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# Early germination physiology in hybrid maize (Zea mays L.) embryos damaged by high-temperature desiccation

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# Early germination physiology in hybrid maize (*Zea mays* L.) embryos damaged by high-temperature desiccation

by

Randall F. Madden

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## TABLE OF CONTENTS

## GENERAL INTRODUCTION

The metabolic potential of a quiescent seed (seed vigor) is expressed during germination and seedling development. Artificial drying of maize (*Zea mays* L.) seed with air temperatures >40°C can reduce this potential and result in significant reductions in seed vigor (Kiesselbach, 1939; Seyedin et al., 1984; Navratil and Burris, 1984; Adegbuyi and Burris, 1989). Characterization of the basis for this injury would be valuable to the commercial seed industry. Furthermore, high-temperature desiccation of maize seed can be utilized as a model injury system to study the basic physiology of seed vigor.

The concept of seed vigor has been extensively debated in the literature (Delouche and Caldwell, 1960; Woodstock, 1965; Pollock and Roos, 1972; Ching, 1973; Grabe, 1973; Bradnock, 1975; Burris, 1976; Abdul-Baki, 1980; Perry, 1980; Perl, 1987), however, no single definition of the term has been universally accepted. Alternatively, some estimation of seed vigor, as a prediction of field emergence, is widely considered to be more important than actual germination percentage. Current laboratory evaluations of seed vigor have little scientific basis for the procedure other than they correlate well with laboratory performance or field emergence. Perl (1987) stated that the amount of seed vigor

that is expressed "depends on a series of biochemical activities and on the presence of available precursors necessary for their activities". He went on to conclude that "the determination of some critical activities and compounds in seeds may predict the quality of seedling performance (field emergence)" or seed vigor. An improved understanding of the specific components or activities which constitute seed vigor would greatly facilitate the development of new physiological evaluation techniques.

The basis for the reduction in seed vigor due to this injury has not been well characterized. Seyedin et al. (1984) speculated that, based on histochemical light microscopy assays, high temperatures resulted in the hydrolysis of starch in the embryonic axis during the early stages of the drying process. Significantly higher electrical conductivity values for leachate from high (50°C) compared to low (35°C) temperature dried seed were also reported. Increased electrolyte leakage has been correlated with reductions in seed vigor due to high-temperature desiccation (Herter and Burris, 1989; Chen and Burris, 1990). This relationship suggests a general role for membrane integrity in the expression of seed vigor.

Genetic tolerance to desiccation injury has been extensively documented. Dimmock (1947) suggested that seed

quality could be improved if inbred lines that were tolerant of high temperatures were used as the seed parents in hybrid seed production. Reiss (1944) reported that, based on vigor evaluations and germination data, the inbred line R4 was more tolerant than the inbred WF9 to high temperatures during drying. More recently, Bdliya and Burris (1988) determined that the maternal influence on tolerance was highly significant. These authors suggested the mitochondria as a possible factor in explaining this inheritance pattern.

A role for mitochondria, in the expression of seed vigor, has been proposed by other investigators. The quantity of mitochondria and rates of activity were reported by McDaniel (1969) to directly influence seed vigor in barley. He later reported (McDaniel, 1973) that measures of mitochondrial function such as ADP:O ratios and respiration rates were well correlated with yield in hybrid barley. He used measures of mitochondrial function as a selection criteria for hybrid vigor. Aged soybean seeds were reported by Abu-Shakra and Ching (1967) to have fewer mitochondria than seedlings resulting from new seed. The rate of oxygen uptake was higher (on a per unit weight of mitochondrial nitrogen) in the aged seeds, however the P/O ratios of mitochondria from these seeds was significantly less than

new seed. Sato and Asahi (1975) reported the isolation of three different fractions of mitochondria from germinating pea seeds. They suggested that the biochemical properties of each of these fractions were different due to the protein density. As the tissue moisture content declines, some of the membrane bound enzymes, such as malate dehydrogenase, detach and are present in the cytoplasm of the mitochondrial matrix in a soluble form. These pre-existing polypeptides were proposed to be re-incorporated into the mitochondrial membrane during imbibition because *de novo* synthesis of proteins was not required for mitochondrial development.

The pattern of mitochondrial development in reserve tissues of germinating seeds has been reported (Morohashi, 1986) to depend on the primary storage compound (lipid or starch). In starch-storing seeds, mitochondrial development was not inhibited by cycloheximide or chloramphenicol, suggesting no requirement for *de novo* synthesis of proteins. In contrast, increases in mitochondrial activity (oxygen uptake) were strongly inhibited during the initial stages of germination in lipid-storing seeds indicating that development depended on the synthesis of new proteins. These results suggest the existence, in the case of reserve tissue, of two distinct patterns of mitochondrial development.

A respiratory transition has been reported by Yentur and Leopold (1975) in germinating seeds of several different species (including maize). They proposed that their observation of changing sensitivity to cyanide and salicylhydroxamic acid represented a shift from primarily alternate pathway during early stages of germination to the cytochrome mediated pathway during later stages. This was suggested to be related to a requirement of something essential that was provided by alternate pathway respiration.

The objective of this investigation was to characterize the mechanism of high-temperature desiccation injury in germinating maize seed and to use this injury system to study the contribution of mitochondria in embryonic tissue to the metabolic potential of whole seed. The samples created by this injury system offer a source of reduced vigor seed, other than that due to ageing, that is more uniform and easily controlled. A better understanding of the basis for vigor reductions in this system will provide a unique perspective in attempting to identify the critical factors that constitute the vigor mechanism.

**Explanation of Dissertation Format**

This dissertation is organized in the alternate format and includes two sections in the main body which will be submitted to Plant Physiology for publication as separate manuscripts. The two papers are followed by a General Summary. References cited in the General Introduction and General Summary are included in the Literature Cited section following the General Summary. Figures that are included in the Appendix are related to information included in the second paper, but will not be part of the manuscript. These documents represent the report of research that was conducted in the Seed Science Center, Iowa State University, by the senior author, Randall F. Madden, who is a Ph.D. candidate under the supervision of Dr. J. S. Burris. The first section is entitled Respiration and Mitochondrial Characteristics in Maize Embryos Damaged by High Temperatures During Desiccation. This section will be submitted for publication first as it is cited as a reference in the second section which is entitled Nucleotide Pools During the Early Stages of Germination of Maize Embryos as Determined by HPLC.

# SECTION I.

## RESPIRATION AND MITOCHONDRIAL CHARACTERISTICS IN MAIZE EMBRYOS DAMAGED BY HIGH TEMPERATURES DURING DESICCATION

**ABSTRACT**

Artificial drying of hybrid maize (*Zea mays* L.) seed using air temperatures in excess of 40°C reduced early seedling dry weight accumulation when compared to seed dried with lower air temperatures. In embryo culture experiments, an extended germination period was observed for excised embryos from high-temperature (45°C) treatments compared to those dried at low-temperature (35°C). This observation coincided with reduced respiration rates of imbibing axis tissue that had been excised from the damaged samples. After 1 h of imbibition, the rate of $O_2$ uptake by axes from low-temperature (LT) treatments was greater than high-temperature (HT) dried samples. Uptake rates continued to increase for LT samples throughout the analysis period while rates for HT axes were constant. Mitochondria isolated from HT treated axis tissue that had been imbibed for 4 h exhibited no respiratory control. However, ADP/O values of 0.79 and 0.77 were recorded for NADH and succinate assays, respectively, for mitochondria from LT treated axes. Electron micrographs of cells from the radicle meristem region showed that mitochondrial development during the initial 24 h of imbibition was impaired in HT treated samples compared to LT tissue. A visible disruption of the matrix structure at 0 and 6 h resulted in the apparent

degeneration of some mitochondria at 24 h while others appear to have achieved repair. Results presented here indicate mitochondrial function, during the initial stages of embryonic tissue hydration, is a key element in determining the rate of germination (whole seed) and subsequent seedling growth.

## INTRODUCTION

Germination and early seedling growth characteristics (seed vigor) of maize (*Zea mays* L.) 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 and dry matter accumulation 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 (Halmer and Bewley, 1984). There continues to be considerable interest in the use of physiological evaluations of vigor to supplement more traditional tests.

The initial uptake of $O_2$ by imbibing seed closely follows hydration of the seed tissues (Bewley and Black, 1985; Attucci et al., 1991) 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 is thought to depend on continued development of mitochondria due, initially, to the import and assembly of preexisting proteins from the cytoplasm (Nawa and Asahi, 1973; Nakayama et al., 1980). This general model has been supported by

ultrastructural analyses showing mitochondria with poorly differentiated internal structure in dry seed (Morohashi et al., 1981; Attucci et al., 1991) followed by dramatic development of the internal structure and increases in the number of cristae during the early stages of germination. However, inherent variation from this model has been reported when comparing storage and embryo tissue (Morohashi et al., 1981). The ability of respiratory measurements of imbibing seed tissue to characterize seed vigor differences due to ageing has been investigated using isolated axes of soybean (Woodstock et al., 1984; Ferguson et al., 1990). Axes tissue has been proposed to be more directly associated with seed vigor than storage tissue. Results indicate that respiration rates and estimates of mitochondrial functionality were significantly lower in axes tissue of low vigor seed.

The purpose of this investigation was to study the contribution of 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. Genetic analysis of tolerance to desiccation injury has indicated that the trait is cytoplasmically inherited with environment having a significant effect on expression of the genetic components

(Bdliya and Burris, 1988). A widely grown inbred B73, which has been characterized as desiccation sensitive, was used as the seed parent in these experiments.

Results indicate that mitochondria from the embryonic axis are damaged by high temperatures during desiccation and their capacity to increase respiratory function during early germination was impaired. The injury is expressed as an extended germination period and reduced seedling dry weight production under standard warm germination test conditions. The results presented here support the theory that the physiological state of mitochondria present in the embryonic axis of a desiccated maize seed is a critical factor in that seed's potential to establish a vigorous seedling.

# 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 X H95 was the pollen parent. Random ear samples were harvested periodically, with husk intact, to obtain ears with kernel moisture contents ranging from 50 to 35% (w/w). At each harvest, ear samples were brought into the laboratory, husked, and immediately placed in thin-layer experimental dryers (Navratil and Burris, 1982). 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 12% (w/w) 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 12% moisture. Each ear sample was hand shelled and the seed stored in a paper envelope 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 x drying temperature treatment. Standard warm germination tests were conducted as previously described (Knittle and Burris, 1976) 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 (Loeffler et al., 1985) was 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 common razor blade was used to remove the endosperm tissue. Embryos were surface sterilized in 1% (v/v) sodium hypochlorite for 30 sec, 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 carefully 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 using a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) calibrated with air-saturated water at 25°C (250 $\mu$M $O_2$). 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 mg of embryonic axis tissue that had been excised from dry seed were imbibed for 4 h at 25°C before being ground by hand using a chilled mortar and pestle. Mitochondria were isolated according to Day and Hanson (1977) with the following modifications. Tes 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 $\mu$L of a reaction mixture consisting of 250 mM sucrose, 10 mM Tes, 1 mM MgCl, 1 mM $KH_2PO_4$ (pH 7.2), 0.1% (w/v) BSA. Triplicate aliquots (10 $\mu$L) of the resuspension were saved for protein determination.

