Adaptative Response of *Vitis* Root to Anoxia

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The effect of anoxia on the energy economy of root cells was studied by measuring heat production, ethanol and ATP production, K^+ fluxes and electrical activity in two *Vitis* species, *V. riparia and V. rupestris*, that differ in their tolerance to anoxia. Anoxia triggered a marked decrease of metabolic activity (measured by microcalorimetry) and of ATP levels in both species. In *V. riparia* after the first 2 h of anoxia, the decrease in the rate of heat production was not associated with a further significant decrease in ATP content, whereas in *V. rupestris* the ATP level continued to decrease until very low values were reached. The concomitant increase in the rate of ethanol production did not compensate for the decreased aerobic ATP supply. In *V. rupestris*, anoxia typically led to energy deficit and ATP imbalance, together with the subsequent disruption of ion homeostasis and cell death. In *V. riparia*, the strong decrease in K^+ membrane permeability together with the fast down-regulation of the electrical signals allowed the cells to avoid severe ion imbalances during prolonged anoxic episodes.

Keywords: Anoxia — Down-regulation — Electrical signals — Hochachka's 'hypoxia defense strategy' — Potassium flux (under anoxia) — *Vitis* spp.

Abbreviations: NS, Nutrient Solution; TZ, transition zone.

Introduction

Higher plants are obligate aerobic organisms needing a continuous supply of oxygen to support both respiration and other oxidation reactions. When air spaces in the soil become saturated with water under adverse environmental conditions (e.g. flooding or water logging), plants can experience oxygen deficiency because of the low solubility and diffusion rate of oxygen in water (Armstrong 1979).

During anoxia, the oxidative phosphorylation by the respiratory chain stops, and glycolysis becomes the only available route for ATP production. Further, the chemical oxidizing power (e.g. NAD^+) must be generated via pathways that use molecules other than oxygen as acceptors of reductant. It appears that fermentation processes such as ethanolic or lactate fermentation are the principal means of supporting the reoxidation and recycling of NADH during anoxic events (Hochachka 1991, Drew 1997).

Under anoxic conditions, plant cells can only produce ATP by anaerobic glycolysis, a process that yields 2–3 mol ATP mol^{-1} glucose. This should be compared with a yield of approximately 24–36 mol ATP mol^{-1} glucose during aerobic oxidative phosphorylation (Gibbs and Greenway 2003). Since during anoxia every molecule of glucose yields less than one-tenth of the ATP produced by oxidative phosphorylation, the energy demand of the plant tissues cannot be met by glycolysis alone. The consequent rapid fall in cellular ATP levels will cause a slow down, and eventually a stop in ion pump activity. Ion homeostasis is therefore lost and ions start running down their electrochemical potential gradients (Armstrong and Drew 2002). Despite this, roots of tolerant species such as *Vitis riparia* are able to tolerate anoxia for several hours/days, suggesting that cells adjust their ATP demand to the impaired ATP production.

Recent studies show that plants can decrease their oxygen consumption in response to oxygen shortage, thus avoiding internal anoxia (for a review see Geigenberger 2003). To offset the loss in energy production, anoxia-tolerant species appear to utilize two different strategies simultaneously: (i) increasing the glycolytic flux (Pasteur effect); and (ii) reducing the rate of ATP consumption (metabolic depression) (Geigenberger 2003, Greenway and Gibbs 2003, Van Dongen et al. 2003).

Although these mechanisms are well known on the basis of three or more decades of work, a number of unexplained problems have remained. In particular, which pathways of ATP consumption are down-regulated and by how much, and how membrane electrochemical potential gradients are stabilized remain unanswered questions. In a previous paper, the oxygen fluxes in the root of *Vitis* spp. with diverse tolerance to hypoxia/anoxia have been measured under low O_2 levels (Mancuso and Boselli 2002). In the present work, we extended our former findings, attempting to identify how *Vitis* root cells maintain energy equilibrium. Changes in metabolic activity that were linked to normoxia to anoxia transitions were quantified by microcalorimetry. The effect of anoxia on K^+ flux and K^+ permeability, and on the electrical activity of root cells was also determined.

The results presented show that the physiological adaptations that allow tolerant *Vitis* species to survive anoxia are
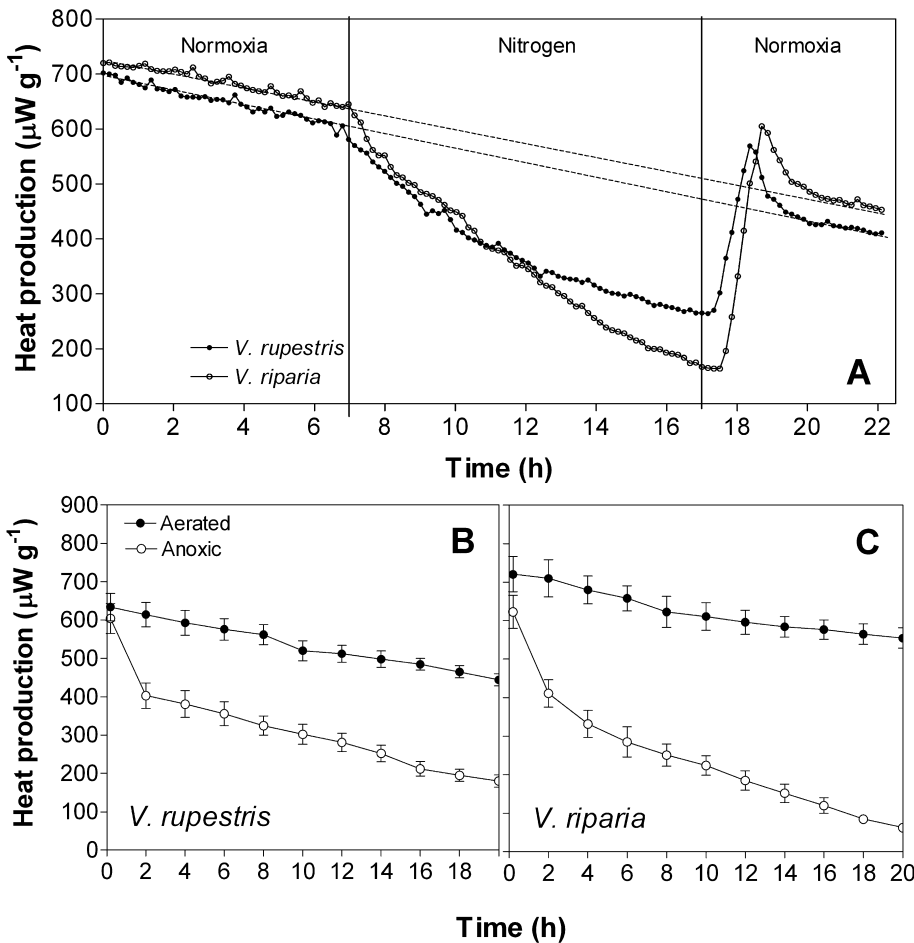
Fig. 1 (A) Representative recording of heat production during anoxia (nitrogen perfusion) for *V. riparia* and *V. rupestris* root segments. Vertical lines indicate the time at which the indicated solution entered the calorimetric chamber. The overshoot in heat output during reoxygenation is also shown. (B) Heat production by normoxic and anoxic root segments of *V. riparia* and (C) *V. rupestris*. Data given are means ± SE ($n = 10$).

