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Simultaneous Substrate Oxidations and Alternative Respiration in Intact *Hoya carnosa* Mitochondria

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Abstract
This research investigated the simultaneous substrate oxidations and alternative respiration with succinate, NADH and NADPH in mitochondria from *Hoya carnosa* leaves. The result shows that *H. carnosa* mitochondria readily oxidized succinate with respiratory control and typical ADP/O ratios. The mitochondria also oxidized both NADH and NADPH with significant rates in the present of Ca^{2+} in which *H. carnosa* mitochondria rapidly oxidized NADH with higher respiration rates than those of NADPH oxidation. Both individual and simultaneous oxidations of succinate, NADH and NADPH in intact mitochondria were very sensitive with cyanide (KCN), indicating that the cytochrome (Cyt) pathway was mostly activated in their electron transport chain (ETC). Also, that most of these oxidations could donate their electrons to Cyt pathways within the ETC of *H. carnosa* mitochondria. In addition, the measurable respiration rate with individual substrate oxidations was less than the total measurable respiration rate with simultaneous substrate oxidations. These results confirm that *H. carnosa* mitochondria are more closely connected with Cyt pathway rather than alternative (Alt) pathway and *H. carnosa* mitochondrial ETC was not fully saturated by a single substrate oxidation.

1. Introduction

Plant mitochondria are the compartments within cells responsible for respiration and oxidative phosphorylation. They contain both an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), each of which is unique in both structure and function. An important difference between these two membranes lies in their permeability. The OMM has pores that are freely permeable to molecules smaller than 10 kDa, allowing free diffusion of small molecules from the cytosol across the membrane and into the inner membrane space. This permeability allows the important energy metabolites of succinate, NADH and NADPH to freely pass through the OMM, but need to pass through the selectively permeable IMM. Normally, succinate is oxidized by succinate dehydrogenase, which is located on the inside surface of the outer membrane and it is well-known as complex II in the ETC. On the other hand, NADH and NADPH are oxidized by NADH dehydrogenase and NADPH dehydrogenase, respectively. Both of these enzymes are located on the outer surface of the IMM [13, 23] and both oxidize their respective metabolites and feed the electrons into the ETC via the ubiquinone (UQ) in C3 and C4 plants [18, 14, 15]. Recently, Hong *et al.* (2008) determined that *H. carnosa* mitochondria readily oxidized malate via a malate-aspartate shuttle and they oxidized NADH and NADPH with high respiration rate in the presence of ion calcium

[7]. More recently, Hong *et al.* (2012) found that complex I, external NAD(P)H dehydrogenases and internal NAD(P)H dehydrogenases were all linked to the electron transport chain [9].

The alternative pathway is well-known as a branch from the Cyt pathway at the ubiquinone (Q) and donates electrons directly to oxygen to form water. The Alt pathway is inhibited by salicylhydroxamic acid (SHAM) and the Cyt pathway is inhibited by KCN [21]. The Alt pathway was known to play an important role during the light period of Crassualacean acid metabolism (CAM) plants. It has been observed that there was a decrease in the Alt pathway of leaf respiration in plants showing CAM, especially during the first daylight hours, suggesting that engagement of the Alt pathway in CAM mitochondria during the light period would allow rapid malate oxidation to proceed simultaneously with photophosphorylation [19]. Our previous study found that *H. carnosa* possessed a malate - aspartate shuttle in the mitochondrial membrane, and during the light time, malic enzyme (ME) and malate dehydrogenase (MDH) systems were operated in parallel to control and regulate the overall malate metabolism, depending on CO_2 requirements and amino acid synthesis. Malate metabolism via the mME system was more connected to alternative (Alt) respiration and this respiration seemed not to produce much energy inside the mitochondria [10]. The relationship between Alt pathways with malic enzyme (ME) or malate dehydrogenase (MDH) activity in malate oxidation during the light was indicated in mitochondria of some plants. In fact, though not all plant mitochondria, which possess high ME activities, are highly KCN resistant [4]. Indeed, it is observed that malate oxidation via MDH was essentially linked to the cyanide sensitive electron transport pathway, whereas malate oxidation via ME is essentially linked to the cyanide-insensitive Alt pathway [20].

