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Effect of Hypoxic Acclimation on Anoxia Tolerance in *Vitis* Roots: Response of Metabolic Activity and K^+ Fluxes

Sergio Mugnai[1,*], Anna Maria Marras[2] and Stefano Mancuso[1]

[1]Department of Plant, Soil and Environmental Sciences, University of Florence, viale delle idee 30, 50019 Sesto F.no (FI), Italy
[2]Department of Pharmaceutical Sciences, University of Florence, via Ugo Schiff 6, 50019 Sesto F.no (FI), Italy
*Corresponding author: E-mail, sergio.mugnai@unifi.it; Fax, +39-055-4574017
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The effect of a hypoxic pre-treatment (HPT) on improving tolerance to prolonged anoxia conditions in two contrasting *Vitis* species (*V. riparia*, anoxia tolerant; *V. rupestris*, anoxia sensitive) was evaluated. The energy economy of root cells was studied by measuring heat production, the activity of pyruvate decarboxylase (PDC) and alcohol dehdrogenase (ADH), ethanol and ATP production, and K^+ fluxes. The results showed that HPT is an effective tool in order to maintain a sustainable metabolic performance in both the species under anoxia conditions, especially in sensitive species such as *V. rupestris*. Our results showed that the improved tolerance was mainly driven by: (i) an enhanced activity of key enzymes in alcohol fermentation (ADC and PDC); (ii) the capability to maintain a higher level of respiration, evidenced by a lesser decrease in heat development and ATP production; and (iii) the maintenance of a better ion homeostasis (highlighted by measurement of K^+ fluxes) and K^+ channel functionality.

Keywords: Anoxia • Cycloheximide • Hypoxic pre-treatment • K^+ fluxes • *Vitis riparia* • *Vitis rupestris*.

Abbreviations: ADH, alcohol dehydrogenase; CX, cycloheximide; DZ, division zone; HPT, hypoxic pre-treatment; MZ, mature zone; NHPT, non-hypoxic pre-treatment; NS, nutrient solution; O_2, oxygen concentration; PDC, pyruvate decarboxylase; TZ, transition zone.

Introduction

Plant tissues normally show an improved tolerance to anoxia when subjected to a preliminary period of acclimation to hypoxia (Saglio et al. 1988, Bouny and Saglio 1996). These hypoxically pre-treated tissues have the ability to maintain a high glycolytic rate during prolonged periods of anoxia, as well as higher ATP levels and energy charge (Johnson et al. 1989, Hole et al. 1992, Xia et al. 1995) in comparison with non-hypoxically pre-treated tissues. As these metabolic adaptations require de novo synthesis of 'anaerobic' proteins (ANPs), expressed when plants are exposed to low O_2 conditions (Dennis et al. 2000), tolerance is usually much better expressed when tissues are first acclimated during hypoxic pre-treatment (HPT), rather that after sudden transfer to anoxia. In particular, a certain number of polypeptides expressed under HPT have been identified as enzymes involved in glycolysis and ethanol fermentation (Gibbs and Greenway 2003).

As ATP generation by oxidative phosphorylation begins to drop due to O_2 limitation, if the cellular ATP demands of cells and tissues in sensitive plant species tend to remain constant, an energetic deficit could be overcome by the activation of anaerobic ATP supply pathways (the so-called 'Pasteur effect'). Because anaerobic ATP production can only temporarily meets the sustained energy demands of the various cellular ATP-consuming processes, finite stores of fermentable substrate together with the accumulation of deleterious end-products impose temporal constraints on anaerobiosis as a long-term solution to severe oxygen limitation in these plants. A solution to prevent the potentially lethal effects of a compromised metabolism would be to decrease the energetic demands of one or more of the major ATP-consuming functions (metabolic depression strategy; Gibbs and Greenway 2003), and to counter-balance such energetic economies with proportional and simultaneous reductions in ATP production. This is exactly the strategy employed by some hypoxia-tolerant plants such as *Vitis riparia* (Mancuso and Marras 2006).

Ion transport ATPases are one of the major ATP sinks in plant cells and tissues (Pang and Shabala 2010). These energy-consuming processes represent a primary cellular target for down-regulating ATP demand in response to O_2 shortage, affecting cell metabolism and the overall plant nutritional status. However, how this process is modified in hypoxically pre-treated root tips, compared with those which are non-hypoxically pre-treated, is the main subject of the present paper. To answer this question, we evaluated the effect of HPT in changing the metabolic activity linked to normoxia to anoxia transitions in two *Vitis* species characterized by a different tolerance to hypoxia/anoxia: *V. riparia* and *V. rupestris* (Mancuso and Boselli 2002). Furthermore, as K^+ significantly affects the activity of vacuolar ion channels, thus playing an important role

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in the maintenance of the cytosolic homeostasis and in the regulation of cell turgor (Shabala and Cuin 2008), the effect of HPT on K^+ uptake in roots subjected to anoxia was evaluated in order to assess the role of acclimation on ion homeostasis. In fact, a steady state in the homeostatic intracellular environment requires the redistribution of ions through the use of ATP-consuming pumping systems (Gibbs and Greenway 2003). Finally, we investigated the effects of the application of a protein synthesis inhibitor (cycloheximide; CX) during the acclimation period, because the de novo synthesis of anaerobic proteins is likely to play an important role in the acclimation to low O_2 levels (Ellis et al. 1999).

