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Respiration and Alternative Oxidase in Corn Seedling Tissues during Germination at Different Temperatures

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ABSTRACT

Respiration rates of *Zea mays* L. seedling tissues grown at 30 and 14°C were measured at 25°C at different stages of seedling growth. Accumulation of heat units was used to define the developmental stages to compare respiration between the two temperatures. At both temperatures, respiration rates of most tissues were highest at the youngest stages, then declined with age. Respiration rates of mesocotyl tissue were the most responsive to temperature, being nearly twofold higher when grown at 14 compared to 30°C. Alternative pathway respiration increased concomitantly with respiration and was higher in mesocotyls grown in the cold. When seedlings were started at 30 then transferred to 14°C, the increase in alternative pathway respiration due to cold was not observed unless the seedlings were transferred before 2 days of growth. Seedlings transferred to 14°C after growth at 30°C for 2 days had the same alternative oxidase capacity as seedlings grown at 30°C. Seedlings grown at 14°C for 10 to 12 days, then transferred to 30°C, lost alternative pathway respiratory capacity over a period of 2 to 3 days. Western blots of mitochondrial proteins indicated that this loss of capacity was due to a loss of the alternative oxidase protein. Some *in vitro* characteristics of mitochondria were determined. The temperature optimum for measurement of alternative oxidase capacity was 15 to 20°C. At 41°C, very little alternative oxidase was measured, *i.e.*, the mitochondrial oxygen uptake was almost completely sensitive to cyanide. This inactivation at 41°C was reversible. After incubation at 41°C, the alternative oxidase capacity measured at 25°C was the similar to when it was measured at that temperature directly. Isolated mitochondria lost alternative oxidase capacity at the same rate when incubated at 41°C as they did when incubated at 25°C. Increasing the supply of electrons to isolated mitochondria increased the degree of engagement of the alternative pathway, whereas lower temperature decreased the degree of engagement. Lower temperatures did not increase the degree of engagement of the pathway in intact tissues. We interpret these observations to indicate that the greater capacity of alternative oxidase in cold-grown seedlings is a consequence of development at these low temperatures which results in elevated respiration rates. Low temperature itself does not cause greater capacity or engagement of the alternative oxidase in mitochondria that have developed under warm temperatures. Our hypothesis would be that the low growth temperatures require the seedlings to have a higher respiration rate for some reason, *e.g.*, to prevent the accumulation of a toxic metabolite, and that the alternative pathway functions in that respiration.

Much of the pioneering experimental work on alternative pathway respiration in plants has been done with model tissues such as thermogenic aroid spadices, ripening fruits, and aged potato slices (10). Spadix tissue of voodoo lilly (*Sauromatum guttatum*) has been an extremely useful source of plant material for the solubilization and identification of the proteins of the alternative oxidase and the production of monoclonal antibodies to them (5, 6). The role of this activity as a thermogenic process during reproductive development has been established as well as its induction by salicylic acid (16). During the current decade, attention has been directed to understanding the occurrence and function of the alternative oxidase in a wide variety of plant species and tissues, including crop species (15).

Evidence is beginning to accumulate which indicates that alternative oxidase capacities in plant tissues are determined by a number of factors such as tissue specificity (5), developmental stage (18), genotype (11), and environmental conditions, particularly temperature (8, 9, 20). Several reports have appeared which indicate that V_{alt}[1] is higher in plants grown at low temperatures (10–15°C) compared to plants grown at near optimal temperatures (25–30°C) (7, 12, 17, 19, 21). Winter wheat varieties have a greater alternative respiratory capacity than do spring varieties (11). Cold resistant corn inbreds had a greater alternative respiratory capacity than sensitive ones (19). Thus it appears that there is some genetic control over the temperature response. Increased respiration and alternative pathway have been observed during cold acclimation of winter rape leaves (17). Leaves of arctic plant species had higher rates of respiration and alternative pathway respiration than did leaves of temperate species (13).

