Response of mitochondria to light intensity in the leaves of sun and shade species

KO NOGUCHI1,2, NICOLAS L. TAYLOR2, A. HARVEY MILLAR2, HANS LAMBERS3 & DAVID A. DAY2

1 Department of Biology, Graduate School of Science, Osaka University, 1–1 Machikaneyama-Cho, Toyonaka, Osaka, 560–0043, Japan, 2 Biochemistry and Molecular Biology, School of Biomedical and Chemical Sciences and 3 School of Plant Biology, the University of Western Australia, Australia

ABSTRACT

The present authors have shown previously that both respiration rates and in vivo activities of the alternative oxidase (AOX) of leaves of Alocasia odora, a shade species, are lower than those in sun species, thereby optimizing energy production under limited light conditions (Noguchi et al., Australian Journal of Plant Physiology 28, 27–35, 2001). In the present study, mitochondria isolated from A. odora leaves were examined in order to investigate the biochemical basis for the differences in respiratory parameters. Alocasia odora and spinach plants were cultivated under both high and low light intensities, mitochondria were isolated from their leaves, and their respiratory properties compared. Mitochondrial content of leaf extracts from the two species was estimated using fumarase activities and antibody detection of porin (the voltage-dependent anion channel of the outer mitochondrial membrane). On a mitochondrial protein basis, spinach leaves showed higher capacities of the cytochrome pathway and cytochrome c oxidase (COX) than A. odora leaves. However, on a mitochondrial protein basis, A. odora showed higher capacities of AOX, which had a high affinity for ubiquinone when activated by pyruvate. Alocasia odora also had larger amounts of mitochondrial protein per leaf dry weight, even under severely shaded conditions, than spinach. Lower growth light intensity led to lower activities of most pathways and proteins tested in both species, especially glycine-dependent oxygen uptake. In the low light environment, most of the AOX protein in A. odora leaves was in its inactive, oxidized dimer form, but was converted to its reduced active form when plants were grown under high light. This shift may prevent over-reduction of the respiratory chain under photo-oxidative conditions.

Key-words: Alocasia odora; Spinacia oleracea; alternative oxidase (AOX); mitochondria; respiration; sun and shade.

INTRODUCTION

Plant mitochondria have two ubiquinol-oxidizing pathways, the cytochrome pathway and the alternative pathway. The latter consists of one enzyme, the alternative oxidase (AOX), and has the potential to catalyse apparently wasteful respiration in higher plant mitochondria. Its role in plants has not been definitively identified, except for heat production in the spadix of Araceae species, but it is thought to prevent production of reactive oxygen species (ROS) in the respiratory chain, especially under environmental stress (Vanlerberghe & McIntosh 1997; Moore et al. 2002; Vanlerberghe & Ordog 2002; Millenaar & Lambers 2003). However, many previous studies have imposed severe physiological stresses on plants or cell cultures to induce changes in AOX activity or expression and consequently the ecophysiological role of AOX remains ambiguous. In vitro regulation of AOX involves alteration of the redox state of the AOX protein, activation by α-keto acids (e.g. pyruvate), and the redox state of ubiquinone (Q) (Day & Wiskich 1995; Siedow & Umbach 2000). However, regulation of AOX activity in vivo remains unclear (Millenaar & Lambers 2003), because (1) AOX protein is more often than not in the reduced, activated form in crude extracts of plant tissues (e.g. Millenaar et al. 2001); (2) the total pyruvate concentration in cells is high enough to fully activate AOX, but the in vivo pyruvate concentration in mitochondria is unknown (Siedow & Umbach 2000); and (3) the in vivo redox state of ubiquinone is rather stable (Millar et al. 1998; Millenaar et al. 2001). These studies have led to the notion that most of the AOX protein is fully activated in vivo. Further analyses are required to understand the in vivo regulation and the physiological significance of AOX.

Shade species show slower rates of leaf photosynthesis and respiration than sun species, contributing to a positive carbon balance under very low light conditions, such as experienced by a forest understorey (Björkman 1981; Noguchi, Sonoiike & Terashima 1996; Noguchi & Terashima 1997). In vivo AOX activity in leaves of Alocasia odora, a shade species, is also low under low light intensities similar to those in their natural habitat (Noguchi et al. 2001). Thus, in A. odora leaves, the respiratory system appears to be geared towards efficient ATP production. In contrast, in leaves of spinach, a high-light adapted plant, in vivo AOX activity was high at the beginning of the night and then decreased during the night, when plants were grown under a high light intensity (Azcón-Bieto, Lambers & Day 1983; Noguchi et al. 2001). Here we investigate the question of whether the leaves of A. odora have a smaller amount of
AOX or whether AOX is inactivated under low-light conditions.