Results

Root survival and growth

Vitis plants subjected to HPT or to non-hypoxic pre-treatment (NHPT) were exposed to 20 h of anoxic stress, followed by a 24 h recovery period at ambient [O_2] in the nutrient solution (NS). After this recovery period, root tip survival was scored (**Fig. 1**). The results confirmed the remarkable tolerance of *V. riparia* to anoxia, compared with *V. rupestris*. In fact, 45% of the roots belonging to *V. riparia* plants survived, whereas in *V. rupestris* this value fell to 3%. HPT significantly increased root survival in both species (95% in *V. riparia* and 60% in *V. rupestris*). In contrast, plants subjected to HPT and those subjected to NHPT and treated with increasing concentrations of CX (10 and 100 μM, respectively) showed a marked loss of vitality in the two species. When [O_2] decreased, the root elongation rate was significantly inhibited (**Fig. 2**). However, a different sensitivity to hypoxia was recorded both in the different species and in the different regions of the root apex. For example, when a mild hypoxic stress ([O_2] = 0.15 mol m^{-3}) was applied, no effect on *V. riparia* roots was detected. In contrast, a reduction in *V. rupestris* root growth occurred in both the mature zone (MZ) and transition zone (TZ), which significantly decreased their elongation rate, whereas the division zone (DZ) maintained a steady growth. After decreasing [O_2] at 0.10 mol m^{-3}, only the TZ elongation rate was strongly affected in *V. riparia* roots, while the other two regions still

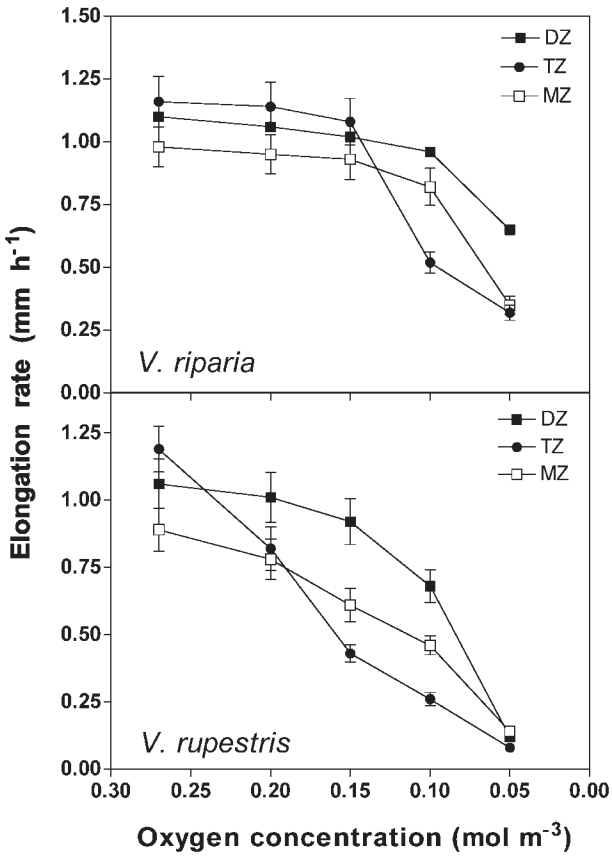

Fig. 2 Elongation rate (mm h^{-1}) measured on the three constituent regions of the root apex at different oxygen concentrations: division zone (DZ), transition zone (TZ) and mature zone (MZ). Data are means ± SD (n = 10).

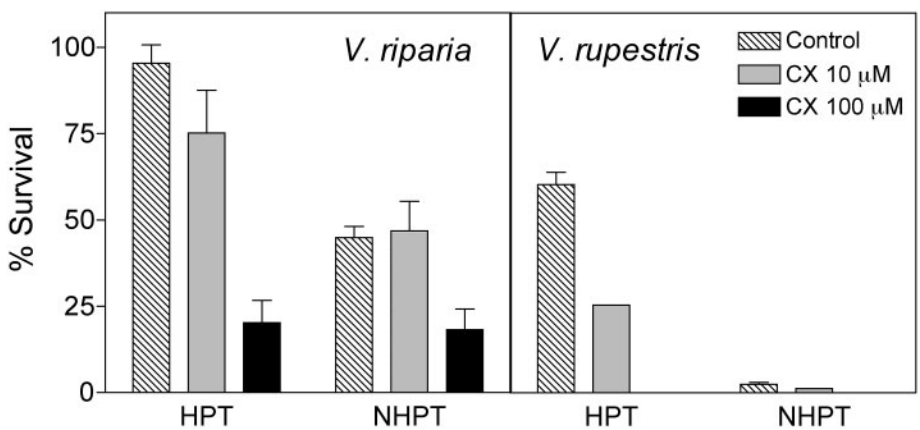

Fig. 1 Root survival rate (%) to 20 h of anoxia, measured after a 24 h recovery period. *Vitis riparia* and *V. rupestris* roots were treated with 0 (control), 10 and 100 μM cycloheximide (CX). Data are means ± SD (n = 10). HPT, hypoxic pre-treatment (5% O_2 for 24 h); NHPT, non-hypoxic pre-treatment.

remained unaffected. At anoxia levels ($[O_2] = 0.05$ mol m^{-3}) *V. rupestris* had completely blocked root growth, while *V. riparia* still maintained a lower but significant growth in all the three constituent regions of the root apex.

Heat production

Heat production of roots subjected to different treatments (aerated control, plants subjected to HPT and NHPT and then subjected to anoxia, and plants subjected to HPT and anoxia and concurrently treated with CX 100 μM) is shown in Fig. 3. After an initial period of a relatively rapid decline in heat production rates, roots maintained a slow steady decline. The slope of this decline did not differ significantly between aerated roots of *V. riparia* and *V. rupestris* and it may reflect a slow loss of cell viability. After 10 h, heat production in aerated roots of *V. riparia* was 562 ± 26 μW g^{-1}, while that of non-hypoxically pre-treated anoxic roots was 325 ± 25 μW g^{-1}, leading to a 42% decrease in heat production. Under the same conditions, the less tolerant *V. rupestris* showed a 60% decrease in heat production (from 623 ± 40 to 251 ± 29 μW g^{-1}). HPT significantly prevented the decline in heat production following anoxia. In fact, the decrease in heat production in roots which had been subjected to HPT was reduced to 32% in *V. rupestris* and only 19% in *V. riparia* after 10 h of anoxia. Similar estimates of depression in heat production were obtained after the total amounts of heat produced during 20 h (corresponding to the area under the curves in Fig. 3) have been calculated (Table 1). As expected, application of CX 100 μM contributed to the disappearance of all the benefits derived from HPT.