Previous studies comparing corn seedlings grown at different temperatures have not documented that the measurements were made on seedlings at comparable developmental stages. Changes in alternative oxidase capacities must occur during seedling development but interactions between growth temperature and development have not been examined. The results of a previous report indicated that estimates of the alternative pathway in intact tissues and in mitochondria both indicate higher levels in cold-grown shoots (7). Corn shoot tissues appear to satisfy the conditions set down by Moller et al. (15) for using intact tissue measurements for estimating the alternative pathway. Thus, both intact corn tissues and mitochondria isolated from them can be used to measure alternative oxidase capacities.

In this paper, we report changes in respiration rates and alternative oxidase capacities of corn seedling tissues during

[1] Abbreviations: V_{alt}, alternative oxidase capacity; v_{alt}, alternative oxidase activity; SHAM, salicylhydroxamic acid.

development at two different temperatures. We have used both intact tissue and isolated mitochondria measurements for this purpose. We also report some *in vitro* effects of temperature on the alternative oxidase in corn mitochondria.

MATERIALS AND METHODS

Inbred corn (*Zea mays* L. B73) seeds were germinated, and seedlings were grown in a vermiculite-peat mixture (Terralite, W. R. Grace & Co.). Growth temperature was either 30± 0.5°C or 14±0.5°C. Intact tissue respiration rates were determined with a YSI Model 5300 Biological Oxygen Moniter as previously described (7). Inhibitor concentrations for intact tissues were 2 mM KCN and 25 mM SHAM.

Mitochondria were isolated by the method of Day and Hanson (3) in which Tes rather than phosphate was used in the extraction buffer. The mitochondrial pellet was suspended in the reaction mixture consisting of 250 mM sucrose, 10 mM Tes, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 1 mg ml^{-1} (w/v) BSA. Unless otherwise specified, the mitochondrial substrate was 10 mM succinate. Succinate was chosen as the substrate for estimating the alternative oxidase because it usually supports the most rapid rates (7). The mitochondria were given substrate followed by ADP for one cycle of phosphorylation to insure adequate uptake of substrate before adding inhibitors. KCN and SHAM were each added to a final concentration of 1 mM in isolated mitochondria reaction mixtures. 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. V_{alt} represents oxygen uptake that is sensitive to SHAM in the presence of cyanide. Alternative oxidase activity (v_{alt}) was determined as oxygen uptake that is sensitive to SHAM alone.

Protein was determined by the Lowry procedure after precipitation with TCA (1). For electrophoresis, blotting 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. The antibodies to the alternative oxidase protein were produced by Dr. Tom Elthon at the University of Maryland, Baltimore County.

Measurements of oxygen uptake were possible throughout the entire range of temperatures from 8 to 41°C because full-scale outputs were obtained from the electrode from aqueous solutions saturated with air at each temperature. The electrode was calibrated with water saturated with air at the measurement temperature. Oxygen concentrations were calculated from the absorption coefficient for oxygen at each temperature.

RESULTS

Respiratory Changes during Growth

Intact tissue respiration rates (measured at 25°C) and growth data of different tissues of corn seedlings grown at 30°C (Table I) and 14°C (Table II) are presented for seedlings of different ages. The columns in Tables I and II are data from tissues of seedlings of ages at which the shoot length and dry weights were similar when grown at the two different temperatures. For the ages shown in the columns, approximately the same number (±20) of growing degree days (heat units) (19) had accumulated at the two different temperatures.

In general, the respiration rates of the growing axis tissues were highest in the youngest tissues. On a per g tissue basis, the rates declined as the axis grew. Respiration rates of the functional but nongrowing scutellum tissue were considerably lower than those of the axis tissue and were not affected by age or growth temperature. Respiration rates (measured at 25°C) of the shoot tissues, especially the mesocotyl, were considerably higher in seedlings grown at 14 compared to those grown at 30°C.

Estimated alternative pathway rates were higher in axis tissues of seedlings grown at 14 compared to 30°C. These higher rates were observed at all stages of growth. The most pronounced effect of growth temperature on the alternative pathway was in mesocotyls, the tissue in which the respiration rate was most responsive to growth temperature. Growth temperature had no effect on the alternative pathway rates in scutella.

