Effects of Chilling on Respiration and Induction of Cyanide-resistant Respiration in Seedling Roots of Cucumber

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ABSTRACT. The effects of chilling stress on respiration and induction of the alternative pathway (AP) were investigated with roots of 3-day-old cucumber seedlings (*Cucumis sativus* L.) grown at 26 °C and then chilled at 2, 10, or 15 °C for 24, 48, 96, 144, or 192 hours. Oxygen uptake by roots exposed to 2 °C was significantly lower than by 10 or 15 °C-treated roots, and the inhibition of oxygen uptake in the presence of SHAM (salicylhydroxamic acid) increased from 29% in unchilled tissue to 60% after 96 hours of treatment. At 10 and 15 °C, the capacity of the AP was nearly double that of the unchilled control and the 2 °C-treated seedlings. A 24 hours, postchilling treatment at 26 °C resulted in greater oxygen uptake at all temperatures as treatment time increased up to 96 hours. At 2 °C, the capacity of the AP was significantly reduced below the level of the 10 and 15 °C-treated tissue and the untreated control. The activity of the AP became fully activated by 96 hours in roots chilled at 2 °C. Results suggest that the capacity of the AP can be affected by low-temperature treatments, and exposure to 2 °C for up to 96 hours leads to a significant loss in capacity of the AP.

Chilling stress results in significant injury to a variety of tropical and subtropical horticultural crops, particularly those grown in temperate environments. Research has focused on fruit and vegetables stored at chilling temperatures with less emphasis on the growing crop, especially during seed germination, radicle development, and early seedling establishment (Herner, 1990). Chilling stress can induce many physiological and biochemical changes at the cellular level (Lyons, 1973; Markhart, 1986; Raison and Orr, 1990) and can result in changes in the respiratory pattern of plants at chilling temperatures (Lyons and Briedenbach, 1990).

Plants contain a cyanide-insensitive respiratory pathway (Bendall and Bonner, 1971; Day et al., 1980; Laties, 1982) located in the inner membrane of the mitochondria. Electrons can be diverted from ubiquinone to this alternative pathway (AP), which is not engaged in ATP synthesis beyond this point of divergence (Lambers, 1990; Wilson, 1988). The physiological implications of the AP are not clear, and the level of activation in plants is variable, depending on the tissue and stage of development (Day et al., 1980). When activated, the AP can account for 30% to 100% of the total respiratory activity depending on the particular tissue, its age, and stress condition (Janes and Wiest, 1982; Lambers, 1982; McCaig and Hill, 1977).

Low temperature is one of several factors that can induce synthesis of alternative respiratory pathway enzymes (Elthon et al., 1986; Leopold and Musgrave, 1979; Kiener and Bramlage, 1981; Vanlerberghe and McIntosh, 1992). This pathway could function as an energy overflow system to complement the normal cyanide-sensitive pathway when cannot handle the flow of respiratory electrons (Lambers, 1982). The respiratory capacity observed when the AP becomes active could be a mechanism to prevent accumulation of toxic metabolites under stress (Day et al., 1980; Graham and Patterson, 1982; Laties, 1982; Rychter et al., 1988).

Titration of AP activity has been conducted with SHAM (salicylhydroxamic acid) in the presence of KCN and expressed as p_{alt} plots as described by Møller et al. (1988). Even though side effects have been reported when using intact tissue and inhibitors of respiration, titration with SHAM remains useful in assessing changes in the activity of the AP (Møller et al, 1988; Bingham and Stevenson, 1995; Atkin et al., 1995).

Chilling stress can occur early in the growing season in many areas in which chilling-sensitive horticultural species are grown. This study was initiated to determine the effects of chilling on root respiration and the relative activity of the cyanide-insensitive pathway during early stages of seed germination and define more precisely the respiratory activity at the time when seedlings lose the ability to resume growth after being returned to nonchilling conditions.

Materials and Methods

PLANT PREPARATION. 'Encore' cucumber (*Cucumis sativus*) seeds were washed in a 1:10 dilution of sodium hypochlorite (v/v), rinsed with distilled water, and placed on moistened paper towels in closed plastic containers for germination at 26 °C for 3 d in the dark.