Activity of fermentative enzymes

To estimate the contribution of ethanol fermentation in recycling NADH/NAD$^+$ in roots during anoxia, we first evaluated the in vitro activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Fig. 4). In roots which had received NHPT, a significant difference in the activities of both the enzymes was noticed in the two species. Furthermore, HPT increased the enzymes' activities in both the species already before the beginning of the anoxia treatment (time 0). After the onset of the anoxia, PDC and ADH activities in roots after both HPT and NHPT increased, but the activities in roots subjected to HPT were much greater than those reported in roots subjected to NHPT. After 20 h of anoxia, PDC activity was 4-fold greater in those roots of *V. rupestris* which had received HPT than in those that received NHPT. Under the same conditions, *V. riparia* was less sensitive to HPT, as the increase in PDC activity was no more than 1.5-fold (Fig. 4A). Roughly the same proportion between activities in roots of *V. rupestris* and *V. riparia* subjected to HPT and NHPT was found for ADH (Fig. 4B).

ATP and ethanol content

When energy production via oxidative phosphorylation is hindered under anoxia, ethanol fermentation appears to be the major pathway for the energy needs in plants. For this reason, ethanol and ATP contents in roots were measured after 20 h under anoxia (Fig. 5). Ethanol production significantly increased after the onset of anoxic conditions in every

Table 1 Total amount of heat produced by roots of *Vitis riparia* and *Vitis rupestris* during 20 h of anoxia

	Total heat production (J g^{-1})	
	Vitis riparia	*Vitis rupestris*
Aerated	35.6 ± 4.2 a	41.1 ± 5.9 a
HPT anoxic	30.1 ± 6.4 b	29.0 ± 4.7 b
NHPT anoxic	21.0 ± 2.3 c	16.8 ± 2.8 c
CX 100 μM	13.1 ± 2.2 d	10.1 ± 1.6 d

Data are reported as means ± SD ($n = 10$). Means were separated by Tukey's multiple comparison test. For each column, different letters indicate statistically significant differences for $P < 0.01$ ($n = 5$).

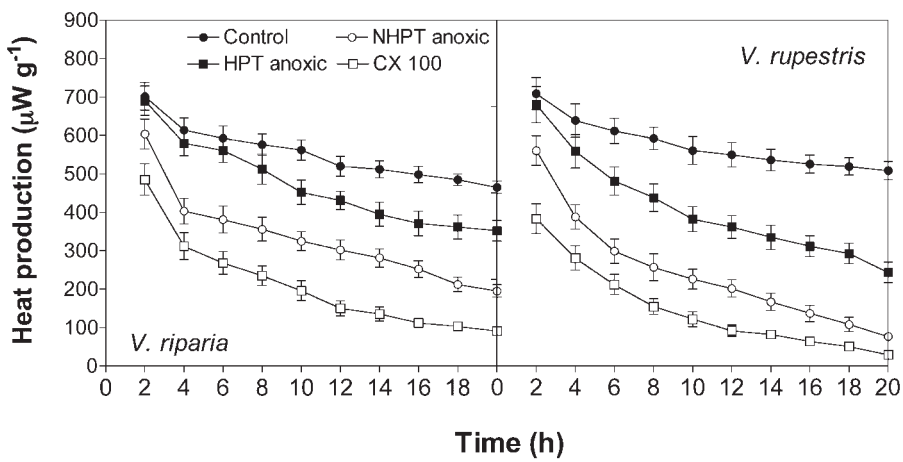

Fig. 3 Heat production by *V. riparia* and *V. rupestris* roots during 20 h after the onset of anoxia treatment in plants subjected to HPT, NHPT, and cycloheximide (CX; 100 μM) and HPT. Control plants were constantly oxygenated, i.e. not subjected to anoxia. Data are means ± SD ($n = 10$).

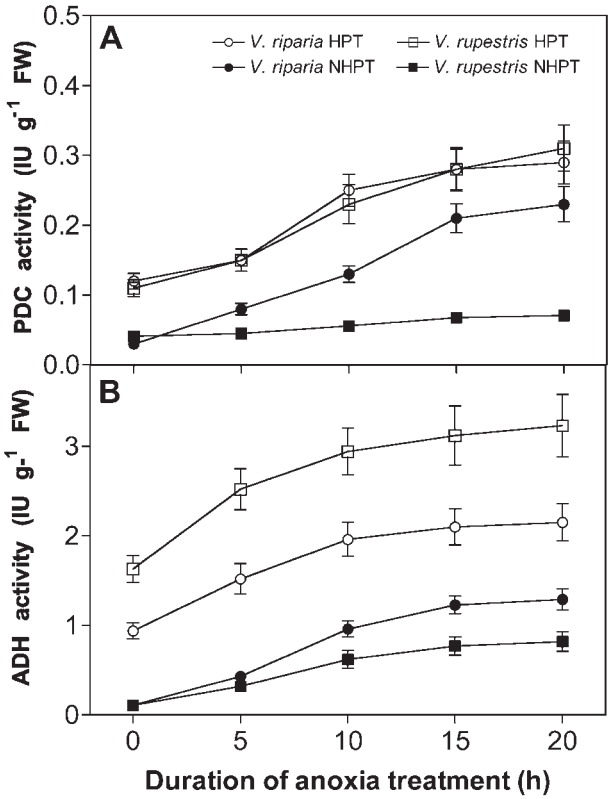

Fig. 4 PDC (A) and ADH (B) activity detected in hypoxic (HPT) or non-hypoxic (NHPT) pre-treated roots, during 20 h of anoxia treatment. Data are means ± SD (n = 10).

