Seed Science Research (2003) **13**, 35–45 DOI: 10.1079/SSR2002122

Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing

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Abstract

Germination is an energy-demanding process that requires the operation of mitochondria, which must survive desiccation in the quiescent seed and become rapidly functional after imbibition to meet the ATP demand. The relationship between germination and mitochondrial performance was addressed by analysing the properties of mitochondria isolated from control, primed and aged pea (*Pisum sativum* L.) seeds. Mitochondria were isolated and purified at early stages of germination (before radicle protrusion), and their oxidative properties, membrane integrity and ultrastructure were examined. Mitochondria isolated after 12 h of imbibition readily oxidized exogenous NADH and Krebs cycle substrates at high rates. However, their phosphorylation efficiency was restricted by poor membrane integrity. After 22 h from the beginning of imbibition, purified seed mitochondria had intact outer membranes and oxidized the substrates at slightly lower rates, but with higher respiratory control (improved capacity for phosphorylation). Purified seed mitochondria were always found to be deficient in endogenous NAD, although the organelles were capable of importing and retaining the cofactor. While the priming treatment appeared to slightly increase the performance of mitochondria, seed deterioration by accelerated ageing strongly affected the oxidative properties of mitochondria, which were badly impaired in ATP production. Outer and inner membrane integrity was identified as the primary target for desiccation and ageing stress. A link between mitochondrial function and seed quality was also corroborated by respiration measurements of seed fragments at the onset of imbibition.

Keywords: mitochondrial membranes, inner membrane, oxidative phosphorylation, seed quality, priming, accelerated ageing, *Pisum sativum*

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Introduction

Germination is a complex phenomenon that encompasses drastic changes in the metabolic activities and in the genetic programme of seeds. Such events require energy, and it is likely that mitochondria are major providers of the cellular ATP during germination. This is suggested by the almost universal requirement for O_2 during germination and for the increase of energy charge that occurs during early germination (Hourmant and Pradet, 1981; Al-Ani *et al*., 1985). In orthodox seeds, which are desiccation-tolerant, mitochondria endure a desiccation stress during maturation, which is followed by a surge of water during imbibition. In the field, germinating seeds (and therefore seed mitochondria) may experience several drying cycles, depending upon weather conditions. As pointed out in a detailed review of respiratory metabolism in seeds (Botha *et al*., 1992), our knowledge concerning the biogenesis of mitochondria and the regulation of mitochondrial metabolism in seeds is still limited.

Observations of cell ultrastructure during seed development led to a general view of poorly differentiated mitochondria in quiescent tissue that evolve, upon imbibition, into healthy looking mitochondria with well-defined cristae and a dense matrix (Bain and Mercer, 1966a, b; Morohashi *et al*., 1981a). Changes in mitochondrial activities (substrate oxidations, ADP/O) during germination, which were monitored using crude preparations of mitochondria, supported the general trend of improvement suggested by cytological observations (Kollöffel and Sluys, 1970; Malhotra and Spencer, 1970; Nawa and Asahi, 1971, 1973; Solomos *et al*., 1972; Morohashi and Shimokoriyama, 1975; Morohashi and Bewley, 1980 a, b; Morohashi *et al*., 1981a). Biogenesis of mitochondria during germination is expected to proceed from structural development of pre-existing mitochondria in starch-storing seeds and from *de novo* synthesis of mitochondria in lipid-storing seeds (Malhotra and Spencer, 1973; Morohashi *et al*., 1981a, b; Morohashi,

1986). Attempts to isolate mitochondria from dry seeds yielded highly damaged mitochondria (Nawa and Asahi, 1971; Wilson and Bonner, 1971; Sato and Asahi, 1975), which were, however, capable of oxidizing substrates (without respiratory control) and generating low amounts of ATP (Attucci *et al*., 1991). Recently, it was proposed that mitochondria from dry maize seeds relied upon cytosolic NADH oxidation to differentiate during imbibition into functional mitochondria (Logan *et al*., 2001). Indeed, the integration of all the previous work is rendered difficult because the experiments were carried out on different types of seeds, focused on different stages of development (most of the studies dealt with post-germinative events), and in most cases used crude organelle fractions.

To gain further insight into the functions of seed mitochondria, we have started to investigate the biochemical and functional properties of germinating pea (*Pisum sativum* L.) seed mitochondria. Here, we report the physiological characterization of mitochondria that were isolated in the initial stages of germination of control, primed and aged seeds. The results highlight the healthy functional state of the organelles that have nevertheless suffered severe dehydration, and identify the outer and inner mitochondrial membranes as the primary targets for stress-induced damage and recovery.

Materials and methods

Seed germination and treatments

Pea seeds (*Pisum sativum* L. cv. Baccara) were produced locally (Fédération Nationale des Agriculteurs Multiplicateurs de Semences, Brain sur l'Authion, France). The seeds, harvested in summer 1999, were naturally dried under ambient conditions to a moisture content of 15–16% (fresh weight basis, fwb) and then sealed in plastic bags and stored at 5°C (70% RH). Another seed lot was harvested in summer 2000 and stored in similar conditions for 4 months. Germination assays were carried out in plastic (polycarbonate) boxes (18.5 × 12.5 × 5.5 cm). One hundred seeds were spread in the folds of pleated paper (Schleicher and Schuell, Ecquevilly, France) moistened with 80 ml of distilled water. This method allowed seeds to imbibe rapidly without being immersed in water. The covered box was incubated in darkness at 20°C. A seed was considered germinated when the radicle pierced the seed coat. The priming treatment consisted of soaking the seeds in aerated, deionized water at 20°C for 16 h. Seeds were then drained rapidly and dried at room temperature for an hour. Subsequent fast drying was achieved by keeping the seeds at 20°C in a ventilated desiccation cabinet containing regenerated silica gel. When seed moisture contents reached 15–16% (fwb), primed seeds were sealed in plastic bags and stored as described above.

