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Effects of KCN, SHAM and Oxygen Concentrations on Respiratory Properties of Purified Mitochondria Isolated from *Ananas comosus* (Pineapple) and *Kalanchoë daigremontiana*

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Abstract : Effects of potassium cyanide (KCN), salicylhydroxamic acid (SHAM), and oxygen concentrations on mitochondrial respiration were investigated in purified mitochondria of a typical phosphoenolpyruvate carboxykinase (PCK) crassulacean acid metabolism (CAM) plant *Ananas comosus* (pineapple) and a typical malic enzyme (ME)-CAM plant *Kalanchoë daigremontiana*. Mitochondria of *A. comosus* oxidized succinate and NADH faster than that of *K. daigremontiana*. Succinate and NADH oxidations in mitochondria of both species were partially inhibited by KCN and SHAM, indicating that these oxidations were connected to cytochrome and alternative pathways in their electron transport chain (ETC). NADH oxidation was more sensitive to KCN than succinate oxidation, suggesting that the ETC from NADH oxidation was less connected to the alternative pathway than that from succinate in mitochondria of both species. Concurrent oxidation of succinate and NADH resulted in much higher rates of cytochrome and alternative respirations than each individual oxidation alone in both species. NADH oxidation in *A. comosus* mitochondria was more connected to the cytochrome pathway, so *A. comosus* could produce much more ATP than *K. daigremontiana*. This capacity might be one of the fitting mechanisms of *A. comosus* to produce a sufficient amount of ATP for cytosolic PCK in the daytime. In addition, the reduction of oxygen concentrations decreased not only the cytochrome respiration, but also the alternative respiration on succinate oxidation in mitochondria of both species, and the decrease was greater in *K. daigremontiana* than in *A. comosus*.

Key words : Alternative pathway, *Ananas comosus*, Cytochrome pathway, *Kalanchoë daigremontiana*, Mitochondria, Oxygen.

Mitochondrial electron transport in higher plants can proceed by the phosphorylating cytochrome (Cyt) pathway or by a largely nonphosphorylating alternative (Alt) pathway (Robinson et al., 1992). The Cyt pathway is the major route for accomplishing ATP-synthesis through oxidative phosphorylation, while the ATP formation through Alt pathway is much limited (Siedow and Umbach, 1995). Cyt pathway is inhibited by KCN, while Alt pathway is inhibited by salicylhydroxamic acid (SHAM) (Siedow and Day, 2000). In plant mitochondria, the activity of the Alt pathway is said to be relatively high in the presence of tricarboxylic acid cycle (TCA) derived substrates (succinate, malate, pyruvate) and lower or even absent in the presence of exogenous NADH as a substrate (Hemrika-Wagner et al., 1986). In mitochondria of *Petunia hybrida* cells, potato tuber callus and Iris bulbs, a combination of NADH and succinate increased the cyanide resistant respiration which is about the sum of the rates in the presence of the substrates added separately (Hemrika-Wagner et al., 1986). In addition, Shugaev and Vyskrebentseva (2001) observed that cooperative oxidation of succinate and NADH by mitochondria

of castor bean hypocotyls was accompanied with the dramatic increase in the rate of oxygen consumption to the level of completely additive oxidation rates of the individual substrates. A similar result was also observed in mitochondria of *K. pinnata* (Hong et al., 2005), but these results have never been reported in mitochondria of PCK-CAM species.

Generally, the Alt and Cyt pathways involved terminal Alt oxidase (AOX) and Cyt oxidase (COX), respectively, and AOX has a relatively low affinity for oxygen (O₂) compared with COX (Paul and Derek, 2003). AOX is a ubiquinone oxidase and it does not pump protons, therefore the energy derived from the oxidation of ubiquinone (UQ) is not conserved as ATP. AOX is not present in animal mitochondria, but is found in the mitochondrial respiratory chain of plant as well as some fungi, yeasts and protozoa. The metabolic rationale behind the presence of AOX may vary with the organism. In typical plants, there is evidence that AOX responds to oxidative stress (Paul and Derek, 2003). Recently, AOX has been reported to play a physiological role as a "survival" protein that allows plants to cope with a stressful

environment (Juszczuk and Rychtr, 2003). Among several environmental conditions controlling AOX abundance *in vivo*, O₂ availability is usually neglected and competition for O₂ as electron acceptor is seldom considered as a factor controlling the electron flow between the Cyt and Alt pathways (Szal et al., 2003). The effect of O₂ concentrations on Cyt respiration and Alt respiration has been reported by some researchers (Ricard et al., 1994; Szal et al., 2003).

In spite of extensive investigations, the physiological role of Alt respiration is not understood in depth, especially in mitochondria of CAM plants. One of the approaches to this problem is to evaluate the contributions of the Cyt and Alt respiratory chain to the total respiration based on the effect of inhibitors on the mitochondrial respiration (Medentsev et al., 2002). The contributions have been identified by the oxygen isotope discrimination method (Robinson et al., 1992). This technique allows measuring the partitioning of electron transport to the Cyt and the Alt respiratory pathways in the plant mitochondria under normal physiological conditions. However, the narrowness between the end points limits the sensitivity of the method, and the time-consuming measurements prevent its use for kinetic studies of electron partitioning in various conditions (Sluse and Jarmuszkiwicz, 1998). Since the mid-1990s, there has been general agreement that AOX can compete with the Cyt pathway for electrons and that this has serious implications for the use of inhibitor to exactly qualify Alt engagement (McDonald et al., 2002). Although the method using inhibitors to assess the Alt pathway in the respiration of isolated mitochondria sometimes considerably underestimates the real Alt pathway, this method is still extensively applied to probe the contribution of Alt pathway to the electron transport chain (ETC) in mitochondria of many plants such as castor bean (Shugaev and Vyskrebentseva, 2001), durum wheat (Pastore et al., 2001), and *Yarrowia lipolytica* (Medentsev et al., 2002). Thus, still now inhibitor titration is useful for analysing the characteristics of ETC in plant mitochondria.