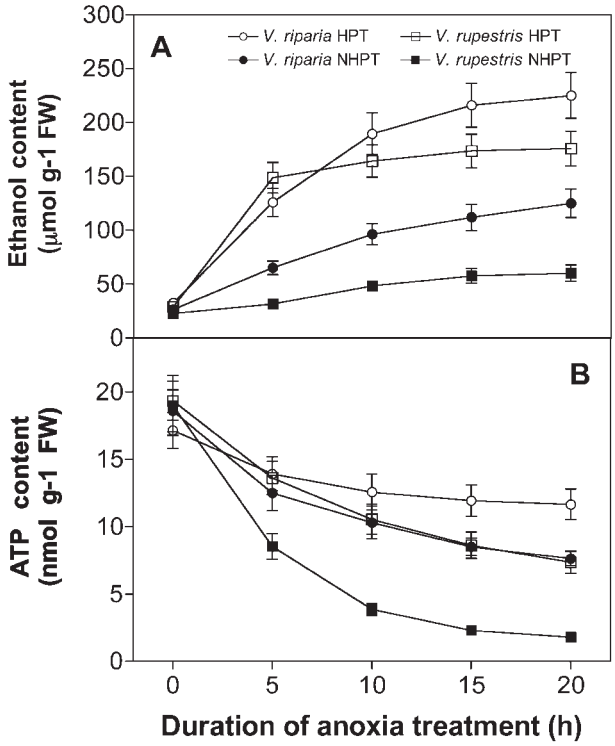

Fig. 5 Ethanol (A) and ATP (B) content detected in hypoxic (HPT) or non-hypoxic (NHPT) pre-treated roots, during 20 h of anoxia treatment. Data are means ± SD (n = 10).

treatment; however, the highest accumulation of ethanol occurred in roots subjected to HPT. In fact, the average ethanol content after 20 h of anoxia in *V. riparia* roots which had undergone HPT and NHPT was 225 and 125 µmol g^{-1} FW, respectively (**Fig. 5A**). A similar pattern, but showing an amplified interval, was found in *V. rupestris* (176 and 60 µmol g^{-1} FW in roots subjected to HPT and NHPT, respectively). The presence of a slight, but detectable, quantity of ethanol in aerated tissues (= time 0) was probably related to the presence of a hypoxic core inside *Vitis* roots. The ATP content decreased in roots which had received HPT and in those which had received NHPT (**Fig. 5B**) during anoxia. However, the decrease was significantly lower in roots which had undergone HPT than in those which had been subjected to NHPT after 20 h of anoxia. Roots of *V. riparia* which had received HPT contained 68% of the ATP contained in aerated roots, whereas the ATP content in roots which had undergone NHPT was 41% of the initial value. The decrease in ATP content was further accentuated in *V. rupestris*. In fact, the roots of plants which had been subjected to HPT contained 38% of the ATP present in aerated roots, but roots which had undergone NHPT contained only 9%. The effect of CX application on ethanol content, ATP content, PDC and ADH activities after 20 h of anoxia has also been evaluated on plants subjected to HPT (**Fig. 6**). By using increasing concentrations of CX, we observed the disappearance of any acclimation process, as demonstrated by the dramatic reduction in both metabolite contents and enzyme activities.

Characterization of K^+ flux and kinetics

Vitis species showed the same spatial organization in net K^+ influx (**Fig. 7**) in normoxic conditions, despite some slight differences in the absolute values. Two distinct peaks characterized K^+ influx: the first was located in the DZ (0.5 mm from the root tip, 32–35 pmol cm^{-2} s^{-1}) and the second in the TZ (1.2–2 mm from the root apex, 42–44 pmol cm^{-2} s^{-1}). In the distal position (>3.5 mm, corresponding to the MZ of the root) K^+ influx was definitely lower (8 pmol cm^{-2} s^{-1}) and remained constant even at further positions (i.e. 10 mm from the root apex). To characterize further the K^+ fluxes in *Vitis* species having different tolerance to anoxia, we also subjected *V. vinifera* to this measurement because it is considered as the model *Vitis* species due to its great economic importance and because of its intermediate tolerance to low oxygen concentrations (Mancuso and Boselli 2002). The three species showed a different behavior when subjected to low oxygen concentrations (0.1 mol m^{-3}) in the NS, in both HPT and NHPT. In hypoxic conditions, plants of *V. riparia* which had undergone NHPT maintained a significant K^+ influx in the TZ. Also, *V. vinifera* showed a lower, but still slight positive value. In contrast, *V. rupestris* was completely unable to maintain any K^+ influx, as only negligible values were detected.

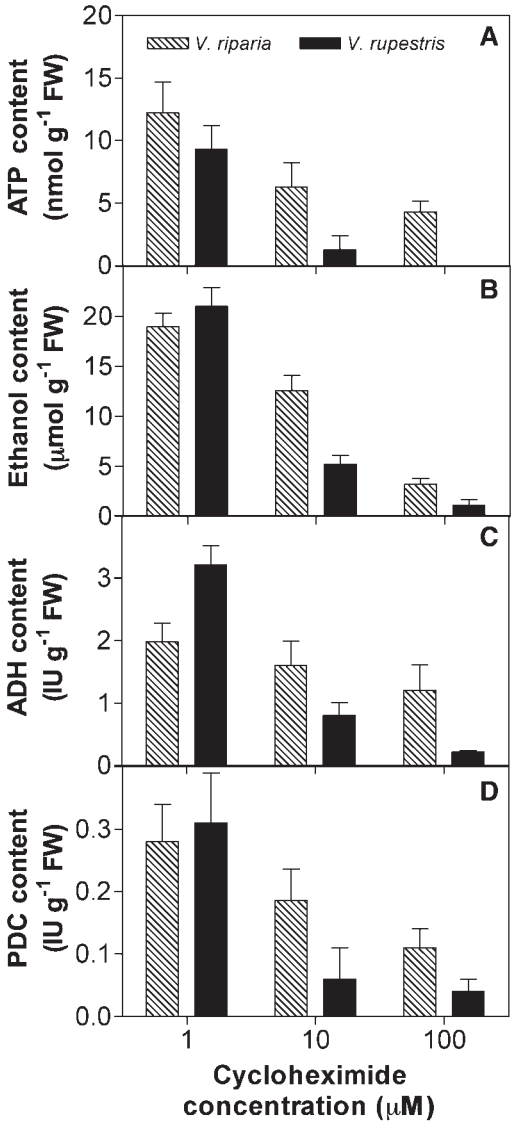

Fig. 6 Effect of cycloheximide application on ATP and ethanol content, PDC and ADH activity measured after 20 h of anoxia in plants subjected to HPT. Data are means ± SD (n = 10).

