Respiration and Mitochondrial Characteristics of Imbibing Maize Embryos Damaged by High Temperatures during Desiccation

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ABSTRACT

Hybrid maize (Zea mays L.) seed artificially dried to 120 g H2O kg⁻¹ moisture in air temperatures of either 35°C (LT) or 45°C (HT) was used to study the effect of desiccation temperature on mitochondrial function during early imbibition. In embryo culture experiments, embryonic growth was delayed for embryos excised from HT treated seed compared with LT samples. After 1 h of imbibition, the rate of O₂ uptake by LT axis tissue was greater than for HT dried samples. Uptake rates continued to increase for LT samples through the first 6 h of imbibition while rates for HT axes remained constant. Mitochondria isolated from LT axis tissue that had undergone imbibition for 4 h exhibited respiratory control with oxidative phosphorylation efficiency values of 0.79 and 0.77 for NADH and succinate assays, respectively. Mitochondria from imbibing HT axis tissue exhibited no respiratory control. Electron micrographs of cells from the radicle meristem region of both treatments showed that mitochondrial development during the initial 24 h of imbibition was impaired in HT samples. These results suggest that mitochondrial function and development, during the initial stages of embryonic tissue hydration, is impaired when maize seed are artificially dried with high air temperatures. We conclude that the development of mitochondrial function during this period is a key element in determining the rate of germination and subsequent seedling growth.

GERMINATION and early seedling growth characteristics (seed vigor) of maize seed lots are routinely used by the seed industry as measures of seed value. During germination, the metabolic potential possessed by a dry quiescent seed is expressed as the ability of that seed to complete germination quickly and produce a seedling that can continue rapid development in a variety of environmental conditions. A comprehensive understanding of the key elements contributing to seed vigor has been complicated by apparent species and tissue source variability (10).

The initial uptake of O₂ by imbibing seed closely follows hydration of the seed tissues (6, 3) and is generally attributed to the presence of a functional mitochondrial enzyme system in the dry seed that becomes active when hydrated. Following complete hydration of the seed, the respiratory rate was thought to depend on continued development of mitochondria due, initially, to the import and assembly of preexisting proteins from the cytoplasm (17, 14). This general model has been supported by ultrastructural analyses showing mitochondria with poorly differentiated internal structure in dry seed (13, 3) followed by dramatic development of the internal structure and increases in the number of cristae during the early stages of germination. However, variation from this model has been reported when comparing storage and embryo tissue (13). The ability of respiratory measurements of imbibing seed tissue to characterize seed vigor differences due to ageing has been investigated with isolated axes of soybean (25, 9). Those studies concluded that respiratory rates and estimates of mitochondrial functionality were significantly lower in axis tissue of low vigor seed.

The purpose of this investigation was to study the contribution of axis tissue mitochondria to the metabolic potential of maize seed. A system of high-temperature desiccation injury was utilized to create samples with reduced seed vigor without significant effect on viability. Maize inbred B73, which has been characterized as sensitive to high temperatures during desiccation (16), was used as the seed parent in these experiments.

MATERIALS AND METHODS

Plant Material

Hybrid maize seed was produced at Iowa State University, Ames, IA. The inbred line B73 was used as the seed parent and single cross H99 × H95 was the pollen parent. Random ear samples were harvested periodically, with husk intact, to obtain ears with kernel moisture contents ranging from 500 to 350 g H₂O kg⁻¹. At each harvest, ear samples were brought into the laboratory, husked, and immediately placed in thin-layer experimental dryers (15). In 1989, samples were taken at random from each harvest for moisture determination (oven method). Dryers were operated with air temperatures maintained at either 35 or 45°C to dry the seed to approximately 120 g H₂O kg⁻¹ moisture. The dried samples were hand shelled, each treatment combined, placed in paper bags, and maintained in cold storage at 10°C and approximately 50% relative humidity until analyzed. In 1990, a moisture determination was made on each ear sample before drying. Dryers were operated at air temperatures of 35, 40, 45, or 50°C until the samples were dried to 120 g H₂O kg⁻¹ moisture. Each ear sample was hand shelled and the seed stored in paper envelopes in cold storage (10°C/50% RH) until analyzed.

