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# Seedling Growth, Mitochondrial Characteristics, and Alternative Respiratory Capacity of Corn Genotypes Differing in Cold Tolerance

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## ABSTRACT

**Four maize (*Zea mays* L.) inbreds representing genetic differences in seedling cold tolerance were used to determine the effect of growth temperatures on dry weight accumulation and mitochondrial properties, especially the alternative oxidase capacity. Seedlings were grown in darkness at 30°C (constant), 14°C (constant), and 15°C for 16 hours and 8°C for 8 hours. Inbreds B73 and B49 were characterized as cold tolerant while G50 and G84 were cold sensitive. Shoot growth rate of cold-sensitive inbreds in the lower temperatures was slower relative to the tolerant inbreds. Mesocotyl tissue was particularly sensitive to low temperatures during growth after germination. There were no significant differences in relative rates of mitochondrial respiration in the cold-tolerant compared to cold-sensitive inbreds measured at 25°C. Mitochondria from all seedlings grown at all temperatures had the ability to phosphorylate as indicated by the observation of respiratory control. This result indicated that differences in low temperature growth were probably not related to mitochondrial function at low temperatures. Alternative oxidase capacity was higher in mitochondria from seedlings of all inbreds grown at 14°C compared to 30°C. Capacities in seedlings of 14°C-grown B73 and G50 were higher than in B49 and G84. Capacities in seedlings grown for 16 hours at 15°C and 8 hours at 8°C were similar to those from 14°C-grown except in G50 which was lower and similar to those grown at 30°C. Mesocotyl tissue was the most responsive tissue to low growth temperature. Coleoptile plus leaf tissue responded similarly but contained lower capacities. Antibody probing of western blots of mitochondrial proteins confirmed that differences in alternative oxidase capacities were due to differences in levels of the alternative oxidase protein. Male sterile lines of B73 were also grown under the three different temperature regimes. These lines grew equally as well as the normal B73 at all temperatures and the response of alternative oxidase capacity and protein to low growth temperature was similar to normal B73.**

Germination and early seedling growth are major determinants of stand establishment. Thus, these developmental events are particularly important for commercial production of maize (*Zea mays* L.). Recent trends in agronomic practices have been to plant maize earlier in the spring to take advantage of more optimal summer rainfall and temperatures and to avoid hot, dry periods during pollination and fertilization. A second recent trend in agronomic practice has been toward various forms of conservation tillage. These practices result in slower warming of soils in the early spring compared to more extensive tillage, particularly that done in the previous fall. Thus, physiological characteristics that improve germination and early seedling growth at low temperatures are increasingly of interest to maize producers (10).

The capacity and activity of the mitochondrial alternative oxidase in plants has been associated with growth at low seedling because of its role in thermogenesis and activity in thermogenic tissue (15). A few papers have attempted to relate the alternative respiratory process to low temperature growth in crop plants (7, 9, 11, 16, 18), calli (8, 20), and arctic plants (12, 13). Compared to capacity in tissues grown at optimal temperature, it appears that any given plant tissue will have a greater alternative respiratory capacity when grown at temperatures near the lower limit of growth. There is some indication that the capacity is higher in genotypes (species or varieties) that show more cold tolerance compared to more sensitive types (11, 16, 18). The role of this oxidase at any temperature is unclear and it is not known why there are higher capacities in plants grown at low temperatures. However, thermogenesis is not likely to be a function because of the small amount of heat generated by the process (12).

In an attempt to determine the extent of genetic control over the low temperature enhanced alternative oxidase capacity and to assess the correlation of the response with cold tolerance, we have studied respiratory characteristics of inbreds differing in cold tolerance. We measured growth rates and alternative oxidase capacities of two cold-tolerant and two cold-sensitive inbreds (10) and made measurements of related mitochondrial characteristics of one cold-tolerant and one cold-sensitive inbred. The practical lower limit for seedling growth is 12 to 14°C. Temperatures below 10°C result in some damage from which the seedling must recover before normal growth is resumed. Seedlings were grown at temperatures near optimal (30°C), near the lower growth limit (14°C), and in a temperature regime which included damaging temperatures but which gave an average temperature to allow growth (16 h at 15°C, 8 h at 8°C). Measurements of alternative oxidase capacity were also made on male steriles grown at these three temperatures because one of them has been reported to lack the oxidase (14).