HPT generally improved K^+ fluxes in hypoxic conditions in all the three species, especially in *V. rupestris*, which reached a very significant peak in the TZ (18.7 pmol cm^{-2} s^{-1}), comparable with those measured in both *V. riparia* and *V. vinifera* (25.4 and 24 pmol cm^{-2} s^{-1}, respectively). Net K^+ uptake measured over a range of $[K^+]$ (1–75 mM in the NS) yielded concentration-dependent kinetics described by the Michaelis–Menten equation regarding both different $[O_2]$ in the NS (**Table 2**) and different treatments (HPT, NHPT, CX; **Table 3**). *Vitis rupestris* dramatically altered the K^+ kinetics curve under $[O_2]$ <0.20 mol m^{-3} (K_m and I_{max}, **Table 2**). In particular, K_m was greatly affected by $[O_2]$ in *V. rupestris*, leading to a strong and significant decrease in substrate affinity. Moreover, I_{max} also dramatically decreased in *V. rupestris* roots when $[O_2]$ decreased. In contrast, lowering $[O_2]$ in the NS had little effect on the K^+ uptake kinetics of *V. riparia* roots. In this species, no net K^+ efflux has ever been recorded at any $[O_2]$ and $[K^+]$, but only a slight decrease in I_{max} values and a slight increase in K_m values were observed. An intermediate behavior was noticed for *V. vinifera* roots, which maintained favorable K^+ kinetics. HPT also produced an efficient increase in I_{max} values and a decrease in K_m values compared with NHPT (**Table 3**) in the roots all the three species. In fact, maximum K^+ influx values after HPT were close to those detected in aerated roots. In contrast, CX treatment dramatically reduced I_{max} and increased K_m values in all the species.

Discussion

The present report completes the results previously obtained by our group (Mancuso and Marras 2006), which described a better tolerance of *V. riparia* to anoxia, compared with *V. rupestris*. *Vitis riparia* plants were able to survive better in anoxic conditions (**Fig. 1**) and to slow down the decrease in the elongation rate during oxygen deprivation (**Fig. 2**) after a period of low oxygen availability. These latter data confirm the root TZ as the most sensitive region of the root apex to environmental stress, as previously reported by Baluška et al. (2010). The maintenance of a higher metabolic performance in *Vitis* species which had been subjected to HPT under anoxia conditions, especially *V. rupestris*, was mainly driven by the following. (i) An enhanced activity of key enzymes in alcohol fermentation (ADC and PDC, **Fig. 4**). Induction of ADH and PDC can increase the survival of plants under flooded conditions (Johnson et al. 1994) and can be considered one of the main strategies for plants to survive in low oxygen conditions (Kato-Noguchi 2000). (ii) The ability to maintain a higher level of respiration, evidenced by a lesser decrease in heat development and ATP production (**Figs. 3**, **5B**). A higher level of ATP is considered to be a significant contributing factor to the subsequent plant survival under anoxia (Kato-Noguchi 2000). (iii) The maintenance of a better ion homeostasis (highlighted by K^+ flux measurement) and K^+ channel functionality when plants were subjected to both decreasing $[O_2]$ in the NS and HPT (**Tables 2**, **3**).

In the present study, the activities of PDC and ADH under anoxic conditions were better expressed in the roots of *V. riparia*, compared with *V. rupestris*, indicating an up-regulation of anaerobic respiration. However, HPT permitted the activities of the two enzymes in both the species to be greatly enhanced, particularly in the less tolerant *V. rupestris*. These results were indirectly confirmed by the measurement of ethanol content, which showed a large increase in both the species after HPT, with a plateau after 12 h. In our case, the in vitro PDC activity during anoxia was around 1/10th of the ADH activity under the same conditions, in agreement with previous studies indicating that the limiting step in ethanolic fermentation is the pyruvate–acetaldehyde conversion (Quimio et al. 2000, Zabalza et al. 2009). A high