Much of the decline in respiration rates (expressed per mg dry weight) with growth shown in Tables I and II was directly related to increases in dry weight. Such data do not present a picture of the development of the respiratory enzymes and organelles. Respiration and alternative pathway rates of mesocotyls as a function of seedling growth are presented in Figure 1, A and B, respectively. In this figure, the rates are expressed per seedling and the developmental course on a heat unit basis. The heat unit basis allows direct comparisons between growth temperatures. Expressing the rates on a seedling basis provide an indication of the developmental rate changes in the tissue that are not complicated by weight changes.

The results in Figure 1 clearly indicate that changes in the alternative pathway paralleled changes in respiration rates during development at both temperatures. Further, the effects of growth temperature were similar for both the overall respiration rate and the alternative pathway. The greater alternative pathway in mesocotyls grown at 14 compared to 30°C was accompanied by greater respiration rates.

Effects of Changing Growth Temperature on Alternative Oxidase Capacity

Since the differences in alternative pathway in corn seedlings grown at different temperatures were affected by different patterns of development, it was of interest to determine changes in the pathway after seedlings were started at warm then transferred to cold temperature. Isolated mitochondria were used for these experiments. The data in Table III indicate that by the time the seedlings were two days old they were no longer capable of responding to the low temperature with increased capacities of the alternative oxidase.

Seedlings grown at low temperature for 12 d had high capacities of the alternative oxidase (Table III). When these seedlings were transferred to 30°C, capacity of the alternative oxidase declined over a 2-d period to the level observed in warm-grown tissue. This decline in activity could have been due to inactivation of the protein in the mitochondria or to a

Table I. *Respiration Rates Measured at 25 °C and Growth Parameters of Corn Seedling Tissues Grown at 30 °C in Darkness*

Respiration rates are the mean of four samples consisting of tissues from five seedlings. Values in parentheses represent the rate that is sensitive to 25 mM SHAM in the presence of 2 mM cyanide (V_{alt}). Growing degree days (heat units) = [(50°F<T°F<86°F)−50](No. of days) (19).

Parameter	Value				
Age	1.25 d (30 h)	1.7 d (40 h)	2.3 d (55 h)	2.7 d (65 h)	3.7 d (89 h)
Shoot length (cm)	1.0 ± 0.2[a]	0.5 ± 0.1[b]	3.0 ± 0.5	5.5 ± 1.5	8.5 ± 1.5
Shoot dry wt (mg)	3.7 ± 0.3[a]	2.0 ± 0.3	3.6 ± 0.2	10.6 ± 0.4	17.4 ± 0.8
Growing degree days (heat units)	45	60	82	97	133
Tissue	**Respiration Rates**				
			μmol min⁻¹ g dry wt⁻¹		
Mesocotyl			3.6 ± 0.2 (1.2)	2.8 ± 0.2 (0.9)	1.2 ± 0.1 (0.6)
Coleoptile + leaf		2.0 ± 0.5 (0.9)[b]	2.0 ± 0.2 (0.7)	2.1 ± 0.2 (0.4)	1.0 ± 0.1 (0.3)
Root	3.1 ± 0.2 (1.1)[a]	4.2 ± 0.5 (1.1)	3.6 ± 0.2 (1.2)	2.9 ± 0.2 (1.5)	2.6 ± 0.2 (1.1)
Scutellum	0.53 ± 0.08 (0.2)	0.65 ± 0.09 (0.2)	0.70 ± 0.04 (0.2)	0.90 ± 0.08 (0.2)	0.82 ± 0.09 (0.2)

[a] Entire root-shoot axis. [b] Entire shoot.

Table II. *Respiration Rates Measured at 25 °C and Growth Parameters of Corn Seedling Tissues Grown at 14 °C in Darkness*

Respiration rates are the mean of four samples consisting of tissue from five seedlings. Values in parenthesis represent the rate that is sensitive to 25 mM SHAM in the presence of 2 mM cyanide (V_{alt}). Growing degree days (heat units) = [(50°F<T°F<86°F)−50](No. of days) (19).

