobic workstations. After 4 h, 1 × 10⁻⁶ m³ gas samples were removed from the headspace and analysed for CO₂ using a Pye Unicam 104 (Cambridge, UK) gas chromatograph (sensitivity approximately 2.0 mmol m⁻³) fitted with a thermal conductivity detector. A standard mixture of ethanol, acetaldehyde, and iso-propanol was chromatographed to determine retention times and relative response factors. These measurements gave instantaneous values of CO₂ production rates. Alternatively, samples were taken every 40 min for up to 6 h. Slopes from time-courses of CO₂ accumulation were used to estimate production rates.

Ethanol, acetaldehyde and lactic acid
The concentration of ethanol and acetaldehyde in the external solution of submerged plants was determined in 1 × 10⁻⁶ m³ samples, to which known volumes of an internal standard (iso-propanol) were added. Aliquots were injected into a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector. A standard mixture of ethanol, acetaldehyde, and iso-propanol was chromatographed to determine retention times and relative response factors.

After the onset of shoot elongation, plants were tested for their growth response to exogenous ethanol by submerging them in unsparged solutions of 0, 10, 50 or 100 mol m⁻³ ethanol in the aerobic workstation. Plants were grown for a further 5 d with solutions refreshed daily to replace any ethanol lost by metabolism.

Lactic acid in the external solution of plants growing anaerobically and aerobically was determined enzymatically using a lactate diagnostic kit (Sigma, UK) (sensitivity approximately 22 mmol m⁻³).

³¹P-NMR of dark-grown internodes of P. pectinatus and Pisum sativum
Batches of P. pectinatus were grown in the dark, in unsparged water, where oxygen concentration fell below ambient (21 kPa) but remained above 8 kPa. These conditions promoted elongation sufficiently to provide stem material long enough to traverse the sampling window of a 10 mm diameter NMR tube. After 5 d, plants were placed overnight in a large tank of continuously aerated water. When required, 25 mm long stem sections were cut and vacuum-infiltrated for 3 min in 10 mol m⁻³ MES/0.1 mol m⁻³ CaSO₄ buffer (pH 6.15). Vacuum infiltration improved resolution and reproducibility of NMR spectra by displacing the air from the gas-filled spaces within the tissue. Air spaces give rise to magnetic field gradients in an NMR magnet, resulting in undesirable line broadening, and this effect was particularly noticeable for the aerenchymatous stem segments of P. pectinatus. After infiltration, 10–12 sections of P. pectinatus were transferred to the NMR tube. MES buffer, sparged with 100% oxygen, was circulated through the system at 6 × 10⁻⁶ m³ min⁻¹ and the circulation was augmented by an airlift system operated with a flow rate of 50 × 10⁻⁶ m³ oxygen min⁻¹. The use of pure oxygen rather than air ensured that the tightly packed sample was fully oxygenated in the NMR tube. Stem tissue was allowed to equilibrate for 30 min before starting the NMR analysis and the spectra were recorded at 121.49 MHz using a double-tuned ³¹P 10 mm diameter probehead as described previously (Fox et al., 1995). Anaerobic conditions were established within the plant tissue by sparging the circulating buffer with nitrogen and by switching the gas flow in the airlift system to nitrogen. The absence of oxygen in the NMR tube was confirmed by passing the outflowing buffer over an oxygen electrode (Radiometer, Copenhagen, Denmark) linked to an oxygen meter (Model 781 Strathkelvin Instruments, Glasgow, Scotland). During the aerobic period, oxygen partial pressure in the outflow buffer was 99.4 kPa. Anaerobic conditions were achieved within 10 min by switching the sparge gas from oxygen to nitrogen. For comparison with anaerobically tolerant P. pectinatus, batches of seven 25 mm long sections from the second internode of 7-d-old dark-grown seedlings of Pisum sativum were also analysed. Pea tissue was not vacuum-infiltrated and the suspending medium comprised 30 mol m⁻³ glucose, 10 mol m⁻³ MES and 0.1 mol m⁻³ CaSO₄ (pH 6.0) in accordance with conditions used previously for successful NMR analysis of pea internode tissue (Talbot et al., 1988).

