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The influence of ascorbic acid on the oxygen consumption and the heat production by the cells of wheat seedling roots with their mitochondrial electron transport chain inhibited at complexes I and III

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Abstract

The influence of exogenous ascorbic acid (AsA) on oxidative phosphorylation was studied using wheat seedling roots. Treatment of them with AsA stimulated the rates of oxygen consumption and the heat production and caused a decrease of the respiratory coefficient. The increase in respiration was prevented by inhibitors of ascorbate oxidase, diethyldithiocarbamate (DEDTC), and of cytochrome oxidase, cyanide (KCN). Exogenous AsA sharply stimulated the rate of oxygen consumption of roots when complexes I and III of the mitochondrial electron transport chain were inhibited by rotenone and antimycin A, respectively, while the rates of heat production did not change significantly. It is concluded that AsA is a potent energy substrate, which can be used in conditions of failing I and III complexes in the mitochondrial electron transport chain.
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Keywords: Wheat seedling roots; Ascorbic acid; Respiratory poisons; Oxygen uptake rate; Heat production rate; Electron transport chain

1. Introduction

AsA is well known to have a great importance in the metabolism of cells because it is involved in redox processes and the transfer of reducing equivalents via the plasma membrane [1]. Ascorbate is present in cells mainly in the reduced form and is found in many different systems [2]. Less is known about the role of AsA in the energy metabolism of cells. It has been shown that ascorbate can be oxidized not only in the cytosol but also in mitochondria by cytochrome *c* and cytochrome oxidase resulting in the formation of ATP [3,4]. AsA is synthesized endogenously in mitochondria at the first and third complexes of the electron transport chain and cytochrome *c* [5]. In addition, ascorbate has a role in cell energetics during the reverse electron transport in mitochondria resulting in the formation of reduced pyridine nucleotides [6]. Ascorbate is quantitatively the predominant antioxidant in plant cells. It is found in all subcellular compartments and has an average cellular concentration of 2–25 mM or more in the chloroplast stroma [7]. An extramitochondrial pathway, in addition the electron transport chain, has recently been proposed for its synthesis [8]. The amount of AsA in chloroplasts greatly increases under stress conditions. It has been suggested that a high concentration of AsA in cells can possibly serve in case of the breakdown of systems providing ascorbate regeneration [9], which is said particularly to be important in stress conditions [5].

Until now there has been no experimental data on the pathways of ascorbate oxidation introduced exogenously or the coupling of ascorbate oxidation with heat production as an indicator of its metabolism in plant cells. Given the fact that some of the AsA is synthesized and oxidized in mitochondria, the main task of this work was to study mitochondrial ascorbate oxidation by measuring the heat production of wheat roots in optimal conditions and following the inhibition of electron transport in mitochondria.

2. Experimental

The excised roots of 5-day-old wheat (*Triticum aestivum* L.) seedlings grown hydroponically in a solution of 0.25 mM $CaCl_2$ were used as the object of the investigation. In all incubation solutions with effectors and inhibitors, $CaCl_2$ as a membrane-stabilizing compound was present. The pH of all solutions was

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adjusted to 7.0. Roots incubated in 0.25 mM $CaCl_2$ only were used as a control.

Potassium release from cells was determined from the change of its content in the solution after incubation of roots and measured with a flame photometer, Phlapho 41 (Carl Zeiss, Jena, Germany).

The heat production by roots was measured using an LKB differential microcalorimeter (LKB-2277 Bio Activity Monitor, LKB-Produkter AB, Bromma, Sweden) at 30 °C. Excised roots in 3 ml of the various solutions were sealed in the glass vials and incubated for 5 h. The reference vial contained only 0.25 mM $CaCl_2$ and there were two test vials. There was only one case in which a compound was added during the experiment (3 mM AsA) and this required removal of the vial from the calorimeter, injecting a concentrated solution of the effector and re-equilibrating the vial to 30 °C. For the graphical representation of the calorimetric data, points were allocated on the heat flow curve with 1 h intervals (using the graphic editor Origin 5.0, not in the actual calorimetrical records) for convenience to show the statistical variables of the various experiments (average value, S.E.) and for comparison of the calorimetric data with those of respiration at these points.