CHILLING TREATMENTS AND MEASUREMENT OF OXYGEN UPTAKE. Germinated cucumber seedlings were transferred to incubators set at 2, 10, or 15 °C for 24, 48, 72, 96, 144, or 192 h. Roots (fresh mass 500 mg) from each treatment were collected, cut into lengths of 1 to 2 mm, and placed in a buffer solution (5 mM MES 2[N-morpholino]ethanesulfonic acid/KOH pH 6.8) for 15 min. The samples were aerated and O₂ uptake was measured polarographically using an oxygen monitor (model 53; Yellow Spring Instrument Co.) at 26 °C.

Root samples were assayed in 3 mL of buffer solution by measuring O₂ uptake for 5 min following a 15-min preincubation with no inhibitors (control), 1 mM KCN, 15 mM SHAM, or both inhibitors. Another sample of cucumber seedling roots received a postchilling treatment at 26 °C for 24 h and O₂ uptake was measured as described above.

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Fig. 1. Oxygen uptake, on a fresh mass basis, by roots of 3-day-old 'Encore' cucumber seedlings exposed to different temperatures. The experiment was repeated three times. Vertical bars represent differences between observed and predicted values. $y = 4.5 - (0.007)(\text{time}) + (0.3)(\text{temp}) + (0.0002)(\text{time})^2 - (0.0009)(\text{time})(\text{temp})$.

To determine the relative activity of the AP (p_{alt}), 1 g of root tissue was exposed to 7.5, 15, 22.5, and 30 mM of SHAM in the absence or presence of 1 mM KCN. The activity of the AP was determined as described by Møller et al. (1988).

Experiments were repeated three times, and at least two observations made each time and averaged. A quadratic equation was obtained by regression analysis to describe changes in respiration and a completely randomized design with a factorial arrangement was used to analyze these data.

Results

For cucumber seedlings exposed at 2 and 10 °C, an increase in root O_2 uptake was observed with increased time of chilling (Fig. 1). Data were analyzed by regression, and values for the time and temperature factors were significant. After 48 h at 2 °C and for the

rest of 192 h of stress, root oxygen uptake of seedlings chilled at 2 °C increased, and the O_2 uptake observed at 192 h at 2 °C was numerically equivalent to that of roots from seedlings kept at 10 °C. This time period (96 to 192 h) also corresponded to the time of tissue collapse of the 2 °C-treated seedlings (Reyes and Jennings, 1994).

Cucumber seedlings stressed at 2 °C for periods longer than 96 h lose their ability to resume growth when returned to 26 °C for 24 h (Reyes and Jennings, 1994). Therefore, for studies on the effect of respiratory inhibitors at 2, 10, and 15 °C, 96 h was chosen as the maximum treatment time to determine any change in the activity of the AP.

Oxygen uptake was reduced when roots of seedlings held at 26 °C were transferred to 2, 10, and 15 °C (Table 1). For seedlings held at

26 °C, root oxygen uptake was inhibited 29% in the presence of 15 mM SHAM and 57% in the presence of 1 mM KCN. The uptake of oxygen was significantly lower at 2 °C than at either 10 or 15 °C throughout the 96 h of treatment. In the presence of CN, there was no change in the capacity of the AP at 2 °C for any treatment period compared to the control (Table 1), but at 10 and 15 °C the capacity of the AP had doubled by 96 h of treatment. In the presence of SHAM, the CN-sensitive pathway (CP) declined to a greater extent at 2 °C than at either 10 or 15 °C after 96 h compared to the control (Table 1). In all treatments, there were no significant differences between 10 and 15 °C with either of the inhibitors.

Oxygen uptake by cucumber seedling roots, treated at 2, 10, or 15 °C, and then transferred to 26 °C for 24 h, increased with longer temperature treatment periods (Table 2). With one exception, no significant interactions between temperatures were observed over time with either of the inhibitors. After 96 h at 2 °C and 24 h rewarming, in the presence of CN, there was a significant reduction in the capacity of the AP compared to the unchilled control and the 10 and 15 °C-treated tissue. Even though there was no statistical difference in oxygen uptake in the presence of SHAM, the capacity of the CP appeared to be lower after treatment at 2 °C for 72 and

Table 1. Oxygen uptake by roots of 3-day-old cucumber seedlings exposed to different temperatures following 15 min of preincubation with either no inhibitors (control), 1 mM KCN, 15 mM SHAM (salicylhydroxamic acid), or both.