## MATERIALS AND METHODS

Four maize (*Zea mays* L.) inbreds, two cold tolerant and two cold sensitive, were chosen for this study. Seeds were

obtained from the Foundation Seed Department of Pioneer Hi-Bred International, Inc. With the exception of B73, the lines used were Pioneer Hi-bred International proprietary lines. B73 and B49 are considered cold-tolerant whereas G50 and G84 are considered cold-sensitive. Field data on cold tolerance of these inbreds is available (10).

Seeds were germinated and seedlings grown in a commercial vermiculite-peat mixture (Terra-lite, W. R. Grace & Co.). Germination and growth temperature was either at 30 ± 0.5°C (constant), 14 ± 0.5°C (constant) or 16 h at 15 ± 0.5°C, 8 h at 8 ± 0.5°C. This alternating temperature regime gives a 13°C average temperature but includes a stressful temperature period (<10°C). Temperature treatments are indicated in the figures and tables. Unless otherwise indicated the measurements were made on seedlings grown until approximately 120 heat units accumulated (3.5 d at 30°C or 17 to 21 d at the lower temperatures) at which time the seedlings were at similar stages of development (17). Heat units = [(50°F < T°F < 86°F) − 50] (No. of days) (19).

Mitochondria were isolated by the method of Day and Hanson (4) with Tes used rather than phosphate in the extraction buffer. The mitochondrial pellet was suspended in the reaction mixture (pH 7.2) consisting of 250 mM sucrose, 10 mM Tes, 1 mM $MgCl_2$, 1 mM $KH_2PO_4$, 1 mg $mL^{-1}$ (w/v) BSA. Oxygen uptake was measured as previously described (8) in the presence of 10 mM succinate. KCN and SHAM[1] were each added to a final concentration of 1 mM. State 4 rates and effects of cyanide and SHAM were determined after one cycle of phosphorylation, *i.e.* one addition of ADP. Alternative oxidase activity was usually expressed as a percent of the state 4 rate to reduce variability among mitochondrial preparations. State 4 rates were more reproducible than state 3 rates and were unaffected by the level of alternative oxidase. Alternative oxidase capacities $V_{alt}$ were measured as the oxygen uptake that was sensitive to SHAM in the presence of cyanide. Protein was determined by the Lowry procedure after precipitation with TCA (1).

For electrophoresis, electrotransfer and antibody probing of mitochondrial proteins, we followed the relatively standard procedures referred to by Elthon and McIntosh (5, 6) except that we used a 12% (w/v) nongradient polyacrylamide resolving gel. Antibody binding was detected with the alkaline phosphatase method. The developed membranes were photographed with high contrast black and white film. The antibodies to the alternative oxidase were produced by Dr. Tom Elthon at the University of Maryland, Baltimore County.

## RESULTS

### Effects of Low Temperature on Seedling Growth

Cold tolerance of the inbreds studied was characterized by growth in a series of increasingly stressful temperatures (Table I). Growth rates of all inbreds were decreased at the colder and more stressful temperatures. The percentage decreases in growth rate when compared to the rate at 30°C were greater for G50 and G84 than they were for B73 and B49. A more detailed analysis of the growth pattern of B73 and G50 (Table II) indicated that the shoot growth of the sensitive inbred, G50 was more affected by the cold than was root growth. Compared to B73, the mesocotyls and coleoptile plus leaf tissue of G50 grew more slowly at the cold temperatures. Although not evident in the dry weight data, the mesocotyls of G50 were considerably shorter at the 15/8 temperature regime compared to those grown at 14°C. From visual appearance, the mesocotyls were the most sensitive to the cold. The mesocotyls of inbred G84 were sensitive to cold. They were as short at 14°C as G50 mesocotyls were at 15/8.