Evaluation of Seedling Vigor

Seedling vigor and early growth characteristics were evaluated for each harvest moisture × drying temperature treatment. Standard warm germination tests were conducted as previously described (11) after which the shoot and root tissue, from those seedlings considered normal, were removed and dried for 24 h at 103°C for dry weight determinations. To obtain a more traditional vigor rating, a soil-free cold test (12) was performed.
also conducted. This test was evaluated after 14 d, the first 7 d at 10°C followed by 7 d at 25°C.

Embryos (embryonic axis plus scutellum) were excised from dry seed for evaluation in embryo culture. A razor blade was used to remove the endosperm tissue. Embryos were surface sterilized in 1% (v/v) sodium hypochlorite for 30 s, rinsed in distilled water, and transferred to sterile petri dishes containing 34.7 g/L Murashige and Skoog Shoot Multiplication Medium B (Sigma Chemical Co., St. Louis, MO) with 8 g/L agar. Plates were sealed and placed in an incubator at 28°C. All sterilization and transfer operations were conducted in a laminar flow transfer hood.

**Embryonic Respiratory Measurements**

Oxygen uptake by excised embryos and embryonic axes was measured polarographically with a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH) calibrated with air-saturated water at 25°C. Full-scale deflection corresponded to a concentration of 250 µM O₂. Embryos and embryonic axes were maintained in the sample chambers in air-saturated water between measurement periods and were subjected to continuous stirring at all times.

**Isolation of Mitochondria**

Two-hundred milligrams of embryonic axis tissue that had been excised from dry seed were imbibed for 4 h at 25°C before being ground by hand with a chilled mortar and pestle. Mitochondria were isolated according to Day and Hanson (1977) with the following modifications. TES [N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid] was used instead of phosphate in the extraction buffer. Samples were ground in 2.5 mL of extraction buffer followed by a 2.5-mL rinsing of the mortar and pestle. The mitochondrial pellet was resuspended in 150 µL of a reaction mixture consisting of 250 mM sucrose, 10 mM TES, 1 mM MgCl₂, 1 mM KH₂PO₄, (pH 7.2), and 0.1% (w/v) BSA (bovine serum albumin). Triplicate aliquots (10 µL) of the resuspension were saved for protein determination by the Lowry procedure (corrected for added BSA) following precipitation with TCA (5).

Oxygen uptake by mitochondria was measured immediately following resuspension of the pellet in a low volume reaction chamber at 25°C. Initially, 25 µL of mitochondrial suspension was added to 500 µL of a reaction mixture in the reaction chamber. State 3 rates were determined following the addition of 25 nmol of ADP in the presence of either 10 mM succinate, 1 mM NADH or 10 mM malate.

**Electron Microscopy**

The embryonic axes were fixed for 18 h at 4°C in 3% glutaraldehyde/3% paraformaldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2. Following fixation, the samples were washed three times in buffer only for 10 min each. Samples were then postfixed with 1% osmium tetroxide in the same buffer for 2 h at 4°C. After rinsing three times in double-distilled water for 10 min each, the samples were stained in 4% aqueous uranyl acetate. Dehydration was then carried out in a graded ethanol series (25, 50, 75, 80, 85, 90, and 95%, v/v, at room temperature) followed by three changes of 100% acetone over a 36-h period. The samples were then embedded in Spurr’s resin, the samples were polymerized at 60°C for 24 h. A Reichert Ultracut E ultramicrotome (Leica Inc., Deerfield, IL) was used to section the embedded samples. Thick sections (2.0 µm) were cut with a glass knife, mounted on slides, and stained with toluidine blue for viewing on a Leitz Orthoplan microscope equipped with bright-field optics. Ultrathin sections were cut with a diamond knife, transferred to 200-mesh copper grids, and stained with 2% aqueous uranyl acetate followed by lead citrate (18). Thin sections were then examined and photographed with a JEOL 1200EX STEM electron microscope (Peabody, MA).