Oxygen uptake by mitochondria was measured immediately following resuspension of the pellet using a low volume reaction chamber at 25°C. Initially, 25 μL of mitochondrial suspension was added to 500 μL of the reaction mixture in the reaction chamber. Subsequent additions of different substrates and inhibitors are described in the legends of the figures. Protein was determined by the Lowry procedure following precipitation with TCA (Bensadoun, A and Weinstein, D., 1976).

**Electron Microscopy**

The embryonic axes were fixed in 3% glutaraldehyde/3% paraformaldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2 for 18 h at 4°C. Following fixation, the samples were washed three times, in buffer only, for ten minutes 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 ten minutes 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). Dehydrated samples were transferred to acetone in graded steps (50, 75, and 100%, 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 low-viscosity embedding medium (Spurr, 1969). Infiltration of the dehydrated samples was carried out with constant rotation using a graded transfer (25, 50, 75, and 100%, v/v, 12 h per step) 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.

A Reichert Ultracut E ultramicrotome 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 (Reynolds, 1963). Thin sections were then examined and photographed with a JEOL 1200EX STEM.

# RESULTS

## Vigor Characterization

Seed viability, as estimated by the warm germination test, was high (>90%) for all treatments (Table I). 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 I) indicate a relatively similar reduction for all 45° treatments when compared to 35°, though the difference was small for the 43.7% 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 I). With the exception of shoot weight for 43.7% harvest moisture, dry weight of shoot and root tissue was greater for each successive reduction in harvest moisture. High-temperature desiccation treatments (HT) reduced shoot dry weight by 40, 32, and 21% for 51.1, 43.7, and 36.1% 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.

**Table I.** Effect of Desiccation Temperature and Harvest Moisture on Seed Viability and Seedling Development in Maize

| Harvest Moisture | Drying Temp | Germination Test | | Seedling Dry Wt[a] | | |
|---|---|---|---|---|---|---|
| | | Warm | Cold | Shoot | Root | Shoot/Root Ratio |
| % | ° | % | | *mg seedling*$^{-1}$ | | |
| 51.1 | 35 | 100 | 94 | 29.2 | 17.3 | 1.69 |
| | 45 | 99 | 81 | 17.5 | 7.2 | 2.43 |
| 43.7 | 35 | 98 | 91 | 28.7 | 26.8 | 1.07 |
| | 45 | 99 | 88 | 19.4 | 10.6 | 1.83 |
| 36.1 | 35 | 100 | 94 | 35.0 | 30.6 | 1.14 |
| | 45 | 94 | 78 | 27.6 | 16.2 | 1.71 |
| $LSD_{0.05}$ | | | | 4.6 | 3.7 | 0.38 |

[a] Dry weight was determined following the warm germination test.

These results indicate that the seed becomes more tolerant to high-temperature desiccation as harvest moisture declines. Shoot/root dry weight ratio is a single index value that has been reported (Navratil and Burris, 1984) to offer an additional quantification of seed vigor. For each harvest moisture, the HT treatment had a higher shoot/root ratio than low temperature (LT).

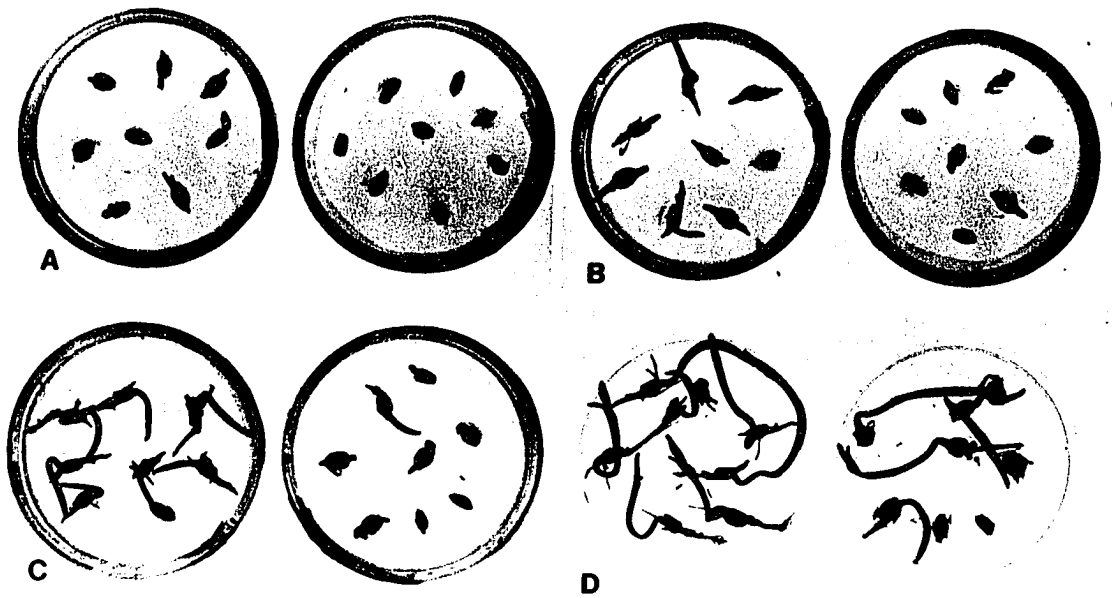
Embryo culture experiments were conducted to focus on the treatment effect on the germination characteristics of excised embryos. Results consistently indicate a delay in

the initiation of elongation of isolated embryos from 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 appear to be developing normally (Fig. 1D). These experiments demonstrate that a significant portion of the injury is localized in the embryo and not an impairment of the reserve mobilization system or pericarp.

**Oxygen Uptake by Excised Embryos**

Respiration patterns for most imbibing seeds have been characterized as tri-phasic (Bewley and Black, 1985). During Phase I, the rate of $O_2$ uptake is directly related to the rate of tissue hydration. Therefore, respiratory measurements of whole seed during this period are sometimes difficult to interpret due to 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, $O_2$ consumption rate during early germination. When $O_2$ consumption assays are conducted utilizing excised embryos (with scutellum) or embryonic axes of maize, the hydration phase (Phase I) is completed within the first few minutes (Ehrenshaft and Brambl, 1990). The following period of imbibition (up until

**Figure 1.** Culture of embryos excised from maize seeds that were dried at 35°C (left petri dish) and 45°C (right petri dish). Time sequence is 48 h (A), 72 h (B), 96 h (C) and 168 h (D) following the initiation of imbibition


A
B
C
D

radicle emergence) represents Phase II. During this "lag phase" (Phase II), respiration becomes more stable with more moderate increases in the rate of $O_2$ uptake being recorded. Consumption during this phase of germination is primarily due to activity of the Cyt-mediated electron transport chain located in the inner membrane system of mitochondria (Ehrenshaft and Brambl, 1990). To eliminate most of the hydration variability and focus on the metabolic activity of the embryonic respiratory system, $O_2$ consumption of embryos and embryonic axes were measured for the first 6 h of imbibition. Rate of $O_2$ 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 (12% moisture).

Initially, experiments were conducted on 36.1% harvest moisture samples created in 1989. Results indicate that embryonic tissue samples from HT dried seeds were not taking up $O_2$ at the same rate as undamaged tissue at any of the early imbibition times measured (Fig. 2). In the case of embryos (Fig. 2A), $O_2$ consumption by damaged embryos at 1 h was 78% that of undamaged. This difference was only slightly more at the 6 h measurement. The rate of uptake continued to increase for both treatments throughout the analysis period

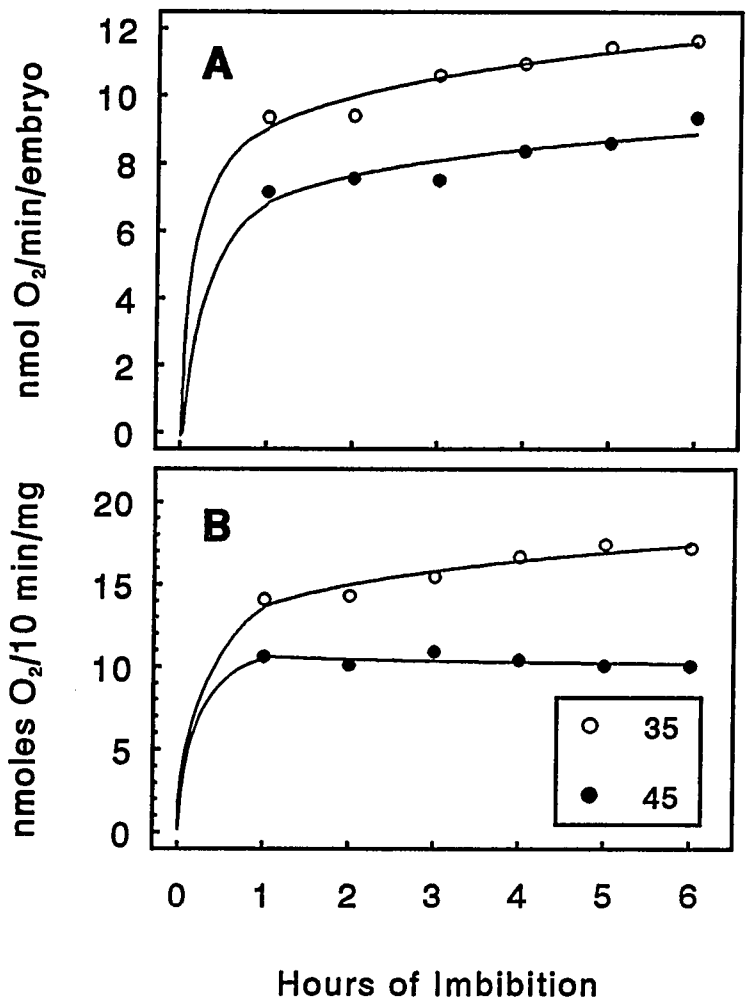



Figure 2. Rates of oxygen uptake by excised embryos (A) and axes only (B) from seed dried at 35 or 45°C. Tissue weights were taken before the initiation of imbibition. The data represent averages of determinations on six different replicates

indicating improvement in some component of the activity. Data from the embryonic axis experiments (Fig. 2B) demonstrated a similar relationship between the temperature treatments. In contrast to the whole embryo, damaged axes did not exhibit any increase in the rate of $O_2$ consumption during the analysis period. These results appear to reflect some impairment of the damaged axes ability to develop their respiratory activity during the very early stage of germination.

Based on traditional seed vigor evaluations, the critical air temperature above which drying injury has been observed is 40°C (Navratil and Burris, 1984). To evaluate the effect of desiccation temperature on early embryonic respiration in more detail, four air temperatures were used as drying treatments in 1990. Individual ear samples were the experimental units to which the treatments were applied. Results of $O_2$ consumption experiments conducted on these samples are consistent with the previously reported concept that drying temperatures above 40°C can result in desiccation injury (Fig. 3). Initial respiratory rates were higher for axis samples from seed dried at 35 and 40°C than either 45 or 50°C samples. Rate of uptake increased gradually throughout the analysis period for the two lowest temperature treatments, however, the 40° treatment was higher than 35°.