The oxygen consumption and carbon dioxide production were measured in parallel using Warburg's manometric method [10,11]. Twenty millilitre glass vessels containing 150 mg of excised roots and 3 ml of incubation solution were shaken at 110 oscillations per minute at 30 °C for 5 h.

For the treatments, the test vials and vessels containing the roots incubated with 0.25 mM $CaCl_2$ or 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A were placed in the calorimeter or Warburg apparatus, respectively. For the latter, vessels were taken out at certain intervals of time in order to add ascorbate, KCN or DEDTC at final concentrations of 5, 3 and 5 mM, respectively. Then the vials were reloaded into the apparatus. CO_2 was absorbed with 20% NaOH and the respiratory coefficient was calculated from the ratio of CO_2/O_2.

The intracellular content of ATP and GTP was assayed by the reverse-phase high-pressure liquid chromatography (HPLC) method as described in Ref. [12], with modifications for plant material. Separation was carried out in an isocratic manner on the Polymer-C_{18} column (Astec, USA) with phosphate buffer as the mobile phase. The intracellular nucleotide content of excised roots was expressed in μmole per gram fresh weight (f.w.).

Rotenone and antimycin A were dissolved in 100 μl of ethanol and then brought up to necessary volume with 0.25 mM $CaCl_2$. The equivalent amount of ethanol was added to the control solutions.

The calorimetric experiments were repeated five times. The other experiments were performed four or five times with three replicates. The standard error (S.E.) was calculated using the program Microcal Origin™ V. 5.0.

3. Results

It can be seen in Fig. 1 that treatment of roots with AsA caused the stimulation of root respiration by 40%. It is necessary to note that the respiratory coefficient (CO_2/O_2) of AsA treated roots was lower compared to that of the controls (Table 1). It is also shown in Fig. 1 that the AsA-induced stimulation of respiration was abolished in the presence of either DETDC or KCN and in fact the respiratory poisons resulted in less respiration than in the control. Simultaneously with the rise in the intensity of respiration, AsA induced a considerable increase in the rate of heat production by roots (Fig. 2), which was inhibited

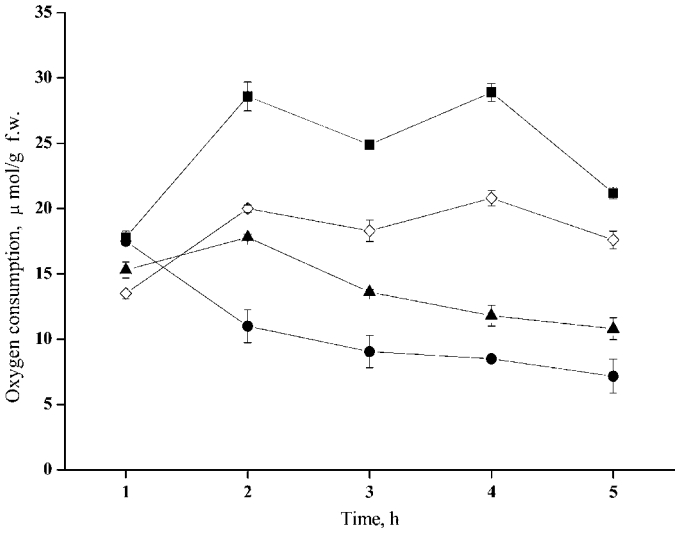

Fig. 1. The effect of AsA, DEDTC and KCN on the respiration of wheat roots: ◊, control (0.25 mM $CaCl_2$); ■ , 0.25 mM $CaCl_2$ + 5 mM AsA; ▲, 0.25 mM $CaCl_2$ + 5 mM AsA + 5 mM DEDTC; ●, 0.25 mM $CaCl_2$ + 5 mM AsA + 3 mM KCN.