It had been indicated that under the physiological conditions, the mitochondria contain more than one respiration substrate, and, when two (or more) respiratory substrates are oxidized simultaneously and donate their reducing equivalents at the various segments of the respiratory chain, the competition for the coenzyme Q binding sites, which are among the fluxes of reducing equivalents derived from various dehydrogenases, is a universal mechanism for the control of ETC functioning. Cooperative oxidation of two or three substrates by mitochondria was accompanied with the dramatic increase in the rate of oxygen consumption in states 3 and 4, with completely additive oxidation rates for the individual substrates [22].

In this study, we further investigated respiratory properties with succinate as a single substrate in *H. carnosa* mitochondria, and the combined oxidation of succinate with one or two substrates of NADH and NADPH. Also, we determined the effects of the KCN and SHAM inhibitors on the Alt pathway in *H. carnosa* mitochondria on the oxidation of various substrates, single and combined. The aim of the present study was to investigate whether there are important differences in the general respiratory properties as well as Cyt and Alt respiration in the simultaneous two or three substrate oxidations, and provide additional information concerning Alt pathway participation in the respiratory metabolism of intact *H. carnosa* mitochondria.

2. Materials and Methods

2.1. Preparation of Plant Materials

Hoya carnosa plants were vegetatively propagated and grown in plastic pots in a greenhouse with a heater under natural light conditions. The leaves of mature plants 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.

2.2. Mitochondrial Isolation and Purification

Mitochondria were isolated and purified as described previously by Hong *et al*. [8, 9]. 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 KH_2PO_4, 5 mM $MgCl_2$, 10 mM KCl, 20 mM HEPES-KOH) and the pH was adjusted at 7.4 with 3 mM KOH. The mitochondria were preincubated with 0.16 mM ATP for 2 min to ensure full activation of succinate dehydrogenase before each assay with succinate oxidation. NADH and NADPH oxidations were investigated at pH 6.8 with 1mM Ca^{2+}.

Respiration control rate (RCR) and ADP/O ratios value were calculated according to Estabrook [5]. The O_2 concentration in air-saturated medium was taken as 258 mM. The protein content was measured by the method of Bradford [2] using BSA as the standard.

2.3. Effect of the Inhibitors

The effect of the inhibitors was investigated within the range of 25 μM of 400 μM for KCN (100 mM stock solutions were made in distilled water) and 1mM for SHAM (0.5 M stock solution in methoxyethanol). Measurements were conducted using an oxygen electrode with 2 mL of reaction medium. Oxygen concentration at zero was adjusted by using $Na_2S_2O_4$. The respiration 4-5 min after the addition of the inhibitor was used to calculate the percent inhibition.

3. Results

3.1. Mitochondria Isolation

We already published a successful protocol to isolate and purify mitochondria with percoll gradient from leaves of *H. carnosa*. The intactness of the outer and inner membranes of *H. carnosa* mitochondria was examined by the latency of both cytochrome oxidase (COX) and NAD^+-malate

dehydrogenase (NAD^+-MDH), and the mitochondria showed about 95% intactness of the outer membrane and 91% of the inner membrane [7, 8]. In this study, we continue to use this protocol to isolate and purify mitochondria from *H. carnosa* leaves, and then we used the mitochondria to investigate the simultaneous substrate oxidations and alternative respiration with succinate, NADH and NADPH in mitochondria from *H. carnosa* leaves.

3.2. Substrate Oxidations

We already published a protocol to isolate and purify *H. carnosa* mitochondria in which isolated mitochondria were not contaminated by other organelles and showed high intactness [7, 8]. We continued to use this protocol to isolate and purify mitochondria from *H. carnosa* leaves for further investigation of the capacity of *H. carnosa* mitochondria in single and simultaneous oxidations with the substrates of succinate, NADH and NADPH. The result in Figure 1 shows that under assay conditions *H. carnosa* mitochondria readily oxidized succinate, NADH, and NADPH with significant rates and coupling. The respiration rate on succinate oxidation of *H. carnosa* mitochondria was the highest in comparison with NADH and NADPH oxidations, and it was about 235 nmol mg^{-1} min^{-1} protein. The respiration control rate (RCR) and the ADP/O ratio in succinate oxidation by *H. carnosa* mitochondria was 1.8 and 1.7, respectively, indicating that succinate oxidation was coupled with two proton-extrusion sites (Figure 1). All of these results suggested that succinate oxidation in *H. carnosa* mitochondria was operated via a typical way and it was similar to what has been described in the mitochondria of the other CAM species such as *Kalanchoe daigremontiana,* and *Ananas cosmosus* [5].