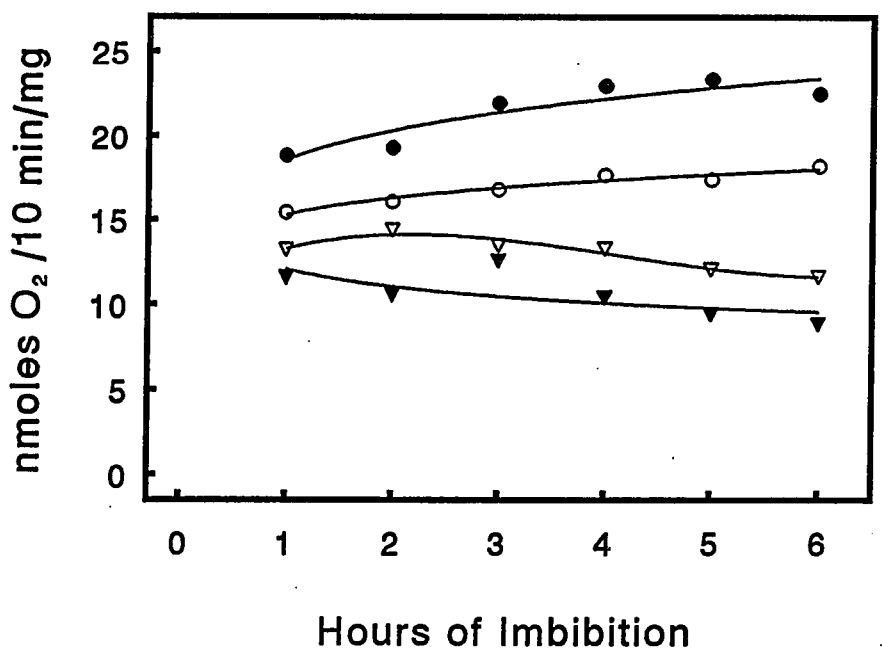



**Figure 3.** Rates of oxygen uptake by maize axes excised from seed that was harvested in 1990 at 50% harvest moisture and dried at 35 (O), 40 (●), 45 (▽), and 50°C (▼). Tissue weights were taken before the initiation of imbibition. The data represent averages of determinations on six different replicates

Consistent with earlier experiments, the rate of uptake did not increase (actually decreased) for damaged axes. Although differences were not large, rate of uptake for axes dried at 50°C were 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 19% less for the HT samples when compared to LT treatments (Table II). This suggests that either the damaged axes yielded fewer mitochondria or that these organelles are less protein dense than those from undamaged tissue. Any combination of these possibilities could contribute to the lower $O_2$ uptake rates that were observed in experiments using the complete axis.

**Table II.** Yield of Mitochondrial Protein from Desiccated Axis Tissue

| Drying Temperature | Mitochondrial Protein[a] |
|---|---|
| *°C* | *μg (200 mg tissue)*$^{-1}$ |
| 35 | 692 |
| 45 | 559 |

[a] Values represent the mean of four isolation procedures. Triplicate determinations were made for each isolation.

To compare mitochondrial activities and thus begin to distinguish between these two possibilities, experiments measuring respiratory function were conducted using the isolated mitochondrial preparations. The rate of $O_2$ uptake was lower in mitochondria isolated from HT embryonic axes (Table III). State 3 rates for the HT treatment were approximately 70% that of LT when either NADH or succinate were used as substrate. A remarkably similar relationship was observed using NADH and succinate as substrates for both temperature treatments.

**Table III.** Effect of Drying Temperature on Functionality of Mitochondria Isolated from Embryonic Axes of Maize Seed

| Substrate | Drying Temp | State 3 Rate[a] | State 4 Rate | Respiratory Control | ADP/O Ratio |
|---|---|---|---|---|---|
| | °C | nmol $min^{-1}$(mg protein)$^{-1}$ | | | |
| NADH | 35 | 92.6 | 59.4 | 1.56 | 0.79 |
| | 45 | 66.8 | | | |
| Succinate | 35 | 63.6 | 49.1 | 1.30 | 0.77 |
| | 45 | 43.1 | | | |
| Malate | 35 | 36.4 | | | |
| | 45 | 38.8 | | | |

[a] Rates were measured at 25°C and represent the mean of determinations from at least three preparations. Excised axes were imbibed for 4 h before being analyzed.

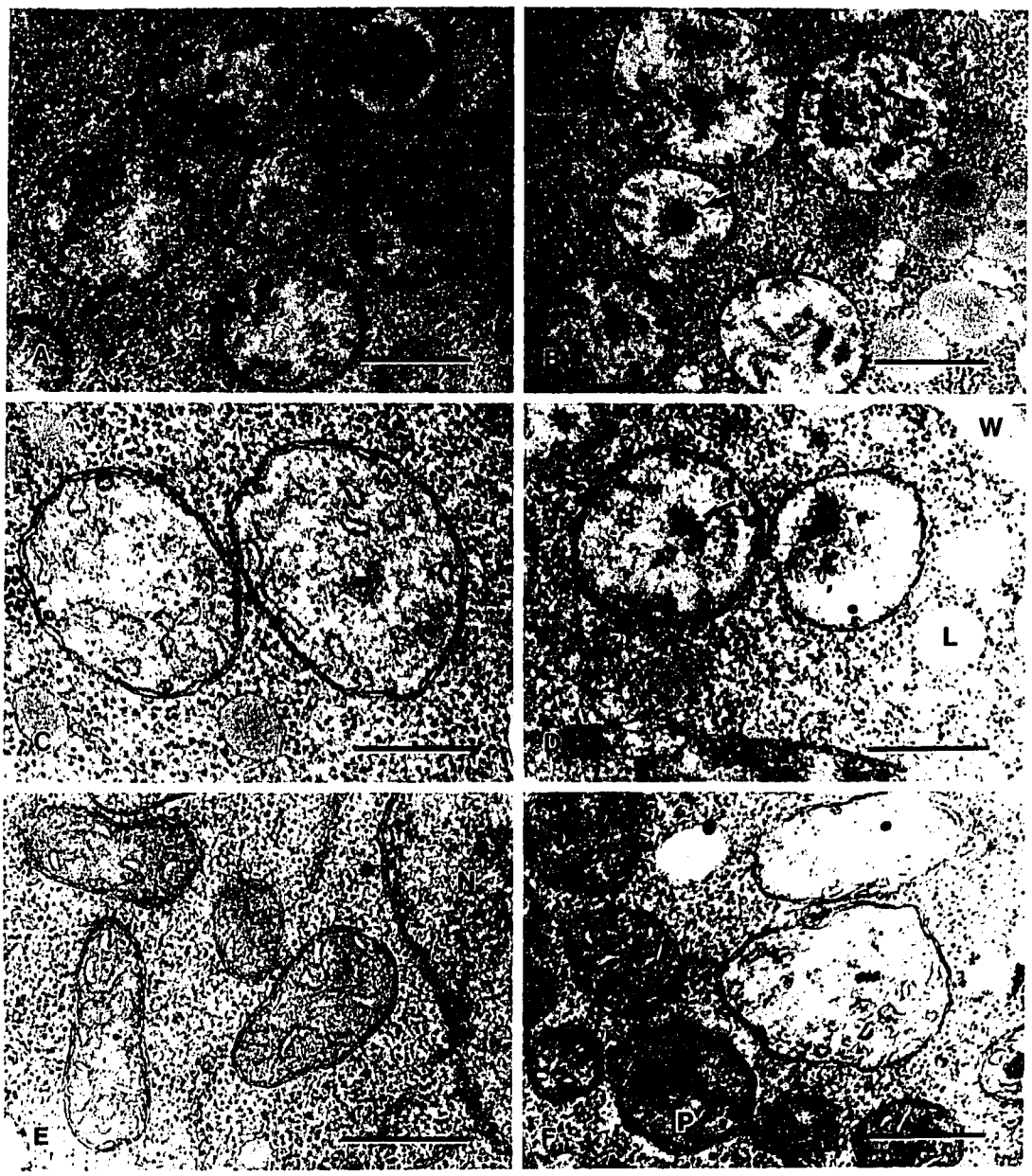
NADH resulted in a 46 and 55% higher state 3 rate than succinate for LT and HT treatments respectively. This is consistent with other reports that the capacity to oxidize exogenous NADH is more developed and then becomes active more rapidly upon hydration (Bewley and Black, 1985). Uptake rates were essentially the same in the presence of 10 mM malate, however, rates were low and more variable.

$O_2$ uptake by mitochondria from the HT treated axes was not stimulated by the addition of ADP (Table III). Alternatively, mitochondria from the LT treated axes preps showed RC and nearly identical estimates of oxidative phosphorylation efficiency (ADP/O ratio). These results suggest that there has been a disruption of the inner membrane integrity in HT treated tissue that decreased the capacity of the electron transport system and impaired the ability of these mitochondria to develop oxidative phosphorylation during the very early stages of germination.

**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 TEM. Mitochondria from the LT treated samples at 0 h imbibition (Fig. 4A) appear swollen and have a homogeneous spherical

**Figure 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 (x37,500; bar, 0.5 μm). B, 45° treatment at 0 h; mitochondrial matrix contains electron dense aggregates (arrow), transparent areas, and a few narrow cristae (x40,000; bar, 0.5 μm). C, 35° at 6 h imbibition; both mitochondrial membranes are present and intact with numerous cristae evident (x67,500; bar, 0.3 μm). D, 45° at 6 h; mitochondrial matrix is electron transparent except for dense aggregates (arrow) with no visible cristae (L = lipid body; W = cell wall) (x42,850; bar, 0.5 μm). E, 35° at 24 h of imbibition; mitochondria have dense matrix, abundant cristae and elongated shape (N = nucleus) (x45,300; bar, 0.5 μm). F, 45° 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 (x40,000; bar, 0.5 μm)


A
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C
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D
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E
P
F

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. Outer and inner membranes are clearly visible and the external shape seems less swollen. The matrix ground substance is very uniform. In contrast, mitochondria from the HT treatments at 6 h (Fig. 4D) still exhibit the matrix aggregation and variable density that was evident at 0 h. Essentially no internal structure or cristae are visible. Even ribosomes in the cytoplasm appear to have formed small aggregates resulting in a less uniform density.