broadly similar to those present in some anoxia-tolerant animals, suggesting, in accord also with Dixon et al. (2006), that in both the animal and plant kingdom a common strategy has evolved to survive anoxia.

Results

Root survival

Vitis plants that had been subjected to hypoxia pre-treatment (5% O_2 for 24 h) were exposed to 24 h of anoxic stress, followed by a 24 h recovery period. At the end of this period, root tip survival was scored. The results confirmed the good tolerance of *V. riparia* which showed 95% root survival, whereas in *V. rupestris* the number of living roots was <16.0%.

Heat production

A typical calorimetric recording is illustrated in Fig. 1A. The results demonstrated that root cells depress heat flow under nitrogen treatment. Reoxygenation reversed the metabolic decrease completely in both the tolerant and the intolerant genotypes. After 10 h of treatment, perfusion of the chamber with air restored the heat flow to the level that would be expected if the slow steady decline in heat production were taken into account (Fig. 1A). This suggests that root cells main-

tained their integrity and homeostasis. Reoxygenation of anoxic roots caused an overshoot in heat production that probably reflects restoration of the original energy levels.

The heat production of oxygenated and anoxic roots of *V. riparia* and *V. rupestris* is shown in Fig. 1B and C. The roots showed a slow steady decline in heat production. The slope of this decline did not differ significantly between aerated roots of *V. riparia* ($1.42 \pm 0.28\%$ h^{-1}) and *V. rupestris* ($1.39 \pm 0.33\%$ h^{-1}), and it may reflect a slow loss of viable cells also under normal (aerated) conditions. The same phenomenon has been noted previously in calorimetric studies on animal tissues (see for example Johansson et al. 1995) and can probably be ascribed to the technique.

After 10 h, heat production of aerated roots of *V. rupestris* was 562 ± 26 μWg^{-1}, while that of anoxic roots was 325 ± 25 μWg^{-1}, corresponding to a 42% decrease. Under the same conditions, the more tolerant *V. riparia* showed a decrease of 59%. Similar estimates for the reduction of heat production were obtained when the total amount of heat produced during 20 h (corresponding to the area under the curves in Fig. 1B and C) were calculated. These values were 35.6 ± 4.2 and 41.1 ± 5.3 Jg^{-1} in aerated solution, and 21.0 ± 2.0 and 16.8 ± 1.3 Jg^{-1} in anoxia for *V. rupestris* and *V. riparia*, respectively.
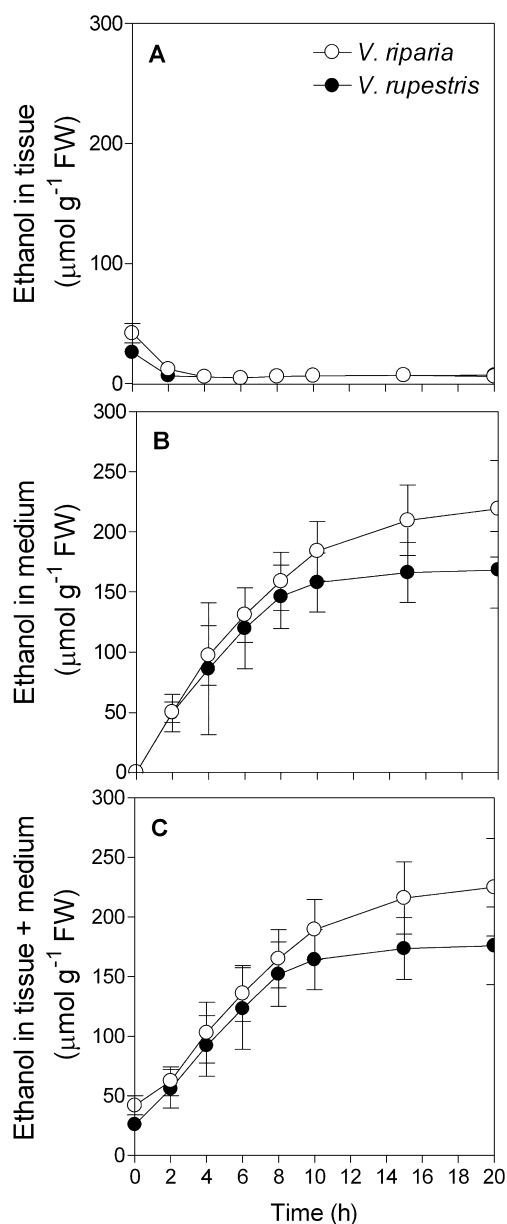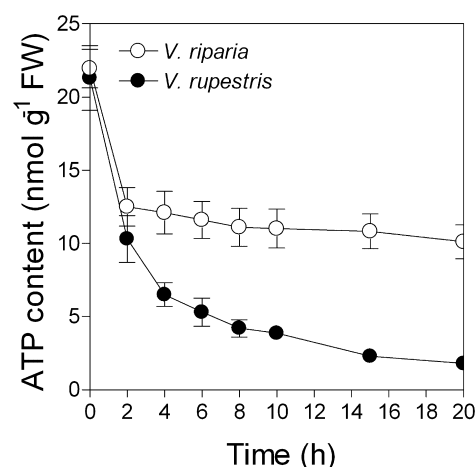
Fig. 2 Effect of anoxia on the ethanol content in apical root segments of *V. riparia* and *V. rupestris* after the onset of anoxia. Data given are means ± SE (*n* = 12). The ethanol content of the tissue (A) and the suspending medium (B) were combined to give the total ethanol production (C).