**RESULTS**

**Vigor Characterization**

Seed viability, as estimated by the warm germination test, was high (>90%) for all treatments (Table 1). The effects of harvest moisture and drying temperature on seed vigor were estimated by the cold germination test and seedling dry weights following the warm germination test. Cold test results (Table 1) indicate a relatively similar reduction for all HT treatments when compared with LT, though the difference was not significant for the 437 g H₂O kg⁻¹ harvest moisture treatment. Harvest moisture had little effect on cold germination results when comparing means within a drying temperature treatment.

The results of seedling dry weight determinations clearly indicate the progressive nature of the severity of the injury as harvest moisture increases (Table 1). HT treatments reduced shoot dry weight by 40, 32, and 21% for 511, 437, and 361 g H₂O kg⁻¹ harvest moisture samples respectively. Root development was even more severely affected by temperature with reductions of 58, 60, and 47% respectively as harvest moisture was reduced. These results indicate that the seed becomes more tolerant of high-temperature desiccation as harvest moisture declines. Shoot/root dry weight ratio is a single index value that has been reported (16) to offer an additional quantification of seed vigor. For each harvest moisture, the HT treatment had a higher shoot/root ratio (indicative of lower vigor) than the LT treatment.

Embryo culture experiments were conducted to focus on the treatment effect on germination characteristics of excised embryos. Results consistently indicated a delay in the initiation of elongation of isolated embryos from acetone to Spurr’s resin followed by three changes of 100% resin over a 36-h period. Following the final change of resin, the samples were polymerized at 60°C for 24 h.

**Table 1. Effect of desiccation temperature and harvest moisture on seed viability and seedling development in maize. Germination values are means of four evaluations of 50 seed each.**

<table>
<thead>
<tr>
<th>Harvest moisture (g H₂O kg⁻¹)</th>
<th>Drying temp. °C</th>
<th>Warm</th>
<th>Cold</th>
<th>Seedling dry weight mg seedling⁻¹</th>
<th>Shoot/root ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Shoot</strong></td>
<td><strong>Root</strong></td>
<td><strong>Shoot</strong></td>
<td><strong>Root</strong></td>
<td><strong>Shoot/root</strong></td>
<td><strong>Ratio</strong></td>
</tr>
<tr>
<td>511</td>
<td>35</td>
<td>99</td>
<td>98</td>
<td>29.2</td>
<td>17.3</td>
</tr>
<tr>
<td>437</td>
<td>35</td>
<td>99</td>
<td>98</td>
<td>28.7</td>
<td>10.6</td>
</tr>
<tr>
<td>361</td>
<td>35</td>
<td>100</td>
<td>94</td>
<td>35.0</td>
<td>30.6</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>NS</td>
<td>7.6</td>
<td>4.6</td>
<td>3.7</td>
<td>1.71</td>
</tr>
</tbody>
</table>
HT desiccated seed. The first visible sign of elongation was delayed from 24 to 48 h for the damaged embryos (Fig. 1A–C). After 1 wk of culture, shoots from several of the damaged embryos appeared to be developing normally (Fig. 1D). These experiments demonstrated that a significant portion of the injury is localized in the embryo and not an impairment of the reserve mobilization system or pericarp integrity.