Shade leaves within crops and other sun species, such as spinach, also show slower respiratory rates than leaves exposed to the sun (Noguchi et al. 1996). In vivo AOX activity in leaves of spinach is also low under these conditions (Noguchi et al. 2001), ensuring high efficiency of respiration, which may be advantageous in shaded environments in which photosynthetic production is low. Slower rates of photosrespiration in shade leaves would also enhance net production of photosynthate (Muraoka et al. 2000). The photosynthetic activity involves chloroplasts, peroxisomes and mitochondria. In the mitochondria, glycine decarboxylase (GDC) converts glycine to serine, and produces NADH and NH₃. We have also investigated the effect of light intensity on GDC and mitochondrial glycine oxidation.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

We used *Spinacia oleracea* L., a sun species, and *Alocasia odora* (Lodd.) Spach., a shade species. Seeds of *S. oleracea* were purchased from a local supermarket in Perth, Australia. The seeds were germinated in soil and 10 d later the seedlings were transferred to 20-L containers with hydroponic nutrient solutions. Plants of *A. odora* were propagated vegetatively from rhizome segments. The mother plant was purchased at a local nursery in Perth. Seedlings gated vegetatively from rhizome segments. The mother seedlings were transferred to 20-L containers with hydroponic nutrient solutions. Twelve seedlings were purchased from a local supermarket in Perth, Australia. The photosynthetic photon flux densities (PPFD) were 490, 200 and 20 μmol photons m⁻² s⁻¹ for high-light (HL), low-light (LL), and very low-light (VLL) conditions, respectively. As spinach did not grow under the VLL condition, we cultivated spinach under HL and LL conditions, respectively. As spinach did not grow under these conditions. The most recently fully expanded leaves were used for all experiments. Leaves of *S. oleracea* were harvested about 1 month after germination and those of *A. odora* about 1 month after propagation.

**Leaf respiration**

Rates of O₂ uptake of leaves were measured polarographically with a Clark-type O₂ electrode (Rank Brothers, Cambridge, UK) at 20 °C in 5 mL of air-saturated solution containing 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulphonic acid (HEPES), 10 mM 2-morpholinoethanesulfonic acid (MES) (pH 6.6) and 0.2 mM CaCl₂ (after Azcón-Bieto et al. 1983). Leaf segments (approximately 5 cm²) were sampled 1 h after the end of daytime, and then incubated for more than 10 min in buffer before the measurements. A piece of nylon netting was used to keep the segments above the stirrer bar and the electrode. The O₂-uptake rates were calculated assuming that the concentration of oxygen in air-saturated buffer at 20 °C was 276 μmol L⁻¹.

**Isolation of mitochondria**

Mitochondria were isolated according to the methods of Day, Neuburger & Douce (1985) and Taylor, Day & Millar (2002), from 20 to 60 g of leaves. Leaves were disrupted with a Polytron (Kinematica, Kriens, Switzerland) for spinach, and with a mortar and pestle for *A. odora*, in 150–250 mL of cold extraction medium [0.3 m sucrose, 25 mM tetra-sodium pyrophosphate, 10 mM KH₂PO₄, 2 mM EDTA, 1 mM glycine, 1% (w/v) polyvinylpyrrolidone (PVP)-40, 1% (w/v) bovine serum albumin (BSA), 20 mM ascorbate, pH 7.5]. The homogenate was filtered through four layers of damp stretcher cloth (Dräger Medical, Adelaide, Australia), and centrifuged for 5 min at 1100 × g. The supernatant was centrifuged for 20 min at 18 000 × g, and the pellet was re-suspended in 200 mL of wash medium [0.3 m sucrose, 10 mM TES, 1 mM glycine, 0.1% (w/v) BSA, pH 7.5], and centrifuged for 5 min at 1100 × g. The supernatant was centrifuged for 20 min at 18 000 × g, and the pellet was re-suspended in 10 mL of wash medium. Aliquots of 5 mL were then layered over 27.5 mL of solution [0.3 m sucrose, 10 mM TES, 1 mM glycine, 0.1% (w/v) BSA, 28% and 32% (v/v) Percoll for spinach and *A. odora*, respectively, and a linear gradient of 0–4.4% (w/v) PVP-40, pH 7.5] in a centrifuge tube, and centrifuged for 40 min at 40 000 × g. The mitochondria were found as a tight light yellow-brown band near the bottom of the tube. The mitochondrial fraction was removed and diluted in 250 mL of wash medium and centrifuged at 31 000 × g for 15 min. The supernatant was removed, and this wash was repeated. The final mitochondrial pellet was re-suspended in approximately 1 mL of wash medium. Mitochondrial integrity was monitored as described by Neuburger et al. (1982) and was consistently better than 90%, in both species. Respiratory control ratios (RCR) with NADH plus succinate as substrates, were always more than two (data not shown).

**Measurements of O₂ consumption and Q redox state in isolated mitochondria**

The O₂ consumption was measured with an O₂ electrode (Rank Brothers) in 1 mL of reaction medium containing 0.3 m sucrose, 10 mM TES-KOH (pH 7.5), 5 mM KH₂PO₄, 10 mM NaCl, 2 mM MgSO₄, and 0.1% (w/v) BSA. Glycine (10 mM), pyruvate (5 mM), malate (1 mM), 2-oxoglutarate...
(5 mM), succinate (5 mM), NADH (1 mM), NAD (0.5 mM), ATP (0.5 mM), ADP (0.1–1 mM), DTT (1.5 mM), n-propyl gallate (30 μM), myxothiazol (1.5 μM) were added as indicated. The O₂-uptake rates were calculated assuming that the concentration of oxygen in air-saturated buffer at 25 °C was 240 μmol L⁻¹. Protein concentrations were determined by the method of Peterson (1977) using BSA as standard. The redox state of ubiquinone (Q) was measured voltametrically with glassy carbon and platinum electrodes according to the method of Moore, Dry & Wiskich (1988).