Accelerated ageing was performed by exposing the seeds to 45°C for different periods of time in tightly closed boxes containing a saturated NaCl solution that maintained the relative humidity at 76% (Byrd and Delouche, 1971). Seeds were then dried rapidly in the desiccation cabinet to their initial water content (15–16 % fresh weight).

Isolation of mitochondria

Mitochondria were isolated from pea seeds 12 or 22 h after the start of imbibition, and purified through Percoll (Amersham Biosciences Europe GmbH, Freiburg, Germany) gradients using a method adapted from the classical procedure established for plant mitochondria (Douce *et al*., 1987). Approximately 250 g of imbibed seeds were rinsed briefly in distilled water and ground (30 s) using a Waring blender containing 400 ml of cold grinding buffer [0.6 M mannitol, 30 mM $Na_4P_2O_7$, pH 7.5, 1 mM EDTA, 3 mM β-mercaptoethanol, 0.5% (w/v) polyvinylpyrolidone-25, 0.4% (w/v) bovine serum albumin (BSA)]. All subsequent operations were carried out at 4°C, and all centrifugations were performed using a JA-20 rotor (Beckman, Fullerton, California, USA). The homogenate was filtered through eight layers of muslin and a 50 µm nylon mesh. The filtrate was submitted to differential centrifugation (1500 *g* for 10 min; 10 000 *g* for 20 min). The pellets were suspended in washing buffer [0.6 M mannitol, 20 mM KH_2PO_4, pH 7.2, 1 mM EDTA, 0.2 % (w/v) BSA], and the suspensions were homogenized gently using a Potter–Elvehjem system (Connie, Gattikon, Switzerland). The suspension was submitted to a second round of differential centrifugation (same conditions). The 10 000 *g* pellets were suspended in a small volume of washing buffer. The suspension was layered on to a discontinuous step gradient (22% and 30% Percoll) in gradient medium [22 or 30% (v/v) Percoll, 0.6 M sucrose, 10 mM KH_2PO_4, pH 7.2, 1 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 0.2% (w/v) BSA]. After centrifugation at 10 000 *g* for 20 min, the 22–30% interface was collected and slowly diluted with washing buffer. The suspension was centrifuged (13 000 *g*, 15 min) and the resulting pellets were suspended in a small volume of washing buffer. The suspension was layered on the top of a tube filled with 27.5% Percoll (22 h imbibed seeds) or 27% Percoll (12 h imbibed seeds) in gradient buffer. The tubes were centrifuged at 40 000 *g* for 40 min with controlled acceleration and no braking, to create a self-generating exponential Percoll gradient. The mitochondria, which were found as a cloudy

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band in the lower third of the gradient, were collected, and the fraction was diluted gently with washing buffer (omitting BSA). Mitochondria were rinsed twice by centrifugation (13 000 *g*, 15 min), and the final pellet was suspended in a minimal volume of washing buffer (without BSA). To estimate contamination by plastid membranes, carotenoids were extracted with 80% (v/v) acetone and quantified spectrophotometrically using an absorption coefficient (mg^{-1} ml cm^{-1}) of 2500 (Davies, 1976).

Oxygraphic measurements

Oxygen consumption of seed fragments or mitochondria was monitored with a calibrated oxygen electrode (OXYTHERM, Hansatech, King's Lynn, UK) at 25°C. Seed fragments of approximately 0.3 mm were obtained by grinding dry seeds for 30 s with a laboratory Mixer Mill (Retsch, Haan, Germany) and selecting the fragments by sieving through a combination of 1, 0.7 and 0.3 mm metal grids. Oxygen consumption was measured in 1 ml of 0.1 M sucrose, 50 mM KH_2PO_4 (pH 7.2) for seed fragments, or in electrode medium containing 0.6 M mannitol, 20 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) (pH 7.5), 10 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$ and 0.1% (w/v) BSA for mitochondria. Substrates were added to provide the following final concentrations: succinate (5 mM), malate (7.5 mM), pyruvate (7.5 mM), 2-oxoglutarate (9 mM), citrate (7.5 mM), NADH (1.5 mM) and NADPH (1.5 mM). Additional cofactors or metabolites required for substrate oxidation were added as required or as indicated in the figure legends: 3 mM ATP (succinate oxidation), 1 mM NAD (pyruvate, oxoglutarate, citrate, malate oxidations), 0.3 mM thiamine pyrophosphate (TPP; pyruvate, oxoglutarate oxidations), 50 µM coenzyme A (pyruvate, oxoglutarate oxidations) and 1.5 mM malate (pyruvate oxidation). Cyanide (1 mM) and 100 µM propylgallate were used as inhibitors of electron transfer, and 2 µM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP) as an uncoupler, when required. For the outer-membrane integrity assay, the substrate was 32 µM cytochrome *c* (Sigma-Aldrich, St Louis, USA) reduced by 8 mM ascorbate, which gave rise to the initial oxygen consumption (rate A) corresponding to the activity of mitochondria with damaged outer membranes. The rupture of the outer membrane in all mitochondria was achieved by the addition of 0.03 % (v/v) Triton X100 (rate B). The final addition of 1 mM cyanide made it possible to measure the non-enzymatic oxidation of cytochrome *c* (rate C). The percentage of integrity was calculated from the rates A, B, C according to the following formula: integrity = 100 – [(A – C/B – C) × 100]. Protein concentration was determined by a modified Lowry assay (RC DC Protein Assay, Biorad, Hercules, CA, USA), using BSA as a standard. Catalase was assayed in electrode buffer (after nitrogen flushing) by adding 5 mM H_2O_2 and recording oxygen evolution.