Parameter	Value				
Age	3 d (72 h)	6 d (144 h)	9 d (216 h)	13 d (312 h)	17 d (408 h)
Shoot length (cm)	1.0 ± 0.2[a]	1.0 ± 0.2[b]	2.5 ± 0.5	5.0 ± 1.0	7.5 ± 1.5
Shoot dry wt (mg)	4.4 ± 0.4	3.4 ± 0.5	5.4 ± 0.4	11.4 ± 0.4	17.2 ± 0.7
Growing degree days (heat units)	21	42	63	91	120
Tissue	**Respiration Rates**				
			μmol min⁻¹ g dry wt⁻¹		
Mesocotyl			5.8 ± 0.3 (2.8)	3.7 ± 0.2 (2.3)	1.6 ± 0.1 (0.9)
Coleoptile + leaf		2.5 ± 0.2 (1.1)[b]	3.4 ± 0.3 (1.7)	2.7 ± 0.3 (1.4)	1.3 ± 0.2 (0.7)
Root	3.1 ± 0.3 (1.5)[a]	3.6 ± 0.3 (2.0)	3.1 ± 0.1 (1.7)	3.7 ± 0.2 (2.2)	1.9 ± 0.3 (0.7)
Scutellum	0.67 ± 0.09 (0.2)	0.7 ± 0.1 (0.2)	0.8 ± 0.1 (0.2)	0.8 ± 0.1 (0.2)	0.7 ± 0.1 (0.2)

[a] Entire root-shoot axis. [b] Entire shoot.

loss of the protein. Figure 2 shows clearly that the proportion of alternative oxidase in mitochondrial protein declines during the period of decline in capacity. The visual appearance of the bands in Figure 2 indicates that the loss in protein was greater than the loss in measured capacity (Table III). Faint bands are recorded even more faintly on photographic prints of the westerns even when high contrast film is used. In unpublished results, we have observed a good correlation between densitometer measured color intensities of bands on Western blots from corn mitochondria and measured capacities.

Effects of Measurement Temperature on Alternative Oxidase in Isolated Mitochondria

Whereas it is clear that growth temperature affects the level of alternative oxidase, measurements of the effects of measurement temperature on the alternative oxidase have not been reported. The oxygen electrode is sufficiently responsive to allow measurements of oxygen uptake throughout the entire range of temperatures that permit corn seedling growth. Here we report effects of temperature on mitochondrial activities including the alternative oxidase capacity and activity.

Figure 3 shows the state 3 and state 4 rates and V_{alt} in mitochondria measured at temperature intervals between 8 and 41°C. These upper and lower limits were chosen because they represent temperatures at which detrimental effects of temperature on germination and growth have been observed. State 3 and 4 rates increased throughout the temperature range as expected. V_{alt}, on the other hand, increased only slightly between 8 and 15°C, then declined throughout the remainder of the temperature range up to 41°C. The optimal temperature for V_{alt} was 15 to 20°C. When the increase in state 4 rate and decrease in V_{alt} were combined, a dramatic decline in V_{alt} as a percent of the state 4 rate was observed. Mitochondrial oxygen uptake at 41°C, was almost completely inhibited by cyanide.

The low V_{alt} measured at 41°C was reversible. To determine whether or not there was an irreversible inactivation of V_{alt} at 41°C, mitochondria were incubated for a period of time at 41°C then measured at 25°C. Similar activity was measured after incubation for as long as 20 min at 41°C as was measured when the mitochondria were incubated at 25°C (Table IV). About one-third of the activity was lost by 33 min at both temperatures. There was very little decline in the state 4 rate