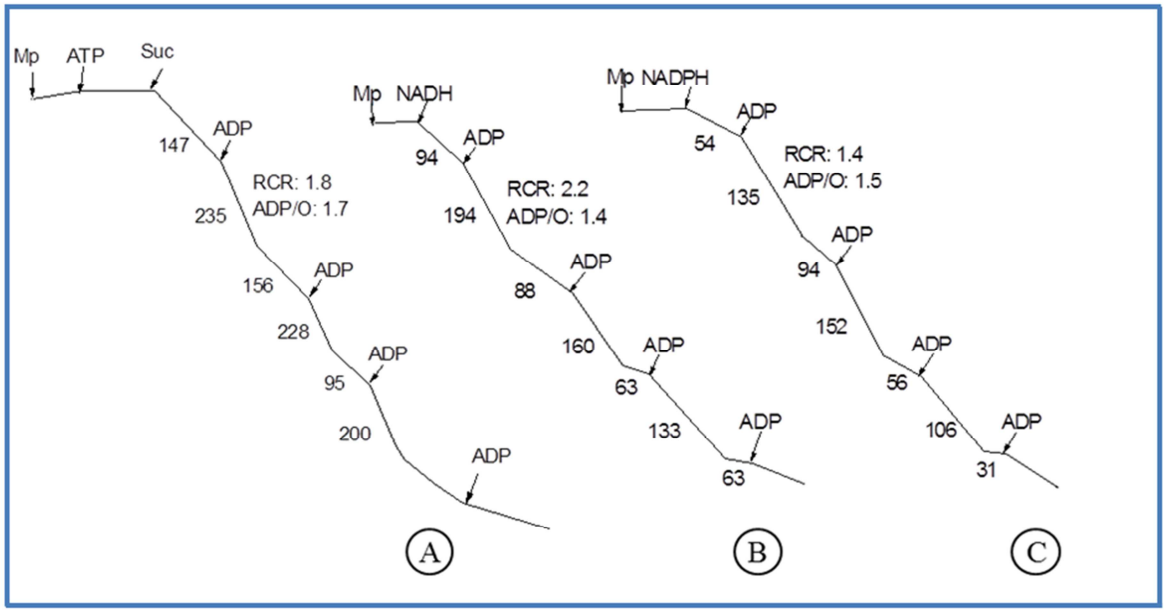

Figure 1. *A typical trace showing the substrates oxidations by H. carnosa mitochondria. Assay conditions were: 0.3 ml mitochondria (Mp), 0.16 mM ADP, 10 mM ATP, 10 mM succinate (A), 2 mM NADH (B), 2 mM NADPH (C). With succinate as a substrate, the mitochondria were preincubated with 0.16 mM ATP for 2 min to ensure full activation of succinate dehydrogenase before each assay. NADH and NADPH were oxidized in the presence of 1 mM $CaCl_2$, at pH 6.8. Numbers along the trace refer to nmol O_2 consumed $min^{-1}mg^{-1}$ protein.*

H. carnosa mitochondria readily oxidized both NADH and NADPH with significant respiration rates in which NADH was oxidized at a higher rate than that of NADPH oxidation. The ADP/O ratios of NADH and NADPH oxidations in figure 1 were less than 2, indicating that both NADH and NADPH oxidation was coupled with two proton-extrusion sites. Among the three investigated substrates of succinate, NADH and NADPH, the lowest respiration rate was recorded with NADPH.