In our previous studies, intact leaves of a typical ME-CAM species *K. daigremontiana* and a typical PCK-CAM species *A. comosus* showed Alt respiration in CAM phase III; however, *K. daigremontiana* showed a clear O₂ requirement and *A. comosus* showed only a low O₂ requirement (Nose et al., 1999, Nose and Takashi, 2001). Under a low O₂ concentration, *K. daigremontiana* lost phase III of CAM-type diurnal gas-exchange, but *A. comosus* still exhibited a pattern of diurnal gas-exchange. In this study, we continued to use these two typical CAM species to isolate and purify mitochondria; then we used the inhibitors of KCN and SHAM to probe the expression of Alt and Cyt respirations in their mitochondrial respiration. Our study was not to evaluate exactly the quantitative contribution of Alt

or Cyt engagement in mitochondrial respiration, but mainly to assess the control of ETC partitioning and to compare the basic characteristics of the Cyt and Alt respirations on the single and combined oxidations of succinate and NADH. We aimed to elucidate functions of respiration under these experimental conditions by using the inhibitors. Simultaneously, we tried to find the roles of Cyt and Alt respiration in total respiration in CAM phase III under normal and low O₂ concentrations in mitochondria isolated from typical ME-CAM and PCK-CAM species.

Materials and Methods

Plants were vegetatively propagated and grown in plastic pots in a greenhouse with heater under natural light conditions. Ten days before the experiments, the plants were transferred to a growth chamber (KG-50 HLA, Koito Industrial Co., LTD., Japan) with a photoperiod of 12 h light and 12 h dark. The temperature in the growth chamber was maintained at 35°C during the light period and 25°C during the dark period. The intensity of photosynthetically active radiation was 420 to 450 μmol m⁻² s⁻¹ at the mid-plant height, and a relative humidity 70%. The leaves were harvested at 6 to 7 h after the beginning of the light period. The harvested leaves were transported to the laboratory, rinsed thoroughly with distilled water and used to isolate mitochondria. The mitochondria were isolated according to the method of Hong et al. (2004b) with slight modifications.

Oxygen consumption was measured using an oxygen electrode (Rank Brothers England) at 25°C in 2 mL of reaction medium [300 mM mannitol, 10 mM KH₂PO₄, 5 mM MgCl₂, 10 mM KCl, 100 mM HEPES-KOH (pH 7.2)]. The mitochondria were preincubated in 0.16 mM ATP for 2 min to ensure full activation of succinate dehydrogenase before each assay for succinate oxidation. NADH oxidations were investigated at pH 6.8 in the presence of 1mM Ca²⁺. Respiratory control ratio (RCR) and ADP/O ratio were calculated according to Estabrook (1967). The O₂ concentration in air-saturated medium was taken as 258 μM. The protein content was measured by the method of Bradford (1976) using BSA as the standard.

Titration of KCN and SHAM was performed for NADH and succinate oxidation, respectively. Effect of the inhibitors was investigated within the range: 10-400 μM for KCN and 0.5-4 mM for SHAM in methoxyethanol. Measurements were conducted in an oxygen electrode with 2 mL of reaction medium. Oxygen concentration at zero was adjusted by using Na₂S₂O₄.

The effects of O₂ on the succinate oxidation in mitochondria of *K. daigremontiana* and *A. comosus* were probed at the three levels of 21, 14 and 8 kPa O₂. Partial pressure of oxygen at 21, 14 and 8 kPa O₂ were set in a mixture of O₂ and nitrogen (N₂) using total

Table 1. Effect of KCN and SHAM on succinate and NADH oxidations in mitochondria of *A. comosus* and *K. daigremontiana*. Assay conditions were the same as in Fig. 1. Results shown are means \pm SE (n=3 - 4) of separate preparations.

Treatments	<i>A. comosus</i>		<i>K. daigremontiana</i>	
	Succinate oxidation*	NADH oxidation*	Succinate oxidation*	NADH oxidation*
no inhibitor	217 \pm 24	147 \pm 16	152 \pm 18	131 \pm 15
+0.5 mM SHAM	145 \pm 16	147 \pm 16	84 \pm 9	100 \pm 8
+0.1 mM KCN	78 \pm 9	0	60 \pm 7	46 \pm 7

* Values are shown as nmol O₂ min⁻¹ mg⁻¹ protein.

volume of 3 liters for each assay. The partial pressure of O₂ was calculated based on the formula as below:

$$Y_{\text{kPa}} = X / (3 - X)$$

$$Z = 3 - X$$

where: Y_{kPa}, O₂ partial pressure for assay; 3, total volume (L/min); X, O₂ volume (L/min); and Z, N₂ volume (L/min).

After mixing O₂ and N₂ gases at the calculated volumes to reach the assay concentration of 21, 14, or 8 kPa O₂, the mixed-gas was provided into the oxygen electrode reaction medium. Inside the reaction medium, the mixed-gas was continuously bubbling, and this bubbling process gradually achieved to the saturated levels. At that time, the mitochondria, substrates, ADP and the inhibitors were added to measure the effects of O₂ concentrations on the respiratory property and Alt respiration pathway in mitochondria of both species. During the assay at each O₂ concentration, each mixed-gas was blown on the surface of the reaction medium. The saturated concentration of O₂ in medium at 21, 14 and 8 kPa O₂ were taken as 258, 172 and 98 μ M, respectively (Estabrook, 1967).