An elongated shape, which is characteristic of metabolically active mitochondria, predominates in LT treated cells after 24 h of imbibition (Fig. 4E). The matrix has stained more dense and abundant cristae are visible. Mitochondria from HT treated radicle cells are extremely

variable in their appearance (Fig. 4F). Most pictures indicate the presence of normal appearing mitochondria as well as some that are still very electron transparent and have poorly differentiated matrices. The number of these organelles with elongated shape is lower, as well. In a few cases, these 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 utilized high air temperatures during the desiccation process to impair the ability of a maize seed to complete germination as rapidly as the controls. In contrast to the extensive research on the effect of ageing on respiration during imbibition (Abu-Shakra and Ching, 1967; Woodstock et al., 1984; Tluczkiewicz, 1980; Ferguson et al., 1990), 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 (Bdliya and Burris, 1988; Navratil and Burris, 1984; Seyedin et al., 1984), the evaluations of seed vigor demonstrate the progressive susceptibility of the seed samples 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 (Stewart et al., 1990), characterize the injury as a limitation in early growth potential. This concept was supported by embryo culture experiments which focused on the metabolic potential of the embryo and eliminated any contributory effect of the pericarp,

endosperm, or aleurone. HT treated embryos exhibited an extended germination time. This observation is consistent with the apparent reduced potential for early growth as indicated by seedling dry weight determinations.

Respiratory measurements during imbibition, using excised axes, have been reported to be well correlated with vigor differences due to deterioration of soybean seeds (Woodstock et al., 1984). We found a similar relationship in that the HT treated embryos were not taking up $O_2$ at the same rate as LT samples after only one hour of imbibition. The rate of $O_2$ uptake increased for both treatments during the remainder of the analysis period. However, excised axes from HT samples essentially maintained a steady uptake rate while LT axes exhibited a trend similar to whole embryos. These apparently conflicting results emphasize the inherent complexity involved when assaying differentiated tissue for relatively minor differences in function. The axis data suggests that HT treatments reduced the initial mitochondrial respiratory activity as well as the potential for early development. This pattern is even more evident when four drying temperatures were used as treatments. Non-damaging temperatures (35 and 40°C) resulted in axis uptake curves indicative of a developing respiratory activity. Damaging treatments (45 and 50°C), however, exhibited a

declining activity throughout the analysis period. These differences 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 that is available in the cytoplasm.

Imbibed axis tissue from HT treated seeds did not yield as much mitochondrial protein as LT tissue. In addition, on a per mg protein basis, $O_2$ uptake was less when either NADH or succinate were used as substrate. These results suggest that HT treatment has effectively reduced the amount of protein in mitochondria during early imbibitional stages as well as the activity. This pattern is consistent with the inability of HT axes to increase $O_2$ uptake because mitochondrial development during imbibition is thought to be the result of the import and assembly of preexisting soluble proteins into the inner membrane. (Nakayama et al., 1980; Bewley and Black, 1985). Additional support for the concept of poorly developed inner structure can be derived from the apparent lack of functional oxidative phosphorylation. ADP/O ratios measured for the LT mitochondria are similar to those reported for mitochondria isolated from imbibed peanut embryos of 0.9 (Wilson and Bonner, 1971), from sunflower seeds of 0.85 (Attucci et al., 1991) and imbibed soybean axes of 1.2 (Woodstock et al., 1984).

Results from the investigation of mitochondrial ultrastructure with TEM provide convincing evidence of the disruption of inner structure in the HT samples. 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 (Vartapetian et al., 1987). 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 are not able to recover and are degenerating. It is not clear whether the presence of metabolically active appearing mitochondria in HT samples at 24 h is representative of organelles that have successfully achieved repair or if they represent a totally different population as has been suggested for peanut cotyledons (Morohashi et al., 1981). There is a noticeable difference in the number of morphologically mature mitochondria in HT samples at 24 h when compared to LT. The results reported in this paper support 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 (Abdul-Baki, 1980). In recent years, mitochondrial activity has been clearly demonstrated to be the primary source of energy during germination (Attucci et al., 1991; Ehrenshaft and Brambl, 1990). 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 high-temperature desiccation injury in maize (Bdliya and Burris, 1988) suggests potential for this system as a model with which to study the protection concept. The resiliency demonstrated by the mitochondria in this study is a striking example of the efficiency of the protection/repair mechanism.

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# SECTION II.

## NUCLEOTIDE POOLS DURING EARLY STAGES OF GERMINATION OF MAIZE EMBRYOS AS DETERMINED BY HPLC

**ABSTRACT**

The role of mitochondria in nucleotide metabolism during the initial stages of imbibition of excised maize axes was evaluated by analyzing nucleotides with high performance liquid chromatography (HPLC). Embryonic axis tissue samples with diminished capacity for mitochondrial activity during imbibition were created by artifically drying hybrid maize (*Zea mays* L.) seed with high air temperatures (>40°C). On a whole seed basis, this injury has been characterized as a decrease in seed vigor without having a significant effect on viability. Analysis of trichloroacetic acid extracts of imbibed axis tissue showed lower triphosphate nucleoside levels in damaged samples. After 2 h of imbibition in air-saturated water, ATP levels in low-temperature (35°C) dried samples had increased dramatically and were 45% larger than high-temperature (45°C) treatments. The size of the ATP pool was reduced by 50% when low-temperature (LT) axes were imbibed for 2 h in 10mM KCN. Though ATP levels increased in high-temperature (HT) treated tissue through 6 h of imbibition, the pool size did not attain the level measured for LT samples at 2 h. Tissue imbibed in KCN maintained the same ATP level throughout the analysis period. A reduced capacity for *de novo* synthesis of adenine nucleotides was also evident in

the HT samples with total adenylates increasing only slightly in the first 6 h of imbibition. The UDPG pool size in HT samples declined to low levels during the initial stages of imbibition and did not show recovery until the 6 h analysis. Adenylate energy charge values for LT samples after 4 h of 0.87 were consistent with metabolically active tissue while HT samples could not attain the same energy status. These early changes in nucleotide levels correlate well with treatment effects on mitochondrial function in germinating maize embryos.

## INTRODUCTION

The initial stages of seed imbibition are characterized by the inception of fundamental cellular activities, many of which require ATP as an energy source. Nucleotide metabolism during this period of development, therefore, has been the focus of extensive investigations (for reviews see refs. Rodaway et al., 1979; Bewley and Black, 1985; Perl, 1986, 1987). The principal pathway that is responsible for ATP synthesis/regeneration during this period and the absolute requirement of a specific source for the successful completion of germination has been disputed (Perl, 1980a, 1980b, 1986). Although oxidative phosphorylation has been reported to be the primary ATP regeneration mechanism in imbibing seeds of several different species (Wilson and Bonner, 1971; Morohashi and Sugimoto, 1988; Attucci et al., 1991), the relationship of this contribution to the energy status of the embryo and ultimately the completion of germination is less clear and appears to vary with different types of seed and seed tissues (Raymond et al., 1985).

The endogenous levels of ATP and other nucleoside triphosphates in embryonic tissue increase dramatically during early imbibition from very low levels present in a dry seed (Obendorf and Marcus, 1974; Rodaway et al., 1979;

Standard et al., 1983). Various measures of the ability of a seed to regenerate ATP and increase the adenylate pool size during this stage of development have been correlated with the vigor of a seed (Ching, 1973; Standard et al., 1983). Although the absolute level of ATP accumulation during imbibition has been reported to be insignificant with respect to an indication of seed vigor potential (Perl, 1986), this type of information, combined with the total adenylate pool size and a profile of the composition of this pool commonly termed adenylate energy charge (AEC), have proven to be useful indicators of the metabolic state of most biological tissues. For example, dramatic increases in the pool size of adenine and uracil nucleotides consistently precede the start of proliferation in tobacco cell culture (Meyer and Wagner, 1985b). Tissue levels of UDPG have also been proposed as indicators of tissue metabolic status. Considering the requirement of this compound as a key intermediate in cell wall biosynthesis and carbohydrate mobilization and transport in imbibing seed (Bewley and Black, 1985), it is not surprising that investigators have reported a strong correlation between levels of this nucleotide sugar and seed vigor (Standard et al., 1983) .

A comprehensive determination of nucleotide content of biological tissues has traditionally involved significant

effort and inherent variability due to the number of different analyses that had to be conducted. The development of a method for separation of nucleotides, nucleosides and bases by high performance liquid chromatography (HPLC), originally developed for analysis of body fluids (Perret, 1982) and product purity (for review see ref. Zakaria and Brown, 1981), provided a single analysis technique for complex biological extracts. This procedure makes it possible to quantify, not only adenylates, but guanine, cytosine and uracil nucleotides and nucleotide sugars in a single analytical scheme. The application of this technique to plant tissues was hampered by the presence of compounds that absorb in the UV and elute with the compounds of interest which are not present in animal extracts (Standard et al., 1985; Dutta et al., 1988). Recent reports have described the adaptation of this approach to the analysis of seed and other plant tissues (Standard et al., 1983; Meyer and Wagner, 1985a; Standard et al., 1985; Bray et al., 1989; Brown and Davies, 1989; Tajima and Kouzai, 1989).

In this study, we modified previously reported procedures of nucleotide analysis by HPLC to study nucleotide metabolism in imbibing maize embryonic axes. A system of desiccation injury was utilized to create samples

that exhibit a range of metabolic potential during germination. The basis for this disfunction has been characterized as an impairment of development of mitochondrial activity in axis tissue during the early stages of imbibition (Madden and Burris, 1991). This model injury system provided a mechanism for a correlative study to relate the effect of mitochondrial integrity with increases in nucleotide levels and AEC during the early stages of imbibition. The results are consistent with the concept that high-temperature damaged axis tissue is less able to attain nucleotide pool levels and AEC comparable to undamaged samples.

## 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 X H95 was the pollen parent. For the first study (1989), ears were harvested with a kernel moisture content of 36% (w/w) as determined by the oven method. Harvested ears were husked and immediately placed in thin-layer experimental dryers (Navratil and Burris, 1982) with air temperatures maintained at either 35 or 45°C until the seed reached a moisture content of approximately 12% (w/w). Dried samples were hand shelled, samples representing each treatment bulked, and placed in cold storage at 10°C and approximately 50% relative humidity until analyzed. Seed for the second study was harvested in 1990 and a moisture determination was made on each ear sample. The seed that was analyzed in this study was 50% (w/w) moisture when harvested and desiccation air temperatures were 35, 40, 45, and 50°C. Dried samples were handled the same as in 1989, except seed from each ear was stored and analyzed individually.

**Nucleotide Extraction**

Nucleotide extraction was carried out using a TCA in ether method essentially as described by Standard et al. (1985). Variations of this method have been described as the most reliable procedure for nucleotide extraction (Hooks et al., 1989). Embryonic axes were carefully excised from dry seed with a common razor blade and stored at 10°C until analyzed. Axis tissue (40 mg) was weighed and then imbibed in small shallow trays for the times described. At the end of the imbibition time, samples were placed in a 1.5 mL microcentrifuge tube containing 0.4 mL of extraction buffer consisting of 8% (w/v) TCA 20% (v/v) methanol and immediately homogenized using a teflon fitted pestle. Insoluble material was removed by centrifugation for 5 min at 9000 x g. The pellet was washed with an additional 0.4 mL of extraction buffer and recentrifuged. The two supernatants were combined before removing the TCA by washing the samples six times in seven volumes of water-saturated diethyl ether. Following neutralization, samples were filtered using a 0.2 $\mu$m filter into sample vials and then frozen until analyzed. The samples were kept in ice during all operations to minimize acid hydrolysis of nucleotides.