**Table I.** *Growth Rates of Four Corn Inbreds Grown at Different Temperature Regimes*

Seeds were germinated and seedlings were grown until approximately 100 heat units had accumulated. Numbers in parentheses represent growth rates as a percent of the rate at 30°C. Values represent mean dry weights of 20 seedlings.

| Growth Temperature | Dry Weight of Inbred | | | |
|---|---|---|---|---|
| | B73 | B49 | G50 | G84 |
| °C | *mg seedling$^{-1}$ d$^{-1}$* | | | |
| 30 (constant) | 7.4 | 5.8 | 6.3 | 5.9 |
| 14 (constant) | 2.2 (30) | 1.9 (33) | 1.6 (25) | 1.4 (24) |
| 16 h at 15/8 h at 8 | 1.9 (26) | 1.8 (31) | 1.3 (21) | 1.1 (19) |
| 16 h at 13/8 h at 4 | 1.3 (18) | 1.2 (21) | 0.9 (14) | 0.6 (10) |
| LSD = 0.05 | 0.62 | 0.18 | 0.46 | 0.26 |

### Effect of Low Growth Temperature on Mitochondrial Characteristics

Even though the mesocotyls were the most sensitive tissue to the low temperature growth, mitochondria isolated from mesocotyls of seedlings grown at the stressful temperatures were equally functional as those grown at 30°C. The rates of oxygen uptake and ability to phosphorylate (respiratory control) were similar in mitochondria isolated from all temperatures (Table III). The capacity of the alternative oxidase measured in these mitochondria was high in the tissue which grew relatively well in the cold, *i.e.* mesocotyls from B73 and G50 grown at 14°C and mesocotyls from B73 grown at 15/8. Mitochondria with high measured alternative oxidase exhibited lower respiratory control (Table III). In all preparations, the residual rate of oxygen uptake after the addition of SHAM and KCN was less than 5% of the control. This small residual rate was probably chemical rather than mitochondrial because of the chemical interactions of these compounds in the presence of reducing agents (our unpublished observations).

Compared to other tissues, the mesocotyl and the root of the corn seedling contained the highest $V_{alt}$. Because the mesocotyls contained the highest capacity, were most responsive to temperature, and were easier to grind and isolate from vermiculite-grown seedlings, most of the measurements of $V_{alt}$ were from mitochondria from mesocotyl tissue. Alternative capacity was measurable in mitochondria from mesocotyls of all inbreds grown at all temperatures (Fig. 1). For comparisons among inbreds and growth temperature regimes, the data are expressed as a percent of the state 4 rate. The percentage basis for comparison was chosen because it corrects for variation

[1] Abbreviations: SHAM, salicylhydroxamic acid; $V_{alt}$, alternative oxidase capacity; $v_{alt}$, alternative oxidase activity; 15/8, growth regime consisting of 16 h at 15°C and 8 h at 8°C; C+L, coleoptile plus leaf.

**Table II.** *Growth Rates of Seedling Parts of Two Corn Inbreds under Different Temperature Regimes*

Values represent mean dry weights of parts of 20 seedlings.

| Growth Temperature | Dry Weight of Inbred | | | | | | LSD = 0.05 |
|---|---|---|---|---|---|---|---|
| | B73 | | | G50 | | | |
| | Roots | Mesocotyls | C+L | Roots | Mesocotyls | C+L | |
| °C | *mg seedling$^{-1}$ d$^{-1}$* | | | | | | |
| 30 (constant) | 3.0 | 3.1 | 3.1 | 3.9 | 2.2 | 2.5 | 0.4 |
| 14 (constant) | 0.5 | 0.6 | 0.7 | 0.6 | 0.3 | 0.4 | 0.1 |
| 16 h at 15/8 h at 8 | 0.6 | 0.6 | 0.7 | 0.6 | 0.3 | 0.4 | 0.2 |