Fig. 3 Changes in the level of ATP in apical root segments of *V. riparia* and *V. rupestris* after the onset of anoxia. Data given are means ± SE (*n* = 12).

Ethanol and ATP content

When energy production via oxidative phosphorylation is hindered under anoxia, ethanol fermentation appears to be the major pathway for the energy needs of the plants.

Ethanol was the principal fermentation product under anoxia for both species; using an assay with a detection limit of 20 nmol g^{-1} FW, lactate was undetectable. Since a different accumulation of ethanol in the tissues could be the reason for the differences in metabolic activity and survival rates, sepa-

rate data on ethanol content of the tissue and the incubation solution are reported.

The results showed a significant increase of ethanol subsequent to the onset of anoxic conditions. After 20 h, the average ethanol produced by roots of *V. riparia* and *V. rupestris* was 225 and 175 μmol g^{-1} FW, respectively (Fig. 2C). The presence of a low quantity of ethanol in aerated tissues (at time zero) was probably due to the presence of a hypoxic core in the *Vitis* roots. No differences were detected in the permeability for ethanol in the two species, that showed no accumulation of ethanol in the root tissues (Fig. 2A, B).

After the onset of the anoxia, the ATP content decreased in the root cells of both species with a clear biphasic pattern, characterized by a first rapid phase, lasting 2 h, followed by a slower second phase. On the whole, there were no significant differences between the two species during the first phase, whilst in the second phase the decrease was considerably lower in *V. riparia* than in *V. rupestris*. As a result, after 20 h of anoxia, root cells of *V. riparia* hold 45% of the normal ATP content whereas in *V. rupestris* during the same period ATP was reduced to <10% (Fig. 3). Moreover, in *V. riparia*, the decline in ATP contents was almost totally limited to the first 2 h of anoxia, being negligible in the following 18 h.

Effects of the low oxygen condition on K$^+$ uptake kinetics

Net potassium uptake measured in aerated cells of the transition zone (TZ) of the root was around 30 pmol cm^{-2} s^{-1} for both *V. riparia* and *V. rupestris*. When subjected to anoxia, the net K$^+$ uptake in *V. riparia* declined in 4 h to approximately 5 pmol cm^{-2} s^{-1} and remained more or less stable at this level for all the time of the anoxia treatment (20 h). Re-aeration of root tissues after 20 h of anoxia elicited in *V. riparia* a fast resumption of K$^+$ net uptake within 2 h (Fig. 4), suggesting that
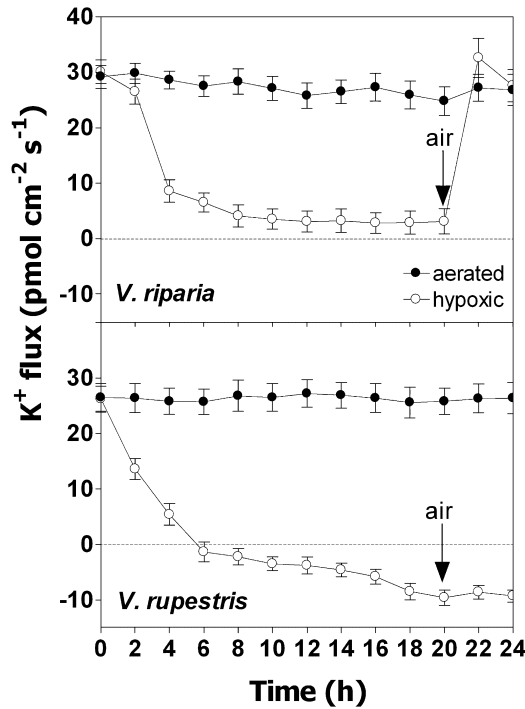
Fig. 4 K$^+$ fluxes (influx positive) in normoxic and anoxic intact roots of *V. riparia* and *V. rupestris*. The data were recorded in the transition zone oscillating the electrode perpendicularly to the root with an amplitude of 20 μm, such that the extremes of the vibration were between 10 and 30 μm from the root surface. Data given are means ± SE (*n* = 12).

Fig. 5 (A) Changes in membrane potential after changes in K$^+$ concentration of the bathing solution in a cell located in the transition zone (around 800–1,000 μm from the root apex) of *V. rupestris*. In this representative recording, from an initial membrane potential of −188 mV, the membrane depolarized in response to increasing the [K$^+$] as KCl of the bathing solution from 10 to 100 M, and then from 100 to 1,000 μM. Increasing the [KCl]$_{ext}$ caused a positive shift in membrane potential, showing that the membrane was more permeable to K$^+$ that the counter-ion Cl$^-$ as is typical for plant cells (B) K$^+$ permeability of root cells of *V. riparia* and *V. rupestris* after the onset of anoxia. The magnitude of the change in the steady-state membrane potential in response to a shift in [K$^+$]$_{ext}$ from 10 to 100 μM, and from 100 to 1,000 μM is reported. Data given are means ± SE (*n* = 12).

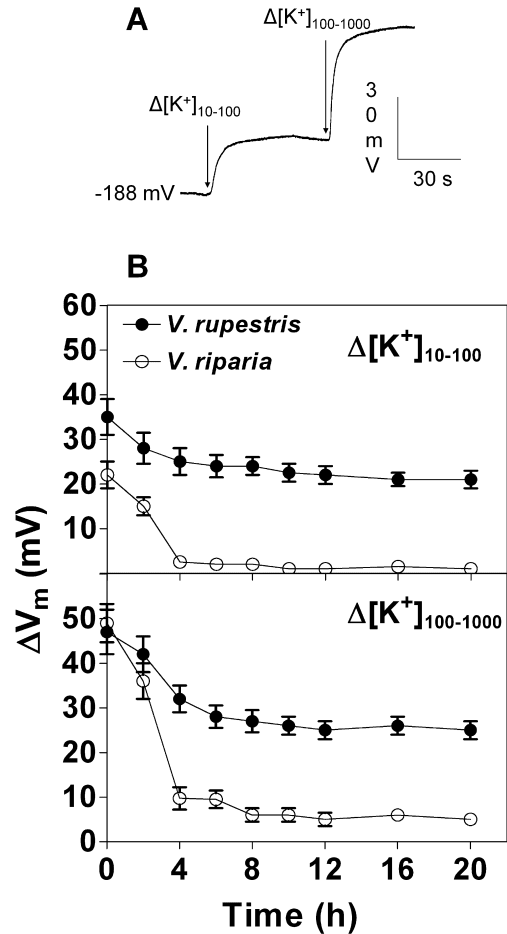no deterioration in membrane integrity or damage in metabolic activities occurred during anoxia.