Electron microscopy

Mitochondrial pellets were suspended in washing buffer (without BSA) containing 4% (w/v) glutaraldehyde and incubated for 1.5 h at 20°C. In the case of imbibed seed fragments, the fixation was carried out in 4% (w/v) glutaraldehyde, 100 mM phosphate buffer (pH 7.4). After centrifugation (5 min, 10 000 *g*), the pellet was post-fixed for 2 h with 2% (w/v) OsO_4 in 100 mM phosphate buffer (pH 7.4). The material was pelleted by centrifugation and dehydrated with a graded ethanol series and propylene oxide.

Samples were embedded in Epon. Ultrathin sections (60 nm) were cut with a diamond knife on a Leica Ultra cut S (Leica, Rueil Malmaison, France) and transferred on to grids coated with a film of collodion. Grids were air dried and stained with uranyl acetate (saturated solution in 50% ethanol) and lead citrate. The grids were carbon coated (10 nm) with a MED 020 (Baltec-Balzers, Liechtenstein). Transmission electron microscopy (TEM) observations were performed at 200 kV with a JEOL JEM 2011 microscope (JEOL Europe, Croissy sur Seine, France).

Results

Pea seed germination, priming and ageing

Seeds of pea var. Baccara, a high-protein-content cultivar, produced locally in 1999, exhibited a high rate of germination (radicle protrusion); the time for 50% germination (T_{50}) was 40 h, and all seeds had germinated 86 h after the start of imbibition (Fig. 1A). The water uptake curve showed the classical triphasic uptake of water (Bewley, 1997), with initial imbibition for 12–16 h, followed by a plateau phase (up to 50 h) and the subsequent increase due to axis elongation (results not shown). Germination was completely inhibited by 100 µM sodium azide (results not shown), a strong inhibitor of respiration. In order to manipulate the germination potential of the seeds, we set up priming and accelerated ageing treatments that are described in the experimental procedures. The priming treatment was performed by allowing the seeds to imbibe for 16 h (water content = 49% fwb) before drying them rapidly to their initial water content (15% fwb). As shown in Fig. 1A, primed seeds germinated faster (T_{50} = 26 h) than the control. Aged seeds germinated slowly (T_{50} = 89 h) and the total

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percentage of germination was reduced (Fig. 1A), indicating deterioration. After 20 d of ageing treatment, all seeds were unable to germinate (results not shown).

Pea seed respiration

Whole seeds were ground to 0.3 mm particles that were imbibed directly inside the electrode chamber, which allowed monitoring of oxygen consumption from the start of imbibition. The control seed particles exhibited an initially high rate of oxygen consumption that rapidly decreased. In contrast, the fragments of primed seeds did not exhibit the same type of kinetics, since a nearly linear rate of oxygen consumption was observed from the onset of imbibition. Aged seeds showed the same type of kinetics as control seeds, but at a much faster rate (Fig. 1B). In a longer-term experiment, the seed fragments were allowed to imbibe on a wet filter paper for 1 h before being introduced into the oxygen electrode chamber. In these conditions, oxygen consumption, which reached a steady state, was similar for control and primed seeds (respectively, 96 and 88 nmol O_2 min^{-1} g dry $weight^{-1}$). However, aged seeds still consumed oxygen at a much higher rate (146 nmol O_2 min^{-1} g dry $weight^{-1}$). The addition of 1 mM cyanide or 10 µM antimycin resulted in a marked (about 70%) inhibition of the O_2 uptake with all seed treatments, and the residual consumption was partly inhibited by 100 µM propyl gallate (results not shown). These inhibitory effects strongly suggest that the measured oxygen consumption is of mitochondrial origin.