Results

1. Effects of KCN and SHAM

Table 1 and Fig. 1 show the effects of KCN and SHAM on succinate and NADH oxidations in mitochondria of *A. comosus* and *K. daigremontiana*. Mitochondria of both species oxidized succinate and NADH at high rates, and mitochondria of *A. comosus* oxidized succinate at a higher rate than that of *K. daigremontiana* (Table 1 and Fig. 2). Succinate oxidation in both species was slightly inhibited by KCN at concentrations less than 50 μ M (Fig. 1A). Addition of KCN at 50-150 μ M mainly inhibited succinate oxidations in mitochondria of both species and the rate of inhibition was significantly lower in mitochondria of *K. daigremontiana* than in those of *A. comosus* (p<0.05, t-test). KCN at 200 μ M inhibited mitochondrial succinate oxidation quite differently

in the two species. KCN at 200 μ M inhibited succinate oxidation completely in *K. daigremontiana*, but only about 82% in *A. comosus* mitochondria (Fig. 1A). In *A. comosus* mitochondria, succinate oxidation was completely inhibited by 400 μ M KCN (Fig. 1A).

The results indicated that NADH oxidation in mitochondria of *A. comosus* was especially sensitive to KCN but that of *K. daigremontiana* was not so sensitive (Fig. 1B). Ten μ M KCN inhibited about 15% of mitochondrial NADH oxidation in *A. comosus* but it did not affect in *K. daigremontiana*. Fifty μ M KCN was sufficient to completely block NADH oxidation in *A. comosus*, but it inhibited only about 30% of the oxidation in *K. daigremontiana*. NADH oxidation in *K. daigremontiana* mitochondria was completely inhibited by 150 μ M KCN (Fig. 1B). In plant mitochondria, the Alt pathway is less connected with NADH oxidation than succinate oxidation (Day et al., 1988). A similar trend was also observed in our study (Fig. 1A, B). NADH oxidation in mitochondria of both species was more sensitive with KCN than in those of succinate oxidations. NADH oxidation was affected by KCN at 10-150 μ M (Fig. 1B) but succinate oxidation at 50-400 μ M (Fig. 1A).

The effect of SHAM on succinate oxidation in mitochondria of *K. daigremontiana* was similar to that in those of *A. comosus* (Fig. 1C). The succinate oxidation in both species was significantly inhibited by 0.5-2 mM SHAM. Addition of 3 mM SHAM completely blocked mitochondrial succinate oxidation in *K. daigremontiana*, about 85% in *A. comosus*, and addition of 4 mM SHAM fully inhibited succinate oxidation in *A. comosus* mitochondria (Fig. 1C). As shown in Fig. 1D, the effect of SHAM on mitochondrial NADH oxidation in *A. comosus* was clearly different from that in *K. daigremontiana*. NADH oxidation of *A. comosus* was not inhibited by 0.5 mM SHAM, but that in *K. daigremontiana* was inhibited about 25%, implicating that in this assay condition, the ETC from NADH oxidation was mainly operated by COX via Cyt pathway in *A. comosus*. NADH oxidation in *A. comosus* and *K.*