Statistical analysis
Computations of least significant differences (LSD, P = 0.05) were based on analyses of variance. Means quoted in the text carry their associated standard errors (SE).

Results
Sucrose content and concentration
Fructose, glucose and galactose were present in extracts of plants taken directly from storage and in plants grown for 6 d in anaerobic or aerobic environments but concentrations were too small to be estimated accurately. However, concentrations of sucrose in tubers taken directly from storage was 13.6 ± 3.8 mg g⁻¹ FW, the average content of each tuber being 1.5 ± 0.5 mg (Fig. 1). After 6 d growth, concentration and content of tubers had decreased to 4.0 ± 0.7 mg g⁻¹ FW and 0.4 ± 0.08 mg per tuber and were similar in both aerobic and anaerobic treatments (P < 0.05). Sucrose concentrations in shoots of plants taken directly from cold storage were much smaller than in tubers. Overall, the amount in shoots was less than 2% of that in tubers. At this time, approximately 80% of all shoot sucrose was located in the stem where the concentration was 1.2 ± 0.3 mg g⁻¹ and the content 0.02 ± 0.003 mg. During 6 d of aerobic conditions, the sucrose content of stems changed very little, although concentration fell by half, partly because of dilution by tissue growth. In contrast, sucrose content of aerobic stems increased 6-fold to 0.12 ± 0.03 mg, although dilution caused by the additional extension growth reduced the impact on concentration to a 1.5-fold increase. The sucrose content of aerobic leaves increased nearly 5-fold over the 6 d of aerobic growth although, because of dilution caused by growth, the concentration remained
similar (anaerobic, 2.13 ± 0.16; aerobic, 1.71 ± 0.53 μmol kg\(^{-1}\) s\(^{-1}\), \(n = 7\), LSD = 0.75)).

In a separate experiment, plants were again grown for 6 d in aerobic or anaerobic conditions. Shoots were separated from tubers in situ, and CO\(_2\) production of each part measured separately every 40 min for 200 min (Fig. 2). Production (mmol kg\(^{-1}\) FW s\(^{-1}\)) was calculated from the mean of slopes of regression lines generated for individual plants. Carbon dioxide production by anaerobic shoots was 3.8 ± 0.45 μmol kg\(^{-1}\) FW s\(^{-1}\) and twice that of aerobic shoots (1.9 ± 0.15 μmol kg\(^{-1}\) FW s\(^{-1}\) (LSD = 1.1)). However, production rates of tubers were similar irrespective of treatment (0.78 ± 0.15 and 1.00 ± 0.20 μmol kg\(^{-1}\) FW s\(^{-1}\) in anaerobic and aerobic conditions, respectively (LSD = 0.58)). Despite faster shoot CO\(_2\) production in anaerobic plants, the output calculated on a whole plant basis was not significantly different under the two treatments (1.83 ± 0.26 and 1.34 ± 0.16 μmol kg\(^{-1}\) FW s\(^{-1}\) in anaerobic and aerobic conditions, respectively (LSD = 0.35)).

Fig. 1. Sucrose content and concentration of tubers, stems and leaves of plants taken directly from 2 °C storage conditions (hatched) and 6-d-old plants grown in aerobic (open) or anaerobic (closed) conditions at 20 °C (\(n = 5\)). Vertical lines represent standard errors. LSDs (\(P = 0.05\)) for sucrose content of the tuber, whole plant, stem and leaf are 0.87, 0.88, 0.053, and 0.011, respectively, and 7.4, 6.6, 1.0, and 0.46 for sucrose concentration.

Fig. 2. Time-course of carbon dioxide evolution from excised shoots and tubers from 6-d-old plants grown in anaerobic or aerobic conditions at 20 °C. Each symbol represents the performance of one of five individuals. For each individual shoot or tuber \(R^2\) values ranged between 0.85 and 0.99. Linear slopes are mean slopes of regression lines generated for each replicate. Units of the calculated mean slope (b) are μmol kg\(^{-1}\) FW s\(^{-1}\).
conditions, respectively, LSD = 0.69), as the shoot constituted only 37% of total fresh weight. Whole plant production rates obtained by combining tuber and shoot results were comparable to those measured in intact plants (see above).