**Determination by HPLC**

The HPLC delivery system (Shimadzu Corporation, Kyoto, Japan) consisted of two Model LC-6A pumps, together with a Model SCL-6A system controller for gradient elution. A Model SIL-6A auto-injector was used to inject the samples and nucleotides were detected with a Model SPV-6AV UV-VIS detector at 254 nm. Chromatograms were processed using a Model C-R3A integrator.

Nucleotides and nucleotide sugars were chromatographed on a 250 x 4.6 mm ID Supelcosil LC-SAX (5 $\mu$m) column (Supelco Inc., Belefonte, PA) eluted at 1.6 mL $min^{-1}$. The elution buffer system consisted of buffer A, 2 mM potassium dihydrogen orthophosphate (HPLC grade, VWR Scientifc) pH 2.75 with 8 or 20% acetonitrile (as indicated in the legends) and buffer B, 0.6 M potassium dihydrogen orthophosphate pH 3.25. A 12 $\mu$L aliquot of each sample was injected into the column and eluted with a concave gradient (20 min) from 100% buffer A to 100% buffer B, followed by a 5 min period of 100% buffer B. The column was regenerated by washing for 5-10 min with buffer A between injections. The gradient elution program was adjusted to achieve desired separation of the nucleotides according to the age of the column. Individual components of the nucleotide pools were

identified using retention time relative to known standards and by injecting internal standards.

# RESULTS

## HPLC Separation Method

To achieve adequate separatation and quantification of AMP and NAD in the TCA extracts, a modification to the previously reported method of Standard et al. (1985) had to be made. The inclusion of 20% acetonitrile in buffer A resulted in adequate (≈ 2 min) separation of the AMP/NAD complex from the interfering material. However, AMP and NAD eluted as a single peak and could not be resolved consistently. The retention time of AMP was reduced with minor decreases in the concentration of acetonitrile in buffer A (Fig. 1). Alternatively the retention time of NAD was not significantly affected by acetonitrile concentrations. With 8% acetonitrile in buffer A, separation was adequate for resolving AMP from NAD and there was still ample elution time separating these compounds from the interfering material. This type of separation could not be consistently achieved by altering the gradient program or pH of either buffer as had been suggested (Standard et al., 1985).

Since separation and resolution of diphosphate nucleosides and nucleotide sugars was not satisfactory with less than 20% acetonitrile (data not shown), all samples were analyzed with two different buffer systems. This

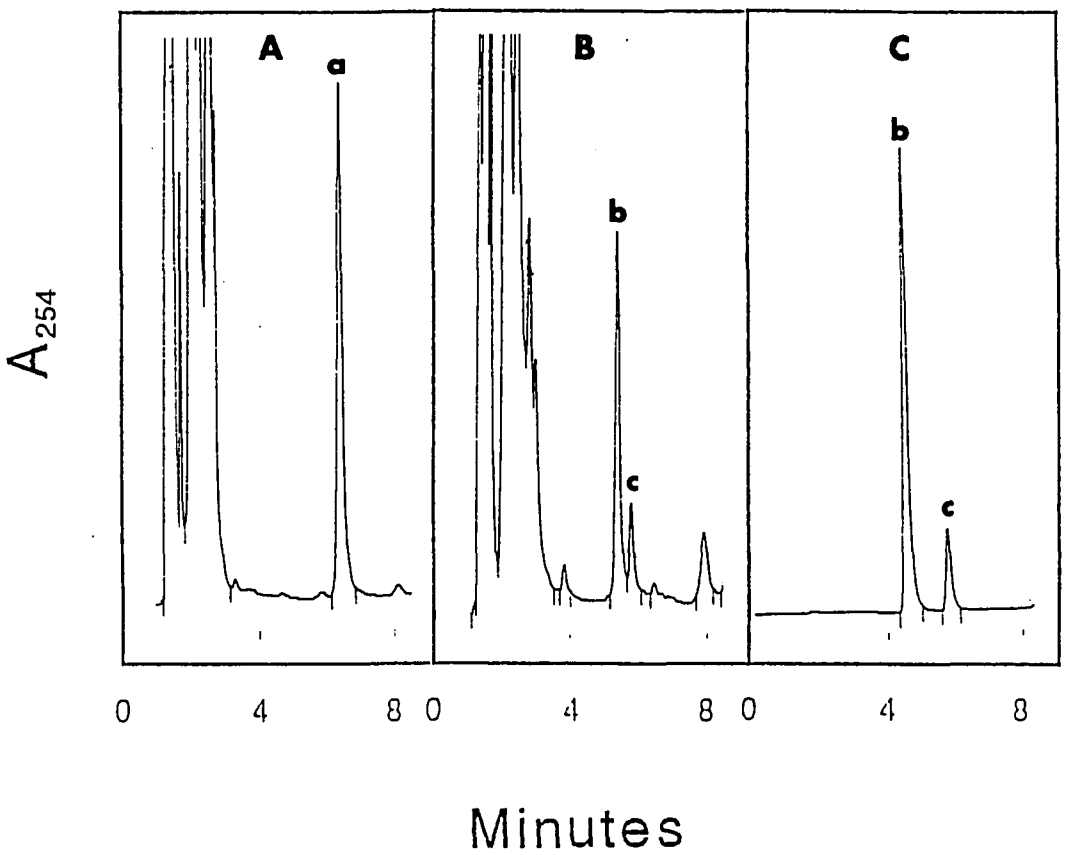



**Figure 1.** HPLC chromatogram showing the effect of different concentrations of acetonitrile in buffer A on separation of AMP/NAD complex. (A) 20% acetonitrile (B) 8% acetonitrile (C) 0% acetonitrile. Key: a, AMP/NAD complex; b, AMP; c, NAD

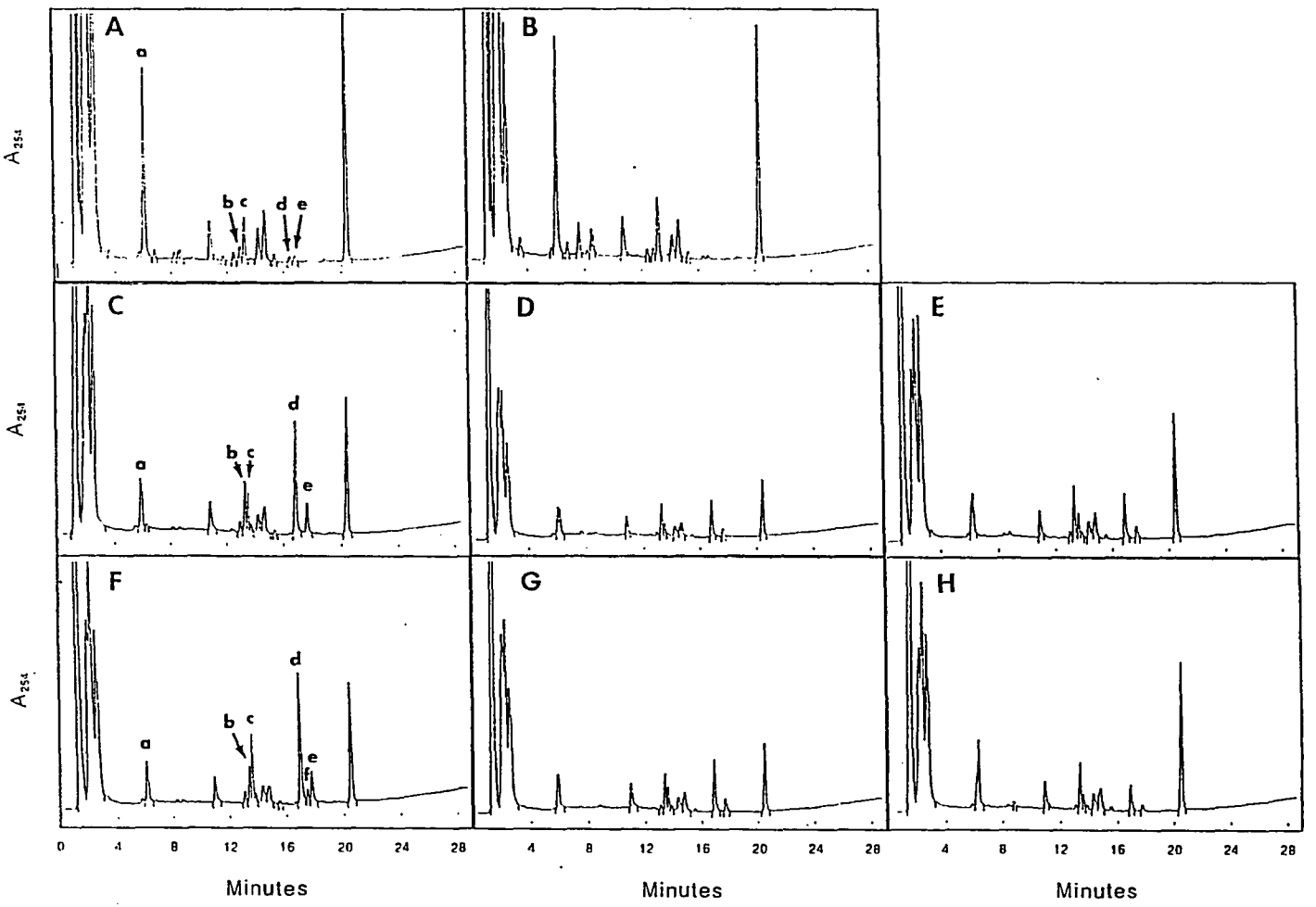
approach provided the most optimum resolution for all compounds in the extracts evaluated in this study. Specific assay conditions, including column age and extract complexity, will require minor alterations in the elution protocol to achieve satisfactory separation.

### Comparison of the Effect of Desiccation Injury and Respiration Inhibitor on Nucleotide Pools During Early Imbibition

A representative HPLC elution pattern (20% acetonitrile in buffer A) for extracts from three different treatments are illustrated in Fig. 2. Embryonic tissue excised from seed that was dried using an air temperature of 45°C has lower mitochondrial activity during the initial stages of imbibition (Madden and Burris, 1991) than seed dried at 35°C. The comparison of these two treatments with axis tissue imbibed in 10mM KCN should, therefore, represent three levels of mitochondrial activity.

Analysis of nucleotide levels in quiescent embryonic axes show nearly identical profiles for the two desiccation treatments (Fig. 2A and B). The AMP/NAD complex elutes as a single major peak over 2 min after the unidentified weakly charged material commonly found in seed extracts (Standard et al., 1983). Nucleoside triphosphate levels are very low and only barely detectable. We were not able to identify

**Figure 2.** HPLC chromatograms showing the effect of high-temperature desiccation and respiration inhibitor on nucleotide pools in imbibing maize axis tissue. Desiccation temperature and imbibition time are indicated for each treatment. (A) 35°C 0 h (B) 45°C 0 h (C) 35°C 2 h (D) 45° 2 h (E) 35°+CN 2 h (F) 35°C 4 h (G) 45°C 4 h (H) 35°+CN 4 h.
Key: a, AMP/NAD complex; b, ADP; c, UDPG; d, ATP; e, GTP; f, UTP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Minutes

the compound that elutes several minutes after ATP and GTP. This compound was present in every sample that was analyzed and appeared to decline with increasing time of imbibition. The strong negative charge and relatively high levels in axis tissue of maize suggest a compound like phytic acid, however phytin does not absorb UV wavelengths.