		Treatment ^z							
Time	Temp	Control	SHAM			KCN			Both
(h)	(°C)	O ₂ ↑	O ₂ ↑	AP (%)	CP (%)	O ₂ ↑	AP (%)	CP (%)	O ₂ ↑
	26 ^y	10.1	7.2	29	71	4.3	43	57	1.0
24	2	7.0 a ^x	4.1 a	41	59	3.3 a	47	53	0.54 a
	10	8.0 b	5.6 b	30	70	6.1 b	76	24	0.69 a
	15	8.2 b	5.9 b	28	72	6.3 b	77	23	0.86 a
48	2	5.9 a	4.1 a	31	69	2.6 a	44	56	0.57 a
	10	9.0 b	5.6 b	38	62	6.5 b	72	28	0.45 a
	15	9.1 b	5.6 b	38	62	6.4 b	70	30	0.60 a
72	2	5.9 a	3.9 b	34	66	2.9 a	49	51	0.52 a
	10	8.5 b	5.1 b	40	60	6.6 b	78	22	0.70 a
	15	8.9 b	5.3 b	40	60	6.4 b	72	28	0.49 a
96	2	6.0 a	3.0 a	50	50	2.5 a	42	58	0.76 a
	10	8.4 b	5.4 b	36	64	6.9 b	82	18	0.81 a
	15	8.9 b	5.9 b	34	66	7.5 b	84	16	0.98 a

² $O_2 \uparrow$ = oxygen uptake on a 0.5-g fresh mass basis ($\mu\text{mol} \cdot \text{h}^{-1}$); AP = alternative pathway; CP = cyanide sensitive pathway

³Values obtained before imposing temperature treatments.

^xMeans represent three replications. Means followed by same letter represent no significant differences in columns (LSD $p < 0.05$).

96 h compared to the unchilled and the 10 and 15 °C-treated tissue.

The effect of 30 mM SHAM on O₂ uptake was measured to determine the maximum inhibition of the AP in the presence of CN (Table 3). For unchilled tissue (26 °C treatment), oxygen uptake was reduced 23% by SHAM (Table 3). After 48 h at 2 °C, inhibition was 36% and after 96 h at 2 °C, inhibition was 60%. When cucumber roots were treated with 30 mM SHAM at 10 °C for 24 h, oxygen uptake was reduced 33% and after 96 h the inhibition was 43%. For roots held at 15 °C, oxygen uptake was inhibited 34% during the first 24 h and 33% after 96 h.

The AP activity in cucumber seedling roots held at 2 °C increased over time (Table 4), becoming fully activated by 96 h at 2 °C ($p_{alt} = 1$). The corresponding p_{alt} value for roots held at 26 °C was 0.52. The activity of the AP in roots held at 10 or 15 °C remained fairly constant over the whole treatment period.

Discussion

Changes in chilling-induced respiration rates have been described as secondary stress events resulting from a chilling time × temperature interaction (Raison and Orr, 1990). A temperature treatment of 2 °C resulted in a decrease in oxygen uptake of seedling cucumber roots during the first 24 h when compared to roots treated at 10 or 15 °C (Fig. 1). This effect has been ascribed to changes in mitochondrial activity and is considered reversible (Lyons and Breidenbach, 1990).

Oxygen uptake of 2 °C-treated roots began to increase after 96 h of chilling and by 192 h was similar to 10 °C-treated roots. Temperature was the most significant factor contributing to this increase in oxygen uptake followed by the interaction of time and temperature ($p < 0.001$) (Fig. 1). The gradual increase in O₂ uptake of 2 °C-treated roots with increased exposure time could be interpreted as an indication of irreversible damage accumulating as a result of alteration of cellular metabolism, disruption of membranes, or the release of cellular components that utilize O₂ as a substrate. The damage accumulated in seedling roots exposed to 2 °C for 96 h or more resulted in total loss of root tissue integrity (Reyes and Jennings, 1994).

The activity of the AP in roots of cucumber seedlings grown at 26 °C was similar to that reported in other studies that used SHAM

to inhibit the AP (Kiener and Bramlage, 1981; Laties, 1982; Lambers et al., 1983). Even though SHAM can be used at high concentrations, the potential for SHAM to stimulate oxygen uptake is also present. However, SHAM has been used to detect changes in the activity of the AP and it is assumed that the level of activity would be maintained unless some external factor caused a reduction of electron transport through the cytochrome oxidase pathway (Lambers, 1982). Oxygen uptake at 10 and 15 °C was greater than expected compared to the control when KCN was used to block the cytochrome pathway in roots of cucumber seedlings (Table 1). This may be the result of electrons being diverted to the AP (Wilson, 1988).