**Ethanol and acetaldehyde measurements**

The concentration of ethanol and acetaldehyde in the external medium of batches of plants growing in anaerobic and aerobic conditions was measured daily for 5 d. There were no statistically significant differences in acetaldehyde concentrations between the two treatments and concentrations remained below approximately 0.2 mol m\(^{-3}\). In contrast, significant differences in ethanol were measured between treatments and on a day-to-day basis. In fully aerated conditions, ethanol concentrations in solution plants were drained, re-filled with fresh deoxygenated water and capped for ethanol analysis. Carbon dioxide evolution was measured at intervals of 40 min for 6 h, starting 3 d after removing from cold storage (Fig. 3). Test-tubes were drained of water and from 0 to 0.48 ± 0.04 mol m\(^{-3}\) (production rate of 0.94 ± 0.15 μmol kg\(^{-1}\) FW s\(^{-1}\)) when compared, ethanol and CO\(_2\) production rates (slopes of the linear regressions in Fig. 3) were statistically similar (LSD = 0.39).

In a separate experiment, ethanol and CO\(_2\) production under anaerobic conditions were measured at intervals of 40 min for 6 h, starting 3 d after removing from cold storage (Fig. 3). Test-tubes were drained of water and either capped and CO\(_2\) in the headspace analysed by gas chromatography or refilled with fresh deoxygenated water and capped for ethanol analysis. Carbon dioxide in the headspace increased linearly over this 6 h period at a rate of 0.64 ± 0.12 μmol kg\(^{-1}\) FW s\(^{-1}\), whilst ethanol concentration in the refreshed solution rose from 0 to 0.48 ± 0.04 mol m\(^{-3}\) (production rate of 0.94 ± 0.15 μmol kg\(^{-1}\) FW s\(^{-1}\)). When compared, ethanol and CO\(_2\) production rates (slopes of the linear regressions in Fig. 3) were statistically similar (LSD = 0.39).

**Re-oxidation of ethanol to acetaldehyde**

To test the effect of re-aeration on acetaldehyde production, two batches of seven plants were first grown anaerobically for 3 d. During this period, acetaldehyde concentrations rose slightly to 0.43 ± 0.05 mol m\(^{-3}\) and ethanol increased to 2.34 ± 0.27 mol m\(^{-3}\). Plants were then transferred to the aerobic workstation, where the water from one batch was aerated with air at 10 × 10\(^{-6}\) m\(^3\) min\(^{-1}\) and sampled approximately every 40 min for 6 h, and again after a further 18 h. Acetaldehyde concentrations fell within the first 30 min to 0.21 mol m\(^{-3}\) and remained stable thereafter. Ethanol concentrations, however, decreased linearly to 1.89 ± 0.22 mol m\(^{-3}\) after 6 h and to 1.20 ± 0.18 mol m\(^{-3}\) after 24 h. The test-tubes containing the second batch of anaerobically-grown plants were drained, refilled with fresh aerated water and capped to prevent potential loss of acetaldehyde in the vapour phase. Acetaldehyde in solution was sampled every 40 min for 6 h. Once more, concentrations remained low and stable at approximately 0.09 mol m\(^{-3}\). Both experiments indicate that when oxygen is re-introduced, accumulation of any acetaldehyde formed from ethanol by re-oxidation is strongly suppressed.

**Ethanol toxicity and lactic acid production**

Plants were treated with solutions of ethanol ranging from 0–100 mol m\(^{-3}\) for 5 d. Treatment solutions were replaced each day. Total shoot elongation at the end of 5 d was unaffected by any of these concentrations (results not shown).

After 5 d growth in aerobic or anaerobic conditions, lactic acid concentration in all external solutions remained very small and below the detection limit of the assay (<22 mmol m\(^{-3}\)).