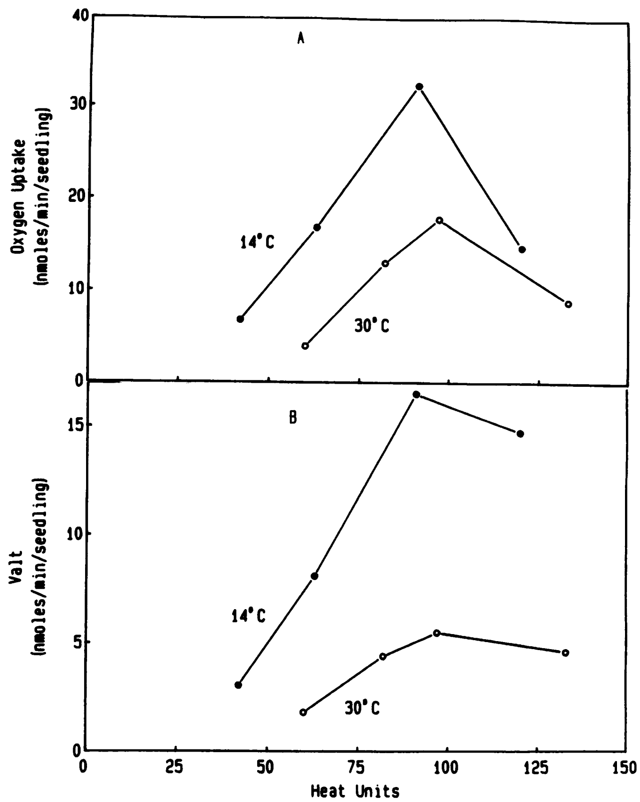

Figure 1. Changes in total (A) and alternative pathway (B) respiration during seed germination and growth at 30 and 14°C. Final concentrations of inhibitors for measurements of V_{alt} were: KCN 2 mM and SHAM 25 mM. SHAM was added in 2-methoxyethanol.

Table III. *Alternative Oxidase in Mitochondria from Mesocotyls of Corn Seedlings Grown under Different Temperature Regimes*

Respiratory control ratios varied from 1.5 in mitochondria with high alternative oxidase to 2.3 in mitochondria with low alternative oxidase.

Growth Conditions		Alternative Oxidase Capacity	
		nmol min⁻¹ mg protein⁻¹	*% of state 4 rate*
1[a]	10[b]	89	60
2	10	23	20
3	10	16	15
4	0	21	22
17[c]	0[d]	85	55
12	0	89	47
12	1	64	36
12	2	30	26
12	3	38	27

[a] Number of days at 30°C before transfer to 14°C. [b] Number of days at 14°C after transfer from 30°C. [c] Number of days at 14°C before transfer to 30°C. [d] Number of days at 30°C after transfer from 14°C.

during incubation at either temperature for the 33 min period. Thus there was a reversible inactivation of V_{alt} at 41°C and a slower irreversible loss of activity at both 25 and 41°C.

We have unpublished results which indicate that the inac-

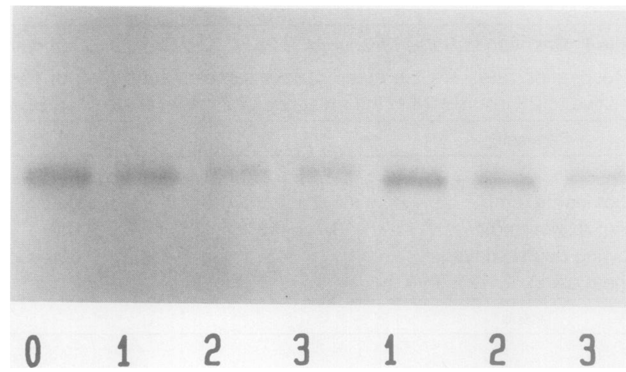

Figure 2. Western blots of mitochondrial proteins from mesocotyls of corn seedlings probed with a monoclonal antibody to the alternative oxidase protein. Numbers in the lanes represent the number of days the seedlings had been growing at 30°C after growing at 14°C for 12 d. Two series of preps are included. One hundred micrograms of protein were added to each lane. The apparent mol wt of the antibody reactive protein was estimated to be 37,000. Mean V_{alt} values for these preparations are presented in Table III. Color on the membranes was developed with the alkaline phosphatase method. The developed membranes were photographed with high contrast black and white film.