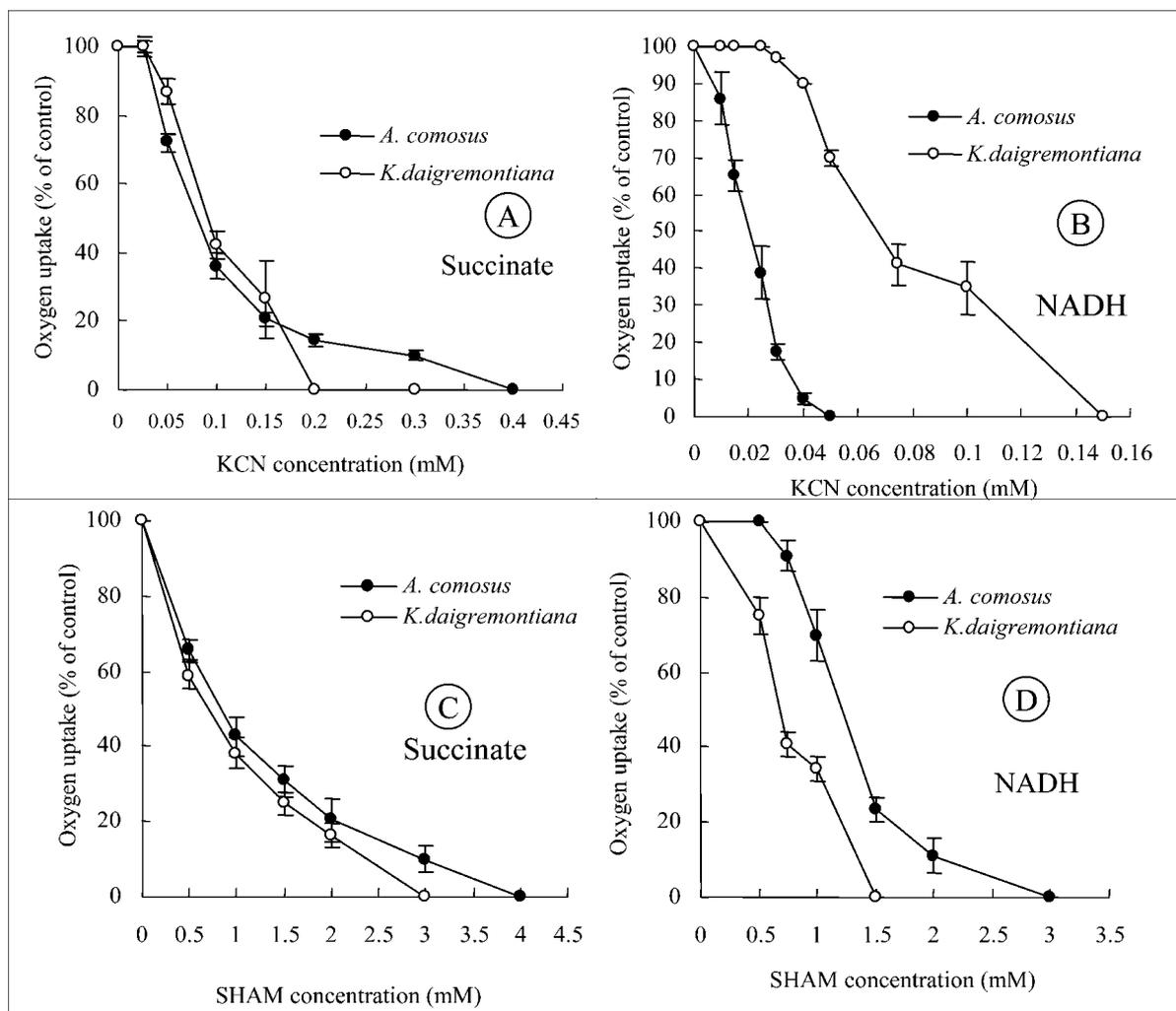


Fig. 1. Effects of KCN and SHAM on succinate and NADH oxidations in mitochondria of *A. comosus* and *K. daigremontiana*. Assay conditions were: 10 mM succinate with 0.16 mM ATP, 1 mM NADH, 1 mM Ca^{2+} , and 0.32 mM ADP. Control values were calculated in the absence of the inhibitors and they were considered as 100% in these assays.

daigremontiana was inhibited about 10% and 60%, respectively, by 0.75 mM SHAM, and about 30% and 68%, respectively, by 1 mM SHAM. Addition of 1.5 mM SHAM completely inhibited NADH oxidation in *K. daigremontiana*, but about 78% in *A. comosus*. SHAM at 3 mM fully inhibited NADH oxidation in *A. comosus* mitochondria.

The results of titration assays clearly indicated that the ETC in mitochondria of these two species engaged on both Cyt and Alt pathways and their engagement levels varied depending on the substrates and species. In general, there were no clear differences between the sensitivities to KCN and SHAM of succinate oxidation in mitochondria of both species. KCN and SHAM had a significant effect on succinate oxidation in the range of 0.05-0.15 mM and 0.5-2 mM, respectively in both species. The inhibitory effects of KCN and SHAM were slightly lower and higher, respectively in *K. daigremontiana* than in *A. comosus* (Fig.

1A, C). By contrast, the effects of KCN and SHAM on NADH oxidations were completely different in the mitochondria of the two species (Fig. 1B, D). NADH oxidation in *A. comosus* mitochondria usually showed a rather higher Cyt respiration rate but much lower Alt respiration rate than those in *K. daigremontiana* mitochondria (Table 1 and Fig. 1B). The effects of KCN (Fig. 1B) and SHAM (Fig. 1D) on NADH oxidation suggest that activity of external NADH dehydrogenase (NDE) was less related with AOX but more with COX in *A. comosus* mitochondria.

2. Effects in combined application of KCN and SHAM

Effects of combined application of 0.1 mM KCN and 1 mM SHAM on the succinate and NADH oxidation also were investigated in both species (Fig. 2). In the presence of 0.1 mM KCN and 1mM SHAM, succinate oxidation in *A. comosus* mitochondria showed about 22% oxygen uptake rate, and further addition of 2 mM

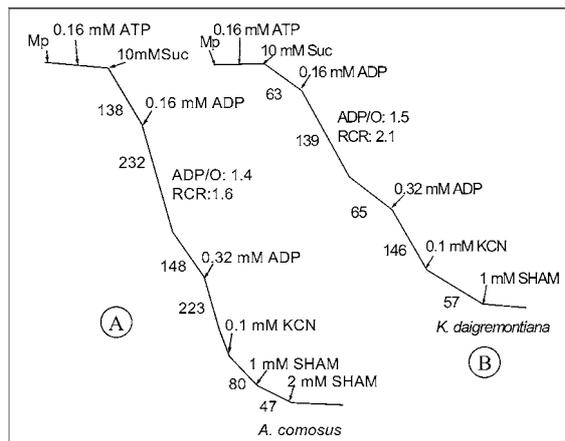


Fig. 2. Effects of combined application of KCN and SHAM on succinate oxidation in mitochondria of *A. cosmosus* (A) and *K. daigremontiana* (B). The presented traces were one of typical electrode traces obtained from three independent experiments. Numbers along the traces refer to nmol O₂ consumed min⁻¹ mg⁻¹ protein. Mp, purified mitochondria; RCR, respiratory control rate.

SHAM fully inhibited this oxidation (Fig. 2A). In *K. daigremontiana* mitochondria, addition of 0.1 mM KCN and 1 mM SHAM completely blocked the residual respiration for NADH oxidation (Fig. 3).

3. Effects of KCN and SHAM on the concurrent oxidation of succinate and NADH

Fig. 4 shows the effects of inhibitors on the concurrent oxidation of succinate and NADH in mitochondria of both species. The respiration rate on concurrent oxidation of succinate and NADH were strongly increased and these respiration rates than that on NADH or succinate oxidation in both species (Fig. 2, Fig. 3 and Fig. 4). This concurrent oxidation of succinate and NADH usually gave the ADP/O ratios of less than 2 indicating that only two translocation sites were operated in these oxidations, and two proton-extrusion sites were utilized (Siedow and Day, 2000).