Roots of cucumber seedlings exposed to 2 °C and treated with 15 mM SHAM, exhibited an increase in AP activity, which remained elevated up to 96 h of temperature treatment (Table 1), similar to other previous reports for different species (Elthon et al., 1986; Leopold and Musgrave, 1979; Vanlerberghe and McIntosh, 1992). Even though 10 and 15 °C also induced a change in the AP activity, it never became fully activated even after 96 h of treatment at these temperatures (Table 4, $p_{alt} = 0.63$ and 0.7 respectively). This suggests that full activation of the AP in cucumber roots was conditioned to a more severe stress. In roots held at 2 °C, the AP became fully active between 72 and 96 h of treatment (Table 4). Damage or changes in the mitochondria in roots held at this temperature may be responsible for the greater activation of this pathway, especially when chilling was longer than 96 h. After 96 h at 2 °C, cyanide-sensitive oxygen uptake was at its highest level and was significantly different from the 10 and 15 °C treatments.

Previous work from our laboratory (Reyes and Jennings, 1994) has shown that seedlings chilled at 2 °C for 96 h or longer irreversibly lose their ability to resume root growth when returned to 26 °C. When 15 mM SHAM was used to inhibit the AP, rewarming for 24 h at 26 °C had no significant effect on cyanide sensitive oxygen uptake (Table 2) at any time or temperature treatment. However, there was a significant reduction in AP activity on rewarming to 26 °C after 96 h at 2 °C when CN was used.

At 30 mM SHAM, the activity of the AP, as percentage of control, was greater in 2 °C-treated cucumber roots after 96 h than in 10 or 15 °C-treated roots (Table 3). It seems likely that severe

Table 2. Oxygen uptake by roots of 3-day-old cucumber seedlings exposed to different temperatures, rewarmed for 24 h at 26 °C and following 15 min of preincubation with either no inhibitors (control), 1 mM KCN, 15 mM SHAM, or both.

		Treatment ^z							
Time	Temp	Control	SHAM			KCN			Both
(h)	(°C)	O ₂ ↑	O ₂ ↑	AP (%)	CP (%)	O ₂ ↑	AP (%)	CP (%)	O ₂ ↑
24	26 ^y	10.1	7.2	29	71	4.3	43	57	1.0
	2	10.9 a ^x	6.5 a	40	60	6.0 a	55	45	0.50 a
	10	9.3 a	4.9 a	47	53	7.5 a	81	19	0.35 a
48	15	9.7 a	8.0 a	18	82	5.8 a	60	40	0.46 a
	2	9.0 a	5.4 a	40	60	6.9 a	77	23	1.0 a
	10	8.5 a	7.0 a	18	82	6.5 a	76	24	1.0 a
72	15	9.3 a	5.9 a	37	63	7.0 a	75	25	0.6 a
	2	11.4 a	5.0 a	56	44	6.6 a	58	42	0.64 a
	10	11.1 a	5.8 a	48	52	6.7 a	60	40	0.98 a
96	15	10.3 a	6.0 a	42	58	7.4 a	72	28	0.77 a
	2	12.0 a	5.4 a	55	45	3.1 a	26	74	0.32 a
	10	10.7 a	6.0 a	44	56	9.1 b	85	15	1.3 a
	15	11.0 a	7.9 a	28	72	7.7 b	70	30	1.0 a

^aO₂↑ = oxygen uptake on a 0.5-g fresh mass basis (μmol·h⁻¹); AP = alternative pathway; CP = cyanide sensitive pathway.

^yValues obtained before imposing temperature treatments.

^xMeans represent values from three replications. Values with different letters represent significant differences in columns (LSD $p < 0.05$).

Table 3. Inhibition of oxygen uptake on a fresh mass basis² for various time × temperature interactions in the presence of SHAM (salicylhydroxamic acid).

Time		Temp (°C)		
(h)	Treatment	2	10	15
		(μmol·h ⁻¹ per 0.5 g)		
24	Control	7.2 ^y	7.9	11.9
	+ SHAM ^x	4.2 (41) ^w	5.3(33)	7.9(34)
48	Control	6.1	8.6	11.1
	+ SHAM	3.9(36)	5.2(39)	8.5(23)
72	Control	5.3	8.1	11.7
	+ SHAM	2.2(58)	4.5(44)	8.1(30)
96	Control	4.8	9.8	12.0
	+ SHAM	1.9(60)	5.6(43)	8.1(33)

²Fresh weight basis = 11.9 μmol·h⁻¹ per 0.5 g for unchilled tissue (26 °C) and 9.1 μmol·h⁻¹ per 0.5 g in the presence of SHAM.