**31P-NMR of dark-grown Potamogeton pectinatus internodes**

*In vivo* 31P-NMR was used to monitor the effect of anoxia on the cytoplasmic pH of the *P. pectinatus* internodes. Figure 4 shows that with a switch from aerobic to anaerobic conditions had only minor effects on the 31P-NMR spectrum of *P. pectinatus* internodes. In contrast to less tolerant tissues, there was only a small decrease in the intensity of the NTP signals (peaks 4, 5 and 8) and only a very small shift, 0.15 ppm, in the pH-dependent position of the cytoplasmic Pi signal (peak 2). The shift in peak 2 corresponded to a fall in pH from 7.48 under aerobic conditions (Fig. 4A) to 7.32 (Fig. 4B) under anaerobic conditions, and in four similar experiments the maximum acidification never exceeded 0.2 pH units.

Figure 5 contrasts the small effect of anoxia on the cytoplasmic pH of *P. pectinatus* with the much larger acidification observed in anoxia-intolerant pea internodes. A 1 h anoxic treatment caused an upfield shift of 0.20 ppm in the cytoplasmic P\(_i\) signal from the *P. pectinatus* inter-
lysis the ratio of anaerobic CO$_2$ production:aerobic CO$_2$ production is 1/3.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$ (1)

$$C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_5OH$$ (2)

Any enhancement of this ratio is known as the Pasteur effect. In whole plants of _P. pectinatus_, this ratio was close to unity, i.e. glycolysis was running three times faster in anaerobic conditions than in air. In extending anaerobic shoots, CO$_2$ production was twice the aerobic rate (Fig. 2), indicating a 6-fold faster rate of glycolysis in this tissue. These are unusually large Pasteur effects for plants. The enhancement of the rate of glycolysis was just 1.5 in germinating rice (Taylor, 1942), 2 in maize root tips when anoxia was first imposed (Hole et al., 1992) and 3 for rice coleoptiles, (Mayne and Kende, 1986). A 4.5-fold increase has been claimed (Small et al., 1989) for germinating _Erythrina caffra_, but does not appear to be substantiated by their actual results. Such increases in the rates of glycolysis will go some way towards compensating for the shortfall in ATP generation inherent in anaerobic fermentation.

**Fueling the Pasteur effect**

Overwintering tubers of _P. pectinatus_ are rich in starch and smaller carbohydrates (Hodgson, 1966), making them a good food source for wildfowl (Kantrud, 1990). Breakdown of the starch to glucose (and on to sucrose for transport) presumably fuels the large Pasteur effect. Although starch breakdown was not measured, carbon dioxide production rates of 2 mmol kg$^{-1}$ FW s$^{-1}$ found in intact plants grown for 6 d in either anaerobic or aerobic conditions allow sucrose consumption by respiration to be calculated. The outcome is 17.3 mmol sucrose per plant (0.2 g) by aerobic respiration over 6 d (1 mol sucrose generating 12 mol CO$_2$) or 51.8 mmol sucrose by anaerobic respiration (1 mol sucrose generating 4 mol CO$_2$) assuming exclusive running of either the Krebs cycle or ethanolic fermentation. These amounts far exceed the decrease in free sucrose (approximately 1 mg or 2.92 mmol per plant) measured over this time in both aerobic and anaerobic plants (Fig. 1). The substantial shortfall can only be provided by new sucrose formation, with starch breakdown being its most likely source. Clearly the rate of starch breakdown would need to be at least three times faster in anaerobic plants which points to a strong promotion of starch hydrolysis by anoxia.

Although the total amount of sucrose in whole plants decreased similarly during 6 d in either anaerobic or aerobic conditions, the content in different shoot tissues depended on aeration treatment (Fig. 1). Leaf sucrose content was higher in plants grown in aerobic conditions, whereas stem sucrose content was higher in plants grown anaerobically. Under aerobic conditions there is substan-
Fig. 5. Resolution enhanced $^3$P-NMR spectra of stem sections of *P. pectinatus* (A–C) and *P. sativum* (D–F) showing the effect of oxygen deprivation. The 30 min spectra were recorded: (A, D) under aerobic conditions; (B, E) during the second 30 min following the switch to anoxia; and (C, F) during either the second 30 min (C) or the third 30 min (F) following reoxygenation after 1 h anoxia. The numbered peaks may be assigned to: 1, several phosphomonoesters, including glucose-6-phosphate (1a) and phosphocholine (1c); 2, cytoplasmic P$_i$; and 3, vacuolar P$_i$. The vertical lines emphasise the upfield shift in the cytoplasmic P$_i$ signal following the switch to anoxia.