**Oxygen Uptake by Excised Embryos**

Respiratory patterns for most imbibing seeds have been characterized as tri-phasic (6). During Phase I, the rate of O₂ uptake is directly related to the rate of tissue hydration. Therefore, respiratory measurements of whole seed during this period are sometimes difficult to interpret because of variability in the rate of tissue hydration. Physical characteristics of the seed such as pericarp integrity, seed size, and shape influence the hydration pattern of seed tissue and, thus, O₂-consumption rate during early germination. When O₂-consumption assays are conducted with excised embryos (with scutellum) or embryonic axes of maize, the hydration phase (Phase I) is completed within the first few minutes (8). The following period of imbibition (until radicle emergence) represents Phase II. During this lag phase (Phase II), the rate of respiration continues to increase, but more slowly than during Phase I. Oxygen consumption during this phase of germination is primarily due to activity of the cytochrome-mediated electron transport chain located in the inner membrane system of mitochondria (8). To eliminate most of the hydration variability and focus on the metabolic activity of the embryonic respiratory system, O₂ consumption of embryos and embryonic axes was measured during the first 6 h of imbibition. Rate of O₂ uptake by embryos is reported on a per embryo basis due to variability in the ratio of scutellum to axis. Uptake data for axis tissue is reported on a per mg of tissue weight as determined before the tissue was imbibed (120 g H₂O kg⁻¹ moisture).

Initially, experiments were conducted on 361 g H₂O kg⁻¹ harvest moisture samples created in 1989. Embryonic tissue samples from HT dried seeds did not take up O₂ at the same rate as undamaged tissue at any of the early imbibition times measured (Fig. 2). In the case of embryos (Fig. 2A), O₂ consumption by damaged embryos at 1 h was 78% that of undamaged. This difference was essentially the same at the 6-h measurement. The rate of uptake continued to increase for both treatments...
throughout the analysis period. Data from the embryonic axis experiments (Fig. 2B) show a similar relationship between the temperature treatments. In contrast to the whole embryo, damaged axes did not exhibit any increase in the rate of O₂ consumption during the analysis period. These results appear to reflect some impairment of the damaged axes' ability to develop respiratory activity during the very early stages of germination.

Based on traditional seed vigor evaluations, the critical air temperature above which drying injury has been observed is 40°C (16). To evaluate the effect of desiccation temperature on early embryonic respiration in more detail, four air temperatures were used as drying treatments in 1990. Initial respiratory rates were higher for axis samples from seed dried at 35 and 40°C than either 45 or 50°C samples (Fig. 3). Rate of uptake increased gradually throughout the analysis period for the two lowest temperature treatments; however, the rate of uptake was higher for the 40°C treatment than for the 35°C treatment. Consistent with earlier experiments, the rate of uptake did not increase (actually decreased) for damaged axes. Although differences were not significant, the rate of uptake for axes dried at 50°C was less than the 45°C treatment at all times measured, suggesting progressive injury as air temperature is increased.

Characteristics of Mitochondria Isolated from Imbibing Axes

The yield of mitochondrial protein, on a fresh weight (before imbibition) basis, was 690 ± 39.4 and 560 ± 65.5 μg (200 mg tissue)⁻¹ for the LT and HT treatments, respectively. This suggests that either the HT tissue yielded fewer mitochondria (damaged mitochondria could be more difficult to isolate) or that these organelles contain less protein than those from undamaged tissue. To compare mitochondrial activities and thus begin to distinguish between these two possibilities, experiments measuring respiratory function were conducted with isolated mitochondrial preparations. NADH supported a 46 and 55% higher State 3 rate than succinate for LT and HT treatments, respectively (Table 2). Uptake rates were much lower and essentially the same, for both treatments, in the presence of 10 mM malate (data not shown). This is consistent with other reports that, in an imbibing seed, the capacity to oxidize exogenous NADH is more developed than for endogenous NADH, and the former becomes active more rapidly upon hydration (6).

Oxygen uptake by mitochondria from the HT treated axes was not stimulated by the addition of ADP (Table 2). State 3 rates for these treatments, therefore, may not represent phosphorylation. Alternatively, mitochondria from the LT treated axes preparations showed RC and nearly identical estimates of oxidative phosphorylation efficiency (ADP/O ratio). The ADP/O ratios for LT mitochondria are similar to those reported for mitochondria isolated from imbibed peanut embryos of 0.9 (24), sunflower seeds of 0.85 (3) and soybean axes of 1.2 (25). These relatively low values reflect the competency of axis mitochondria following desiccation.