Elution profiles of extracts after 2 h of imbibition show significant differences in the pool size of several nucleotides (Fig. 2C and D). Nucleotide pools in axis tissue from 45°C dried seeds were intermediate to 35°C treatments and CN. Furthermore, the profiles of CN and 45° treatments are relatively similar. ATP and UDPG pools are visibly smaller for 45° treatments than 35° and smaller yet for the CN treatments. The same relationship is evident in 4 h profiles (Fig. 2F and G). ATP and UDPG pool sizes appear to decrease steadily from 35° treatments to CN. In 35° extracts, the UTP peak is now visible and representative of further improvement in the energy status of axis tissue.

Quantification of nucleotide levels was achieved by the use of standard curves (Table I). Increased ATP levels during the first 2 h of imbibition were observed for all treatments. However, differences are already evident at 2 h with a pool in 35° treated tissue that is nearly double that of the CN treatment and 45% larger than 45° samples.

**Table I.** Effect of Desiccation Temperature and Cyanide on Nucleotide Pools in Imbibing Maize Embryonic Axis Tissue

| Imbibition Time | Nucleotide | Desiccation Temperature 35° | 45° | 35°+KCN |
|---|---|---|---|---|
| *h* | | *nmol (g fresh wt )$^{-1}$* | | |
| 0 | AMP | 609.9 | 738.8 | **[b] |
| | NAD | 112.7 | 105.1 | ** |
| | ADP | 77.0 | 68.2 | ** |
| | UDPG | 228.9 | 268.9 | ** |
| | ATP | tr.[a] | tr. | ** |
| | GTP | tr. | tr. | ** |
| 2 | AMP | 85.7 | 124.2 | 299.9 |
| | NAD | 96.5 | 85.9 | 87.8 |
| | ADP | 384.8 | 364.9 | 507.4 |
| | UDPG | 159.1 | 49.9 | 51.2 |
| | ATP | 1172.7 | 809.7 | 607.4 |
| | GTP | 607.7 | 430.0 | 395.0 |
| | Total Adenylates | 1643.2 | 1298.8 | 1414.7 |
| 4 | AMP | 61.9 | 117.0 | 173.5 |
| | NAD | 102.1 | 90.2 | 81.5 |
| | ADP | 342.1 | 374.5 | 469.0 |
| | UDPG | 335.7 | 71.5 | 72.2 |
| | ATP | 1431.0 | 818.4 | 691.3 |
| | GTP | 666.5 | 457.8 | 423.8 |
| | Total Adenylates | 1835.0 | 1309.9 | 1333.8 |
| 6 | AMP | 58.3 | 82.4 | 154.1 |
| | NAD | 111.3 | 97.6 | 87.6 |
| | ADP | 376.6 | 413.2 | 502.4 |
| | UDPG | 464.0 | 224.5 | 108.8 |
| | ATP | 1461.4 | 936.1 | 641.9 |
| | GTP | 681.2 | 518.8 | 384.5 |
| | Total Adenylates | 1896.3 | 1431.7 | 1298.4 |

[a] Peak evident but too small for integration.
[b] Data is the same as 35° 0 H.

Corresponding to decreases in the ATP levels is the reverse effect on the pool size of AMP. This relationship is also observedin the 4 and 6 h analyses. When considering the 35° treatment, the ATP pool size appears to reach a maximum between 2 and 4 h and then remain constant at 4 and 6 h. Relatively small increases in the ATP levels of the 45° treatment were recorded, though after 6 h these axes had not yet equalled the 2 h level of the 35° treatment. CN treatments did not significantly increase the ATP pool size and total adenylate levels actually declined. A clear indication of the inhibitory effect of CN on oxidative phosphorylation is the large ADP pool that is maintained, relative to the other samples, by CN treatments at all imbibition times.

The level of UDPG in imbibing embryonic tissue has been suggested to be a critical factor in initiating growth and elongation during germination (Rodaway, 1979). The 3-fold difference at 2 h and nearly 5-fold at 4 h between 35 and 45° treatments in the UDPG pool size suggests a greatly impaired potential to initiate elongation mechanisms. Some recovery is evident for the 45° treatment by 6 h when the pool size is 2-fold higher than CN treatments. NAD levels are similar in all extracts.

**Effect of Desiccation Temperature on Nucleotide Pools in Imbibing Axis Tissue**

The range of desiccation temperatures was expanded to better characterize the effect of this injury on nucleotide metabolism during imbibition. Elution patterns of nucleotide extracts (Fig. 3) clearly establish the critical temperature above which, the injury becomes increasingly more severe. After 2 h of imbibition, elution profiles are similar, except for the area representing the triphosphate nucleotides where lower levels are evident for 45 and 50° treatments compared to either 35 or 40° treatments. This relationship is even more distinct after 4 h when larger differences have developed in UDPG and UTP in addition to ATP.

Determination of the concentration of nucleotides in these samples (Table II) shows differences between the temperature treatments in the high energy nucleotides at both imbibition times evaluated. In addition to triphosphate pool size, higher AMP levels in the high-tememerature treated seeds provide convincing evidence of a reduced capacity to improve the energy status in these damaged tissues. The 50° treated samples produced lower levels of ATP than 45° samples indicating increasingly more severe injury.

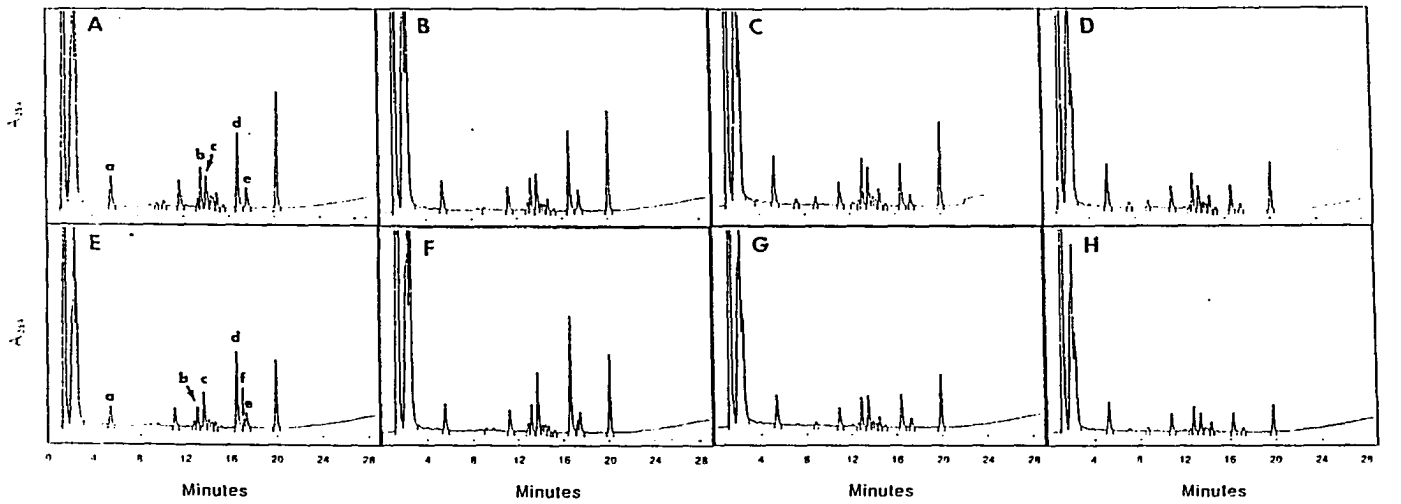


**Figure 3.** HPLC chromatograms showing the effect of desiccation temperature on nucleotide pools in imbibing maize axis tissue. Desiccation temperature and imbibition time are indicated for each treatment. (A) 35°C 2 h (B) 40°C 2 h (C) 45°C 2 h (D) 50° 2 h (E) 35 4 h (F) 40°C 4 h (G) 45°C 4 h (H) 50° 4 h.
Key: a, AMP/NAD complex; b, ADP; c, UDPG; d, ATP; e, GTP; f, UTP

**Table II.** Effect of Desiccation Temperature on Nucleotide Pools in Imbibing Maize Embryonic Axis Tissue (B73 X H99xH95) Harvested at 50% Moisture

| Imbibition Time | Nucleotide | Desiccation Temperature 35° | 40° | 45° | 50° |
|---|---|---|---|---|---|
| *h* | | *nmol (g fresh wt )*$^{-1}$ | | | |
| 2 | AMP | 86.4 | 71.3 | 155.9 | 174.3 |
| | NAD | 101.6 | 109.8 | 134.7 | 94.6 |
| | ADP | 457.0 | 392.1 | 541.9 | 441.2 |
| | UDPG | 240.4 | 225.1 | 196.9 | 121.0 |
| | ATP | 1126.4 | 1147.9 | 791.8 | 614.0 |
| | GTP | 596.4 | 576.9 | 449.2 | 399.2 |
| | Total Adenylates | 1669.8 | 1611.3 | 1489.6 | 1229.5 |
| 4 | AMP | 52.9 | 38.2 | 83.9 | 103.3 |
| | NAD | 84.5 | 97.9 | 87.6 | 61.1 |
| | ADP | 314.3 | 295.8 | 345.9 | 281.5 |
| | UDPG | 276.8 | 337.1 | 154.5 | 88.3 |
| | ATP | 1295.8 | 1405.8 | 672.5 | 521.7 |
| | GTP | 533.2 | 538.6 | 409.7 | 376.1 |
| | Total Adenylates | 1663.0 | 1739.8 | 1102.3 | 906.5 |

**Adenylate Energy Charge**

Average AEC values for samples from the first experiment are illustrated in Fig. 4. For all analysis times, the 45° treatment AEC values are intermediate compared to 35° and CN treatments. Although values increased during imbibition for 45° treated samples, the energy status at 6 h had not yet attained the level of 35° at 2 h. Similar results were obtained from samples in the second experiment (Fig. 5). AEC values of 0.87 and 0.89

were recorded for 35 and 40° treatments, respectively, after 4 h of imbibition. In contrast, 50° treated tissue registered values only marginally better than CN imbibed tissue from the first experiment.