3.3. Simultaneous Substrate Oxidations

Previous study found that *H. carnosa* mitochondria possessed the external NAD(P)H dehydrogenases in their membrane so they could oxidize NAD(P)H and donate their electron to ETC [9].). In this study, we checked again the capacity of NADH and NADPH oxidations as single substrates in order to compare and further investigate the simultaneous oxidations of two of these substrates together with succinate as well as the effect of the inhibitors to single and simultaneous oxidations of these two substrates with succinate in *H. carnosa* mitochondria. The average respiration rates in state 3 of the individual and simultaneous substrates oxidations in *H. carnosa* mitochondria were showed in Figure 2.

It had been indicated that in the mitochondria of many vascular plants such as cauliflower [4, 5], the *Iris* bulb [6], *Arum italicum* spadices [24], the potato [1] and castor bean (*Ricinus communis* L.) seedlings [22]. *K. daigremontiana,* and *A. cosmosus* [8] that the multiple substrate oxidations normally lead to an increase in the respiration rate. This rate

was higher than the individual rates but lower than the sum of individual rates. In this study, we also found that the addition of NADPH or NADH as the second substrates in succinate oxidation of *H. carnosa* mitochondria mainly stimulated the respiration rate that was significantly higher than the individual rate. Also, the addition of NADPH to NADH oxidation as a second substrate stimulated the respiration rate in the simultaneous oxidation of NADH and NADPH (Figure 2).

Based on these results, it is possible suggest that simultaneous substrates' oxidation normally produced O_2 uptake rates far in excess of those obtained with the single substrates oxidations. In our experiments, all of the simultaneous oxidation of substrates gave ADP/O ratios of lower than 2, indicating that two proton-extrusion sites were utilized.

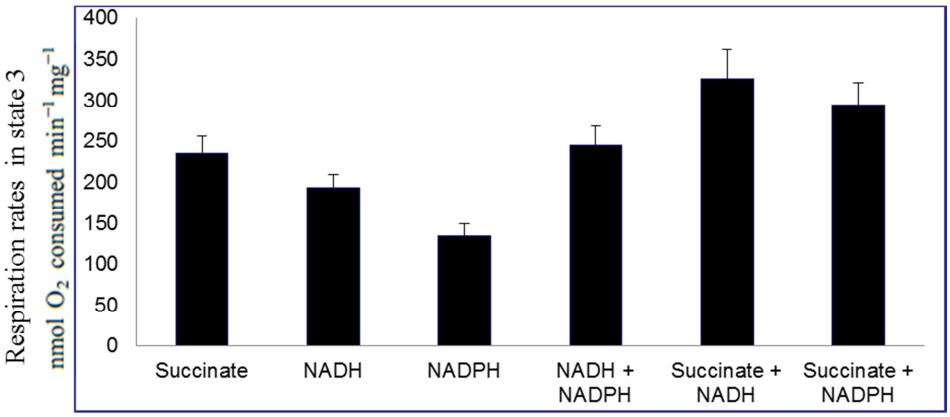

Figure 2. *Respiration rates in state 3 of the individual and simultaneous substrates' oxidations in H. carnosa mitochondria*

Oxygen uptake was measured as described in "Materials and Methods". Concentrations used were: 10 mM succinate, 2 mM NADH, 2 mM NADPH, 320 nmol ADP. State 3 refers to the respiration rate of O_2 uptake in the presence of ADP. Each value was the average of four or five independent experiments. Unless otherwise indicated, other assay conditions were as shown in Figure 1.

3.4. Effect of KCN on Single Substrate Oxidations

In *H. carnosa* mitochondria, the effect of KCN on the succinate oxidation was completely different with NADH and NADPH oxidation (Figure 3). Though all three single substrate oxidations were inhibited by KCN, succinate oxidation was less sensitive to KCN than those of NADH and NADPH oxidations (Figure 3). Adding 25 µM KCN to single substrate oxidations of *H. carnosa* mitochondria caused an inhibition about 22,14% of state 3 respiration rate for succinate oxidation whereas these inhibition respiration rates for NADPH and NADH oxidations were about 49,12% and 56,73%, respectively (Figure 3). Also, the oxidations of NADH and NADPH were completely inhibited by 200 µM KCN while succinate oxidation was inhibited about 88,6% by 200 µM KCN (Figure 3). NADH oxidation in *H. carnosa* mitochondria was completely inhibited by 100 µM KCN while NADPH oxidation was inhibited about 94,47% by 100 µM KCN, indicating that NADPH oxidation was more sensitive with KCN than NADH oxidation (Figure 3).