**Fumarase activity**

The maximal activity of fumarase was determined for crude extracts as described by Noguchi et al. (1996). Crude extracts of segments were prepared by the method of Makino & Osmond (1991). Frozen leaf segments were ground to a powder in liquid nitrogen and extracted in a buffer that contained 100 mM HEPES-KOH (pH 7.5), 10 mM KH₂PO₄, 0.5 mM EDTA, 10% (v/v) glycerol and 10 mM DTT with a chilled mortar and pestle. The ratio of leaf to buffer was 1 : 7 (w/v). After centrifugation at 15,000 × g for 3 min, the supernatant was used for measurements. Ten microlitres of the supernatant was placed in a cuvette containing 990 μL of a reaction mixture consisting of 70 mM KH₂PO₄–NaOH (pH 7.5), 4 mM DTT, 50 mM malate and 0.05% (v/v) Triton X-100. For isolated mitochondria, 10 μL of mitochondria solution was placed in a cuvette containing the above mixture except for DTT. The increase in absorbance at 240 nm due to the conversion of malate to fumarate was monitored with a spectrophotometer. A molar extinction coefficient of 2.25 mM⁻¹ cm⁻¹ was used (Hatch 1978).

**SDS-PAGE and Western blotting**

For isolated mitochondria, aliquots containing 40 μg of protein were solubilized in sample buffer [2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] including 50 mM DTT and boiled for 5 min. For isolated leaf membranes, approximately 250 mg fresh weight of A. odora leaf was crushed with a mortar and pestle in 1 mL of grinding medium [0.3 M sucrose, 25 mM tetra-sodium pyrophosphate, 10 mM KH₂PO₄, 2 mM EDTA, 1 mM glycine, 1% (w/v) PVP-40, 1% (w/v) BSA, 20 mM ascorbate, a protease inhibitor tablet (Roche, Mannheim, Germany), pH 7.5]. The homogenate was centrifuged for 5 min at 11,000 × g. The supernatant was centrifuged for 5 min at 10,000 × g and the pellet was re-suspended in 100 μL of sample buffer, and boiled for 5 min. Five milligrams of diamide (azobis-dimethylformamide) in 1 mL of 50 mM Tris-HCl, pH 7.0, was used to grind leaf samples for oxidation of samples, and the grinding medium and the sample buffer included 50 mM DTT for reduction. For whole-tissue extracts, 250 mg fresh weight of leaves was snap frozen in liquid nitrogen, glass beads were added, and the sample was crushed to a fine powder with a mortar and pestle. Samples were then solubilized in 1 mL of the sample buffer, boiled for 5 min, and centrifuged at 10,000 × g for 5 min; 40 μL of the supernatant was loaded per lane for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Twenty microlitres of sample was separated by electrophoresis under denaturing reducing conditions on 0.1% (w/v) SDS-12% (w/v) polyacrylamide gels according to Laemmli (1970). For immunoreaction experiments, proteins were electroblotted from SDS-PAGE gels onto nitrocellulose membranes and blocked. A 1/1000 dilution of the serum raised against H-protein of GDC (Dr Steve Rawsthorne, John Innes Centre, Norwich, UK), 1/50 dilution of the ‘AOA’ monoclonal culture supernatant reacting with AOX (Elton, Nickels & McIntosh 1989), 1/7500 dilution of serum raised against subunit II of cytochrome c oxidase (Daley et al., 2002), 1/10 000 dilution of serum raised against uncoupling protein (Considine, Daley & Whelan 2001), and 1/500 dilution of monoclonal culture supernatant reacting with the voltage-dependent anion channel, porin (PM035, from Dr Tom Elthon, Lincoln, NE, USA) were used as primary antibodies. Chemiluminescence was used for detection of horseradish peroxidase-conjugated secondary antibodies and visualized using LAS 1000 (Fuji, Tokyo, Japan). The blots were quantified using the IMAGE GAUGE v3.0 software (Fuji).

**Statistical analyses**

All data were analysed with StatView (ver. 5.0 J; SAS, Cary, NC, USA).

**RESULTS**

**Leaf respiration**

In general, growing both spinach and A. odora under low light resulted in slower rates of leaf respiration. Alocasia odora leaves showed significantly slower respiratory rates on a dry weight (DW) basis than those observed in spinach leaves, under all growth light conditions (Table 1). Spinach leaves showed significantly different rates of respiration between light conditions when the data were expressed on a leaf area basis, but not when expressed on a DW basis. For A. odora leaves, respiration rates of leaves in VLL were significantly slower than those of leaves in HL.