In the concurrent oxidation of succinate and NADH, concomitantly with the increase in the Cyt respiration rate, an increase in the Alt respiration rate was also observed (Fig. 4). Addition of 0.1 mM KCN usually inhibited about 64% and 61% of the total respiration on succinate oxidation (Table 1 and Fig. 2) whereas this addition inhibited about 54% and 47% of total respiration rate on concurrent oxidations of succinate and NADH in mitochondria of *A. cosmosus* and *K. daigremontiana*, respectively (Fig. 4A, B). In the presence of 0.1 mM KCN and 1 mM SHAM, succinate oxidation in *K. daigremontiana* mitochondria was fully inhibited whereas the concurrent oxidation of succinate and NADH gave about 22% residual respiration rate (Figs. 2B and 4B). In *A. cosmosus*

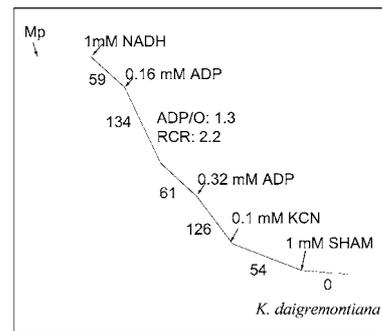


Fig. 3. Effects of combined application of KCN and SHAM on NADH oxidation in *K. daigremontiana* mitochondria. The presented trace was one of typical electrode traces obtained from three independent experiments. Numbers along the traces refer to nmol O₂ consumed min⁻¹ mg⁻¹ protein.

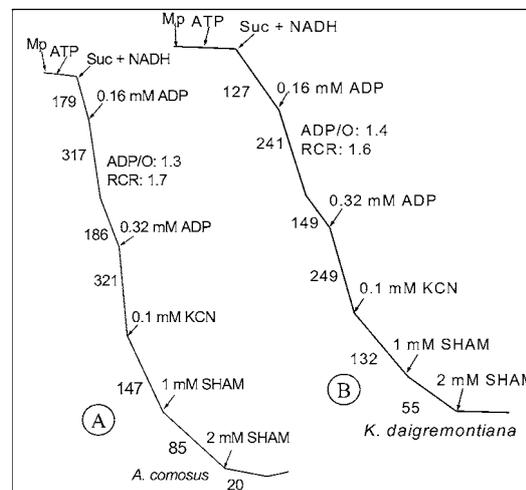


Fig. 4. Effects of combined application of KCN and SHAM on oxidation of both succinate plus NADH in mitochondria of *A. cosmosus* (A) and *K. daigremontiana* (B). Unless otherwise indicated, assay conditions were the same as in Fig. 1. One of typical electrode trace obtained from three independent experiments is shown. Numbers along the traces refer to nmol O₂ consumed min⁻¹ mg⁻¹ protein.

mitochondria, addition of 0.1 mM KCN and 1 mM SHAM inhibited about 80% of the respiration on succinate oxidation whereas this addition inhibited about 74% of the respiration of concurrent succinate and NADH oxidation (Figs. 2A and 4A).

4. Effects of oxygen concentrations on mitochondrial succinate oxidation

The effect of O₂ concentration was probed at 21, 14 and 8 kPa O₂ for succinate oxidation in mitochondria of *K. daigremontiana* and *A. cosmosus* (Fig. 5) and the results are summarized in Table 2. Mitochondria of both species oxidized succinate under all O₂ partial pressures, showing a significant respiration rates and respiratory control ratio (RCR). These oxidations gave an ADP/O ratio less than two indicating that two

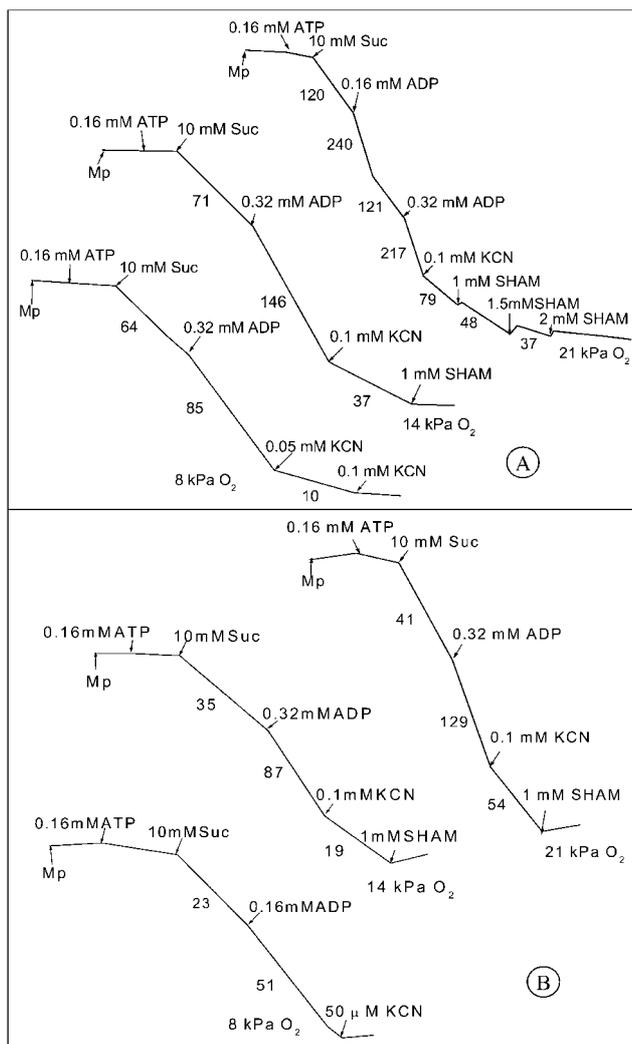


Fig. 5. Effect of low oxygen concentration on succinate oxidation in *A. cosmosus* mitochondria (A) and *K. daigremontiana* (B). The presented traces were one of typical electrode traces obtained from three independent experiments. Numbers along the traces refer to nmol O₂ consumed min⁻¹ mg⁻¹ protein. The O₂ saturated concentration in medium at 21, 14 and 8 kPa were 258, 172 and 98 μ M, respectively.