In *V. rupestris*, the decline of the K$^+$ uptake was much faster, and after 6 h of anoxia a net loss of K$^+$ began, increasing to 10 pmol cm^{-2} s^{-1} after 20 h of treatment. At this time, no change was observed after re-aeration of root tissues (Fig. 4).

Effect of anoxia on K$^+$ membrane permeability

According to Spalding et al. (1999), the changes in membrane potentials (ΔV_m) in response to the shifts in the K$^+$ concentration of the bathing solution are related to the K$^+$ permeability of the plasmalemma (see Materials and Methods for further details).

Vitis riparia and *V. rupestris* showed very similar values of V_m under aerobic conditions (−152 and −146 mV). Remarkably, also after 2 h of anoxia, the value of the membrane potential remained similar in the two species (−80 mV in *V. riparia* and −78 mV in *V. rupestris*).

K$^+$-induced shifts in membrane potential were measured in *V. riparia* and *V. rupestris* to determine if anoxia affected K$^+$ permeability of the membrane, as evidenced by the measurements of the K$^+$ fluxes. The results in Fig. 5 show that after a 4 h anoxia treatment, the *V. riparia* root cells were able to decrease the K$^+$ permeability by 89% in the low concentration

interval ($\Delta[K^+]_{10-100}$) and by 71% in the high concentration interval ($\Delta[K^+]_{100-1,000}$). The same 4 h anoxia treatment had a much smaller effect in *V. rupestris*, resulting in a decrease of the K$^+$ permeability of 40% in both the low and high concentration interval.

Electrical signals in the root cells

When a leaf is damaged with a heat wound, there is a rapid electrical response in the root cells. A few seconds after the stimulus, the microelectrodes placed in the epidermal cells of the TZ of the root (approximately 1 mm from the root apex)
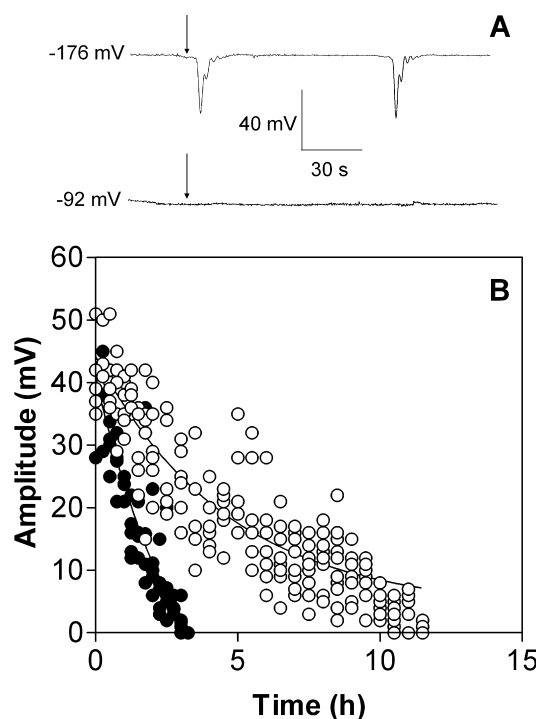
Fig. 6 (A) Kinetics of wound-induced electrical signals in root cells of the transition zone of *V. riparia* in aerated conditions (top) and after 3 h of anoxia (bottom). At the time indicated by the arrows, a small area of one leaf was heated using a metal block which had been heated for 10 min in boiling water. (B) Changes in the amplitude of wound-induced electrical signals measured in root cells of the transition zone of *V. riparia* (filled circles) and *V. rupestris* (open circles) after the onset of anoxia. Values are from 18 different trials.

recorded a rapid negative shift of around 40 mV, followed by a recovery to the initial values (Fig. 6A). When plants were enclosed and wounded in an N₂ atmosphere, the amplitude of the wound-induced electrical signals decreased with a different velocity in the two species (Fig. 6B). In *V. riparia*, no electrical signals were induced after 3 h (Fig. 6A, B), whereas in *V. rupestris* the complete inhibition of electrical signals required around 10 h (Fig. 6B).

Discussion

When plants are faced with a serious deprivation of oxygen, the only response that can be called upon is to shut down metabolic operations as much as possible in order to remain active, but at a reduced rate fuelled by non-oxidative energy-producing pathways (Bucher and Kuhlemeier 1993). Since glycolysis, which is the only energy source during anoxia, yields <10% of the ATP produced by aerobic metabolism (Zhang and Greenway 1994), there are only two possible ways to maintain ATP levels in the absence of oxygen, the first being to increase the rate of glycolysis (the so-called glycolytic strategy or Pasteur effect) and the second to depress the rate of ATP

use (the metabolic depression strategy). However, while the Pasteur effect is often strong in animal cells (Hochachka 1991), in plants the increase in glucose consumption is usually 3- to 4-fold at the most (Mayne and Kende 1986, Small et al. 1989), which is far from the increase of 10- to 18-fold required to match the level of energy production in air (Gibbs and Greenway 2003). Thus, tolerant root cells are required to depress their metabolic state to survive during the anoxic episodes.

The rate of heat production of apical root segments from both species was clearly responsive to O₂ availability (Fig. 1). When root apices of *V. riparia* and *V. rupestris* were under anoxic conditions, they reduced their heat production by 59 and 41%, respectively, as a consequence of metabolic depression. These are low values compared with similar microcalorimetric studies made on animal tissues where a 70–90% decrease in heat production has been seen (Jackson 1968, Doll et al. 1994), while these are quite close to the value of down-regulation measured in rice coleoptiles by Colmer et al. (2001).