**Table III.** *Properties of Mitochondria Isolated from Mesocotyls of Seedlings Grown at Different Temperatures*

$V_{alt}$ is the rate that is sensitive to SHAM in the presence of cyanide. $V_{alt}$ is the rate that is sensitive to SHAM alone. Rates were measured at 25°C and represent the mean of the determinations from at least three preparations.

| Inbred | Growth Conditions | State 3 Rate | State 4 Rate | $V_{alt}$ | $V_{alt}$ | Respiratory Control | Engagement |
|---|---|---|---|---|---|---|---|
| | °C | *nmol min$^{-1}$ (mg protein)$^{-1}$* | | | | | $V_{alt}/V_{alt}$ |
| G50 | 30 | 389 | 170 | 33 | 18 | 2.3 | 0.5 |
| B73 | 30 | 456 | 195 | 30 | 30 | 2.3 | 1.0 |
| G50 | 14 | 346 | 186 | 80 | 51 | 1.9 | 0.6 |
| B73 | 14 | 188 | 130 | 58 | 38 | 1.4 | 0.7 |
| G50 | 15/8 | 394 | 172 | 35 | 32 | 2.3 | 0.9 |
| B73 | 15/8 | 284 | 198 | 88 | 75 | 1.4 | 0.8 |

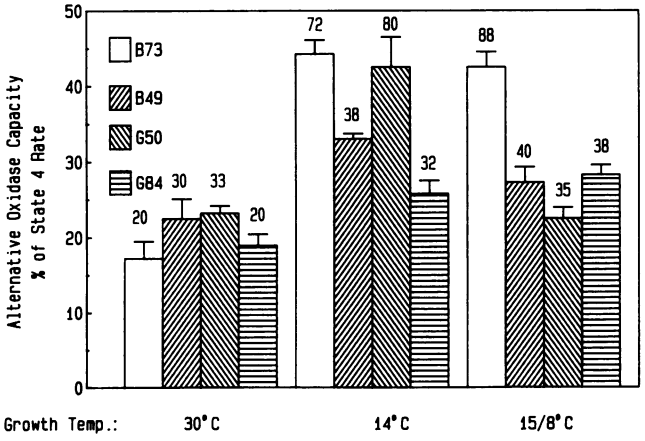


**Figure 1.** Alternative oxidase capacities in mitochondria from mesocotyls of seedlings of four corn inbreds grown under three different temperature regimes. $V_{alt}$ was determined as the oxygen uptake that was sensitive to SHAM in the presence of cyanide. The number above each bar represents the $V_{alt}$ (nmol min$^{-1}$ mg protein$^{-1}$) corresponding to the percentage value represented by the bar. The data represent averages of duplicate determinations on three replicate preparations.

among preparations due to isolation artifacts. The comparisons were made on the basis of the state 4 rate because it is the most reproducible among all the treatments. It appeared to be less affected than the state 3 rate by the levels of alternative oxidase. Typical absolute rates for each inbred and growth regime are indicated above the bars in the figures. Levels were similar among inbreds when they were grown at 30°C (Fig. 1). All inbreds responded to low temperature with higher $V_{alt}$ at 14°C than observed in seedlings grown at 30°C. Capacities in mitochondria from G50 mesocotyls grown at 15/8°C were considerably lower than at 14°C and similar to the 30°C level.

As described above, mesocotyls from seedlings grown in the cold were shorter and thicker than those grown at 30°C. Those which did not grow well were quite short and many mesocotyls contained damping-off lesions just above the attachment to the seed. No pathogenic organisms appeared to be associated with these lesions when they first appeared and the lesions disappeared when the seedlings were transferred to 30°C. Thus for G50 and G84, the lower $V_{alt}$ at low temperature compared to B73 was associated with poorer growth. B49 was different in that the $V_{alt}$ did not increase as much at the lower temperature even though the seedlings appeared to grow as well as the other inbreds. The $V_{alt}$ of mitochondria from B49 mesocotyls grown at 15/8°C was not lower than in mitochondria from mesocotyls grown at 14°C. Thus, the $V_{alt}$ responded to temperature in all inbreds. With the exception of B49, the levels at low temperature appear to reflect the ability to grow at the low temperature.