**Tissue mitochondria content**

The polypeptide profiles of mitochondria from the leaves of both species were virtually identical (Fig. 1a), indicating that the purity of the organelle preparations was not significantly different. Figures 1b and c show immunoblots of porin in isolated mitochondria and in whole leaf extracts, respectively. On a protein basis, the antibody produced a significantly different. Figures 1b and c show immunoblots of porin in isolated mitochondria and in whole leaf extracts, respectively. On a protein basis, the antibody produced a significantly different response in isolated spinach and A. odora leaf mitochondria, further attesting to the similar levels of purity of the different preparations. However, the amount of porin (the outer membrane voltage-dependent anion...
Table 1. Respiratory rates of leaves of *S. oleracea* and *A. odora*

<table>
<thead>
<tr>
<th></th>
<th><em>S. oleracea</em></th>
<th><em>A. odora</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL</td>
<td>LL</td>
</tr>
<tr>
<td>Leaf area basis (μmol m⁻² s⁻¹)</td>
<td>0.483 ± 0.079a</td>
<td>0.181 ± 0.031b</td>
</tr>
<tr>
<td>Leaf DW basis (nmol g⁻¹ s⁻¹)</td>
<td>14.7 ± 2.3a</td>
<td>11.2 ± 2.0 a</td>
</tr>
</tbody>
</table>

The rates were measured 1 h after the end of daytime. Averages ± standard deviations (n ≥ 3). Different letters mean significant differences among conditions (Tukey–Kramer’s multiple comparison test, \( P < 0.05 \)).

channel) in mitochondria isolated from VLL *A. odora* leaves was smaller than that in the mitochondria from the higher light environment. There was no difference in the amounts of porin in spinach leaves between light conditions. In contrast, when expressed on a leaf fresh weight (FW) basis, the amount of porin in spinach leaf extracts was much smaller than that in *A. odora* leaf extracts (Fig. 1c), suggesting differences in leaf mitochondrial content between the two species. The light conditions also influenced the amount of porin in *A. odora*, with higher light resulting in larger amounts of porin protein. On the other hand, in spinach leaves, there were small differences between light conditions. These results were confirmed in repeated experiments (results not shown). Both of the immunoblots of porin in isolated mitochondria and in total leaf extracts, and the ratio of DW to FW of leaf tissues (data not shown) gave an estimate of relative contents of mitochondrial protein on a leaf DW basis (Table 2).

![Figure 1](image-url)
The TCA cycle enzyme, fumarase, was also used as a marker activity mitochondrial protein content in leaves. Fumarase activities of mitochondria isolated from spinach leaves were much higher than those from A. odora leaves (Table 2). In both species, the activity in isolated mitochondria from plants grown under lower light conditions was less than in those grown at high light intensity, but the difference was not statistically significant. In contrast, fumarase activity in whole leaf extracts of A. odora was greater than that in spinach leaves, when plants were grown under HL. Under LL conditions, the difference between species was not significant. There was no difference in the activities of leaf extracts between light conditions for spinach, but the activity of A. odora leaves was significantly lower under VLL than that under higher light. The fumarase activity in leaf extracts divided by that in isolated mitochondria, gives an estimate of the mitochondrial protein content of leaf tissue (Table 2). On this basis, tissue mitochondrial content of A. odora leaves was significantly greater than that of spinach leaves, even under VLL. There was no significant difference in tissue mitochondrial content between HL and LL conditions in the leaves of either species, but the mitochondrial content of A. odora grown at VLL was lower than when grown at higher light. These results confirm those obtained with the porin immunoblots and independently indicate that light intensity affects mitochondrial protein content in A. odora.

### Respiratory electron transport chain activity in isolated mitochondria

Maximal electron flux via the respiratory chain was estimated in isolated mitochondria using a combination of NADH and succinate as substrates, in the presence of ADP, to optimize flux through the cytochrome chain, and pyruvate and DTT, to optimize flux via AOX. Under these conditions, spinach mitochondria showed higher rates of oxygen uptake on a protein basis than those from A. odora leaves, when plants were grown under HL conditions (Table 3). Under LL conditions, the differences between the two species were smaller. Significant differences were observed when malate and glutamate or glycine were substrates. Glycine-dependent oxygen uptake rates were affected greatly by light levels during growth of spinach, decreasing dramatically at the lower light level. Glycine-dependent oxygen uptake rates were substantially less in A. odora mitochondria, especially under VLL (Table 3). In spinach mitochondria, glycine-dependent oxygen uptake rates were faster than those with any other single substrate under HL condition, but under LL conditions, succinate-dependent oxygen uptake was highest. In A. odora mitochondria, the oxygen-uptake rates of LL plants were faster than those under other light conditions, except with malate as a substrate. In A. odora mitochondria, under all light conditions, the NADH-dependent oxygen-uptake rate was faster than that with other substrates.