Interestingly, in both species, heat production and ATP levels showed a similar biphasic pattern characterized by a strong decrease in the first phase lasting for 2 h, followed by a second phase in which the two species differed greatly in their response. In *V. riparia* after the first 2 h of anoxia, the decrease in the rate of heat production was not associated with a further significant decrease in ATP content, whereas in *V. rupestris* the ATP level continued to decrease until very low values were reached (Fig. 3). The ability to depress the metabolism rapidly during anoxic episodes, avoiding a severe decline in ATP levels, is a fundamental adaptive response of *V. riparia* roots to oxygen deprivation. After 20 h of anoxia, the ATP decrease in *V. riparia* accounted for 55% of the 'aerobic level', whereas under the same conditions *V. rupestris* showed a reduction of 92% (Fig. 3).

As no significant differences in the rate of ATP production by glycolysis can be inferred from the ethanol output of the two species (Fig. 2C), a drastic, balanced, suppression of ATP demand must occur in *V. riparia* in order to maintain ATP levels relatively constant even while ATP production greatly declines. Protein synthesis and ion-transporting ATPases are the dominant energy-consuming processes of root cells (Van der Werf et al. 1992, Bouma and De Visser 1993), so decreases in ATP demand in these processes under energy deficit status are thought to be largely responsible for enabling the down-regulation of energy turnover in metabolically depressed states. Furthermore, the ATP-consuming processes seem to be arranged in a hierarchy, with protein synthesis and RNA/DNA synthesis falling off rather sharply as energy becomes limiting (Chang et al. 2000). This implies that ATPases which drive ion transport are likely to become the dominant energy sinks in anoxic root cells. In effect, the primary cause of anoxia-induced death in plants is cell dysfunction due to a loss of ionic integrity of the cell membranes. Ion leakage across cell membranes occurs as a result of both intracellular and extracellular
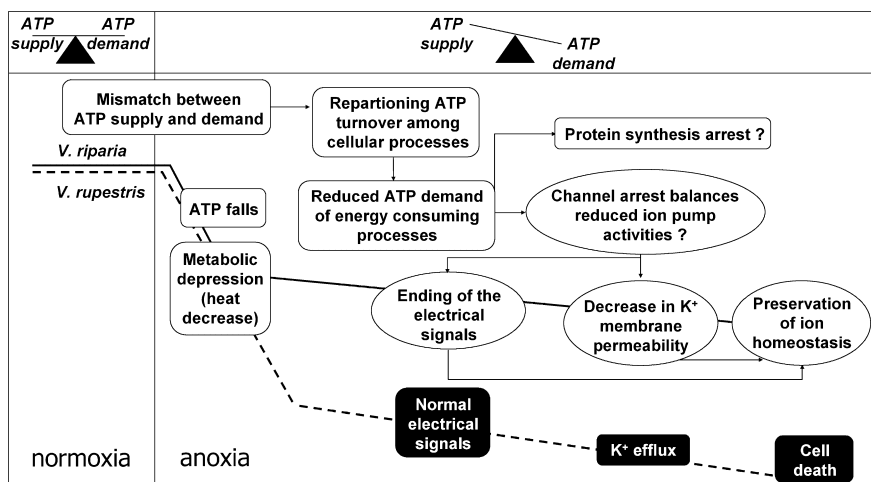
Fig. 7 A generalized model of the responses of the hypoxia-tolerant *V. riparia* (solid line) and hypoxia-sensitive *V. rupestris* (dashed line) metabolism to anoxia. Common responses to anoxia are shown in rectangular boxes; *V. riparia* responses are shown in elliptical boxes and *V. rupestris* responses are shown in black rectangular boxes. In sensitive species, anoxia typically led to an energy deficit and ATP unbalancing, subsequent disruption of ion homeostasis and cell death. The decrease in K^+ membrane permeability with consequent stabilization of transmembrane ion gradients by a balanced reduction in ion pumping and channel conductance, and the balanced cessation of ATP synthesis and ATP demand, leads to the anoxia tolerance of *V. riparia*.

ions drifting toward thermodynamic equilibrium. Maintenance of a homeostatic intracellular environment therefore requires the redistribution of these ions through the use of ATP-consuming pumping systems. These can consume 40–60% of the cell's resting metabolic rate (Greenway and Gibbs 2003).

The main difference in the response of *V. riparia* and *V. rupestris* to anoxia is in their ability to cope with ion-transporting ATPases. After 4 h of anoxia, root cells of the anoxia-tolerant *V. riparia* eliminated the K^+ membrane permeability in the low concentration range (10–100 μM) and dramatically reduced it in the higher concentration range (100–1,000 μM). Similar findings in the high concentration range have been shown in rice coleoptile cells where a 17-fold reduction of the membrane permeability in anoxia has been estimated (Colmer et al. 2001). Inspection of the *V. rupestris* data provides a different picture, with just a small decrement in the K^+ membrane permeability (Fig. 5). Interestingly, root cells are more permeable to K^+ than to any other cation (Spalding and Goldsmith 1993), and in anoxic cells of hypoxia-tolerant animal species the inhibition of the K^+ channel activity and the consequent reduction in ion permeability have been described as a major energy-saving response to low oxygen conditions (Hochachka 1991).

The ability of *V. riparia* in maintaining a functional ion homeostasis during prolonged anoxia episodes is also confirmed by the sustained K^+ uptake (Fig. 4). Unlike *V. riparia*, the anoxia-intolerant *V. rupestris* was unable to preserve ion homeostasis and efflux of K^+ was evident after 6 h of anoxia (Fig. 5). In accordance with our results in *V. riparia*, Colmer et al. (2001) reported for rice coleoptiles a sustained net uptake of K^+ over a prolonged anoxic period, indicating that the preservation, or the quick recovery, of ion homeostasis during anoxia is an attribute of tolerant plants. A link between K^+ permeability and anoxia in root cells could include ATP-sensitive K^+ channels of the sort identified in *Arabidopsis* (Spalding and Goldsmith 1993). Their activity could represent a direct response to changes in the intracellular energy charge or oxy-

gen-sensitive K^+ channels such as have been identified in animal cells (Lopez-Barneo 1994).