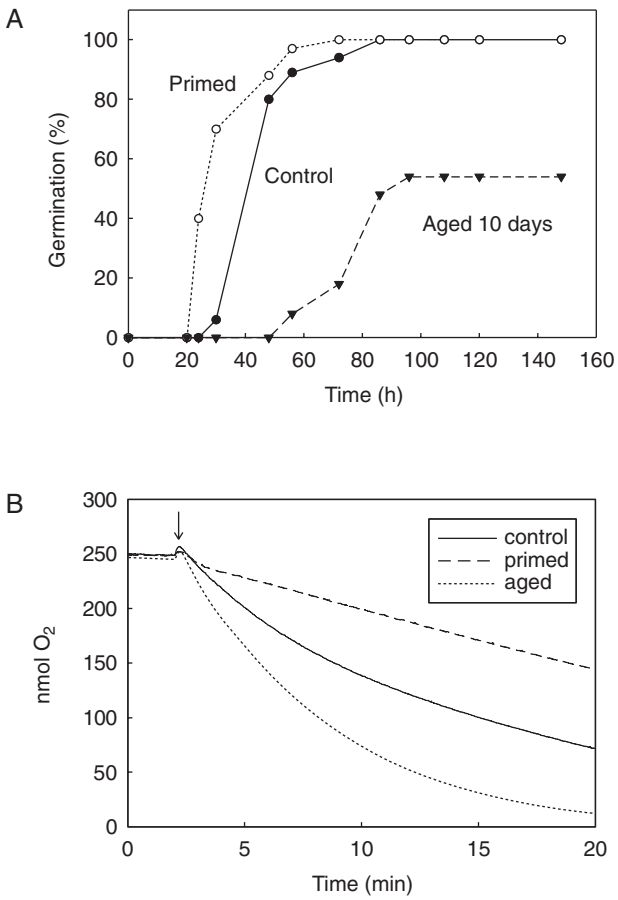

Figure 1. Germination and initial respiration of control, primed and aged pea seeds. (A) Germination curves of pea seeds at 20°C (● control, ○ primed, ▼ accelerated ageing for 10 days). (B) Oxygen consumption by seed fragments during imbibition. Dry seed fragments (100 mg) of approximately 0.3 mm size were introduced (arrow) in the electrode chamber filled with sucrose phosphate buffer (pH 7.2). The electrode chamber was closed immediately to follow the oxygen consumption as a function of time.

Isolation of mitochondria from pea seeds

Mitochondria were isolated from imbibed pea seeds by using established methods for plant mitochondria (Douce *et al.*, 1987) that were optimized for seed tissue. Crude mitochondria were prepared from the seed extract by differential centrifugation, and the mitochondria were then purified using a combination of step and exponential Percoll gradients. Starting with 250 g of imbibed seeds, mitochondria (15–30 mg protein) were isolated and formed a yellowish-brown pellet after the final washing step. As far as can be judged from electron microscopy (see below), the mitochondria were highly purified, except for slight contamination with membrane vesicles or occasional dark organelles, which were possibly protein bodies. Indeed, a proteomic analysis of seed mitochondria purified from another variety of pea revealed contamination by storage proteins (Bardel *et al.*, 2002). Catalase activity in the mitochondrial preparation was always in the range of 2–4 µmol min^{-1} mg $protein^{-1}$. Taking into account the high specific activity of plant catalase, which can reach 120 mmol min^{-1} g $protein^{-1}$ (Yamaguchi and Nishimura, 1984), the amount of catalase in mitochondrial fractions was very low (1–3 ng per mg mitochondrial protein). Thus, the contamination by peroxisomes appeared minor, and, moreover, this activity could presumably be attributed to a genuine mitochondrial enzyme, considering that a mitochondrial catalase has been found in maize (Scandalios *et al.*, 1980). The contamination by intact plastids appeared negligible because glucose-6-phosphate dehydrogenase activity was not detectable (results not shown). However, the yellow colour discernible in the mitochondrial pellets prompted us to measure carotenoids, pigments that would reveal

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contamination by plastid membranes. Small amounts (30–60 ng mg protein^{-1}) of carotenoids indicated slight contamination. Even if this contamination was due to intact plastids, it would be rather low; since the mass ratio of protein to carotenoids in non-green plastids isolated from sycamore cells or cauliflower buds is 200 (Dr A.J. Dorne, personal communication), the contamination by intact plastids would only amount to 6–12 µg mg protein^{-1}.

Physiological analysis of mitochondria

The function of pea seed mitochondria with regards to carbon and energy metabolism was studied using classical polarography techniques. The overall results presented in this section summarize more than 70 purifications of mitochondria from pea seeds. Although the same pattern was always found for mitochondria extracted from the same set of seeds, we observed some variations in the absolute rates of respiration. Such variations undoubtedly mirror the unavoidable difficulties inherent to the preparation and analysis of organelles, the natural ageing of pea seeds, and small variations within biochemical products and assays. In order to minimize such hazards, the experiments designed to compare the effect of different treatments (time of imbibition, priming or accelerated ageing) were performed within a single week. Mitochondria were isolated from control seeds at defined times of imbibition that correspond to the end of the first phase of imbibition (12 h) and to the end of germination (22 h), just before radicle protrusion.

Mitochondria extracted after 22 h of imbibition (control) possessed highly intact outer membranes (98% integrity), as deduced from an integrity assay performed with exogenous reduced cytochrome *c* (Fig. 2). The high integrity of the inner membrane was reflected by the respiratory controls (RC), which ranged from 3 to 4 with most substrates (Table 1). Inner membrane integrity was also substantiated by the effect of the uncoupler FCCP on state 4 respiration (NADH as a substrate), which increased the oxygen consumption by a factor of 5.4 (Fig. 3). As shown in Table 1, these mitochondria readily oxidized the classical Krebs cycle substrates in the following decreasing order: succinate, oxoglutarate, pyruvate or citrate, and malate. The highest oxidation rate was obtained with exogenous NADH, while NADPH was oxidized at a much slower rate (Table 1). Preincubation of mitochondria with micromolar amounts of calcium only slightly stimulated NADPH oxidation (results not shown). The isolated mitochondria did not contain significant amounts of