Using the tissue mitochondria contents calculated from the fumarase activities, we expressed the mitochondrial activities on a leaf DW basis. Despite the higher specific activities in mitochondria isolated from spinach, because of the higher mitochondrial protein content in A. odora leaves, optimized respiratory rates expressed on leaf DW basis were generally higher in A. odora, when plants were grown at HL or LL (Table 3). The very low mitochondrial content of VLL A. odora leaves meant that optimized respiration rates were much less than in the other plants (Table 3).

### Cytochrome c oxidase and cytochrome pathway capacity

On a mitochondrial protein basis, cytochrome c oxidase (COX) capacity (KCN-sensitive and cytochrome c-dependent oxygen-uptake rate) in A. odora leaves was significantly lower than that in spinach leaves under HL conditions (Table 4). In spinach leaves, COX capacity under HL was higher than that under LL, but in A. odora leaves the reverse was seen. We calculated COX capacity on a leaf DW basis using the data on tissue mitochondria content (Table 2). There was no significant

---

**Table 2.** Fumarase activity in isolated mitochondria and crude extracts of leaves of *S. oleracea* and *A. odora*.

<table>
<thead>
<tr>
<th></th>
<th><em>S. oleracea</em></th>
<th><em>A. odora</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL</td>
<td>LL</td>
</tr>
<tr>
<td><strong>Fumarase activities</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated mitochondria (nmol mg(^{-1}) protein min(^{-1}))</td>
<td>503 ± 95a</td>
<td>406 ± 100a</td>
</tr>
<tr>
<td>Leaf extract (mol g(^{-1}) DW min(^{-1}))</td>
<td>10.8 ± 0.4a</td>
<td>9.69 ± 0.86a</td>
</tr>
<tr>
<td>Tissue mitochondria content (mg protein g(^{-1}) DW)</td>
<td>21.6 ± 3.8a</td>
<td>23.9 ± 3.5a</td>
</tr>
<tr>
<td>(relative units g(^{-1}) DW)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porin abundance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue mitochondria content (relative units g(^{-1}) DW)</td>
<td>1.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Tissue mitochondria contents were calculated using both activities. Values in parentheses were relative contents. Averages ± standard deviations; n ≥ 3. Relative tissue mitochondria contents were calculated using both quantified data of immunoblots of porin with isolated mitochondria and whole leaf extracts. Different letters mean significant differences among conditions (Tukey–Kramer’s multiple comparison test, P < 0.05).
The COX capacity was measured as KCN-sensitive, cytochrome c-dependent O2 uptake. The capacities of CP and AOX were measured in the presence of NADH and succinate as substrates. The CP capacity was also measured in the presence of ADP and n-propyl gallate, and the AOX capacity in the presence of myxothiazol, DTT and pyruvate. Data are presented on a mitochondrial protein basis (a; nmol mg−1 protein min−1) and a leaf dry weight basis (b; nmol g−1 DW s−1). Averages ± standard deviations are shown; n ≥ 3. Different letters mean significant differences among conditions (Tukey–Kramer’s multiple comparison test, P < 0.05). succ; succinate; pyr; pyruvate.

AOX capacity and the ratio of AOX to cytochrome pathway

Optimized AOX activity was measured in isolated mitochondria with NADH plus succinate as substrates, in the presence of myxothiazol (a complex III inhibitor) and the AOX activators DTT and pyruvate (Day & Wiskich 1995).

Table 3. Oxygen-uptake rates with different substrates in mitochondria isolated from leaves of *S. oleracea* and *A. odora*

<table>
<thead>
<tr>
<th></th>
<th><em>S. oleracea</em></th>
<th></th>
<th><em>A. odora</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HL</td>
<td>LL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. oleracea</em></td>
<td></td>
<td><em>A. odora</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HL</td>
<td>LL</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VLL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Mitochondria protein basis substrate(s)</td>
<td>NADH</td>
<td>94.7 ± 18.7a</td>
<td>65.5 ± 24.0ab</td>
<td>47.8 ± 9.7b</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>80.5 ± 9.6a</td>
<td>67.9 ± 13.2ac</td>
<td>35.2 ± 9.0b</td>
</tr>
<tr>
<td></td>
<td>NADH + succinate</td>
<td>140 ± 28a</td>
<td>106 ± 21a</td>
<td>67.3 ± 12.2bc</td>
</tr>
<tr>
<td></td>
<td>NADH, succ, pyr, DTT</td>
<td>150 ± 26a</td>
<td>128 ± 23ac</td>
<td>78.5 ± 12.0b</td>
</tr>
<tr>
<td></td>
<td>Malate + glutamate</td>
<td>75.5 ± 12.1a</td>
<td>53.5 ± 13.7b</td>
<td>39.0 ± 6.0bc</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>111 ± 0.5a</td>
<td>54.1 ± 18.1b</td>
<td>26.1 ± 0.7bc</td>
</tr>
<tr>
<td>(b) Leaf dry weight basis substrate(s)</td>
<td>NADH</td>
<td>34.0 ± 6.7a</td>
<td>26.1 ± 9.6a</td>
<td>78.9 ± 16.0b</td>
</tr>
<tr>
<td></td>
<td>Succinate</td>
<td>28.9 ± 3.5a</td>
<td>27.0 ± 5.2ad</td>
<td>58.2 ± 14.9b</td>
</tr>
<tr>
<td></td>
<td>NADH, succinate</td>
<td>50.3 ± 10.2a</td>
<td>42.2 ± 8.2a</td>
<td>111 ± 20b</td>
</tr>
<tr>
<td></td>
<td>NADH, succ, pyr, DTT</td>
<td>53.7 ± 9.4a</td>
<td>51.0 ± 9.1a</td>
<td>130 ± 20b</td>
</tr>
<tr>
<td></td>
<td>Malate, glutamate</td>
<td>27.3 ± 6.1a</td>
<td>21.3 ± 5.5a</td>
<td>64.8 ± 9.9b</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>40.0 ± 0.2a</td>
<td>21.6 ± 7.2b</td>
<td>43.1 ± 1.2a</td>
</tr>
</tbody>
</table>