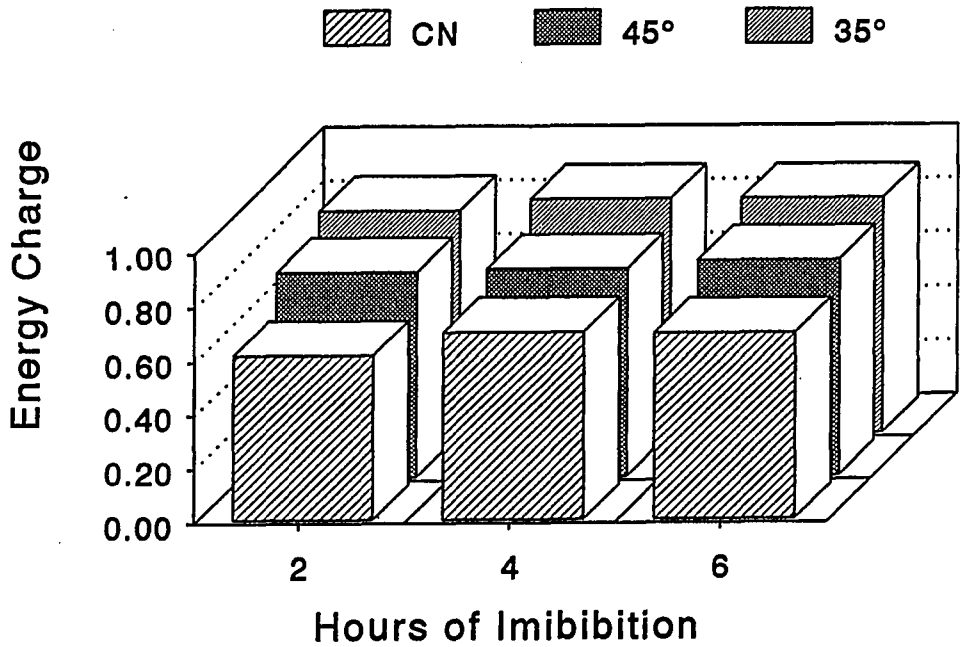


**Figure 4.** Effect of high-temperature desiccation and respiration inhibitor on adenylate energy charge of imbibing maize axis tissue

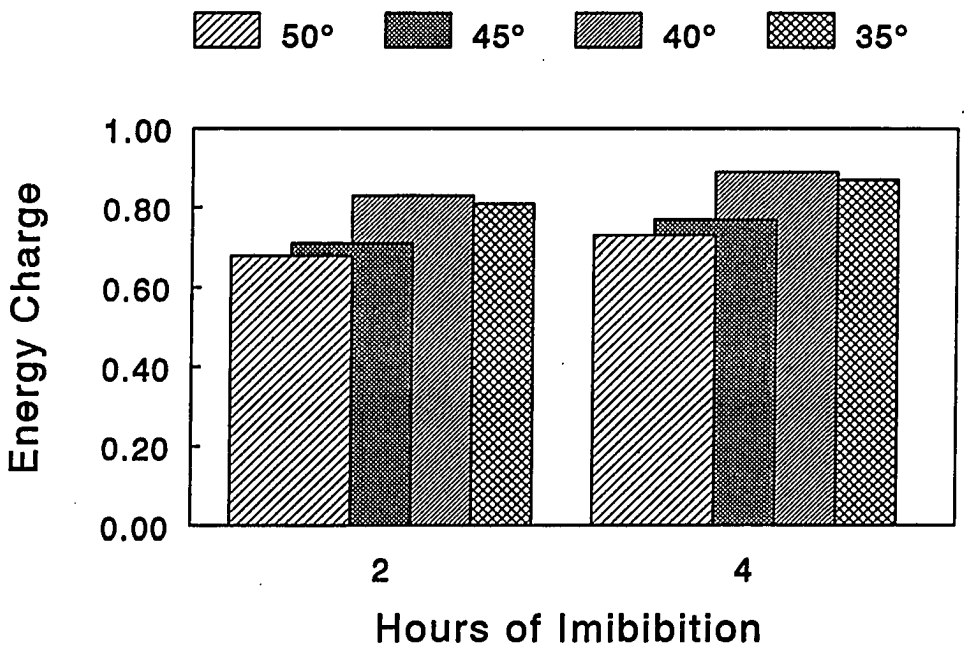


Figure 5. Effect of desiccation temperature on adenylate energy charge of imbibing maize axis tissue

## DISCUSSION

There are a number of reports in the literature of rapid increases in ATP levels in seed tissues following the initiation of imbibition (for review see ref. Perl, 1986). However, relatively few investigations have comprehensively analyzed nucleotide metabolism during early germination (Standard et al., 1983; Standard et al., 1985; Bray et al., 1989). In the present study, we modified a previously described procedure (Standard et al., 1985) for the extraction and quantification of all major nucleotides and nucleoside sugars from small amounts of seed tissue by a single HPLC analysis. This procedure allowed the specific evaluation of the contribution of mitochondria to nucleotide metabolism during imbibition. A similar investigation using traditional analysis methods could not determine a complete nucleotide profile from a single extract and would require the commitment of substantial resources.

Oxidative phosphorylation by mitochondria isolated from dry sunflower seed was recently reported (Attucci et al., 1991). However, the magnitude of this contribution to the high energy nucleotide pools in embryonic tissues has not been clearly demonstrated. In an earlier report (Madden and Burris, 1991), we characterized a high-temperature desiccation injury system that resulted in reduced

mitochondrial function in axis tissue during the early stages of imbibition. The samples created with this injury system provided an opportunity to study the effect of this impairment of mitochondrial development on nucleotide levels in imbibing tissue.

The first experiment compared damaged samples and samples imbibed in KCN with control axis tissue. These results support our theory that the desiccation injury results in a disruption of mitochondrial function since the nucleotide profile of 45° desiccated samples is very similar to the CN treatments. Furthermore, the significant contribution of mitochondria to the attainment of maximum triphosphate nucleoside and nucleotide sugar levels during the first hours of imbibition is demonstrated by the differences between 35° treatments and either 45° or CN. An indication of the amount of *de novo* synthesis of adenine nucleotides is evident in total adenylate values which show the damaged tissue failing to increase this pool and CN treated tissue levels declining. This observation is consistent with other reports of reduced *de novo* synthesis of adenylates in seeds exhibiting reduced vigor (Standard et al., 1983; Bray et al., 1989).

Increases in triphosphate nucleosides that were recorded for CN treatments could be the result of several

different possibilities. Incomplete inhibition of Cyt-mediated respiratory pathway in the axis tissue could result in a low level of oxidative phosphorylation. However, 98% inhibition of oxygen uptake by excised axes of maize during this same time period was demonstrated with a similar system (Ehrenshaft and Brambl, 1990) indicating the efficiency of the uptake of cyanide in imbibing tissue. A more likely explanation for a portion of the increase in ATP pool is the suggestion that ATP can be produced during this period by adenylate kinase through an equilibration of the adenylate pool (Raymond et al., 1983). A third possibility would include any contribution to ATP regeneration by fermentation which has been reported to be higher in starch storing seeds such as maize compared to lipid storing seeds (Raymond et al., 1985).

The level of UDPG at 0 h was similar in both treatments though these levels were higher than that reported for wheat embryos (Standard et al., 1983). Under the conditions of a cold germination (stress test), the level of UDPG in these wheat embryos was found to be correlated with whole seed vigor ratings. In this study, the significant difference between 35 and 45° treatments in the level of this compound after 2 h of imbibition is an indication of the reduced potential to initiate growth mechanisms. Nucleotide sugar

pools are dominated by the level of UDPG in germinating embryo tissue (Rodaway et al., 1979). The level of this pool in high vigor seed increases steadily through all phases of germination. Alternatively, a much slower rate of accumulation in 45° treated tissue is likely one of the factors in extending the time to complete germination that has been observed in embryo culture (Madden and Burris, 1991).

The energy status of living cells has been characterized extensively in the literature by the concept of AEC which normally falls within a range from 0.75 to 0.95 (Wiskich, 1980). In germinating seed, values from 0.8 to 0.9 are commonly recorded (Raymond et al., 1985). The AEC data for maize axis tissue reported in this study are within this range. These values are higher than those reported for whole maize seed of the same genotype (VanToai et al., 1988), but these values were determined with the luciferin/luciferase assay. Differences between the desiccation treatments are not large, though the apparent lag in the ability of the 45° samples to achieve the same status as 35° tissue is characteristic of a reduced metabolic potential (Raymond et al., 1987).

Support for the effects of desiccation temperature on nucleotide metabolism was provided by the second experiment.

As determined by numerous other evaluations, the critical temperature for desiccation injury to occur to this genotype is between 40° and 45°C. Temperatures above this result in a reduction of all the high energy nucleotide pools during the early stages of germination.

All these observations indicate that mitochondrial function makes a major contribution to nucleotide metabolism during the initial stages of imbibition. Samples created by a desiccation injury system demonstrate the effect that reduced mitochondrial activity has on nucleotide metabolism in a way that has not previously been reported. These results support the theory that oxidative phosphorylation is the primary mechanism for ATP regeneration early in the germination process of seeds.

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## GENERAL SUMMARY

Artificial drying of hybrid maize seed using air temperatures in excess of 40°C can result in the reduction of seed quality or vigor. Tolerance to this injury has been observed in some genotypes (Bdliya and Burris, 1988). In this study, a susceptible genotype B73 was used as the seed parent to produce hybrid seed that was used for analysis. Seed was harvested over a range of moisture contents and dried with a variety of air temperatures to create samples with high viability and reduced vigor potential. These samples were used to study the effect of this injury on early germination physiology of maize embryos. Results from some of the early experiments suggested that the injury mechanism may involve the respiratory activity/capacity of the embryonic axis. This concept developed into a working hypothesis and subsequent experiments were designed to test this theory.

The desiccation injury system was ultimately used as a model system to create samples with which to study mitochondrial development in imbibing maize axis tissue. This area of seed physiology has been the focus of extensive research effort for a long time and continues to be debated in the literature. It has been suggested that oxidative phosphorylation is not a significant contributor of energy

during early imbibition (Perl, 1986). This proposal has been repeatedly rebuffed by reports demonstrating mitochondrial activity during this stage of development that is coupled to the phosphorylation of ADP to ATP (Attucci et al., 1991).

In this study, desiccation injury was localized to mitochondria in the axis by utilizing a number of experimental techniques. Initially, the effect that each treatment had on whole seed characteristics was determined. Embryo culture was then used to eliminate any possible contribution of the endosperm or pericarp to the treatment effect. This technique demonstrated a significant reduction in the rate of germination for damaged tissue and provided convincing evidence that a majority of the injury was expressed in embryo tissue. All ensuing experimental procedures were performed using embryonic tissue excised from dry seed. The issue of tissue uniformity in seed related experiments is extensive in the literature and was a fundamental component of this effort.

Differences in the respiratory activity of imbibing embryo tissue were determined with oxygen uptake experiments. Assays using axis tissue demonstrated an impairment of the ability of high-temperature treated samples to increase oxygen uptake during phase II of

imbibition. This observation narrowed the focus of the investigations exclusively to axis tissue during the first few hours of imbibition which is the foundation of this effort. The cost, in terms of extra time to excise the tissue, was offset by the additional tissue specificity that was attainable.

Three different basic physiological evaluations of mitochondrial function during imbibition characterized the desiccation injury as a disruption of mitochondrial function during imbibition. Mitochondria isolated from imbibing axis tissue of damaged seed demonstrated a lack of coupling and yielded less protein than control samples. Nucleotide analysis of extracts from axis tissue during the same time period indicated that the level of high energy nucleotides was lower in damaged tissue. Ultimately, electron microscopy provided satisfying support for the theory of mitochondrial disruption. Visual differences in mitochondrial development in imbibing axis tissue, combined with the results of previous experiments, were convincing evidence of mitochondrial damage.