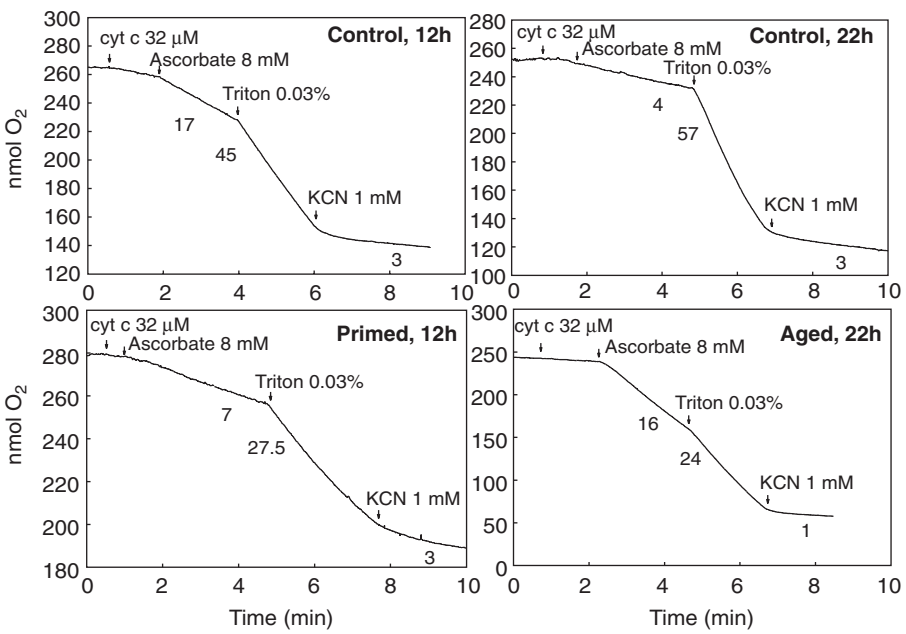

Figure 2. Outer membrane cytochrome *c* permeation assay. Oxygraphic analysis of outer membrane integrity of mitochondria from control seeds (12 h and 22 h imbibition), primed seeds (12 h imbibition) and artificially aged seeds (22 h imbibition). The experiments were carried out in 1 ml of electrode buffer. The arrows show the addition of compounds with their final concentrations. Numbers under the traces indicate the rate of oxygen consumption in nmol O_2 min^{-1} mg protein^{-1}.

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Krebs cycle intermediates, since the addition of a small amount of malate (1.5 mM) was required to initiate the oxidation of pyruvate (results not shown). Without prior addition of the cofactor TPP (0.3 mM), the mitochondria were unable to oxidize pyruvate or oxoglutarate, thus indicating a lack of TPP in the purified sample. The mitochondria were also depleted in endogenous NAD, since oxidation of all the NAD-dependent substrates (malate, pyruvate, citrate, oxoglutarate) was strictly dependent on the addition of the cofactor (result not shown). The imported NAD was retained in the organelles, because mitochondria that were incubated with 1 mM NAD for 15 min on ice and then washed twice by centrifugation in NAD-free medium no longer relied on NAD addition for the oxidation of malate or pyruvate (results not shown). Cyanide strongly inhibited (>90%) the oxidation of succinate or NADH, and had a lesser effect (75% inhibition) upon pyruvate or citrate oxidation, thus revealing alternative oxidase activity, known to be up-regulated by pyruvate and citric acid (Vanlerberghe *et al.*, 1995). With a high concentration of mitochondria (several mg protein) in the electrode chamber, we could detect a very small rate of glycine oxidation (1–2 nmol O_2 min^{-1} mg $protein^{-1}$), which could be reasonably attributed to the functioning of the glycine cleavage system, since it was inhibited by 0.5 mM carboxymethylamine (result not shown), a potent inhibitor (Sarojini and Oliver, 1985). We could also detect a low, but significant, oxidation of formate (12 nmol O_2 min^{-1} mg $protein^{-1}$), which is probably due to the operation of formate dehydrogenase, a mitochondrial enzyme known to be highly expressed in non-green tissues such as roots, tubers or etiolated shoots (Colas des Francs-Small *et al.*, 1993).

In comparison to the 22 h preparation, mitochondria that were extracted after 12 h of imbibition exhibited slightly higher rates of oxidation with most substrates, except for malate (Table 1). However, the RC was lower with all substrates (Table 1), and, accordingly, FCCP exerted a lesser stimulation of the NADH state 4 oxidation (Fig. 3). The outer membrane integrity measured with cytochrome *c* was lower (66%) (Figs 2 and 3). Interestingly, when mitochondria were isolated from primed seeds after 12 h of imbibition, they exhibited membrane integrities and oxidation rates intermediate between their counterparts with 12 h and 22 h of imbibition (Fig. 3). However, such an improvement could not be confirmed with RCs, since with several substrates they were found to be equal or slightly lower than those measured with mitochondria from non-primed seeds imbibed for 12 h (Table 1, Fig. 3).

Mitochondria were also isolated after 22 h of imbibition from seeds that had been aged. These mitochondria exhibited reduced oxidation for several substrates (NADH, succinate, oxoglutarate, malate), as shown in Table 1. Inner membrane quality was dramatically reduced, since the RCs dropped with all substrates (Table 1). For instance, the RC was 1.9 instead of 4.3 for NADH (Table 1), and this was reflected by the much lower uncoupling effect (2.8 instead of 5.4) of FCCP on state 4 respiration (Fig. 3). The outer membrane integrity of aged seed mitochondria was very low (Fig. 2 and 3), reaching only 35% in the assay. Since the intrinsic quality of

Table 1. Respiratory activities and respiratory control of pea seed mitochondria. The state 3 respiration rate (nmol O_2 min^{-1} mg $protein^{-1}$) and the respiratory control (value between brackets) were measured for the oxidation of each of the indicated substrates by mitochondria isolated from control seeds (12 h and 22 h of imbibition), primed seeds (12 h imbibition) or artificially aged seeds (22 h of imbibition). With oxoglutarate, the RC value is not indicated since it does not reflect the actual RC, because ADP acts not only as a P_i acceptor of oxidative phosphorylation but also as a co-substrate of the following Krebs cycle reaction (succinyl-CoA synthetase) that recycles the CoA used as a substrate by oxoglutarate dehydrogenase (Douce, 1985). The data were obtained within a single week to prevent variation in chemicals, protein determination and seed age that could influence the comparison. The respiratory rates are the averages of 2–3 sets of oxygraphic data (± SE) obtained with the same batch of mitochondria. The RC, which is independent of the absolute rate of respiration, has SEs ranging from 0.1 to 0.2.