Inbreds B73 and G50 were selected for more detailed studies of mitochondria from various tissues and for growth studies. Mitochondria were isolated from coleoptile plus leaf tissue of seedlings grown at the different temperatures. Although the alternative oxidase capacities were lower than in the mesocotyls, the response of this tissue to temperature was similar to that of the mesocotyls (Fig. 2).

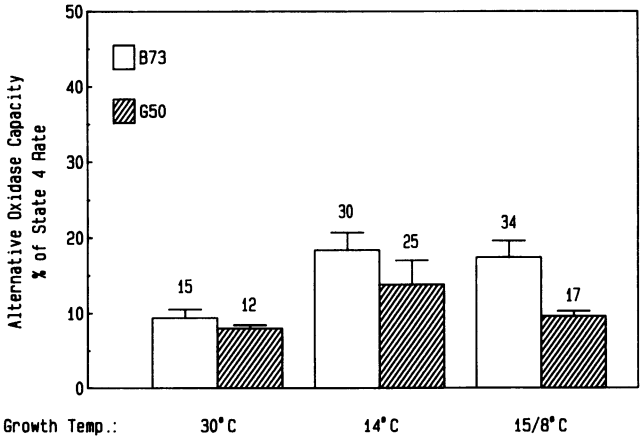


**Figure 2.** Alternative oxidase capacities in mitochondria from coleoptile plus leaf tissue of seedlings of two corn inbreds grown under three different temperature regimes. $V_{alt}$ was determined as the oxygen uptake that was sensitive to SHAM in the presence of cyanide. The number above each bar represents the $V_{alt}$ (nmol $min^{-1}$ mg $protein^{-1}$) corresponding to the percentage value represented by the bar. The data represent averages of duplicate determinations on three replicate preparations.

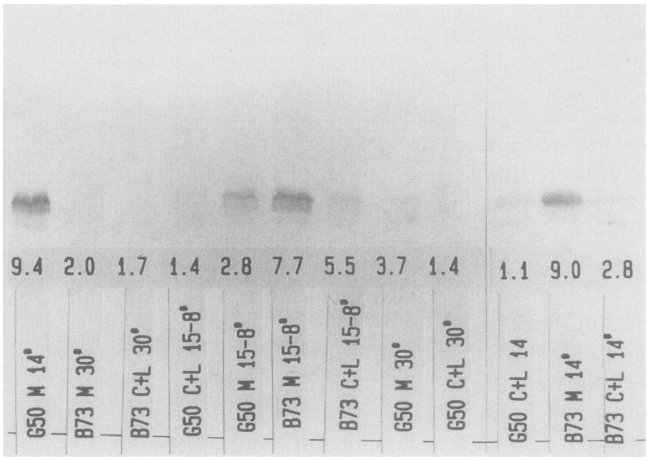


**Figure 3.** Western blots of mitochondrial proteins from various tissues probed with a monoclonal antibody to the alternative oxidase protein. Numbers beneath the bands represent $V_{alt}$ in the aliquot added to the gel (nmol/min). Amounts of protein added to the gel ranged from 80 to 120 $\mu$g. M refers to mitochondria from mesocotyls, C+L refers to mitochondria from coleoptile plus leaf tissue. The apparent mol wt of the antibody reactive protein was estimated to be 37,000.

Amounts of alternative oxidase protein were measured by immunoblots of the mitochondrial proteins, which had been separated on polyacrylamide gels, probed with a monoclonal antibody to the alternative oxidase. Mitochondrial preparations from those tissues reported in Figures 1 and 2 as having the highest measured $V_{alt}$, also had the highest amount of binding to the antibody to the alternative oxidase (Fig. 3). The antibody reactive bands correspond to those of similar mol wt from voodoo lily mitochondria, *i.e.* 35,000 to 37,000 (6, 7). Mitochondria from mesocotyls of B73 seedlings grown at 14 and 15/8°C and G50 seedlings grown at 14°C stand out as having the most alternative oxidase protein. In general, the intensity of the band correlated well with the capacity loaded on the gel and that reported for the various tissues in Figures 1 and 2. In those preparations containing low capacities, the antibody reaction was too faint to show in the photographs (Fig. 3). There are quantitative anomalies in band intensities that are not explained by the activities, e.g., B73 C+L 15/8. Protease inhibitors were not added to preparations and some were thawed more than once after storage at −80°C so there was the possibility for some protein degradation.