Electron Microscopy

The ultrastructure of cells from the meristematic region of the radicle of imbibing maize axes, with special emphasis on mitochondrial development, was studied with transmission electron microscopy. Mitochondria from the LT treated samples at 0-h imbibition (Fig. 4A) appear swollen and have a homogeneous spherical shape. The outer and inner membranes appear intact with distinct cristae evident. The ground substance of the matrix is moderately uniform in electron density. Alternatively, while the external shape and membrane integrity are similar for the HT samples at 0 h (Fig. 4B), matrix properties are distinctly different. These mitochondria are characterized by large areas of electron transparent ground substance in the matrix combined with very electron dense aggregates and few visible cristae. After 6 h of imbibition, mitochondria from LT treated radicles (Fig. 4C) have well developed internal structure with numerous cristae present. Both envelope membranes are clearly visible, the external shape seems less swollen, and the matrix ground substance is very uniform. In contrast, mitochondria from the HT treatments at 6 h (Fig. 4D) continue to exhibit the matrix aggregation and variable density that was evident at 0 h. Essentially no internal structure or cristae are visible.

Table 2. Effect of drying temperature on functionality of mitochondria isolated from embryonic axes of maize seed. Rates were measured at 25°C and represent the mean ± SD of determinations from at least three preparations. Excised axes were imbibed for 4 h before being analyzed.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Drying temp.</th>
<th>State 3 rate</th>
<th>State 4 rate</th>
<th>Respiratory control</th>
<th>ADP/O ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate</td>
<td>35°C</td>
<td>63.6 ± 17.5</td>
<td>49.1 ± 16.1</td>
<td>1.30 ± 0.16</td>
<td>0.77 ± 0.07</td>
</tr>
<tr>
<td>NADH</td>
<td>35°C</td>
<td>92.6 ± 23.9</td>
<td>59.4 ± 13.0</td>
<td>1.56 ± 0.16</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>66.8 ± 19.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35°C</td>
<td>43.1 ± 11.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45°C</td>
<td>43.1 ± 11.7</td>
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</tbody>
</table>
A heterogeneous, elongated shape, which is characteristic of metabolically active mitochondria, predominates in LT treated cells after 24 h of imbibition (Fig. 4E). The matrix is stained more densely and abundant cristae are visible. Mitochondria from HT treated radicle cells are extremely variable in their appearance (Fig. 4F). Most pictures indicate the existence of normal appearing mitochondria (though very few are elongated) as well as some that still have electron transparent matrices. In a few cases, the damaged mitochondria appear to be degenerating.

DISCUSSION

Various components of respiration and mitochondrial activity have been studied during germination in a wide variety of seeds and seed tissues. In this study, we used high temperature during the desiccation process to impair the ability of a maize seed to complete germination. In contrast to the extensive work that has focused on respiration in relation to seed vigor differences due to ageing (1, 25, 22, 9), this system offers a distinctly different injury mechanism from which to identify key elements in seed germination. Consistent with early studies using this injury system and genotype (4, 16, 19), the traditional evaluations of seed vigor demonstrated the progressive susceptibility of maize seed to injury as harvest moisture and desiccation temperature increase. Seedling dry weight determinations, which are often used for growth analysis during this phase of development in maize (21), characterized the injury as a limitation in