proton-extrusion sites were utilized in all investigated succinate oxidation in mitochondria of both species. Our results showed that low O₂ concentrations caused a significant decrease in the respiration on succinate oxidation in mitochondria of both species. The respiration on succinate oxidation in mitochondria of *A. cosmosus* and *K. daigremontiana* was decreased by the falling of O₂ concentration. However, the decrease in mitochondria of *K. daigremontiana* was rather faster than that in *A. cosmosus* (Table 2). In the presence of 0.1 mM KCN, succinate oxidation of *K. daigremontiana* was inhibited about 61.3 \pm 5.6% at 21 kPa O₂, but this oxidation was inhibited about 75.5 \pm 6.9% at 14 kPa O₂. In *A. cosmosus* mitochondria, addition of 0.1 mM KCN inhibited the respiration rates about 65.9 \pm 4.1%

and 71.7 \pm 3.9% on succinate oxidation at 21 and 14 kPa O₂, respectively. At much lower O₂ concentration of 8 kPa O₂, addition of 0.1 mM KCN completely inhibited succinate oxidation in mitochondria of *K. daigremontiana*, while this addition inhibited succinate oxidation about 93.4 \pm 2.8% in *A. cosmosus* (Table 2).

Discussion

The present study indicated that the succinate and NADH oxidations in mitochondria of *A. cosmosus* and *K. daigremontiana* were partially inhibited by KCN and SHAM, indicating that these oxidations were connected with both Alt and Cyt pathways in their ETC. In our study, however the effects of KCN and SHAM on succinate and NADH oxidations strongly differed. The mitochondrial respiratory property and Alt respiration varied with the substrate and species, in which these capacities were lower with NADH as the substrate than with succinate in both species (Table 1).

In our study, the NADH and succinate oxidations in mitochondria of both species showed that the respiration rates in the absence of inhibitor was similar to the sum of the respiration rates in the presence of 0.5 mM SHAM and 0.1 mM KCN (Table 1). This suggests that the activities of AOX and COX were accurately evaluated in our experiments. Because the succinate oxidation in mitochondria of *A. cosmosus* and *K. daigremontiana* was significantly affected by 0.1 mM KCN and 0.5-2 mM SHAM, we fixed KCN at 0.1 mM and SHAM at 1 mM or 2 mM to probe the mutual effect of KCN and SHAM on the individual and concurrent oxidation of succinate and NADH as described in Fig. 2 to Fig. 5. A previous study with mitochondria of the ME-CAM plant *Sedum praealtum* indicated that the O₂ uptake was completely inhibited by 0.1 mM KCN and 1 mM SHAM (Arron et al., 1979). In our study, also, the respiration rate on succinate oxidation was completely blocked by the same treatment in mitochondria of *K. daigremontiana* (Fig. 2B), but not in *A. cosmosus* (Fig. 2A).

Although there remained problems in the quantification of Alt respiration by the combined use of an oxygen electrode and inhibitors, as reported previously (McDonald et al., 2002), the differences in responses of mitochondrial respiration to different substrates or species obtained in the present study were intriguing. We will discuss the species and substrate specific Alt and Cyt respiration referring to the connotative problems of the inhibitor experiment following the ETC in mitochondrial matrix and membrane illustrated in Fig. 6.

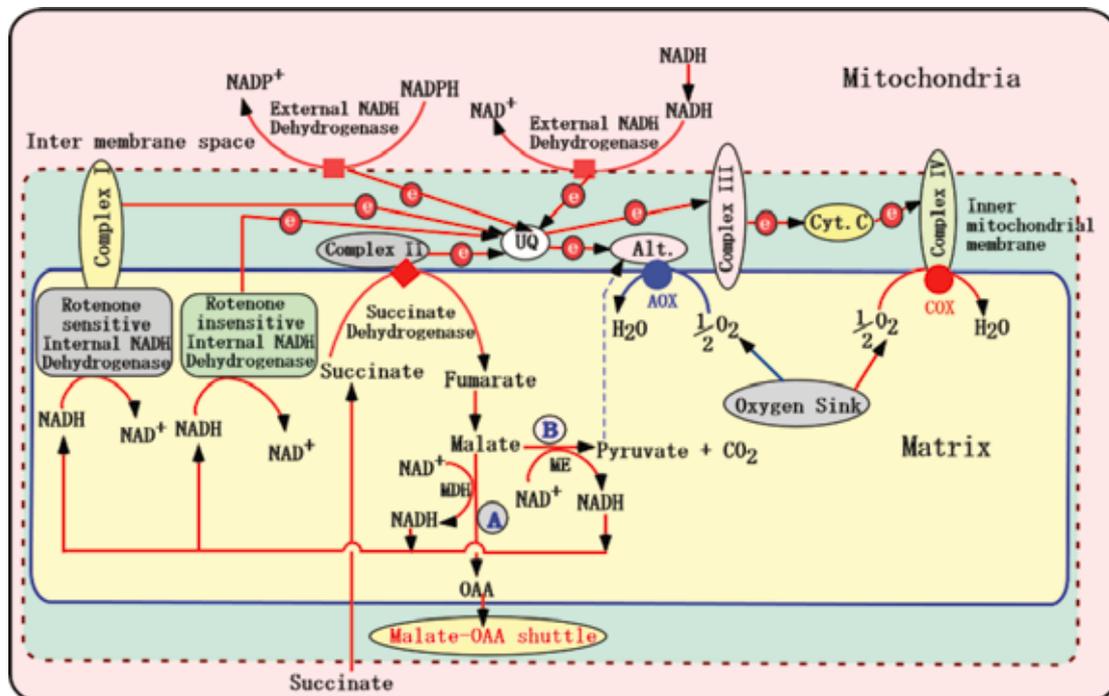
Plant mitochondrial respiration conserves energy by linking NADH oxidation and electron-couple proton translocation for ATP synthesis, through a core pathway involving three larger complexes denoted by Complex I, III, and IV. NADH is oxidized by two different dehydrogenases. An external

Table 2. Effects of oxygen on respiratory property and alternative (Alt) respiration on succinate oxidation in mitochondria of *K. daigremontiana* and *A. comosus*. Assay conditions were 10 mM succinate, 0.16 mM ADP and 0.1 mM KCN. State 3 refers to the respiration rate of O₂ uptake in the presence of ADP; state 4 refers to the rate upon depletion of ADP. Respiratory control ratio (RCR) was calculated as the ratio of the rate in state 3 to that in state 4. The O₂ saturated concentration in medium at 21, 14 and 8 kPa O₂ were taken as 258, 172 and 98 μM, respectively. Results shown are means ± SE (n=3 - 4) of separate preparations.