Potential elongation by the leaf but very little by the stem but, in anaerobic conditions, more growth occurs in the stem (Summers and Jackson, 1994). Thus, sucrose is seemingly translocated preferentially to the fastest growing organs where substrate demand will be greater. In anaerobic plants, the accumulation of stem sucrose was reflected in an increased sucrose concentration. This contrasts with the aerobic leaf, where an increase in content (mass $\times$ concentration) was not associated with an increase in concentration. This stronger concentration indicates that, in anaerobic conditions, sucrose supply to the stem (a combination of synthesis and transport—both ATP requiring processes) not only matches, but exceeds the requirements for both the extra extension growth and the 6-fold enhancement of the rate of glycolysis. Clearly, anaerobic shoots are less sucrose-limited than are aerobic shoots.

Despite the large Pasteur effect in *P. pectinatus*, a considerable decrease in ATP production must inevitably occur under anaerobiosis and yet stem extension proceeds at a faster rate (Summers and Jackson, 1994, 1996). It is difficult to reconcile these seemingly contradictory attributes. Extension growth under anaerobic conditions may be less energy consuming than in aerobic conditions, in part, because cell division rates are halved (Summers and Jackson, 1994). Energy savings to support tuber respiration may also be achieved by using different enzymatic pathways to degrade starch. In seeds, including those of anoxic rice, degradation of starch seems to be mostly by hydrolytic enzymes that produce glucose (Perata et al., 1992, 1997, 1998), whereas starch-storing tubers largely mobilize starch by means of phosphorylase action. Here, phosphorylase catalyses the cleavage of α-1→4-glucosidic linkages in the presence of orthophosphate to produce glucose-1-phosphate directly (Stitt and Steup, 1985) without the need for ATP, thus by-passing the first step in glycolysis. The enzyme can operate at low oxygen partial pressures (Zhou and Solomos, 1998) and is active in rhizomes of *Schoenoplectus lacustris* during spring growth when oxygen levels can be small (Steinmann and Brändle, 1984).

**Anaerobic metabolism**

From measurements of ethanol concentration in the solution surrounding anaerobic plants, production was estimated at between 0.63 and 0.95 μmol kg$^{-1}$ FW s$^{-1}$. These rates are similar to those of anoxia-tolerant marsh species (Studer and Brändle, 1984), although five to six times slower than those obtained with anaerobically ger-
minating *Trapa natans* (Menegus *et al*., 1992). The stoichiometry of alcoholic fermentation is such that 2 mol each of carbon dioxide and ethanol are formed during anaerobic respiration of each glucose molecule (equation 2 above). Thus, the roughly equimolar ratio of ethanol:carbon dioxide production in anaerobic *P. pectinatus* (Fig. 3) suggests no substantial diversion of carbon into other pathways that generate carbon dioxide (e.g. pentose phosphate pathway). However, it is possible that some carbon was routed into pathways that do not generate carbon dioxide, e.g. synthesis of proteins and cell wall polymers and metabolites such as lactate, alanine, γ-aminobutyric acid (GABA), and malate. Production of lactate was minimal, but any synthesis of the other products listed above would only serve to increase the already substantial Pasteur effect since it would require still more sugar to be catabolized than is indicated by CO₂ and ethanol analyses alone.