Table 1
Influence of AsA on the respiratory coefficient (CO_2/O_2) of wheat roots

Treatment	Time (h)				
	1	2	3	4	5
Control	1.04	0.92	1.06	1.02	1.06
AsA (5 mM)	0.71	0.63	0.87	0.75	0.86

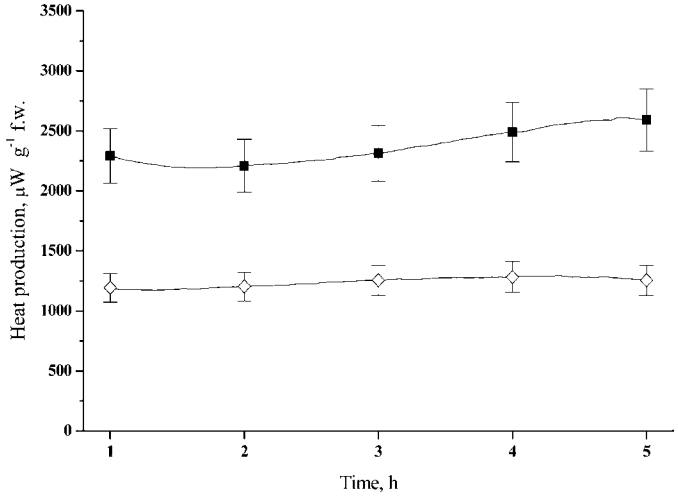

Fig. 2. The effect of AsA on the heat production of wheat roots: ◊, control (0.25 mM $CaCl_2$); ■ , 0.25 mM $CaCl_2$ + 5 mM AsA.

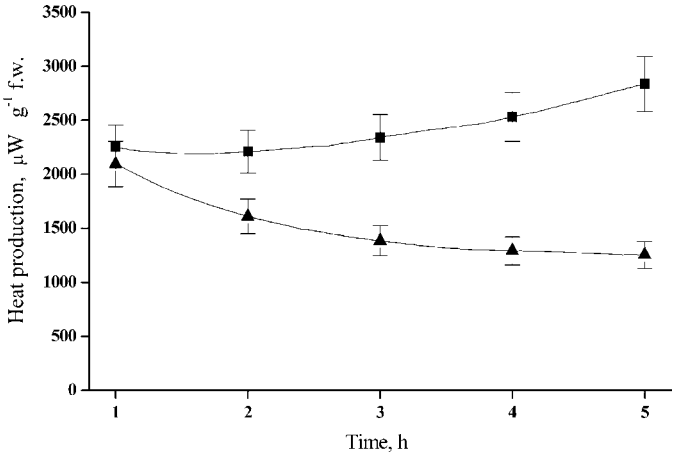

Fig. 3. The effects of AsA and DEDTC on the heat production of wheat roots: ■, 0.25 mM $CaCl_2$ + 5 mM AsA; ▲, 0.25 mM $CaCl_2$ + 5 mM AsA + 5 mM DEDTC.

by DEDTC (Fig. 3) and KCN (Fig. 4). It can be seen that heat production in the AsA-treated roots (Figs. 2–4) varied between experiments. This was because in a living system there is biological variability. The important finding is that the AsA control and roots treated with inhibitor were significantly different. It should be noted that AsA had no effect on membrane permeability judged by the fact that in the presence of ascorbate the level of potassium loss by root cells was similar to that in the control (0.6 ± 0.2 and 0.4 ± 0.1, respectively).

Data for the influence of AsA on the respiration of roots when complexes I and III of the mitochondrial electron transport chain were inhibited by rotenone and antimycin A are presented in Fig. 5. When they were added to the roots at the beginning of the incubation period, the rate of respiration initially was less than in the control (1–3 h) but then increased to give a similar value to it (Fig. 5). After incubation of the roots with the poisons for 3 h, AsA was added to the medium and it caused a sharp increase in the oxygen consumption of up to 80%. This reaction was sensitive to KCN (Fig. 5).

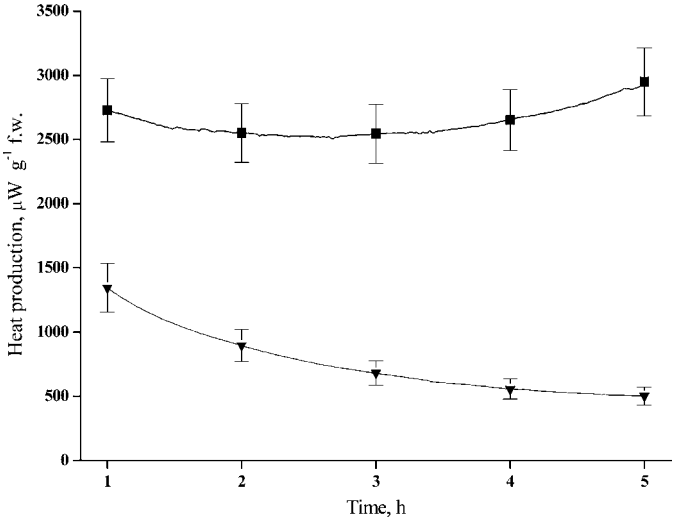

Fig. 4. The effects of AsA and KCN on the heat production of wheat roots: ■, 0.25 mM $CaCl_2$ + 5 mM AsA; ▼, 0.25 mM $CaCl_2$ + 5 mM AsA + 3 mM KCN.