Species	Respiration rate (nmol O ₂ min ⁻¹ mg ⁻¹ protein)		RCR	ADP/O	Oxygen uptake in the presence of KCN	
	+ADP	-ADP			Respiration rate	% inhibited
	State 3	State 4			rate	**
<i>K. daigremontiana</i>						
21 kPa O ₂	142 ± 18	67 ± 6	2.1 ± 0.2	1.5 ± 0.3	55 ± 8	61.3 ± 5.6
14 kPa O ₂	87 ± 12 (61)*	43 ± 8	2.0 ± 0.4	1.6 ± 0.3	21 ± 6	75.5 ± 6.9
8 kPa O ₂	52 ± 9 (37)*	24 ± 5	2.2 ± 0.3	1.8 ± 0.2	0	100
<i>A. comosus</i>						
21 kPa O ₂	217 ± 24	110 ± 18	2.0 ± 0.3	1.4 ± 0.3	74 ± 9	65.9 ± 4.1
14 kPa O ₂	152 ± 18 (70)*	81 ± 10	1.9 ± 0.4	1.7 ± 0.2	43 ± 6	71.7 ± 3.9
8 kPa O ₂	105 ± 21 (48)*	46 ± 9	2.4 ± 0.2	1.8 ± 0.2	7 ± 3	93.4 ± 2.8

*: Numbers in parentheses are percentages to the value in state 3 under 21 kPa O₂

** : Values are percentages of inhibited respiration rate in the presence of 0.1 mM KCN to respiration rate at state 3 under each O₂ condition.



---> Effect of pyruvate on alternative oxidase activity

Fig. 6. Electron transport chain during NADH and succinate oxidation in mitochondria of *A. comosus* (A) and *K. daigremontiana* (B). UQ, ubiquinone; Alt, alternative; AOX, Alt oxidase; Cyt, cytochrome; COX, Cyt oxidase, MDH, malate dehydrogenase, ME, malic enzyme; OAA, oxaloacetate.

NADH dehydrogenase (NDE) enables cytoplasmic NADH to be used directly, while an internal NADH dehydrogenase (NDI) catalyses a rotenone-insensitive oxidation of mitochondrial NADH generated by TCA cycle (Wood and Hollomon, 2003). NADH is unable to pass through the inner mitochondrial membrane so it is oxidized outside of the mitochondria by NDE and donate their electron directly to ubiquinone (UQ) pool (Siedow and Day, 2000; Møller, 2002). Differing from NADH, succinate permeates the inner mitochondrial membrane easily (Paul and Derek, 2003), and is oxidized in the matrix by succinate dehydrogenase to produce fumarate, donating an electron to complex II which is linked to the UQ pool. The fumarate is converted to malate, which is further oxidized via NAD-ME to produce pyruvate and/or via malate dehydrogenase (MDH) to produce oxaloacetate (OAA). This process also produces NADH in the mitochondrial matrix, which is oxidized by NDI, and donates an electron to UQ (Fig. 6). As a result, all of these processes could produce more electrons donating to UQ, increasing the transporting capacity of electrons flux from UQ to Cyt and Alt pathways during succinate oxidation, whereas NADH oxidation may not. Furthermore, the NADH produced in mitochondrial matrix during the above process might also become the substrates for enzyme reaction in the matrix and these activities further contributes to increase the respiration rates. This might be a reason why succinate was oxidized faster than NADH in mitochondria of both species (Table 1).

Our previous study showed that mitochondrial malate was further oxidized mainly by mitochondrial MDH via malate-OAA shuttle to produce OAA in *A. comosus*, while it was oxidized mainly via NAD-ME to produce pyruvate in *K. daigremontiana* (Hong et al., 2004a, b). Under assay conditions of this study, we did not supply external Acetyl-CoA into mitochondria, so probably the pyruvate and OAA formed by malate oxidation could not be used in the next reactions in the TCA cycle, and be accumulated in the mitochondrial matrix of *K. daigremontiana* and *A. comosus*. In fact, the pyruvate generated intramitochondrially during oxidation of malate and succinate could also activate AOX (Day et al., 1994), so pyruvate might accumulate in *K. daigremontiana* mitochondria further contribute to Alt respiration whereas OAA was still accumulated in matrix of *A. comosus*, resulting in a slightly higher Alt respiration rate in mitochondria of *K. daigremontiana* than in mitochondria of *A. comosus* (Fig. 6).

In this study, we emphasized that the effect of KCN on NADH oxidation in mitochondria of *A. comosus* was completely different from that in mitochondria of *K. daigremontiana*. The concentration of KCN effective for NADH oxidation in *A. comosus* mitochondria was lower than 50 μM , but it was around 50-150 μM

in *K. daigremontiana* mitochondria (Fig. 1B). These results indicated that NADH oxidation in *A. comosus* mitochondria was more sensitive to KCN than that in *K. daigremontiana* mitochondria (Fig. 1B).