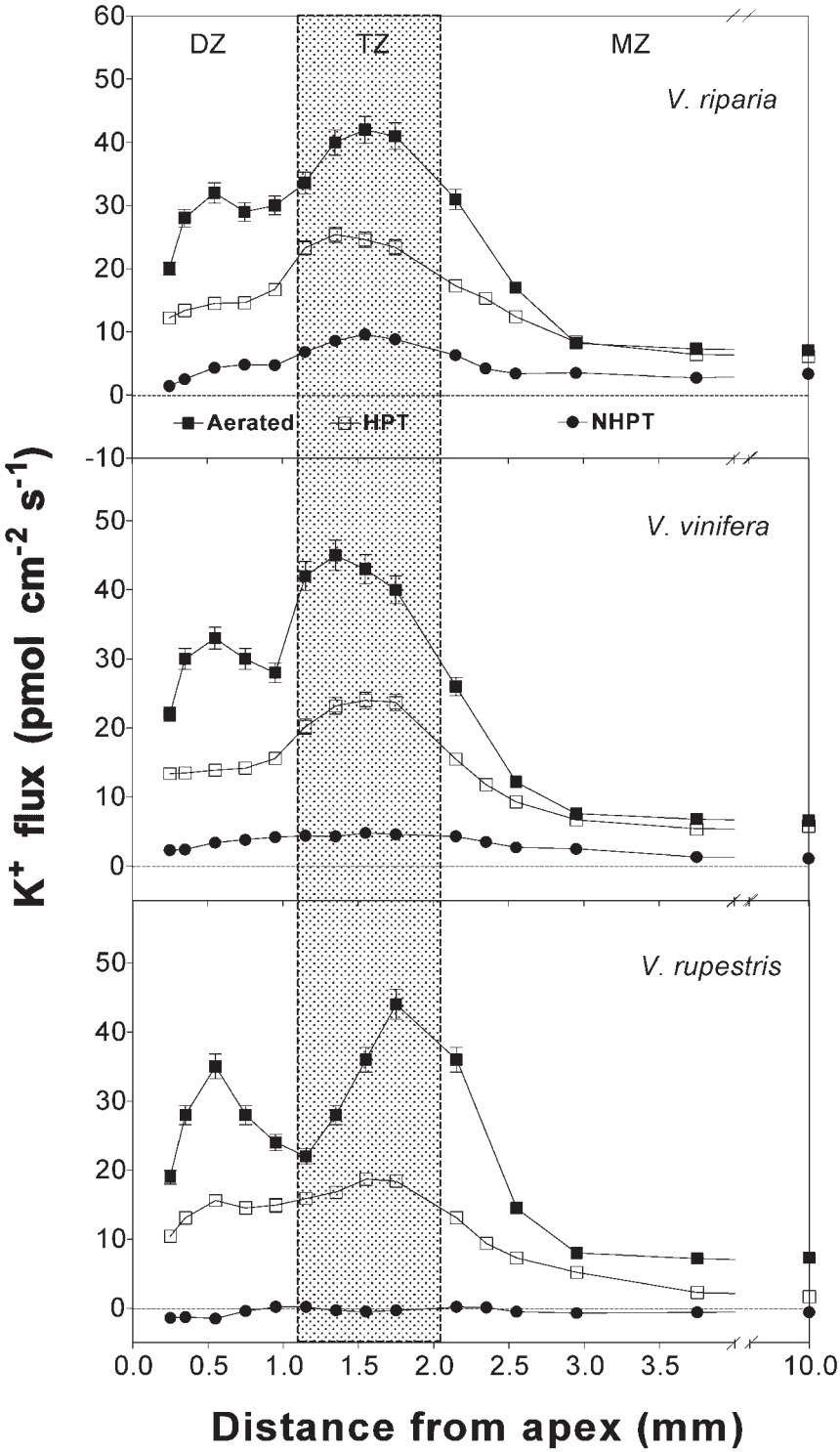

Fig. 7 K^+ fluxes map obtained by measurements in different regions (DZ, division zone; TZ, transition zone; MZ, mature zone) of intact root apices belonging to three *Vitis* spp. grown subjected to different conditions: normoxic (filled squares), HPT (hypoxic pre-treatment, open squares) and NHPT (non-hypoxic pre-treatment, filled circles). Data are means ± SD ($n = 10$).

ADH/PDC ratio has been reported to be a decisive factor in preventing accumulation of potentially toxic acetaldehyde (Gibbs and Greenway 2003). In the present study, a few pieces of conflicting evidence were found: after 20 h of anoxia the ADH/PDC ratio in roots of the tolerant *V. riparia* subjected to NHPT was 5.6, half of the value measured in the sensitive *V. rupestris* (11.7). Interestingly, HPT had no effect on the ADH/PDC ratio in *V. rupestris* (11.7 in roots after NHPT and

Table 2 Kinetics of K^+ uptake measured in the transition zone of intact roots over a range of oxygen content in the nutrient solution

Oxygen content (mol m^{-3})	*V. riparia*		*V. vinifera*		*V. rupestris*	
	I_{max}	K_m	I_{max}	K_m	I_{max}	K_m
0.27	29.1	10.2	27.9	11.2	26.2	12.5
0.20	26.5	11.8	23.7	12.8	22.0	16.7
0.15	24.4	12.1	20.8	13.4	11.6	18.5
0.10	22.0	14.8	16.2	21.4	6.0	30.0

I_{max}, maximum K^+ uptake rate; K_m, half-saturation constant.

Table 3 Kinetics of K^+ uptake measured in the transition zone of intact roots over a series of treatments (HPT, hypoxic pre-treatment; NHPT, non-hypoxic pre-treatment; CX100, cycloheximide at 100 μM)

Treatments	*V. riparia*		*V. vinifera*		*V. rupestris*	
	I_{max}	K_m	I_{max}	K_m	I_{max}	K_m
Aerated	28.8	10.3	27.7	10.8	26.4	11.6
HPT	24.8	11.0	24.2	13.1	20.7	14.7
NHPT	22.5	14.4	21.3	16.5	12.0	27.2
CX100	16.0	27.0	17.1	28.0	5.7	30.5

HPT, NHPT and CX100 measurements were performed under hypoxic conditions (0.1 mol m^{-3} of oxygen). I_{max}, maximum K^+ uptake rate; K_m, half-saturation constant.

10.4 after HPT), while HPT permitted a significant increase in the ADH/PDC ratio in *V. riparia* (from 5.6 in roots after NHPT to 7.4 after HPT). Therefore, it is tempting to conclude that the absolute value of the ADH/PDC ratio appears to be less important than the capability of plants to change the relative weight of the numerator and denominator as a determining factor for the induction of anoxia tolerance.

CX dramatically blocked root acclimation and the subsequent survival to hypoxia. Chemical inhibition of protein synthesis during HPT is likely to prevent the induction of hypoxia-inducible genes, indicating that de novo translation of proteins that respond to hypoxic conditions is required. Hence, acclimation mechanisms to hypoxia are strongly dependent on protein synthesis (Ellis et al. 1999, Bond et al. 2009).