In hypoxia-tolerant animals, several lines of evidence have shown a down-regulation of cellular electrical activity as one of the major mechanism for metabolic depression (for a review see Lutz and Nilsson 1994). In plants, the reduction of the coordinated activity of ion channels responsible for action potential generation (Wayne 1994) could be part of a synchronized process of energy conservation. To test this hypothesis, we recorded the stimulus-induced electrical activity under anoxia, in cells of the TZ of the root apex. We studied the TZ because of its intense signal activity. In fact, this zone of the growing root apex (Baluška et al. 1994) is known to be a kind of sensory zone very responsive to many environmental parameters (exhaustively reviewed in Baluška et al. 2001) such as touch, extracellular calcium (Ishikawa and Evans 1992, Baluška et al. 1996), and water and salt stresses (Sharp et al. 1988, Wu and Cosgrove 2000).

Under anoxic conditions, both *Vitis* species showed an inhibition of the wound-induced electrical signals (Fig. 6). The different evolution of the inhibitory response, much faster in *V. riparia* (3 h) than in *V. rupestris* (>10 h) (Fig. 7), suggests the existence of an energy-saving mechanism in *V. riparia* only. Interestingly, inhibition of the wounding response at low oxygen concentrations (Butler et al. 1990, Rumeau et al. 1990, Geigenberger et al. 2000) as well as the role of the electrical signals in gene expression in response to the wound (Stankovic and Davies 1996) have both been verified, suggesting a causal link between the two events.

Conclusion

The main conclusions of this study are presented below and summarized schematically in Fig 7. One anoxic defence mechanism shared by plants and animals is the ability to reallocate cellular energy between essential and non-essential ATP-demanding processes as energy supplies become limiting (Dixon et al. 2006). Following this criterion, the critical differ-

ence between the two species studied is that the anoxia-sensitive *V. rupestris* show no reduction in the absolute ATP-demand of the ion-transporting ATPases in response to lack of O_2. In contrast, cells of anoxia-tolerant *V. riparia* exhibit large-scale reductions in metabolic activities during anoxia. Although this might normally be interpreted as pump failure in a plant cell, such decreases in ion motive activity in root cells of *V. riparia* are brought about without any disruptions in electrochemical potentials, cellular ion levels or ATP concentrations. Thus, quite apart from there being any failure of ionic homeostasis, the reduction in K^+ flux in anoxia-tolerant cells of *V. riparia* is part of a coordinated process of energy conservation wherein the lack of O_2 initiates a generalized suppression of ion channel densities. The net result is that cell membrane permeability is reduced, thereby lowering the energy costs of maintaining transmembrane ion gradients. This phenomenon, so-called 'channel arrest' (Hochachka 1986), serves as a potent mechanism for actively down-regulating the ATP demands of cells in potentially energy-limited states.

Materials and Methods

Plant material and growth conditions

Cuttings from 1-year-old shoots, 15–20 cm long and with six leaves, were taken from 7-year-old stock plants of *V. riparia* Michx. and *V. rupestris* Scheel. In order to favour rooting, the lower portion of each cutting was immersed, to a depth of 5 mm, in an aqueous KIBA (3-indolebutyric acid potassium salt) solution at 3,600 μg ml^{-1} for 5 s. The cuttings were then placed in an inert medium of perlite and kept in a greenhouse under conditions of high relative humidity (mist propagation), daylight for 12 h d^{-1} and average diurnal and nocturnal temperatures of 22 and 15°C, respectively. Rooting took 6 weeks. Plants were brought into the laboratory 4 d before the experiment, and additional light was furnished to the plants (from fluorescent tubes; 400 μmol m^{-2} s^{-1} at canopy height). After removing the perlite, the root systems of entire plants were immersed for 48 h, under weak illumination, in a vigorously aerated nutrient solution (NS) composed of 1 mM KCl; 0.905 mM NaH$_2$PO$_4$; 0.048 mM Na$_2$HPO$_4$; 1 mM Ca(NO$_3$)$_2$; 0.25 mM MgSO$_4$; and 100 mM glucose. The solution temperature was maintained at 21 ± 1°C for the immersion period. The plants were then used for the experiments.

Experimental procedure

Plants with root length 2–3 cm were transferred to a chamber containing 100 ml of NS. The shoots were maintained in air while the root system of the plant and the measuring chamber were placed in a glove-bag. A low oxygen concentration was obtained using pressurized gases comprising 5% O_2 and 0.1% CO_2, the remaining volume being N_2. Plants were pre-treated hypoxically by continuously passing a stream of 5% O_2 through the chamber at a rate of 100 ml min^{-1} for 24 h. Anoxic treatments were achieved by flushing N_2 continuously through the chamber at a rate of 100 ml min^{-1}. The bulk solution oxygen concentration in the measuring chamber was recorded polarographically using a Clark type electrode for pO_2. Control plants were maintained in the NS.

Survival determination

After treatment, the plants were tested for root survival. Roots were arranged in a plastic holder at the top of a Plexiglas box 40 cm long, 8 cm wide and 20 cm tall containing NS. Following the method adopted by Verslues et al. (1998), the roots were allowed to grow downward through transparent root guides made from plastic drinking straws (i.d. 7 mm), which facilitated measurements of root elongation. The root guides were perforated with several holes (diameter 1 mm) to allow exchange of solution. The roots were considered to have survived if they resumed elongation after anoxic treatment. Under normal conditions (aerated nutrient soultion), roots of both species showed no symptoms of damage.

Metabolite measurements

The plants were removed from the chamber, and the roots were harvested, frozen immediately with liquid N_2 and stored at –80°C until extraction. Frozen roots were placed in a mortar containing liquid N_2 and ground to a fine powder using a pestle. ATP was determined spectrophotometrically according to Mohanty et al. (1993). The overall recovery of ATP added to the extraction medium containing root powder before homogenization was 79 ± 5% (mean ± SE), as calculated from seven replications. Quantification of ethanol was carried out by gas chromatography according to the method of Kato-Noguchi (2000). Internal standards for ethanol added to the extraction medium before sample homogenization showed 81 ± 8% (mean ± SE), as calculated from five replications.

Microcalorimetry

Heat production measurements were carried out using a heat conduction multichannel microcalorimeter (TAM 2277, Thermometric, Järfälla, Sweden). The heat produced by the excised roots was measured either for 20 h in oxygenated NS or for 20 h in anoxic NS. The reference ampoules also contained anoxic or oxygenated NS. Before all measurements, the ampoules were kept in the heat-equilibrating position of the calorimeter for 60 min to allow them to warm up to 20°C (the temperature of all the measurements). After the ampoules had been placed in the measuring chambers, control experiments (both ampoules containing NS but no roots) showed that it took around 10 min for the system to equilibrate. Thus, heat measurements for the first 10 min were discarded.