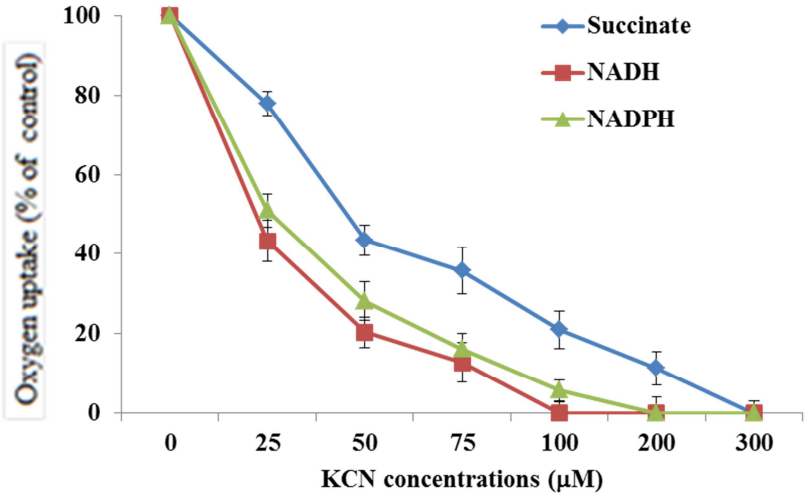

Figure 3. *Effect of KCN on succinate, NADH and NADPH oxidations in Hoya carnosa mitochondria. Assay conditions were: 10mM succinate with 0.16 mM ATP, 1 mM NADH, 1 mM NADPH, 1mM Ca^{2+} and 0.32 mM ADP. Control values were calculated in the absence of KCN and they were considered as 100% in these assays.*

3.5. Effect of both KCN and SHAM on Single Substrate Oxidations

Effects of both KCN and SHAM on succinate, NADH and NADPH oxidations were shown in Figure 4. In the presence of 50 µM KCN, succinate oxidations in *H. carnosa* mitochondria showed about 56,58% of the oxygen uptake rates. Adding 100 µM KCN to substrate oxidations of *H. carnosa* mitochondria completely blocked NADH and NADPH oxidations (data was not shown), while this concentration only inhibited about 79.4% of the oxygen uptake in state 3 for succinate oxidation. Concurrent addition of 50 µM KCN and 1mM SHAM on succinate oxidation leading to a complete inhibition of total respiration rate (Figure 4A). For NADH and NADPH oxidations in *H. carnosa* mitochondria, addition of 50 µM KCN and 1mM SHAM completely blocked the residual respiration (Figure 4B and Figure 4C). Based on the inhibition by both KCN and SHAM in these three substrate oxidations, it is possible to suggest that similar oxidations of mitochondria in other vascular plants, The mitochondria of an intermediate CAM plant such as *H. carnosa* not only possessed the Cyt pathway but also the Alt pathway in their ETC.