Chang et al. (2000) observed that the difference in anoxia tolerance between plants subjected to HPT or NHPT is not limited only to increases in the levels of glycolytic enzymes, such as PDC and ADH. The net uptake of solutes is also important in order to maintain cell turgor and a consequent positive elongation rate. Together with sugars, K^+ represents the major solute contributing to the osmotic pressure of the cell sap, with obvious consequences on cell growth (Huang et al. 2003).

K^+ fluxes have been extensively evaluated in herbaceous plants under environmental stress such as salinity (Chen et al. 2007, Pandolfi et al. 2010) and hypoxia (Pang et al. 2006). As K^+ uptake is disturbed by oxygen deprivation (Pang and Shabala 2010), an improved ability for K^+ uptake in roots may therefore be critical to overall plant performance under hypoxia. For all these reasons, we evaluated the effect of anoxia conditions and HPT on the capability of *Vitis* roots to maintain a high level of K^+ homeostasis during stress conditions. The uptake of K^+ from soil and its transport within the plant require the presence of membrane-bound transport proteins. A large number of these transporters have now been identified and their characterization is currently under investigation (Britto and Kronzucker 2008, Szczerba et al. 2009). The relationship between the ion uptake rate and its external concentration, described by the kinetic parameters of the transporters such as K_m and I_{max}, is usually affected by environmental stresses. Our results confirmed the sensitivity of K^+ transporters to stresses, as hypoxia strongly increased K_m, leading to a decreased affinity of the carrier site for K^+, and concurrently decreased I_{max}. This latter is considered to be a more useful parameter for comparing the competitive abilities of various species for a limiting nutrient or for assessing the effect of an environmental stress on ion transport (Harrison et al. 1989).

Interestingly, the major changes in the K^+ fluxes were detected in the TZ of the root (**Fig. 7**), confirming that this region is highly sensitive to oxygen deprivation phenomena. The TZ of root apices is a unique part of the root (Baluska et al. 2010). The cells of the TZ have the highest rate of oxygen respiration of the whole root (Mancuso et al. 2000), their auxin transport shows the highest degree of activity (Mancuso et al. 2005) and K^+ uptake in this region also shows its maximum activity (Mancuso and Marras 2006). In addition, data obtained from the TZ revealed a synchronous and oscillatory electric activity in this zone only (Masi et al. 2009), supporting the concept that TZ serves some, so-far unknown, but apparently specific purpose for the integration of internal and external signaling (Baluska et al. 2004).

K^+ uptake is an energy-demanding process because, for every K^+ ion absorbed, a slight depolarization of the membrane will occur, with a subsequent energy-dependent electrical compensation by the plasma membrane H^+-ATPase. Anoxia conditions reduce ion transport through ion channels mainly due to the limiting supply of ATP to the plasma membrane proton-pumping ATPase (Elzenga and van Veen 2010), as occurred in *Vitis* plants subjected to NHPT. In our experiment, however, HPT allowed maintenance of a significant K^+ influx (**Fig. 7**) because of the higher ATP production under low oxygen concentrations compared with plants after NHPT, especially in the less tolerant *V. rupestris* (**Fig. 5B**). The main difference in the response to anoxia of *V. riparia* and *V. rupestris* is the ability of *V. riparia* to maintain a functional ion homeostasis during prolonged anoxia episodes, due to a positive K^+ membrane permeability. This ion homeostasis can be also restored by HPT on the hypoxia-sensitive *V. rupestris* as no efflux was ever detected at any [K^+] in plants after HPT. In this case, the improved activity of K^+ channels could represent a direct response to changes in the intracellular energy, derived

from the stimulated production of ATP following HPT, as ion-transporting ATPases are the dominant energy-consuming processes of root cells (Van der Werf et al. 1992, Bouma and De Visser 1993,). It is also known that both hypoxia and anoxia rapidly decrease the K^+ membrane permeability in *V. riparia*, avoiding or reducing K^+ leakage under stress (Mancuso and Marras 2006), whereas K^+ membrane permeability in *V. rupestris* under stress remains unaffected, thus leading to a strong K^+ efflux. Our results support the hypothesis that HPT is able to reduce K^+ membrane permeability significantly in *V. rupestris*, preserving ion homeostasis and permitting a better survival in stressful conditions.

Materials and Methods

Plant material and growth conditions

Cuttings from 1-year-old shoots with six leaves, 15–20 cm long, were taken from 7-year-old stock plants of *V. riparia* Michx. and *V. rupestris* Scheel. The lower portion (5 mm) of each cutting was immersed in an aqueous KIBA (3-indolebutyric acid potassium salt) solution at 3.6 g l^{-1} for 5 s to promote rooting, then placed in perlite and kept in a greenhouse under conditions of high relative humidity (mist propagation), daylight for 12 h and 22°/15°C average day/night temperatures. Rooting occurred in 6 weeks. At the end of the rooting period, plants with uniform root length (3–5 cm), thickness (2–3 mm) and color (white) were chosen. Plants were brought into the laboratory 4 d before the beginning of the experiment, and additional light from fluorescent tubes (110 mmol m^{-2} s^{-1} at canopy height) was provided. The root system was carefully washed in order to remove the perlite residue, and then immersed in an aerated nutritive solution [1 mM KCl, 0.905 mM NaH_2PO_4, 0.048 mM Na_2HPO_4, 1 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$]. The solution temperature was maintained at 21 ± 1°C during the immersion period. After that, plants were utilized for the experiments.