Membrane potential and K^+ membrane permeability measurements

The membrane potential of individual root cells was measured using intracellular 1 M KCl microelectrodes (tip diameter <0.5 μm) and Ag/AgCl reference electrodes in the flowing bath solution. Electrodes were connected to a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA, USA) interfaced with a computer. A drop of water at 0°C, which was added to the recording chamber at the end of each experiment, produced the expected large depolarization mediated by Ca^{2+} and Cl channels, a test of proper cell impalement (Lewis et al. 1997). Membrane permeability in epidermal root cells of the TZ of the root apex was measured with concomitant shifts in the external potassium concentration ([KCl]$_{ext}$) following the method of Spalding et al. (1999). After the membrane potential stabilized, the bathing solution was changed from one containing 10 μM K^+ to one containing 100 μM K^+ (Δ[K^+]$_{10-100}$) and then to 1,000 μM K^+ (Δ[K^+]$_{100-1,000}$). According to Spalding et al. (1999), the changes in membrane potentials (ΔV_m) due to the shift in [K^+]$_{ext}$ are indicative of the relative permeability of the plasma membrane to K^+ assuming that an imposed shift in [KCl]$_{ext}$ does not affect the H^+-ATPase activity of the two *Vitis* species differently under anoxia. Since the depolarization of the membrane potential under anoxia was not different between the two *Vitis* species (72 ± 6 mV in *V. riparia* and 68 ± 10 mV in *V. rupestris*), we assumed that under anoxia the activity of the proton pump was equally influenced in both species.

Anoxia was obtained by eliminating the oxygen from 1.5 liters of stock NS (with an adequate KCl concentration) by bubbling with N_2. The time necessary for this operation was ascertained by measuring the content of O_2 in the solution with a Clark type electrode for pO_2 placed directly in the measuring chamber containing the tissue. Bubbling for 25 min was necessary to reduce the O_2 content to 0 mg l^{-1}.

Electrical signals recording

The occurrence of wound-induced electrical signals was measured with intracellular microelectrodes (tip diameter <0.5 μm) in the TZ of the root apex as described in Mancuso (1999). After electrode connection, a settling time of 1 h was allowed prior to the start of the experiment. Electrical signals were induced by heating the leaves with a metal block, which had been heated for 10 min in boiling water and set for 10 s on the leaf. Anoxia was obtained by enclosing the root system of the plants in polyethylene bags and blowing N_2 through the bag. The bulk solution oxygen concentration in the measuring chamber was recorded polarographically using a Clark type electrode for pO_2.

Potassium flux measurement

The design and mode of the vibrating microelectrode system used was described previously (Mancuso et al. 2000). In brief, micropipettes were pulled from borosilicate tubing and then silanized. The tips were broken to a tip diameter of 3–5 μm. Commercially available K^+ ionophore cocktails (Fluka catalog No. 60031) were used to fill the tips after back-filling with 100 mM KCl. All electrodes were calibrated using sets of standards and confirmed to be Nernstian prior to use. Electrodes with a response of <50 mV per decade were discarded. An Ag/AgCl reference electrode completed the circuit in solution by way of a 3 mol l^{-1} NaCl–3% agar bridge.

During recording from the TZ of the root apex, the microelectrode was oscillated in a square wave parallel to the electrode axis over a distance of 20 μm with a frequency of 0.1 Hz. The nearest position of these oscillations was 10 μm above the root surface. The electrode tip and the root were monitored under a microscope throughout the experiments. Potassium fluxes were calculated using Fick's first law of diffusion, assuming cylindrical diffusion geometry.

Acknowledgments

References

Armstrong, W. (1979) Aeration in higher plants. *Adv. Bot. Res.* 7: 225–232.

Armstrong, W. and Drew, M. (2002) Root growth and metabolism under oxygen deficiency. *In* Plant Roots: The Hidden Half. Edited by Waisel, Y., Eshel, A. and Kafkafi, U. pp. 729–761. Marcell Dekker, Inc., New York.

Baluška, F., Barlow, P.W. and Kubica, Š. (1994) Importance of the post-mitotic growth (PIG) region for growth and development of roots. *Plant Soil* 167: 31–42.

Baluška, F., Volkmann, D. and Barlow, P.W. (1996) Specialized zones of development in roots: view from the cellular level. *Plant Physiol.* 112: 3–4.

Baluška, F., Volkmann, D. and Barlow, P.W. (2001) A polarity crossroad in the transition growth zone of maize root apices: cytoskeletal and developmental implications. *J. Plant Growth Regul.* 20: 170–181.

Bouma, T.J. and De Visser, R. (1993) Energy requirements for maintenance of ion concentrations in roots. *Physiol. Plant.* 89: 133–142.

Bucher, M. and Kuhlemeier, C. (1993) Long-term anoxia tolerance. Multi-level regulation of gene expression in the amphibious plant *Acorus calamus* L. *Plant Physiol.* 103: 441–448.

Butler, W., Cook, L. and Vayda, M.E. (1990) Hypoxic stress inhibits multiple aspects of the potato tuber wound response. *Plant Physiol.* 93: 264–270.

Chang, W.W., Huang, L., Shen, M., Webster, C., Burlingame, A.L. and Roberts, J.K. (2000) Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. *Plant Physiol.* 122: 295–318.

Colmer, T.D., Huang, S. and Greenway, H. (2001) Evidence for down-regulation of ethanolic fermentation and K^+ effluxes in the coleoptiles of rice seedlings during prolonged anoxia. *J. Exp. Bot.* 360: 1507–1517.

Dixon, M.H., Hill, S.A., Jackson. M.B., Ratcliffe, R.G. and Sweetlove, L.J. (2006) Physiological and metabolic adaptation of *Potamogeton pectinatus* L. tubers support rapid elongation of stem tissue in the absence of oxygen. *Plant Cell Physiol.* 47: 128–140.

Doll, C.J., Hochachka, P.W. and Hand S.C. (1994) A microcalorimetric study of turtle cortical slices: insights into brain metabolic depression. *J. Exp. Biol.* 191: 141–153.

Drew, M.C. (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 223–250

Geigenberger, P. (2003) Response of plant metabolism to too little oxygen. *Curr. Opin. Plant Biol.* 6: 247–256.