Fig. 4. Electron micrograph of radicle meristem region from imbibing maize axes. (A), 35°C treatment at 0-h imbibition; mitochondrial matrix is uniform with a few narrow cristae (×37 500; bar, 0.5 μm). (B), 45°C treatment at 0 h; mitochondrial matrix contains electron dense aggregates (arrow), transparent areas, and a few narrow cristae (×4 000; bar, 0.5 μm). (C), 35°C at 6 h imbibition; both mitochondrial membranes are present and intact with numerous cristae evident (×67 500; bar, 0.3 μm). (D), 45°C at 6 h; mitochondrial matrix is electron transparent except for dense aggregates (arrow) with no visible cristae (L = lipid body; W = cell wall) (×42 850; bar, 0.5 μm). (E), 35°C at 24 h of imbibition; mitochondria have dense matrix, abundant cristae and elongated shape (N = nucleus) (×45 300; bar, 0.5 μm). (F), 45°C at 24 h; mitochondria are highly variable in appearance. Some appear normal with a dense matrix; others have no matrix and may be degenerating. Plastid (P) has several plastoglobules (×40 000; bar, 0.5 μm).
early growth potential. This concept was supported by embryo culture experiments which focused specifically on the metabolic potential of the embryo and eliminated any contributory effect of pericarp, endosperm, or aleurone. Respiratory measurements during imbibition, with excised axes, have been well correlated with differences in vigor due to deterioration of soybean seeds (25). We found a similar relationship, in that neither HT treated embryos nor axis tissues were taking up O2 at the same rate as LT samples after only 1 h of imbibition. We interpreted this observation, combined with recent reports that mitochondrial respiration is the predominant O2-uptake process during early germination (3, 8), to indicate that a portion of the high-temperature injury is expressed as reduced mitochondrial activity-capacity.

During the initial stages of imbibition, the respiratory activity of LT axis tissue increased while that of the HT tissue remained steady or declined slightly. This pattern was even more evident when four drying temperatures were used as treatments. Non-damaging temperatures (35 and 40°C) resulted in axis O2-uptake curves indicative of developing respiratory activity. Alternatively, declining activity throughout the analysis period was observed in seeds from the damaging treatments (45 and 50°C). The injury mechanism could be attributable to direct thermal instability of all or part of the respiratory enzyme complex or simply a limitation in the amount of substrate available in the cytoplasm.

The inability to measure respiratory control in mitochondria isolated from HT treated tissue combined with lower State 3 rates suggests that the inner membrane system has been impaired. This theory was strengthened by ultrastructural analysis of imbibing axis tissue. The round swollen appearance, presence of a double outer membrane, and limited number of visible cristae for mitochondria from 0 h for both treatments is typical of dry maize seed (23). However, the presence of visible condensed aggregates in the matrix of HT treatments at both 0- and 6-h imbibition times would seem to indicate significant damage to the inner membrane structure, the extent of which was unexpected. It seems apparent from the 24-h results that many of these damaged mitochondria were not able to recover and were degenerating. It is not clear whether the apparently metabolically active mitochondria in HT samples at 24 h is representative of organelles that have successfully repaired or whether they represent a totally different population, as has been suggested for peanut cotyledons (13).

Our results indicate that mitochondria from the embryonic axis are damaged by high temperatures during desiccation and that their capacity to increase respiratory function during early germination is impaired. The injury is expressed as an extended germination period and reduced seedling dry weight accumulation under standard warm germination test conditions. These observations are consistent with the concept that the widely used and unwieldy measure of seed quality commonly referred to as vigor is essentially a function of the time that it takes for mitochondria in the axis to develop efficient oxidative phosphorylation (2) and/or their total respiratory capacity. In recent years, mitochondrial activity has been clearly demonstrated to be the primary source of energy during seed germination (3, 8). All these observations indicate a mechanism for stabilizing or protecting the mitochondrial inner membrane during desiccation of the seed as a critical element of germination. The genetic variability and inheritance patterns that have been reported for tolerance to dryer injury in maize (4) suggest potential for this system as a model with which to study this concept.

ACKNOWLEDGMENTS

The authors thank Dr. H. T. Horner and the Bessey Microscopy Facility, Iowa State University, Ames, IA, for providing equipment and advice for the microscopy work reported.

REFERENCES