Experimental procedure: HPT, anoxia treatment and CX application

The $[O_2]$ of the solution in the measuring chamber was modified from 0 to about 0.27 mol m^{-3}, covering the whole range of the $[O_2]$ that can occur in soils (Smit and Stachowiak 1988). Plants with roots length of 2–3 cm were transferred to a chamber containing 100 ml of a modified Hoagland's NS (pH 5.5, EC 1.3 mS cm^{-1}, NO_3^- 102 p.p.m., NH_4^+ 5 p.p.m., PO_4^{3-} 29 p.p.m., K^+ 153 p.p.m., Ca^{2+} 88 p.p.m., Mg^{2+} 18 p.p.m., SO_4^{2-} 37 p.p.m.). Shoots were maintained in air while the root system and the measuring chamber were placed in a glove-bag. The different $[O_2]$ was obtained by using pressurized gases containing different oxygen content (from 0 to 21%), 0.1% CO_2 and the balance as N_2 (Palta and Nobel 1989). HPT was obtained 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} for 20 h. Bulk solution $[O_2]$ in the measuring chamber was recorded polarographically using a Clark type electrode for pO_2 (E.C.D., model 0225) connected to an oxygen monitor (E.C.D., model 8602). In some experiments, 1 μM (CX-1), 10 μM (CX-10) or 100 μM (CX-100) CX solution (Sigma-Aldrich), a protein translation inhibitor, was added to the liquid medium during HPT. CX was then washed off with distilled water at the end of the treatment.

Survival determination and root elongation measurement

Eight plants with 3–4 adventitious roots of uniform length (3–5 cm) and elongation velocity (around 1 mm h^{-1}) were arranged in a plastic holder at the top of a Plexiglas box (40 × 8 × 20 cm) containing NS. Following the method adopted by Verslues et al. (1998), roots were left to grow downward through transparent root guides made from plastic drinking straws (i.d. 7 mm), which facilitated the measurement of the root elongation rate. Root guides were perforated with several holes (diameter 1 mm) to allow exchange of solution. The solution was vigorously aerated through a perforated plastic tube. The root elongation rate was measured after 24 h at different $[O_2]$ (0.27, 0.20, 0.15, 0.10, 0.05 mol m^{-3}) obtained by mixing pressurized gases containing from 0 to 21% O_2, 0.1% CO_2 and the balance as N_2. The root elongation rate was determined following the method of Peters and Bernstein (1997). Roots were considered to have survived if they resumed elongation after anoxic treatment. Under aerated conditions, roots of both species showed no symptoms of damage.

Microcalorimetry

Heat production was measured by a heat conduction multichannel microcalorimeter (TAM 2277, Thermometric) on excised roots. The heat was measured for 20 h in oxygenated or in anoxic NS, both on roots after HPT and after NHPT. The reference ampoules also contained anoxic or oxygenated NS. Before each measurement, the ampoules were kept in the heat-equilibrating position of the calorimeter for 60 min to allow them to warm up to 20°C. After the ampoules were placed in the measuring chamber, control experiments (both ampoules containing NS but no roots) showed that the system equilibrated in 10 min. Thus, the results obtained during the first 10 min were discarded.

K^+ flux measurements

K^+ flux measurements were performed on three different *Vitis* spp.: the two species selected for this study together with *V. vinifera*. The latter species was introduced in this set of measurements because it is considered as the model *Vitis* species due to its great economic importance and because of its intermediate tolerance to low oxygen concentrations. The design and operating mode of the vibrating probe system have been previously and extensively described by Mugnai

et al. (2006). In brief, micropipets were pulled from borosilicate tubing and then silanized. The tips were broken to a tip diameter of 3–5 mm. 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 in sets of standard 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 a 3 mol l^{-1} NaCl–3% agar bridge. The K^+-selective microelectrodes were mounted on a manual micromanipulator providing three-dimensional positioning. The distance between the root surface and the electrodes was changed during the measurements by fixing the chamber to a three-way hydraulic micromanipulator (WR-6, Narishige) driven by computer-controlled stepper motors (type I 5PM-K004-01, Minebea Co.). The electrodes were connected by screened cables to a high input impedance (10^{14} Ω) electrometer (home built based on an AD 645 JN operational amplifier). The output signal was amplified, low-pass filtered and connected via a multichannel A/D convertor card (Lab-PC-1200; National Instruments) to a personal computer. Prior to study of K^+ fluxes, *Vitis* spp. roots were carefully washed with deionized water. A single root from the root system still attached to the plant was anchored to the bottom of the measuring chamber containing NS so that the air–water surface was about 1 cm from the root surface. Samples were secured to the chamber bottom using a Plexiglas block fixed with silicone grease. A settling time of 1 h prior to the start of the experiment was allowed. Experiments were performed at 25 ± 0.25°C. To prevent possible artifacts coming from temperature fluctuations, the temperature inside the measuring chamber was recorded during all the experiments. All flux measurements for the characterization of K^+ fluxes in the two species were made in the apical part of the root, from 0.25 to 10 mm from the root tip, whereas the measurements for the K^+ kinetic curves in response to both decreasing $[O_2]$ and HPT were performed in the TZ. During recording, 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 experiment. The difference in electrode voltage at the two extremes of vibration was measured by digitizing the electrode signals in order to compute the potential difference. For each electrode position, the first 2 s after the movement began were automatically discarded to eliminate stirring effects and movement artifacts, and to allow the re-establishment of the gradient; the remaining signal was then averaged. The computer calculated the difference between this average and the previous one at the other extreme position and finally calculated a moving average of these differences over any desired time period. Data were collected at a rate of 1,000 data points s^{-1}. K^+ fluxes were calculated using Fick's first law of diffusion, assuming cylindrical diffusion geometry.

Metabolite measurements

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 81 ± 5% (mean ± SE, $n = 7$). 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 homogenation gave values of 84 ± 8% (mean ± SE, $n = 5$). ADH (EC 1.1.1.1) and PDC (EC 4.1.1.1) activities were determined spectrophotometrically by monitoring the change of absorbance at 340 nm following reduction of NAD^+ by ethanol according to Bergmeyer (1974).

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