### Alternative Oxidase Capacity in Cytoplasmic Male Steriles

Because of a previous report (14) that one of the cytoplasmic male steriles of B73 lacked the alternative oxidase, we considered that these lines might be useful in assessing the role of $V_{alt}$ in seedling cold tolerance. Thus, we measured $V_{alt}$ mitochondria from seedlings of male steriles of B73 grown under the three temperature regimes. All male steriles contained low $V_{alt}$ values and they responded to temperature similarly to the normal B73 (Fig. 4). Immunoblots of these preparations are presented in Figure 5 and the results confirm the rate measurements of Figure 4. Detection of the antibody binding was too faint to show in the photographs of the blots of mitochondrial protein from seedlings grown at 30°C because of the low amount of activity in the preparation added to the gel. The proteins were readily detectable in the preparations from cold grown seedlings.

## DISCUSSION

Differences in cold tolerance among inbreds used in this research refer to relative differences in the rate of seedling dry weight accumulation at low temperatures. This kind of tolerance is rather specific and differs from tolerance to chilling temperatures during germination such as those temperatures

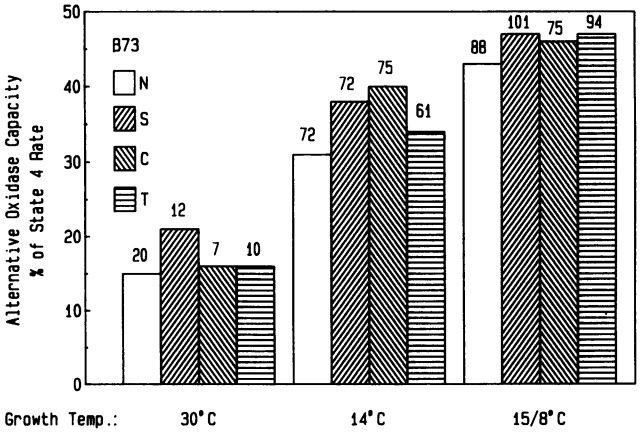


**Figure 4.** Alternative oxidase capacity in mitochondria from mesocotyls of normal and male sterile inbreds of B73 grown under three different temperature regimes. The number above each bar represents the $V_{alt}$ (nmol $min^{-1}$ mg $protein^{-1}$) corresponding to the percentage value represented by the bar. The data represent averages of duplicate determinations on a single preparation.

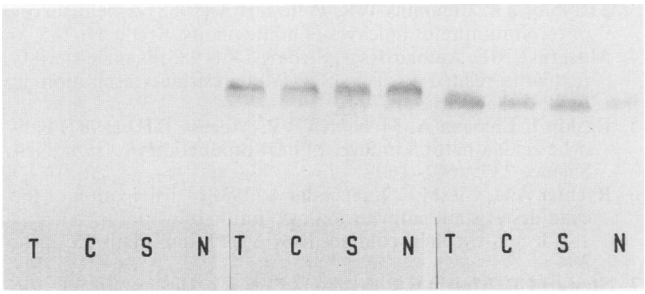


**Figure 5.** Western blots of mitochondrial proteins from mesocotyls of normal (N) and male sterile (T, C, and S) cytoplasms in inbreds of B73 grown under three different temperature regimes. One hundred micrograms of mitochondrial protein were added to each lane. The $V_{alt}$ contained in each lane was 0.1 times the values given above the bars in Figure 4. The apparent mol wt of the antibody reactive protein was estimated to be 37,000.

which cause imbibitional chilling injury (2). Since those seedlings which emerge more rapidly tend to have higher stands and better vigor (R Baker, Pioneer HiBred, personal communication), seedling growth rate at low temperature is an important determinant of stand establishment in cold, wet soils. These differences which exist among inbreds make them useful in testing hypotheses regarding mechanisms of cold tolerance.