Substrate	Control, 12 h imbibition	Primed, 12 h imbibition	Control, 22 h imbibition	Aged 10 d, 22 h imbibition
NADH	100 ± 9 (3.9)	84 ± 8 (3.9)	79 ± 8 (4.3)	31 ± 7 (1.9)
NADPH	9 ± 2 (4.6)	9 ± 2 (3.2)	6 ± 2 (5.7)	6 ± 2 (2.1)
Pyruvate	17 ± 3 (2.7)	11 ± 3 (2.9)	14 ± 2 (3.6)	12 ± 3 (2.4)
Citrate	16 ± 3 (2.4)	10 ± 2 (2.4)	14 ± 2 (3.1)	15 ± 3 (1.7)
Oxoglutarate	47 ± 7	30 ± 6	36 ± 7	23 ± 5
Succinate	81 ± 6 (3.0)	86 ± 7 (3.2)	56 ± 7 (3.5)	27 ± 6 (1.8)
Malate	8 ± 2 (2.8)	8 ± 2 (2.6)	12 ± 2 (3.5)	8 ± 2 (2.2)

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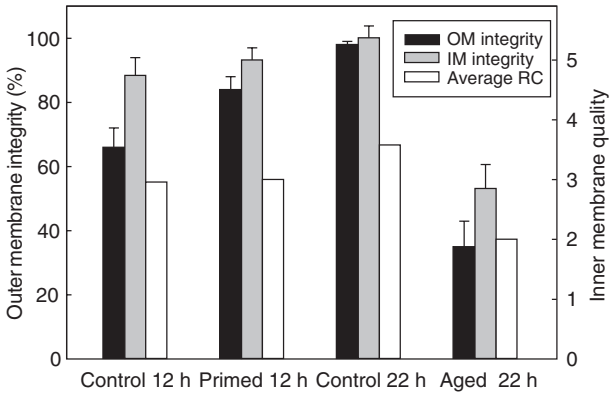

Figure 3. Outer and inner mitochondrial membrane integrity. The outer and inner membrane integrities were evaluated for the mitochondria from control seeds (12 h and 22 h of imbibition), primed seeds (12 h of imbibition) and artificially aged seeds (22 h of imbibition). The graph shows the percentage of outer membrane (OM) integrity (black bars) calculated from the cytochrome *c* permeation assay. An estimate of the level of integrity of the inner membrane was made by measuring the ratio of uncoupled respiration (2 µM FCCP) versus state 4 respiration using NADH as the substrate (grey bars). The ratio increases as the inner membrane impermeability to protons increases. White bars show the average respiratory control (RC) of the rapidly oxidized substrates (NADH, succinate, pyruvate, malate) with the exception of oxoglutarate (see Table 1). SE is indicated for OM integrity and FCCP stimulation (three repetitions).

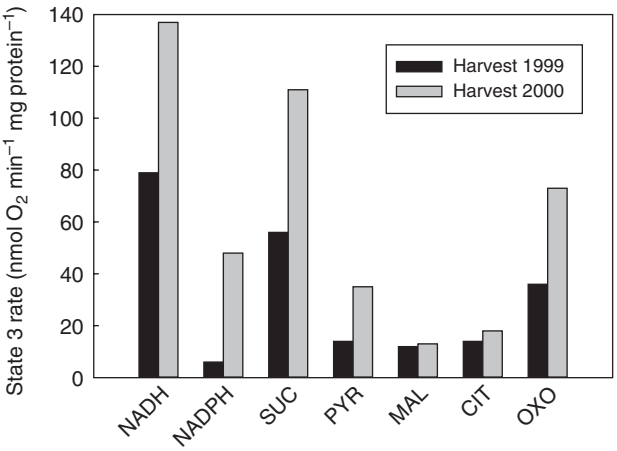

Figure 4. Comparison of the substrate oxidation profiles of two seed lots of pea. State 3 oxidation rates of different substrates for mitochondria isolated after 22 h of imbibition from a seed lot harvested in 1999 and stored for 11 months (black bar), and from a seed lot harvested in 2000 and stored for 4 months. SUC, succinate; PYR, pyruvate; MAL, malate; CIT, citrate; OXO, oxoglutarate.

seeds appeared to be major determinant of mitochondrial performance, we compared the oxidative properties of mitochondria isolated after 22 h of imbibition from the control seeds (harvest 1999, 11 months storage) with those of another seed lot harvested in 2000 and stored for 4 months only. The mitochondria of the 2000 and 1999 seed lots showed similar physiological properties (cofactor deficiency, substrate oxidation profiles). However, oxidation rates of mitochondria from the 2000 harvest were almost two times as high with most substrates, except malate and citrate, which were slowly oxidized (Fig. 4). Intriguingly, NADPH was oxidized at an eightfold higher rate in the preparation from the 2000 harvest (Fig. 4). These results were confirmed on three independent preparations of mitochondria isolated from the 2000 harvest seed lot.