Data are presented on a mitochondrial protein basis (a; nmol mg−1 protein min−1) and leaf dry weight basis (b; nmol g−1 DW s−1). Averages ± standard deviations are shown; n ≥ 3. Different letters mean significant differences among conditions (Tukey–Kramer’s multiple comparison test, P < 0.05). succ; succinate; pyr; pyruvate.
Under these conditions, there was no significant difference in AOX capacity between mitochondria isolated from the two species, when expressed on a protein basis. Likewise, there was no significant difference in the capacity of AOX in mitochondria isolated from leaves of *A. odora* grown under different light regimes, although rates in VLL mitochondria tended to be less. Similar results were obtained when we used duroquinol as a substrate: AOX capacity in *A. odora* mitochondria was slightly higher than that in spinach mitochondria, but the difference between the two species was not significant (results not shown). When malate and glutamate, or glycine, were used as substrates, AOX rates were slower than those in the presence of NADH and succinate, but again, no significant differences were obvious between species or growth conditions (results not shown).

In contrast to the results with isolated mitochondria, when AOX capacity was expressed on a leaf DW basis, it was significantly higher in *A. odora* than in spinach, irrespective of mitochondrial substrates (Table 4). For *A. odora*, AOX capacity under VLL conditions was significantly lower than that under other light conditions. These results reflect the greater density of mitochondria in the leaves of *A. odora* compared with spinach (see above). In spinach leaves, there was no difference in AOX capacity between light conditions. The ratio of maximal AOX activity to that of the cytochrome pathway (AOX/CP) was significantly higher in *A. odora* mitochondria than in spinach mitochondria (Table 4). This is in contrast to measured whole leaf respiratory rates (Table 1 and Noguchi et al. 2001). The ratio in VLL was lower than that under other light conditions for *A. odora* leaves.

**Q redox status**

The relationship between the redox status of ubiquinone (Q) and electron transport rates was investigated using the so-called Q electrode simultaneously with an oxygen electrode (Moore et al. 1988). With succinate as substrate, in both the presence and absence of ADP, oxygen-uptake rates in *A. odora* mitochondria were slower than those in spinach (Fig. 2 and Table 3). In the absence of malonate, a succinate dehydrogenase (complex II) inhibitor, there was little difference in the ratio of reduced quinone to total quinone (Qr/Qt) between the two species. In the presence of myxothiazol alone, the oxygen-uptake rates via AOX were low, and Qr/Qt was high in mitochondria of both species. In the absence of malonate, pyruvate increased oxygen-uptake rates, and decreased Qr/Qt in both species, as shown and discussed previously (Millar et al. 1993; Umbach, Wiskich & Siedow 1994). Titration with malonate decreased both oxygen-uptake rate and Qr/Qt. In the presence of myxothiazol alone, the intercept of the x-axis was similar for mitochondria of both species, whereas, in the presence of myxothiazol and pyruvate, the intercept of the x-axis was different between the two species. The intercept was more than 0.2 for spinach mitochondria, whereas it was about 0.1 for *A. odora* mitochondria. That is, in *A. odora* mitochondria, AOX was active at lower values of Qr/Qt than in spinach mitochondria. Changing the light conditions during plant growth had little effect on these results.

Data obtained with the Q and oxygen electrodes in the absence of malonate, reflect the properties of the quinone-reducing pathway (in this case, succinate dehydrogenase; Millar et al. 1998). We fitted a Michaelis–Menten curve to the data, and estimated $V_{\text{max}}$ and $K_m$ (the ratio of oxidized quinone to the total quinone pool) of the quinone-reducing pathway (Fig. 2 and Table 5). The $V_{\text{max}}$ and $K_m$ values obtained with spinach mitochondria were greater than those of *A. odora* mitochondria, except when *A. odora* was grown under VLL. That is, succinate dehydrogenase has a lower maximal activity in *A. odora* mitochondria but a higher affinity for oxidized quinone than that in spinach. HL spinach mitochondria showed a lower $K_m$ and a higher $V_{\text{max}}$ than LL mitochondria.