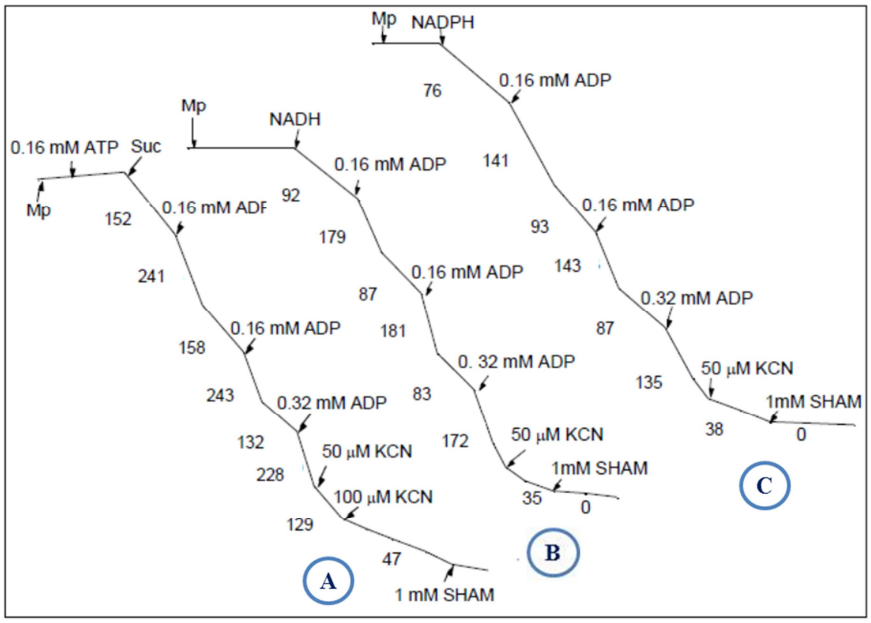

Figure 4. *The effects of both KCN and SHAM on the oxidations of 10 mM succinate (A), 2 mM NADH (B), and 2 mM NADPH (C) in H. carnosa mitochondria. The numbers along the traces refer to nmol O_2 consumed min^{-1} mg^{-1} protein. Mp that is purified mitochondria. Unless otherwise indicated, other assay conditions were as shown in Figure 1. The values were presented from a typical result of three independent experiments.*

3.6. Effects of KCN and SHAM on Concurrent Substrate Oxidations

The results in Table 1 show the effects of KCN and SHAM on concurrent oxidations of NADPH, NADH, and succinate as the substrates. In our experiments, the simultaneous oxidations of the above two substrates were coupling with the higher respiration rates than those in comparing with individual substrate oxidations. Actually, treatment of 50 µM KCN and 1 mM SHAM completely blocked individual NADH oxidation and NADPH oxidations (Figure 4B and 4C). However, this concentration inhibited about 94.4% of the state 3 rates in the simultaneous oxidations of NADPH and NADH (Table 1). The responses of simultaneous oxidations of succinate and NADH or succinate and NADPH were inhibited about 83.6% and 83% by addition of 50 µM KCN and 1 mM SHAM, respectively (Table 1).

Table 1. *The effect of KCN and SHAM on substrate oxidations in H. carnosa mitochondria.*

Substrate	Respiration rate in state 3 (nmol O_2 $min^{-1}mg^{-1}$ protein)		
	No inhibitor	+ 50 µM KCN	+ 1mM SHAM
NADH + NADPH	214 ± 18	74 ± 11	12 ± 3
Succinate + NADH	293 ± 17	53 ± 11	48 ± 7
Succinate + NADPH	247 ± 13	74 ± 09	42 ± 6
Succinate + NADH + NADPH	328 ± 19	85 ± 8	64 ± 9

4. Discussion

In this study, *H. carnosa* mitochondria easily oxidized succinate, NADH and NADPH as individual substrates with significant respiration rates (Figure 1), and cooperative oxidation of succinate and NADH or succinate and NADPH were accompanied with the dramatic increase in the rate of oxygen

consumption with completely additive oxidation rates for the individual substrates (Figure 2). These results indicated that external NADH and NADPH were oxidized by two different enzymes of exogenous NADH and NADPH dehydrogenases located on the outer surface of the inner membrane and these NAD(P)H dehydrogenases were activated in the cooperative oxidations of succinate with NADH or NADPH.

According to the published evidence on animal and yeast mitochondria, when two (or more) respiratory substrates are oxidized simultaneously and donate the reducing equivalents at the various segments of the respiratory chain, the competition for the coenzyme Q binding sites among the fluxes of reducing equivalents derived from various dehydrogenases is a universal mechanism for the control of ETC functioning. This competition must hamper the oxidation of each particulate substrate, with the less active dehydrogenase inhibited to the highest extent [22].