The specific sensitivity to KCN of NADH oxidation in *A. comosus* mitochondria might be caused by one of the following four reasons (1) NDE activity was closely related with COX; (2) NDE activity was inhibited by KCN; (3) KCN also inhibited AOX; and (4) COX was more active than AOX. If KCN could inhibit specially AOX in NADH oxidation, this inhibition would also be observed in succinate oxidation. However, the effects of KCN on succinate oxidation and NADH oxidation were different (Fig. 1A, B), so the third reason is unsuitable. Furthermore, Wood and Hollomon (2003) reported that NDE lack inhibitors, so that the second and third reasons can also be eliminated. The effect of SHAM on NADH oxidation (Fig. 1D) supported reason 1 in which the activity of NDE was closely related to COX in NADH oxidation of *A. comosus* mitochondria.

Wood and Hollomon (2003) indicated that in plant mitochondria, the electron flow through NDE is divided into the Cyt and Alt pathways at the UQ pool depending on AOX and COX activities. Day et al. (1988) observed that the ETC through the NDI was not rapid enough to engage with the AOX. These results suggest that though the electron from NDE in mitochondria of *K. daigremontiana* and *A. comosus* input in the same UQ pool, they were differently branched at UQ where the ETC in mitochondria of *A. comosus* was more clearly connected with the Cyt pathway than that of *K. daigremontiana*. It may be possible that COX was more active and AOX was less active in NADH oxidation in *A. comosus* mitochondria than that in *K. daigremontiana* mitochondria. This result may reflect the ATP synthesis during the CAM phase III in *A. comosus* mitochondria. It is very clear that during the light period, *K. daigremontiana* uses cytosolic ME to decarboxylate malate, generating pyruvate and CO_2 , whereas *A. comosus* uses cytosolic MDH to convert malate to OAA, and then OAA is further converted to PEP and CO_2 by cytosolic PCK. (Winter and Smith, 1996). Our previous study revealed that NADH oxidation in *A. comosus* mitochondria significantly contribute to ATP production for cytosolic PCK activity during the daytime by a malate-OAA shuttle (Hong et al., 2004b). In this study, NADH oxidation in *A. comosus* was more closely connected with Cyt respiration than with ATP respiration, suggesting that NADH oxidation was also related to mitochondrial ATP synthesis to provide energy for cytosolic PCK in the daytime in *A. comosus*.

Similar to *K. pinnata* mitochondria (Hong et al., 2005), we also found that a concurrent oxidation of two substrates, succinate and NADH not only dramatically enhanced the Cyt respiration but also

increased the Alt respiration in mitochondria of *A. comosus* and *K. daigremontiana* (Fig. 4). These results are in agreement with previous reports that mitochondrial respiration and Alt pathway were not fully saturated by a single substrate in mitochondria of some plant species (Shugaev and Vyskrebentseva, 2001, Hong et al., 2005).

Oxygen is essential as the terminal electron acceptor in the oxidative phosphorylation pathway, that provides the majority of ATP for cellular metabolism. Ricard et al. (1994) indicated that under low O₂ concentration, COX activity was limited, leading to decrease in the respiration rate. In potato tuber, when O₂ concentration is decreased to 12 kPa or 8 kPa O₂, the decrease in adenylate energy status and respiratory flux are significantly decreased (Geigenberger, 2003). In our study also when the concentrations of O₂ were reduced to 14 kPa or 8 kPa O₂, the O₂ uptake in mitochondria of *K. daigremontiana* and *A. comosus* was significantly decreased (Table 2). Furthermore, Affourtit et al. (2001) showed that decreasing O₂ concentrations may result in a low AOX activity. In our study, the low O₂ concentrations decreased the Alt respiration on succinate oxidation in both species (Fig. 5 and Table 2). It is probably that under low O₂ concentrations when COX and AOX activities become O₂ limited, ETC from UQ to Cyt or Alt pathway are inactivated, leading to a decrease in the O₂ uptake and Alt respiration.

Our results indicated that the Cyt respiration and Alt respiration on succinate oxidation were decreased markedly by the reduction of O₂ concentrations in mitochondria of both species. Generally, the Alt respiration rate on succinate oxidation at 21, 14 and 8 kPa O₂ in *A. comosus* was about 1.5 times higher than that in *K. daigremontiana*. The effects of O₂ concentration on the Alt respiration in the presence of 0.1 mM KCN were similar in the two species, and the low O₂ concentration in the presence of KCN significantly decreased the respiration in each species. Our results indicated that mitochondria of *A. comosus* oxidized succinate faster than *K. daigremontiana* under similar conditions and the Alt respiration rate was decreased by decreasing O₂ concentrations more strikingly in *K. daigremontiana* than in *A. comosus* (Table 2). Under the O₂ concentration of 8 kPa O₂, the Alt respiration rates on succinate oxidation were strongly decreased, and mitochondria in both species lost the Alt respiration pathway. This might be because under this condition, AOX activity in mitochondria was completely inactivated and the ETC from succinate dehydrogenase did not connect with Alt pathway.

Our study indicated that the effects of SHAM and KCN on NADH oxidation in mitochondria of *A. comosus* differed from those in mitochondria of *K. daigremontiana* (Figs. 1B and 1D). External NADH oxidation in mitochondria of *A. comosus* was more

connected with the Cyt pathway and less with the Alt pathway, so this oxidation could produce much more ATP in *A. comosus* than in *K. daigremontiana*. This might be one of the mechanisms in *A. comosus* to provide sufficient ATP for cytosolic PCK in the daytime. The decrease in Alt respiration on succinate oxidation in mitochondria of both species under a low O₂ concentration may have an adaptive function in responding to environmental changes. These species can decrease their O₂ consumption in response to low O₂ concentrations to avoid internal anoxia and increase their adaptive capacity.

In the present study, NADH oxidation in *A. comosus*, a typical PCK-CAM plant, was more sensitive to KCN than that in mitochondria of *K. daigremontiana*, a typical ME-CAM plant. We will continue to work with other species belonging to the two CAM groups in order to prove the different expressions of the Cyt respiration and Alt respiration as well as their roles in the ME-CAM and PCK-CAM species.

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