Geigenberger, P., Fernie, A.R., Gibon, Y., Christ, M. and Stitt, M. (2000) Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers. *Biol. Chem.* 381: 723–740.

Gibbs, J. and Greenway, H. (2003) Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Funct. Plant Biol.* 30: 1–47.

Greenway, H. and Gibbs, J. (2003) Mechanisms of anoxia tolerance in plants. II. Energy requirement for maintenance and energy distribution to essential processes. *Funct. Plant Biol.* 30: 999–1036.

Hochachka, P.W. (1986) Defense strategies against hypoxia and hypothermia. *Science* 231: 234–241.

Hochachka, P.W. (1991) Metabolic strategies of defence against hypoxia in animals. *In* Plant Life under Oxygen Deprivation. Edited by Jackson, M.B., Davies, D.D. and Lambers, H. pp. 121–128. SPB Academic Publishing, The Hague, The Netherlands.

Ishikawa, H. and Evans, M.L. (1992) Induction of curvature in maize roots by calcium or by thigmostimulation. Role of the postmitotic isodiametric growth zone. *Plant Physiol.* 100: 762–768.

Kato-Noguchi, H. (2000) Abscissic acid and hypoxic induction of anoxia tolerance in roots of lettuce seedlings. *J. Exp. Bot.* 51: 1939–1944.

Jackson, D.C. (1968) Metabolic depression and oxygen depletion in the diving turtle. *J. Appl. Physiol.* 24: 503–509.

Johansson, D., Nillsson, G.E. and Tornblom, E. (1995) Effects of anoxia on energy metabolism in crucian carp brain slices studied with microcalorimetry. *J. Exp. Biol.* 198: 853–859.

Lewis, B.D., Karlin-Neumann, G., Davis, R.W. and Spalding, E.P. (1997) Ca^{2+}-activated anion channels and membrane depolarizations induced by blue light and cold in arabidopsis seedlings. *Plant Physiol.* 114: 1327–1334

Lopez-Barneo, J. (1994) Oxygen sensitive ion channels: how ubiquitous are they? *Trends Neurosci.* 17: 133–135.

Lutz, P.L. and Nilsson, G.E. (1994) The Brain Without Oxygen, Causes of Failure and Mechanisms for Survival. R.G. Landis Co., Austin, Texas.

Mancuso, S. (1999) Hydraulic and electrical transmission of wound-induced signals in *Vitis vinifera. Aust. J. Plant Physiol.* 26: 55–61.

Mancuso, S. (2000) Electrical resistance changes during exposure to low temperature and freezing measure chilling tolerance in olive tree (*Olea europaea* L.) plants. *Plant Cell Environ.* 23: 291–299.

Mancuso, S. and Boselli, M. (2002) Characterisation of the oxygen fluxes in the division, elongation and mature zone of *Vitis* roots: influence of oxygen availability. *Planta* 214: 767–774.

Mancuso, S., Papeschi, G. and Marras, A.M. (2000) A polarographic, oxygen-selective, vibrating-microelectrode system for the spatial and temporal characterisation of transmembrane oxygen fluxes in plants. *Planta* 211: 384–389.

Mayne, R.G. and Kende, H. (1986) Glucose metabolism in anaerobic rice seedlings. *Plant Sci.* 45: 31–36.

Mohanty, B., Wilson, P. and Ap Rees, T. (1993) Effects of anoxia on growth and carbohydrate metabolism in suspension cultures of soybean and rice. *Phytochemistry* 34: 75–82.

Rumeau, D., Maher, E.A., Kelman, A. and Showalter, A.M. (1990) Extensin and phenylalanine ammonia-lyase gene expression are altered in potato tubers in

response to wounding, hypoxia and *Erwinia carotovora. Plant Physiol.* 93: 1134–1139.

Sharp, R.E., Silk, W.K. and Hsiao, T.C. (1988) Growth of the maize primary root at the low water potentials. I. Spatial distribution of expansive growth. *Plant Physiol.* 87: 50–57.

Small, J.G.C., Potgieter G.P. and Botha, F.C. (1989) Anoxic seed germination of *Erythrina caffra*: ethanol fermentation and response to metabolic inhibitors. *J. Exp. Bot.* 40: 375–381.

Spalding, E.P. and Goldsmith, M. (1993) Activation of K$^+$ channels in the plasma membrane of Arabidopsis by ATP produced photosynthetically. *Plant Cell* 5: 477–484.

Spalding, E.P., Hirsch, R.E., Lewis, D.R., Qi, Z., Sussman, M.R. and Lewis, B.D. (1999) Potassium uptake supporting plant growth in the absence of AKT1 channel activity: inhibition by ammonium and stimulation by sodium. *J. Gen. Physiol.* 113: 909–918.

Stankovic, B. and Davies, E. (1996) Both action potentials and variation potentials induce proteinase inhibitor gene expression in tomato. *FEBS Lett.* 390: 275–279.

Van der Werf, A., Van der Berg, G., Ravenstein, H.J.L., Lambers, H. and Eising, R. (1992) Protein turnover: a significant component of maintenance respiration in roots? *In* Molecular, Biochemical and Physiological Aspects of Plant Respiration. Edited by Lambers, H. and van der Plas, L.W.H. pp. 483–492. SPB Academic Publishing, The Hague, The Netherlands.

Van Dongen, J.T., Schurr, U., Pfister, M. and Geigenberger, P. (2003) Phloem metabolism and function have to cope with low internal oxygen. *Plant Physiol.* 131: 1529–1543.

Verslues, P.E., Ober, E.S. and Sharp, R.E. (1998) Root growth and oxygen relations at low water potentials. Impact of oxygen availability in polyethylene glycol solutions. *Plant Physiol.* 116: 1403–1412.

Wayne, R. (1994) The excitability of plant cells with a special emphasis on *Characean* internode cells. *Bot. Rev.* 60: 265–367.

Wu, Y. and Cosgrove, D.J. (2000) Adaptations of roots to low water potentials by changes in cell wall extensibility and cell wall properties. *J. Exp. Bot.* 51: 1543–1553.

Zhang, Q. and Greenway, H. (1994) Anoxia tolerance and anaerobic catabolism of aged beetroot storage tissue. *J. Exp. Bot.* 43: 897–905.