The possibility of damaging hybrid seed with high temperatures during desiccation has been well documented and appropriate precautions are commonly employed by the seed industry. The objective of this study was to localize and

characterize the injury mechanism and utilize this system as a model with which to study the basic physiology of germination. Results clearly implicate the mitochondria in axis tissue as a significant component of the injury mechanism and establish the potential of this model system in the study of mitochondrial development during imbibition. Further, the desiccation temperature treatments result in relatively minor modifications in the maturation process which, ultimately, should lead to a clearer understanding of the relationship between mitochondrial integrity and natural seed maturation including the transition to desiccation tolerance.

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# APPENDIX

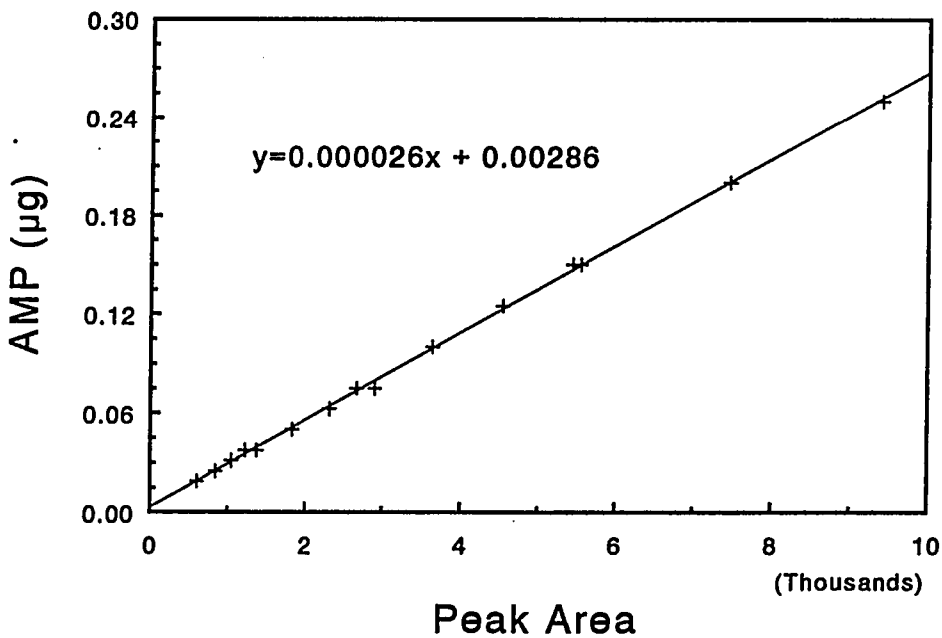


Figure A-1. Standard curve for AMP as determined by HPLC

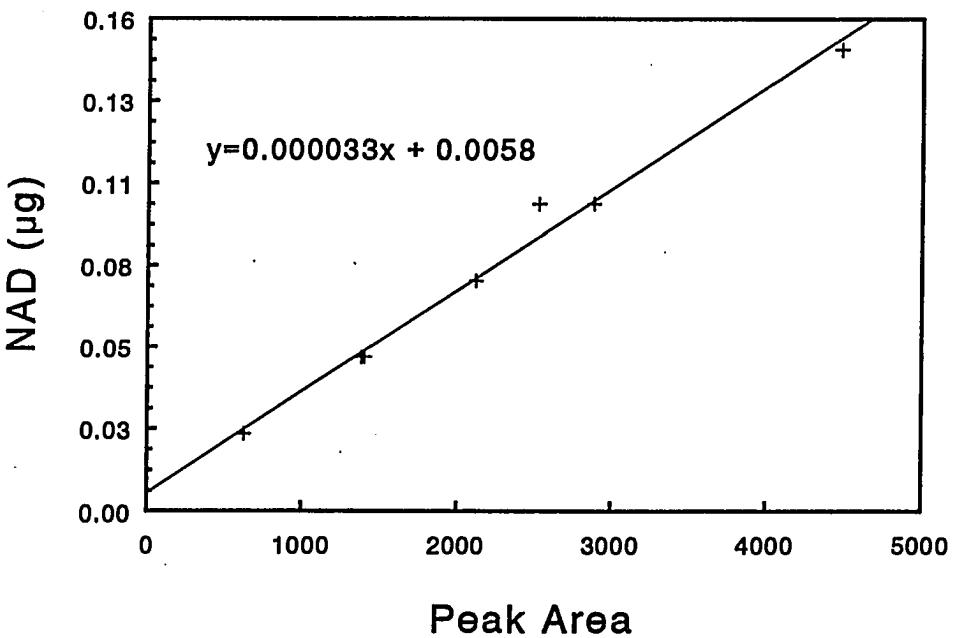


Figure A-2. Standard curve for NAD as determined by HPLC

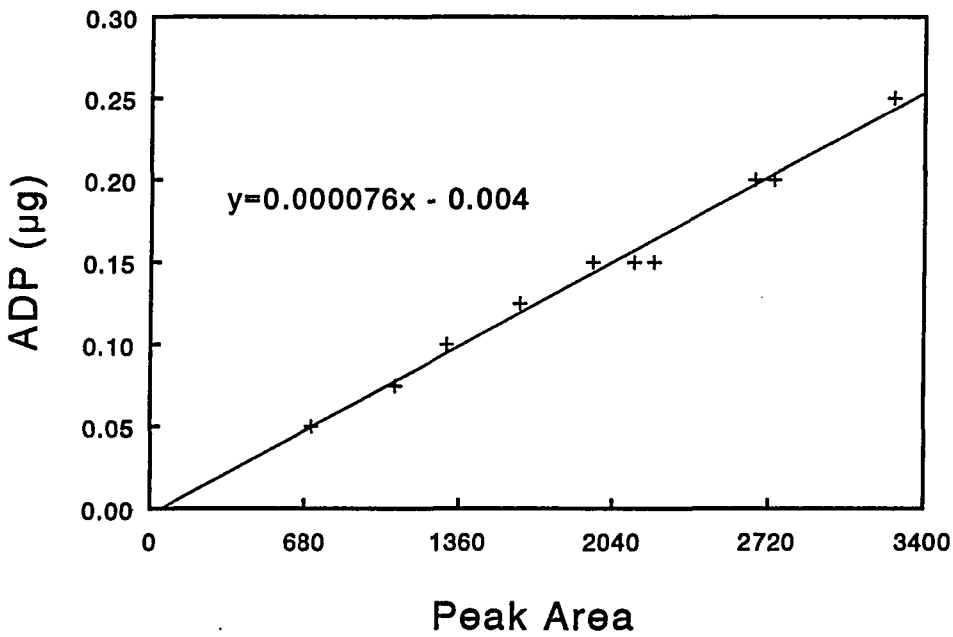


Figure A-3. Standard curve for ADP as determined by HPLC

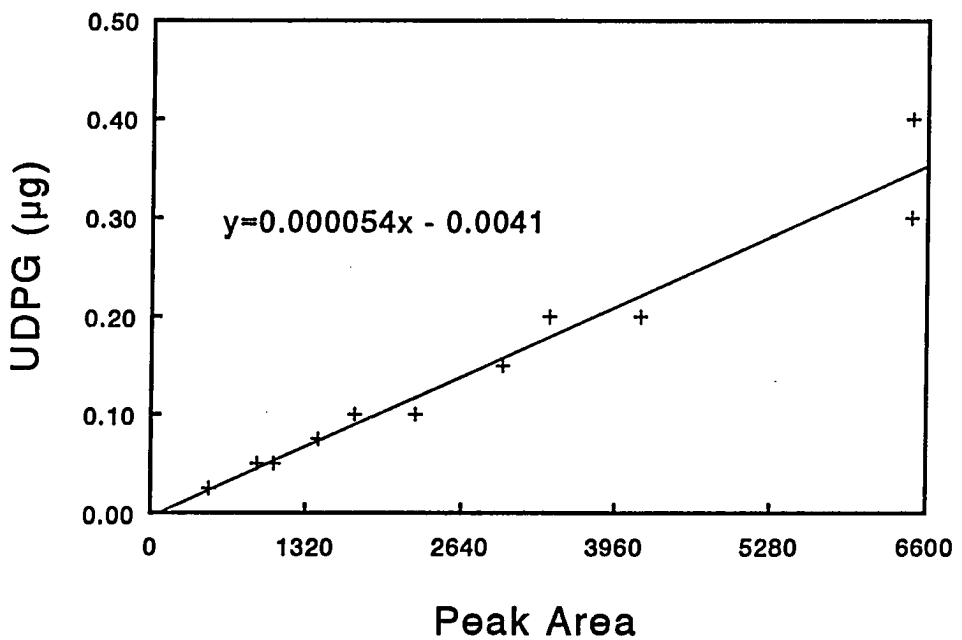


Figure A-4. Standard curve for UDPG as determined by HPLC

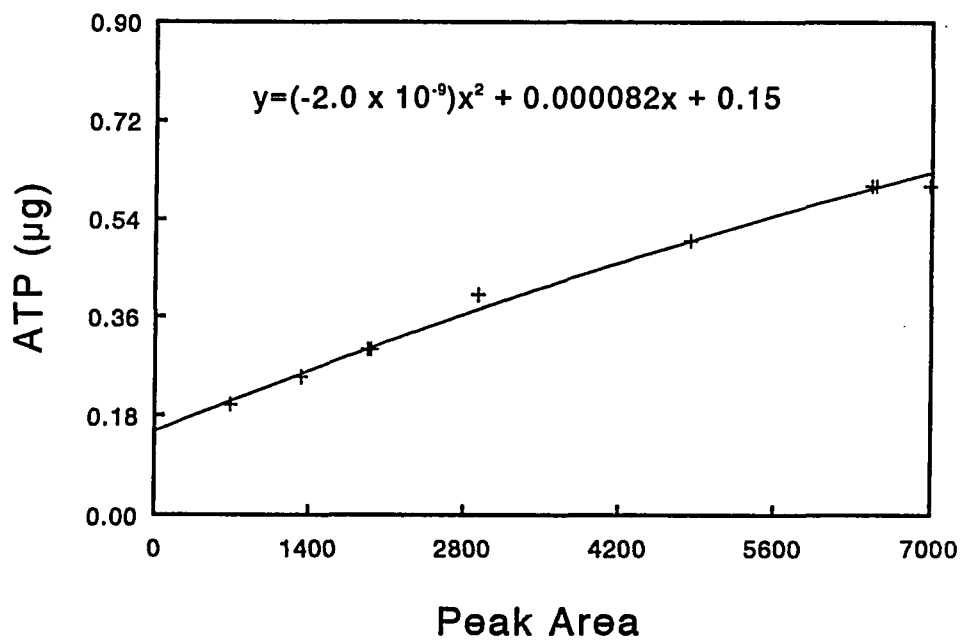


Figure A-5. Standard curve for ATP as determined by HPLC