**Regulation of acetaldehyde accumulation**

Ethanol is synthesized vigorously in anoxia-tolerant plants (Setter and Ella, 1994; Setter *et al.*, 1994). However, acetaldehyde, its immediate precursor, is much more toxic than ethanol (Perata and Alpi, 1991). Although ethanol was plentiful in the bathing medium where *P. pectinatus* was grown anaerobically, almost no acetaldehyde was detected. Thus, the *in vivo* activity of alcohol dehydrogenase (ADH) must match that of pyruvate decarboxylase (PDC) under anaerobic conditions. Moreover, there was no increase in acetaldehyde when tubers were moved to well-oxygenated conditions, despite previously high anaerobic ethanol production. Such re-exposure to oxygen is potentially hazardous (Crawford, 1992) as oxidation of anaerobically accumulated ethanol can cause rapid surges of acetaldehyde (Monk *et al.*, 1987; Pfister-Sieber and Brändle, 1994; Zuckermann *et al.*, 1997). Such high levels of acetaldehyde are thought to be produced by the ADH- and NAD⁺-dependent reverse conversion of ethanol and/or by hydrogen peroxide-dependent catalase-controlled peroxidation of ethanol. Clearly, *P. pectinatus* achieves an effective suppression of post-anoxic acetaldehyde accumulation thereby avoiding possible self-poisoning by this metabolite.

**Cytoplasmic acidification**

An acidification of the cytoplasm is commonly observed in anaerobic plant tissues (Ratcliffe, 1995, 1997, 1999) and prolonged acidosis in anoxia-intolerant plants leads to cell death (Roberts *et al.*, 1984a, 1985). However, in keeping with its remarkable adaptation to anoxia, stem segments of *P. pectinatus* showed only a minimal acidification of the cytoplasm in the absence of oxygen (Figs 4, 5). The fall of ≤0.2 pH units following a switch to anoxia is comparable to the 0.2 pH units reported for rice coleoptiles (Fan *et al.*, 1992), and it is much smaller than the 0.5–0.6 pH units observed for flooding-intolerant maize root tips (Roberts *et al.*, 1984a, b, 1985; Fox *et al.*, 1995) and the 0.7 pH units observed for pea internodes (Fig. 5). Moreover, the cytoplasmic pH of the *P. pectinatus* stem segments recovered much more rapidly than the pea internodes following re-oxygenation (Fig. 5). One practical consequence of the much greater sensitivity of the pea internodes to oxygen deprivation was that it was not possible to use the vacuum infiltration technique to eliminate the air spaces from the tissue before the NMR measurements. *P. pectinatus* stem segments recovered rapidly from the vacuum-infiltration, whereas vacuum-infiltrated pea internodes gave spectra with a poorly defined cytoplasmic Pᵣ signal corresponding to a range of cytoplasmic pH values (data not shown).

The relative stability of the cytoplasmic pH under anoxia is likely to be the net result of several biochemical and biophysical mechanisms (Ratcliffe, 1999) and these will need to be investigated in future work. However, one possibly significant observation reported here is the lack of detectable lactate in the growth medium of anaerobic plants. Assuming that this does not merely reflect the absence of a lactate carrier in the *P. pectinatus* plasma membrane, then it is possible that fermentation to lactate is only a minor pathway and this would have implications for the extent of cytoplasmic acidosis under anoxia. Caution is required, because the correlation between the extent of lactate production and cytoplasmic acidosis is not straightforward (Ratcliffe, 1995, 1997, 1999). But, it is at least possible that the limited acidification of the cytoplasm in *P. pectinatus* stems is the result of avoiding the utilization of the lactate fermentation pathway. Certainly other flooding tolerant species, such as *Trapa natans* and rice (Menegus *et al.*, 1989, 1991), produce little lactate, and *P. pectinatus* may be similar. The small cytoplasmic acidification in *P. pectinatus* also raises an important question about the regulation of PDC, since it has been established in maize roots that the pH change under anoxia plays a critical role in the activation of ethanol production (Roberts *et al.*, 1984b; Fox *et al.*, 1995). This will also have to be investigated further.

It is concluded that anaerobic promotion of stem extension in *P. pectinatus* is underpinned by vigorous fermentation associated with a large Pasteur effect, particularly in the shoot. Fermentation is largely ethanolic and associated with sucrose generation presumably derived from anaerobic breakdown of tuber starch. There is no marked accumulation of potentially toxic acetaldehyde under anaerobic and post-anoxic conditions and no evidence that other respiratory pathways such as lactate fermentation are strongly active. This may help to explain the absence of potentially damaging cytoplasmic acidification, a feature characteristic of less anoxia-tolerant species.
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