It has been also showed that in mitochondria of *Petunia hybrida*, Potato and Iris [6] or mitochondria of castor bean hypocotyls (Shugaev and Vyskrebentseva, 1999), the simultaneous oxidation of two or three substrates accompanied with the dramatic increase in the rate of oxygen consumption and Alt respiration with completely additive oxidation rates for the individual substrates in mitochondria of these species. Shugaev and Vyskrebentseva [22] explained that cooperative oxidation of several substrates, that is, the simultaneous functioning of two or more dehydrogenases in the respiratory chain of the mitochondria, was accompanied by a considerable enhancement of the oxygen consumption, which have completely additive rates for oxidation of the individual substrates

In this study, we found that normally the cooperative oxidation of two or three substrates of succinate, NADH and NADPH by *H. carnosa* mitochondria not only increased the respiration rates but also increased the Alt respiration (Table 1, Figure 3 and Figure 4). Succinate oxidation in *H. carnosa* mitochondria always showed much higher total respiration rates and the Alt respiration rate in cooperative succinate and NADH or succinate and NADPH oxidations were higher than those of individual substrate oxidations. As a result, not only total respiration rate but also Alt respiration was increased in simultaneous oxidations. The characteristics by the additive of the individual rates in the simultaneous oxidation of two or three substrates indicated that the oxygen uptake in *H. carnosa* mitochondria was not saturated with one substrate. These results provided the evidence for previous reports that mitochondrial respiration and Alt pathway were not fully saturated with single substrate in plant mitochondria [22, 24]). Previous study found that in the mitochondria of other CAM plants such as *Crassula lycopodioides* (Lam), the cyanide resistance respiration on succinate or NADH oxidations in the presence of 500 μM KCN were about 11-17% or 14%, respectively [17]. However, NADH and NADPH oxidations in *H. carnosa* mitochondria were completely blocked in the presence of 200 μM KCN and succinate oxidation were completely blocked in the presence of 400 μM KCN, indicating that cyanide insensitive pathway of respiration is not very activated in the mitochondria of *H. carnosa* plants.

5. Conclusion

Based on the results of this research, it is possible to suggest that under conditions of these experimentations, *H. carnosa* mitochondria readily oxidized succinate, NADH, and NADPH in significant rates and with coupling in two of the proton-extrusion sites. Furthermore, *H. carnosa* mitochondria oxidize the simultaneous substrates with respiration rates much higher than that with singe substrate and substrate oxidations in *H. carnosa* mitochondria were very sensitive with KCN.

Acknowledgments

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Abbreviations

Alt., alternative; CAM, crassulacean acid metabolism; Cyt., cytochrome; ETC, electron transport chain; KCN, cyanide; Mp, purified mitochondria; RCR, respiratory control ratio; SHAM, salicylhydroxamic acid.

References

[1] Arrabaca, J. D., Ribeiro-Lima, I. M. R., Tenreiro, A. M. (1992). Interaction between the oxidation of external NADH and NADPH in plant mitochondria. In: Lambers, H., Van der Plas, L. H. W. (ed.): *Molecular, Biochemical and Physiological Aspects of Plant Respiration*. pp. 125-131. SPB Academic Publ., The Hague.

[2] Bradford MM. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analysis Biochemistry*, 72: 248-254.

[3] Day DA, Millar AL, Dry IB, Wiskich JT, Whelan J. (1994). Regulation of alternative oxidize activity by pyruvate in soybean mitochondria. *Plant Physiology*, 106: 1421-1427.

[4] Day DA, Rayner JR, Wiskich JT. (1976). Characteristics of external NADH oxidation by beetroot mitochondria. *Plant Physiology*, 58: 3-42.

[5] Estabrook, R. W. (1967). Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. *Methods Enzymol*, 10: 41-45.

[6] Hemrika-Wagner, A. M., Gude, H., Marissen, N., Vander Plas, L. H. W., Verleur, J. D.: Compartmentation of alternative oxidase in plant mitochondria. - Plant Cell Physiology, 27: 499-503, 1986.

[7] Hong H. T. K., Nose A., Agarie S., Yoshida T. (2008). Malate metabolism in *Hoya carnosa* mitochondria and its role in photosynthesis during CAM phase III. *Journal of Experimental Botany*, 59: 1819-1827.

[8] Hong H. T. K., Nose A., Agarie S. (2005). Effects of KCN, SHAM and low oxygen conditions on respiratory properties of purified mitochondria isolated from pineapple and *K. daigremontiana*. *Plant Production Science*, 8: 383-392.