Respiratory processes are extremely important in early seedling heterotrophic growth. Thus the mitochondria have been the focus of many studies on the effects of low temperatures upon seedling growth (11, 16, 18, 20). In this research, the mitochondria from seedlings of inbreds differing in cold tolerance were not significantly different in their respiratory activity nor their ability to phosphorylate. Even mitochondria from tissues in which growth was reduced and the tissue was somewhat damaged (mesocotyls of G50 grown at 15/8) were as functionally competent as mitochondria from the same inbred grown at warmer temperatures and as competent as mitochondria from the cold tolerant inbred, B73, at all temperatures. The only obvious difference was in the capacity of the alternative oxidase. We have unpublished data which indicate that intact tissue respiration rates of these inbreds have similar temperature responses.

Alternative oxidase capacities in corn seedling tissues were affected by growth temperature, genotype and tissue type. There were also interactions among these factors in that different tissues and genotypes responded differently to temperature. There are few studies in which the capacity of the alternative oxidase has been studied under as many conditions as is reported here. There are also developmental changes which we have reported in a separate paper (17).

Mitochondria from all tissues and genotypes of corn grown at all temperatures contained some level of $V_{alt}$. All tissues of the genotypes studied had higher levels of $V_{alt}$ when seedlings were grown at 14°C compared when they were grown at 30°C. There was not a consistent relationship between cold tolerance and the levels of $V_{alt}$ at any temperature studied. Furthermore there was no consistent relationship between cold tolerance and the effects of temperature on $V_{alt}$. All inbreds studied had similar levels of $V_{alt}$ at 30°C. The fact that $V_{alt}$ was higher in all inbreds when grown at the low temperature implies that this protein probably has an essential function at all temperatures and that higher levels are necessary when seedlings grow in the cold. However, the specific level of $V_{alt}$ does not appear to be a single critical determinant of cold tolerance. The function of the oxidase is not known in nonthermogenic tissues but a role in the utilization of excess substrate has been suggested (3). Another way of stating this function is that this oxidase allows respiration to continue when it would otherwise be restricted by a proton gradient across the inner mitochondrial membrane, *i.e.* regulation by the need for cellular energy. Continued respiration might be necessary to use up a toxic metabolite or to produce needed carbon skeletons for biosynthesis or for heat production in thermogenic tissues.

Differences in $V_{alt}$ have not previously been confirmed with the reaction of the protein to an antibody in tissues other than voodoo lily. Fortunately, the corn alternative oxidase reacts well with the antibody produced against the voodoo lily oxidase. This antibody was useful in this study in confirming that $V_{alt}$ measurements reflect levels of the protein. It is likely that the antibody will be useful for similar studies in a variety of species.

The presence of alternative oxidase in S, C, and T type cytoplasmic male sterile inbreds of B73 confirms the earlier report (14) that it was present in the S and T male steriles. The data showing the lack of the activity in cm-C was with intact tissue measurement on root tissue. The low level of activity observed in seedlings grown at warm temperatures would be difficult to detect using inhibitors on intact tissues. However the male steriles responded to growth temperature similarly to the normals, *i.e.* measured capacity and protein levels are higher in mitochondria from seedlings grown at low temperature than in those grown at warm temperature. Since the male steriles respond similarly as the normal, they are not useful in testing hypotheses regarding the role of the alternative oxidase in cold tolerance.

The results reported in this paper are consistent with the concept that the ability of corn seeds to germinate and grow in cold temperatures is determined by a number of genetic determinants. Inbreds differing in cold tolerance do not rank the same in various tests of germination and growth under stress conditions (12). If this concept is correct, then levels of a single enzyme such as the alternative oxidase would not be expected to correlate with cold tolerance.

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