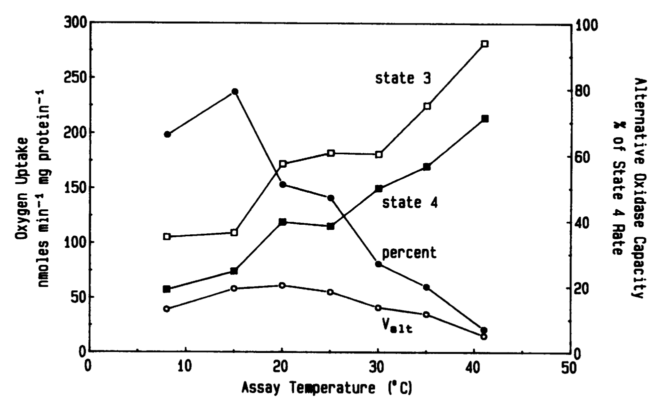

Figure 3. Effect of temperature on mitochondrial processes in mitochondria isolated for mesocotyls of corn seedlings grown for 17 d at 14°C.

Table IV. *Alternative Oxidase Activity in Mitochondria Isolated from Mesocotyls Grown at 17°C Measured at 25°C after Incubation in Reaction Mixture at 25°C and 41°C for Varying Times*

Activity measured at 41°C was <5% of the state 4 rate. Activity after 1 h on ice was the same as 0 time.

Incubation Time	Alternative Oxidase Activity at Incubation Temperature:	
	25°C	41°C
min	*% of state 4 rate*[a]	
0	45	45
14	43	38
22	43	42
33	32	31

[a] Average state 4 rate = 128 nmol min⁻¹ mg protein⁻¹.

tivation of V_{alt} observed in isolated mitochondria was not observed in intact tissue respiration measurements at 41°C. The portion of the respiration rate that was inhibited by SHAM in the presence of cyanide (V_{alt}) at 41°C was similar to that at 25°C. Those measurements required incubation of tissue at the measurement temperature for about 1 h. Thus, had there been an inactivation of V_{alt} in intact tissue during this period, no measurable SHAM-sensitive oxygen uptake in the presence of cyanide would have been observed.

Effect of Temperature on Engagement of Alternative Oxidase in Isolated Mitochondria

The degree of engagement that was commonly observed in mitochondria from cold grown tissue ranged from 0.5 to 1.0 with an average of 0.75 (Table V). Lowering the temperature from 25 to 8°C decreased the degree of engagement of the alternative pathway in isolated mitochondria. However, increasing the electrons supplied to the electron transport system at 25°C by including additional substrates, increased the engagement to 1.0 which indicates that the alternative oxidase was operating at full capacity in the presence of excess substrate. When excess substrate was supplied at 8°C, the degree of engagement increased but was less than the 1.0 observed at 25°C (Table V). However, adding excess substrate to mitochondria at 8°C did not increase the oxygen uptake rate as it did at 25°C.

DISCUSSION

Alternative Oxidase and Respiratory Changes

The model plant systems that have been used to study the alternative oxidase are the *Arum* lilies (5, 6, 16), aged storage tissues (10), and ripening fruits (10). Recently, there has been interest in alternative pathway respiration in leaf tissue (18). In all these systems, the relatively large capacity of alternative pathway respiration appears to accompany or follow a developmental period of high respiration rates. Respiration rates increase dramatically in the *Arum* spadix causing heating (16). During aging of storage tissues, increases in respiration rates are probably part of the overall process of growth resumption after being more or less quiescent (10). During ripening, the respiratory climacteric is part of the sequence of events that define ripening in some fruits (2). Leaf development is characterized by a rapid rate of respiration during expansion prior to the acquisition of photosynthetic competence. Once the leaf becomes an exporting organ, respiration is necessary only for tissue maintenance. In all these systems, the period of rapid respiration is followed by a period during which the respiration rate declines and the capacity of the alternative pathway remains high, particularly when expressed as a proportion of the overall rate.