**Immunodetection of selected mitochondrial proteins**

Using the AOX antibody, apparent molecular weights of AOX in spinach and *A. odora* leaves were 32 and 34 kDa, respectively. On a mitochondrial protein basis, the amount of AOX protein in spinach leaf mitochondria appeared to be much lower than in *A. odora* leaves (Fig. 3). However, since the affinity of the antibody for AOX might differ between the two species, these results must be interpreted cautiously, especially given the similarity in maximal AOX activity in mitochondria isolated from the two species (Table 4). Within the same species, however, the blots can be used to estimate protein levels. Higher growth light intensity induced higher amounts of AOX in mitochondria in both species, but the differences were insignificant (*A. odora*, ANOVA, $F = 2.51$, $P = 0.161$). On a leaf DW basis, on

![Table 5.](https://example.com/table5.png)

Table 5. $V_{\text{max}}$ and $K_m$ of the Q-reducing pathway in mitochondria isolated from leaves of *S. oleracea* and *A. odora*, using the non-linear least square method

Averages ± standard errors are shown.
the other hand, the amount of AOX in leaves of *A. odora* grown under VLL conditions was significantly lower than that of leaves exposed to higher light.

Apparent molecular weights of COX subunit II (COX II) in spinach and *A. odora* leaves were 31 and 30 kDa, respectively. On a mitochondrial protein basis, the amount of COX II in leaves of HL spinach was significantly higher than that of LL spinach, whereas, in *A. odora* leaves, LL mitochondria had a significantly larger amount of COX II than under other light conditions (Fig. 4). These results agree with the activity measurements of COX (Table 4). On a leaf DW basis, similar trends were observed.

Apparent molecular weights of uncoupling protein (UCP) in spinach and *A. odora* leaves were 31 and 30 kDa, respectively. On a mitochondrial protein basis, the amount of UCP in leaves of HL spinach was also higher than that of LL spinach.

**Figure 2.** Succinate-dependent oxygen uptake rate as a function of $Q_r/Q_t$ in isolated mitochondria of leaves of *S. oleracea* and *A. odora*. Data points are in the presence (downward triangles) and absence (upward triangles) of ADP, and in the presence of myxothiazol with (circles) or without (squares) added pyruvate. Open symbols denote data in the absence of malonate; filled symbols are data from malonate titration of succinate oxidation. Curves were fitted through open symbols that represent the activity of succinate dehydrogenase with a non-linear least square method.
of LL spinach (Fig. 5), but the difference was insignificant ($P = 0.0518$). In $A. odora$ leaves, the growth light intensity significantly influenced the amount of UCP (ANOVA, $F = 11.47$, $P = 0.0089$), but we did not observe any significant difference between conditions using the Tukey–Kramer multiple comparison test. On a leaf DW basis, the amount of UCP in VLL leaves of $A. odora$ was significantly lower than that in leaves from plants grown under the other light conditions.

The H protein of GDC in spinach mitochondria did not react with the antibody available, but the apparent molecular weight of H protein in $A. odora$ leaves was 16 kDa. In $A. odora$, the amount of H protein in VLL mitochondria was significantly lower than that under higher light conditions, on both a mitochondrial protein and a leaf DW basis (Fig. 6). This agrees with the rates of oxygen uptake with glycine as a substrate (Table 3).

**Estimation of AOX redox state in vivo**

Given the difference between the amount of AOX protein and its capacity in isolated mitochondria (this study), and previous estimates of in vivo AOX activity in $A. odora$ leaves (Noguchi et al. 2001), we examined the in vivo redox status of AOX protein in $A. odora$ leaves by rapidly isolating a membrane fraction from a leaf homogenate (Fig. 7). This was necessary because although we were able to detect reduced AOX monomers on Western blots of whole leaf extracts, the oxidized dimer was difficult to detect reliably. We added DTT or diamide to the samples, to either reduce or oxidize, respectively, all of the AOX protein in the leaf membrane samples. During subsequent membrane isolation, no obvious shifts between oxidized dimers and reduced monomer forms were observed (Fig. 7a, lanes 1 and 2). We also took the precaution of mixing previously purified mitochondrial membranes, in which AOX was partially oxidized and partially reduced, with the leaf extracts.
before membrane pelleting (Fig. 7b). No obvious change in AOX redox state was observed during membrane isolation. Having ascertained that the procedure did not artifactually oxidize AOX protein, we performed Western blots on membrane fractions from leaves grown under different light regimes (Fig. 7a, lanes 3–6). In leaves from plants grown under low light, all of the AOX protein was in the oxidized, inactive form. In contrast, when grown under higher light conditions, all of the AOX protein was in the reduced, active form (Fig. 7a, lanes 5 and 6). Transfer of plants grown under low to high light conditions also caused AOX protein to be reduced (results not shown). That is, changing the light intensity during plant growth changed the activation status of AOX.