Electron microscopy observations

To further investigate whether the variations of seed mitochondrial properties could be observed at the ultrastructural level, we examined purified mitochondria from control and artificially aged seeds by TEM. The appearance of the isolated mitochondria in the electron micrographs confirmed the purity of the organelles, only contaminated by occasional small, dark, single-membrane organelles, which may have been protein bodies. The mitochondria isolated from control seeds after 22 h of imbibition showed classical morphology, with well-defined inner and outer membranes, cristae and a dense matrix (Fig. 5b). In comparison, the mitochondria isolated after 12 h of imbibition exhibited only a few cristae, and the matrix space was less homogeneous and more granular, suggesting the presence of numerous ribosomes (Fig. 5a). Inner and outer membranes were easily distinguished, but their appearance was not as sharp as those of mitochondria isolated at 22 h (Fig. 5a, b). The mitochondria from aged seeds isolated after 22 h of imbibition were severely altered in their morphology. The matrix appeared much less dense, and the outer membrane was hardly distinguishable in most of the organelles (Fig. 5c). The inner membrane, although blurred, was visible, and cristae were almost undetectable (Fig. 5c).

Discussion

We investigated the oxidative properties and membrane integrity of pea seed mitochondria isolated from whole seeds during early germination, and examined the relationship between seed quality and mitochondrial performance. The work was carried out with whole seeds, and since after 12 or 22 h imbibition cotyledons represent 98% (fwb), it is

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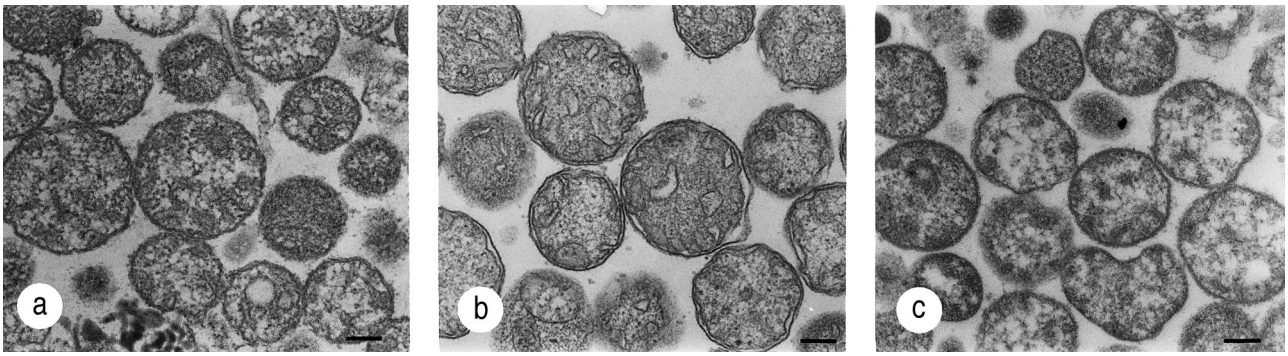

Figure 5. Electron micrographs of pea seed mitochondria. The micrographs represent mitochondria purified from control seeds (harvest 1999) after 12 h (a), or 22 h (b) of imbibition, or from artificially aged seeds after 22 h of imbibition (c). The horizontal scale bar in all micrographs corresponds to 200 nm.

likely that the results are mostly derived from cotyledon mitochondria. Mitochondria were isolated from fully imbibed seeds (12 h) and at the end of germination (22 h), just before radicle protrusion. Isolated mitochondria were capable of oxidizing most classical substrates, and outer membrane integrity increased from 70% at 12 h to 98% at 22 h. A similar improvement was measured for inner membrane coupling efficiency. Although substrate oxidation rates decreased, the efficiency of mitochondrial ATP production was improved during the course of germination. As in any study involving isolated organelles, an issue that should not be brushed aside is whether the results are relevant to the *in vivo* situation. Hoekstra and van Roekel (1983) pointed out that purified organelles can be altered by isolation injuries. However, we believe that the nature and extent of isolation injuries are highly dependent upon the physiological and structural properties of the organelles within their original cellular environment.

According to RC values, seed mitochondria were efficient in generating ATP through the oxidation of the Krebs cycle substrates and exogenous NAD(P)H. Although the oxidation rates were lower (two- to threefold) than the typical rates of leaf or tuber mitochondria, particularly for malate, citrate and pyruvate, they were, nevertheless, consistent with those generally reported for seed mitochondria (Douce, 1985). Much higher rates have been reported with succinate, malate and oxoglutarate when mitochondria were extracted several days after germination from pea cotyledons, suggesting that mitochondrial improvement continues after germination, even in cotyledons (Solomos *et al*., 1972). The substrate oxidation by mitochondria from two different lots of seeds (harvest year, storage period) was compared. Although the profiles were similar, the oxidation rates of mitochondria from the younger seed lot were significantly higher. A remarkable change was a dramatic increase in NADPH oxidation, which is difficult to explain. We can suggest that this might be related to the stability or turnover of external NADPH dehydrogenase. These differences exhibited by mitochondria from the two seed lots cannot be simply related to seed age, because other agronomic factors during seed production and processing were not controlled. Therefore, it is possible that the wide range of oxidation rates reported in the literature for seed mitochondria of the same species is due to differences in seed quality. In most cases, no mention is made about the harvest, age or storage conditions of seeds.