^aData shown were included in the statistical analysis of Table 4.

^bPlus SHAM = 30 mM of SHAM.

^cPercent inhibition in parentheses.

stress, such as 2 °C for 96 h, is responsible for the increased activity (Table 4). Neither 15 nor 30 mM SHAM caused an increase in the activity of the CP, compared to the control, at any time or temperature treatment (Tables 1 and 3). However when CN was used to inhibit oxygen uptake, a significant increase was revealed in AP activity at 10 and 15 °C, but not at 2 °C, for all treatment times (Table 1). Wilson (1988) provided evidence that electrons can be diverted from the AP to the cytochrome oxidase pathway and vice versa in mung-bean mitochondria. Atkin et al. (1995) showed that the cytochrome oxidase pathway does not necessarily need to be fully saturated before the AP becomes active. Thus, if the cytochrome oxidase pathway becomes saturated or inactivated under chilling conditions, it is possible for electrons to be diverted to the AP (Ribas-Carbo, et al. 1995).

An increase in the AP may have a stress-protective role (Chauveau et al. 1978). Kiener and Bramlage (1981) associated the increase in AP activity observed in cucumber hypocotyls with a process that compensated for the damage to cytochrome oxidase,

Table 4. Engagement of the alternative respiratory pathway induced by temperature. Calculation of relative activity of the alternative pathway (ρ_{alt}) values were obtained by plotting values obtained by titration with SHAM (salicylhydroxamic acid) in the absence and presence of 1 mM KCN.

Time (h)	Parameter	Temp ² (°C)		
		2	10	15
24	ρ_{alt}	0.68	0.66	0.66
	R^2	0.97	0.95	0.99
	$p>$	0.002	0.005	0.003
48	ρ_{alt}	0.56	0.69	0.40
	R^2	0.80	0.99	0.86
	$p>$	0.039	0.0004	0.02
72	ρ_{alt}	0.97	0.83	0.61
	R^2	0.93	0.93	0.94
	$p>$	0.007	0.007	0.007
96	ρ_{alt}	1.0	0.63	0.70
	R^2	0.98	0.97	0.94
	$p>$	0.001	0.003	0.007

²Values for tissue at 26 °C before temperature treatments: ρ_{alt} = 0.52, R^2 = 0.83 and $p > 0.03$.

^aValues of ρ_{alt} : 1.0 = full engagement and 0 = no activity.

which is likely to occur when extreme stress is imposed on plant tissue. They reported an initial increase in AP activity when the tissue was restored to 25 °C, which gradually declined to control levels within 24 h. The contribution of AP activity in cucumber seedling roots almost doubled the control level at 10 and 15 °C after 96 h (Table 1). During a 24 h rewarming treatment, CN caused a diversion of electrons to the AP in the 10 and 15 °C-treated roots (Table 2). A similar response was observed in the 2 °C-treated roots up to 72 h of treatment, after which time the AP activity dropped below the control level. This suggests a collapse in AP activity coinciding with the length of the 2 °C treatment (96 h) beyond which seedling root tissue has been reported to collapse when returned to 26 °C (Reyes and Jennings, 1994). The cause of this collapse of AP activity after 96 h at 2 °C is unclear, but appears to be temperature dependent in that a similar effect was not observed after 96 h at either 10 or 15 °C.

Other possible explanations for the increase in activity of the AP is to maintain a metabolic balance and prevent the buildup of otherwise toxic end products (Lambers, 1982) or to prevent the accumulation of active oxygen species (Purvis and Shewfelt, 1993). Diversion of electrons to the AP may be one way to prevent the generation of oxygen-free radicals. Thus, the increased AP activity in cucumber seedlings might be functioning to reduce the level of the oxygen-free radicals induced in roots held for up to 96 h at 10 or 15 °C, but not at 2 °C.

Considering the sensitivity of cucumber seedlings to chilling temperatures, the amount of stress incurred after 96 h at 2 °C may be at a level that exceeds the AP's ability to reduce the accumulation of active oxygen species. The decline in the AP's capacity on rewarming after 96 h at 2 °C may indicate cumulative damage to the pathway that is irreversible. However, additional research is needed to correlate the AP with such a protective role during chilling or with other stress effects.

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