**DISCUSSION**

We have examined the differences in mitochondrial properties between a shade plant, *A. odora*, and spinach, which are adapted to growth at higher light intensities. Measurement of oxygen consumption by leaf segments confirmed previous studies which have shown that leaves of *A. odora* respire more slowly than those of spinach, at all light intensities. Increasing light intensity during growth of both species increased in vivo respiration rates (Table 1). Respiratory rates of isolated mitochondria, with all substrates tested, were also higher in spinach when expressed on a mitochondrial protein basis (Tables 3 & 4). However, unexpectedly, we found that total mitochondrial content (mg protein per leaf DW) was much higher in *A. odora* leaves than in spinach, even when grown at the very low light intensities likely to be found in the natural habitat of the shade plant (Table 2). These estimated mitochondria contents using the fumarase activities were supported by the data estimated using the porin blots (Fig. 1 & Table 2). Consequently, when mitochondrial activities were expressed per leaf DW, the respiratory capacities of both the cytochrome and alternative pathways were substantially greater in *A. odora* leaves (Tables 3 & 4). These
results indicate that *A. odora* leaves maintain a capacity for rapid respiration but carefully regulate this under their usual growth conditions.

The finding of higher AOX capacity in *A. odora* leaves was particularly surprising given the very low AOX activity measured in vivo using oxygen discrimination techniques (Noguchi et al. 2001). It should also be noted that the ratio of AOX capacity to that of the cytochrome pathway in *A. odora* leaves was greater than that in spinach leaves, and UCP was also present (Fig. 5). This shows that *A. odora* leaves have the potential for rapid uncoupled respiration, even under very low light intensities. However, in vivo, when plants were grown under heavily shaded conditions, all of the detectable AOX protein was in the inactive, oxidized form (Fig. 7), explaining the lack of AOX activity in the previous study (Noguchi et al. 2001). When grown at higher light intensity, most of the AOX protein was in the reduced, active form, possibly in response to increased availability of respiratory substrates and increased reducing power in the mitochondria. The extent to which AOX contributes to total leaf respiration under these conditions remains to be determined and requires oxygen discrimination measurements.

It had previously been assumed that shade plants have low respiratory capacity and little AOX activity, in order to minimize respiratory losses under a strictly limited carbon budget (Noguchi et al. 2001). It now seems that respiratory capacity is relatively high in these plants but they restrict AOX activity by inactivating the enzyme. Under very low light, the availability of substrates is also likely to restrict respiration via the cytochrome chain. Only when plants are grown under stressful higher light intensity, AOX activity is activated and respiratory rates rise [under HL conditions, the $F_v/F_m$ of *A. odora* leaves was lower than that at LL (data not shown) indicating that photoinhibition had occurred]. This presumably is a strategy to allow very rapid respiratory response to changing environmental conditions (such as sun flecks) in the shade plant. In this context it is worth noting that AOX is active at lower $Q_r/Q_t$ in *A. odora* leaf mitochondria (Fig. 1 and Table 5). Rapid activation of AOX would help to maintain lower reduced quinone pools and decrease the formation of ROS.

Notwithstanding this, *A. odora* also increases its total respiratory capacity, both via AOX and the cytochrome pathway, when grown at higher light intensities, but this is achieved by a general increase in leaf mitochondrial content rather than an increase in specific activity of electron transport components. An exception to this is the ability of *A. odora* mitochondria to oxidize glycine, which is severely diminished on a mitochondrial protein basis under very low light conditions (Table 4), presumably in response to low photorespiratory flux under these conditions (Muraoka

Figure 7. (a) Immunoblots of AOX in rapidly extracted membrane fractions isolated from *A. odora* leaves. Lane 1, a sample of VLL leaves treated in the presence of 50 mM DTT; lane 2, a sample of VLL leaves treated in the presence of 5 mM diamide; lanes 3 and 4, samples consisted of only VLL leaf membrane fractions; lanes 5 and 6, samples consisted of only HL leaf membrane fractions. (b) Immunoblots of AOX in rapidly extracted membrane fractions and/or mitochondria isolated from *A. odora* leaves. Lane 7, a sample consisted of only VLL leaf membrane fractions; lane 8, a sample of VLL leaf membrane fractions, added with a mitochondrial extract from HL leaves just before the extraction; lane 9, mitochondrial sample isolated from HL leaves.
et al. 2000). This was also true of leaf mitochondria from spinach grown at LL, which oxidized glycine at about half the rate of mitochondria from HL plants.

Concluding remarks

The respiratory activity of both sun and shade plants respond to different light intensities during growth. Alocasia odora, a shade plant adapted to growth under very low light intensities, regulates its respiratory capacity by manipulating the mitochondrial content of its leaves when grown under different light intensities. It also maintains a relatively high AOX capacity but controls its activity by keeping AOX protein as the inactivated, oxidized dimer under very low light.

ACKNOWLEDGMENTS

We would like to thank Christel Norman, Greg Cawthray and Robert Creasy for their kind help in our study. K.N. was supported by a Fellowship from the Japan Society for the Promotion of Science for Research Abroad. H.L., A.H.M. and D.A.D. received support form the Australian Research Council, A.H.M. is funded by an ARC Queen Elizabeth II Fellowship.

REFERENCES


Received 22 October 2004; received in revised form 1 December 2004; accepted for publication 10 December 2004

© 2005 Blackwell Publishing Ltd, Plant, Cell and Environment, 28, 760–771

Respiration in sun and shade species