Isolated seed mitochondria appeared to be depleted in thiamine pyrophosphate, Krebs cycle intermediates and endogenous NAD. The lack of cofactors has often been observed in plant tissues (e.g. tuber, inflorescences), and it has been suggested that the control of NAD transport into mitochondria could participate in a coarse control of metabolism (Douce and Neuburger, 1989). Further experiments will be required to establish a potential role of NAD as a regulator of mitochondrial metabolism in seeds. Since external NADH and succinate were the most rapidly oxidized substrates, they may constitute the primary fuel of seed mitochondria during imbibition, thus driving efficient ATP production without requiring a functional Krebs cycle. Indeed, the oxidation of these substrates by the inner membrane does not require the sophisticated multi-enzyme and cofactor machinery operating in the matrix space. Recently, Logan *et al*. (2001) suggested that exogenous NADH oxidation could drive ATP synthesis in pro-mitochondria, from germinating maize embryos deficient in Krebs cycle enzymes (including succinate), which would later differentiate into fully active mitochondria. However, in our study the oxidation of the other substrates was already substantial after 12 h of imbibition, and rather

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decreased (state 3 rate) 10 h later. This does not favour a major biogenesis of Krebs cycle machinery supported by a NADH-dependent energy supply during the studied period. It would be interesting to determine whether this could be the case at earlier times of imbibition, or whether the mitochondrial biogenesis scheme differs between maize and pea germination. While priming treatment enhanced pea germination, it did not clearly improve mitochondrial performance, although outer membrane integrity was higher than in the control after 12 h of imbibition. The positive effect of priming upon germination is likely to result from the advance of a great variety of cellular events along the road to germination, including mitochondrial improvement.

A general question that can be raised concerns the time needed for mitochondria to become operational after imbibition. In previous work, mitochondria that were isolated from dry seeds using aqueous buffers appeared to be highly damaged, but they were capable of some ATP synthesis, suggesting functional oxidative phosphorylation from the onset of imbibition (Attucci *et al*., 1991; Logan *et al*., 2001). Our attempts to isolate mitochondria from dry pea seeds failed, probably because the grinding of seed tissues in aqueous buffers presents too abrupt an osmotic and biophysical shock to isolate representative organelles. In any case, we decided against isolating mitochondria at imbibition times shorter than 12 h because of the imbibition anisotropy imposed by the size of pea seeds, which results in a heterogeneous water content and hydration age of the tissues. As an alternative to direct investigation of mitochondrial activity at the onset of imbibition, we measured the initial oxygen consumption of seed fragments. The results indicated that as soon as water was available, oxygen consumption started, which was likely due to mitochondrial activity, since it was inhibited by cyanide and antimycin A. Similar observations were made by Parrish and Leopold (1977) with fragmented soybean seeds, and comparable kinetics were obtained with maize embryo axes (Ehrenshaft and Brambl, 1990). In addition, we observed that the kinetics differed markedly according to seed quality. Primed seed fragments exhibited a slower and rather linear rate of respiration in comparison with the control, suggesting a tighter regulation of respiratory metabolism. It should be noted that, in contrast with these results, primed seeds (lettuce, sunflower) have been shown to respire at higher rates than the control (Cantliffe *et al*., 1984; Chojnowski *et al*., 1997). However, these experiments were carried out on whole seeds for longer periods of time, and we could also measure higher rates of respiration with primed seed fragments when the imbibition time was extended to several hours (result not shown). Surprisingly, aged seed fragments exhibited a faster rate of oxygen consumption, which might correspond to an increase in energy demand and a possible uncoupling of mitochondrial energy transfer.

We further investigated the effect of deterioration of seed quality upon mitochondria. Several ultrastructural studies of seeds had indicated mitochondria as targets in the deterioration of stored seeds (reviewed by Smith and Berjak, 1995), and a study of soybean seed respiration during simulated pre-harvest deterioration led the authors to propose mitochondria as a site of the primary lesion (Amable and Obendorf, 1986). Mitochondria isolated from aged seeds after 22 h of imbibition appeared severely affected in their functions, since they exhibited reduced oxidation rates and respiratory controls. According to integrity assays and TEM observations, the outer and inner membranes could be the primary targets of injury resulting from the ageing treatment. These results suggest that mitochondria from aged seeds are hampered in ATP production, which might be related to the slow rate of germination and low germination percentage of the seeds. Such mito-chondrial damage could be responsible for the high rates of O_2 consumption measured for aged seed fragments. The increased respiration rate of aged seed fragments contrasts with other observations that showed a decrease in respiration rates after accelerated ageing of soybean and sunflower seeds (Amable and Obendorf, 1986; Chojnowski *et al*., 1997). Apart from the different species and models (whole seed, fragments), such a discrepancy probably lies in the time course of the experiments, since we focused our measurements on very short times of imbibition (less than 1 h).

In conclusion, the characterization of the oxidative properties of purified pea seed mitochondria has illustrated their improvement during early germination and highlighted a possible correlation between seed quality and mitochondrial functioning. The outer and inner membranes appeared as potential targets for desiccation and ageing injuries of seeds. Considering the high integrity and the oxidative capacity demonstrated by mitochondria from imbibed seeds, it will be of primary interest to determine whether they are protected against desiccation stress, and to decipher the biosynthetic and repair mechanisms involved in mitochondrial maintenance during germination. Such issues require investigations at the molecular level, and, interestingly, a recent proteomic survey of plant mitochondria suggested the occurrence of seed-specific mitochondrial proteins (Bardel *et al*., 2002).

Acknowledgements

This work was supported by the Program 'Semences-CER 2000–2003' from Région Pays-de-la-Loire. We are

https://doi.org/10.1079/SSR2002122 Published online by Cambridge University Press

grateful to Robert Filmon and Maurice Lesourd (SCIAM, Université d'Angers) for their expert assistance with electron microscopy.

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Received 29 April 2002
accepted after revision 8 November 2002

Nitrogen Fixation: Global Perspectives

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February 2002 576 pages Hardback
ISBN 0 85199 591 8
£85.00 (US$149.00)

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