The changes in mesocotyl respiration rate during early seedling growth and accompanying changes in alternative pathway respiration reported in this paper are interpreted to be somewhat analogous to the changes observed in the model systems mentioned above. Seedlings grown in the cold achieved a higher respiration rate and a higher V_{alt}. We interpret these results to mean that the capacity of a tissue for alternative pathway respiration is determined by the previous developmental history of the tissue. This interpretation is consistent with data demonstrating that seedlings transferred from warm to cold temperatures after the respiratory changes were well underway failed to achieve high capacities of the alternative pathway. It appears that the tissue must be exposed to the cold at the early stages of development in order for higher V_{alt} values to be attained. Thus the capacity of the alternative oxidase may be determined during mitochondrial development and very little if any increase is observed after the mitochondria have been formed.

The loss of alternative oxidase activity and protein when seedlings were transferred from cold to warm temperatures indicates that there was turnover of the protein. The fact that losses in activity and protein occurred without loss of overall mitochondrial function further indicates some specificity in changes in mitochondrial function. The fact that it took 2 to 3 d for the levels to approach that of warm-grown seedlings indicates that this loss proceeded slowly. Low temperatures apparently prevent this loss.

In Vitro Inactivation of Alternative Oxidase

Two different kinds of inactivation of V_{alt} were observed in isolated mitochondria. The reversible inactivation that occurred when the mitochondria were in the reaction mixture at 41°C may be due to a reversible dissociation of subunits or dissociation of the protein from other membrane components. The irreversible inactivation that occurred over a period of 30 minutes or so was probably due to denaturation. This inactivation occurred at 25 as well as 41°C and V_{alt} declined faster than the state 4 rate which is primarily cyto-

Table V. *Effect of Measurement Temperature and Substrate on Engagement of Alternative Pathway in Mesocotyl Mitochondria from Seedlings Grown at 14°C*

V_{alt} = oxygen uptake that is sensitive to SHAM in presence of cyanide; V_{alt} = oxygen uptake that is sensitive to SHAM alone. Concentration of succinate was 10 mM, malate 10 mM, NADH 1 mM.

Measurement Temperature	Substrate	State 3 Rate	State 4 Rate	V_{alt}/V_{alt}
°C		*nmol min^{-1} mg protein^{-1}*		
25	Succinate	169	134	0.75
25	Succinate, malate, NADH	234	198	1.0
8	Succinate	55	81	0.35
8	Succinate, malate, NADH	57	83	0.5

chrome mediated. The significance of this inactivation is unclear because our unpublished measurements of V_{alt} of intact tissues indicate that it represents a similar proportion of the total respiration at 41 and 25°C. However, it is noteworthy that 41°C is near the upper limit of temperature reached by *Arum* lily spadices during thermogenesis when the alternative pathway is the functional (14). The *in vitro* inactivation is different from that which occurs over a period of days in intact tissue.

Effect of Low Temperature on Engagement of Alternative Oxidase

There are some data indicating that engagement of the alternative pathway in isolated mitochondria is related to the relative supply of electrons and the capacity of the cytochrome oxidase (4). Low temperature did not cause an increase in v_{alt}/V_{alt} whereas increasing the supply of electrons from dehydrogenases did. Low temperature did decrease v_{alt}/V_{alt} even when excess substrate was present. The fact that the addition of extra substrate did not increase electron transport at 8°C like it did at 25°C indicates that low temperature decreased the capacity of the electron transport system more than it did the dehydrogenases.

CONCLUSIONS

The fact that transferring seedlings that have developed under warm temperatures to cold temperatures does not increase the capacity of the alternative oxidase suggests that it is not temperature itself that is the factor which causes the elevated capacities of the alternative oxidase. The fact that low temperature does not increase the participation of the alternative oxidase in isolated mitochondria, further supports this conclusion. It appears to us that the capacity of the alternative oxidase reflects the respiratory capacity of the tissue during its development. Presumably, as the tissue develops in the cold a greater respiratory capacity is needed. It is not likely that heat production is the reason for the higher respiration rate or the greater alternative oxidase capacity because of the small amount of heat produced (12, 13). It is more likely that the increase rates are required to prevent the accumulation of metabolites that would otherwise accumulate to toxic levels.

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