[9] Hong HTK, Nose A. (2012). Characteristics of external and internal NAD(P)H dehydrogenases in *Hoya carnosa* mitochondria. *Journal of Bioenergetics and Biomembranes,* 44 (6): 655-664.

[10] Hong H. T. K., Nose A., Agarie S. Yoshida T. (2008). Malate metabolism in *Hoya carnosa* mitochondria and its role in photosynthesis during CAM phase III. *Journal* of Experimental Botany, 59: 1819-1827.

[11] Hong H. T. K., Nose A., Agarie S. (2004). Respiratory properties and malate metabolism in Percoll-purified mitochondria isolated from pineapple, *Ananas comosus* (L.) Merr. cv. smooth cayenne. *Journal of Experimental Botany,* 55: 2201-2211.

[12] Hong H. T. K., Nose A., Agarie S. (2005). Effects of KCN, SHAM and low oxygen conditions on respiratory properties of purified mitochondria isolated from pineapple and *K. daigremontiana. Plant Production Science,* 8: 383-392.

[13] Johansson F. I., Michalecka A. M., Møller I. M., Rasmusson A. G. (2004). Oxidation and reduction of pyridine nucleotides in alamethicin permeabilized plant mitochondria. *Biochemical Journal,* 380: 193-202.

[14] Møller IM. (1977). The oxidation of cytosolic NAD(P)H by external NAD(P)H dehydrogenases in the respiratory chain of plant mitochondria. *Physiologia Plantarum*, 100: 85-90.

[15] Møller, I, M. (2002). A new dawn for plant mitochondrial NAD(P)H dehydrogenases. *Trends in Plant Sciences,* 7: 235-237.

[16] Møller IM. (1997). The oxidation of cytosolic NAD(P)H by external NAD(P)H dehydrogenases in the respiratory chain of plant mitochondria. Physiologia Plantarum. 100: 85-90.

[17] Peckmann K., Eillert D. J. V., Martin C. E., Herppich W. B. (2012). Mitochondrial respiration in ME-CAM, PEPCK-CAM, and C_3 succulents: comparative operation of the cytochrome, alternative, and rotenone-resistant pathways. *Journal of Experimental Botany*, 63 (8): 2909-2012.

[18] Rasmusson, A. G., Møller, I. M. (1991). Effect of calcium ions and inhibitors on internal NAD(P)H dehydrogenase in plant mitochondria. *European Journal of Biochemistry,* 202: 617-623.

[19] Robinson S. A., Yakir D., Ribas-Carbo M., Giles. L, Osmond C. B., Siedow J. N., Berry J. A. (1992). Measurements of the engagement of cyanide-respiration in the crassulacean acid metabolism *Kalanchoë daigremontiana* with the use of on-line oxygen isotope discrimination. *Plant Physiology*, 100: 1087-1091.

[20] Rustin P., Queiroz-Claret C. (1985). Changes in oxidative properties of *Kalanchoë blossfeldiana* leaf mitochondria during development of Crassulacean acid metabolism. *Plant Physiology,* 164: 415-422.

[21] Siedow JN, Day DA. (2000). Respiration and photorespiration. BB Buchanan, W Gruissem, and RL Jones (eds). In *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologists, Rockville, Maryland, USA. 676-725.

[22] Shugaev A. G., Vyskrebentseva E. I. (1999). Cooperative substrate oxidation by mitochondria from Castor been hypocotyls. *Russian Journal* of *Plant Physiology*, 48: 165-170.

[23] Smith C., Barthet M., Melino V., Smith P., Day D., Soole K. (2011). Alterations in the Mitochondrial Alternative NAD(P)H Dehydrogenase NDB4 Lead to Changes in Mitochondrial Electron Transport Chain Composition, Plant Growth and Response to Oxidative Stress. Plant Cell Physiology 52 (7): 1222-1237.

[24] Tenreiro A. M., Vaz-Pinto, Arrabaca, J. D. (1922). Simultaneous oxidation of substrates and the effects of specific inhibitors in mitochondria from *Arum italicum* spadices. - In: Lambers, H., Van der Plas, L. H. W. (ed.): *Molecular, Biochemical and Physiological Aspects of Plant Respiration*. Pp: 117-123. SPB Academic Publ., The Hague.