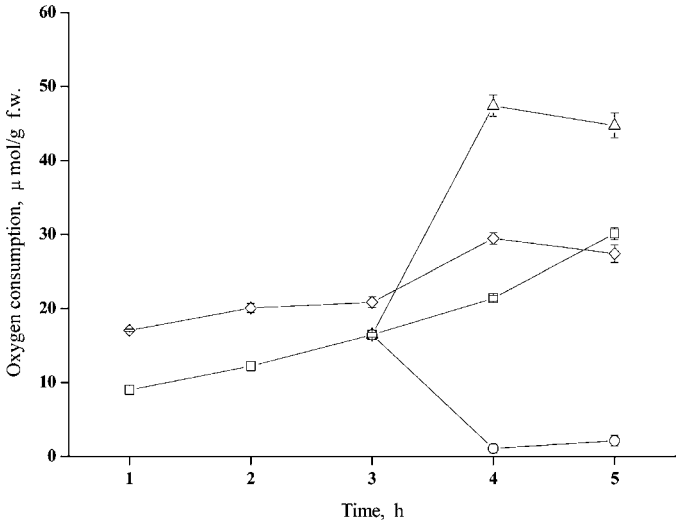

Fig. 5. The effect of AsA on the respiration of wheat roots in presence of rotenone and antimycin A: ◊, control (0.25 mM $CaCl_2$); □, 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A; △, 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A + 5 mM AsA. After 3 h measuring the rates of oxygen consumption by roots in a solution of 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A, AsA was added from a stock solution to give a final concentration of 5 mM; ◯, 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A + 5 mM AsA + 3 mM KCN. After 3 h measuring the rates of oxygen consumption by roots in solution of 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A, AsA was added from a stock solution to give a final concentration of 5 mM and KCN was added from stock solution with final concentration of 3 mM. The appropriate amount of 0.25 mM $CaCl_2$ was added to the control vessels.

Rotenone and antimycin A also caused a decrease in the heat production between 1 and 3 h of incubating the roots with them (Fig. 6), but by 4–5 h they had become markedly less effective inhibitors. The exogenous application of AsA to roots after 3 h of incubation with rotenone and antimycin A only caused a 20% increase in heat production (Fig. 7), compared to the 80% rise in oxygen consumption (see Fig. 5).

The amount of intracellular ATP slightly increased after the addition of AsA from 0.13 ± 0.01 μmol/g f.w. with rotenone

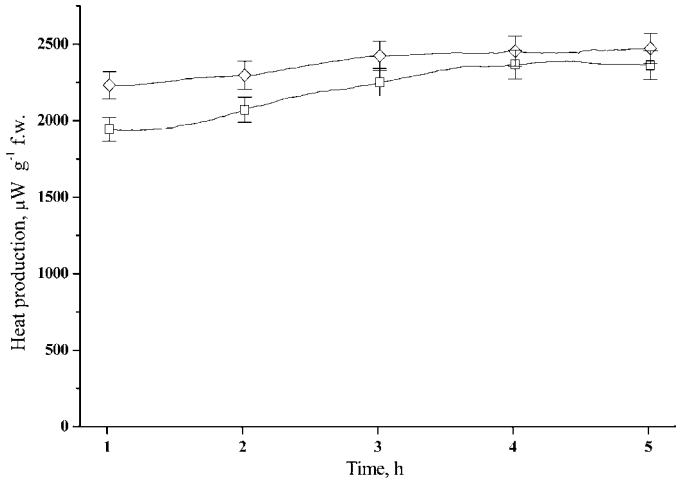

Fig. 6. The effects of rotenone and antimycin A on the heat production of wheat roots: ◊, control (0.25 mM $CaCl_2$); □, 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A.

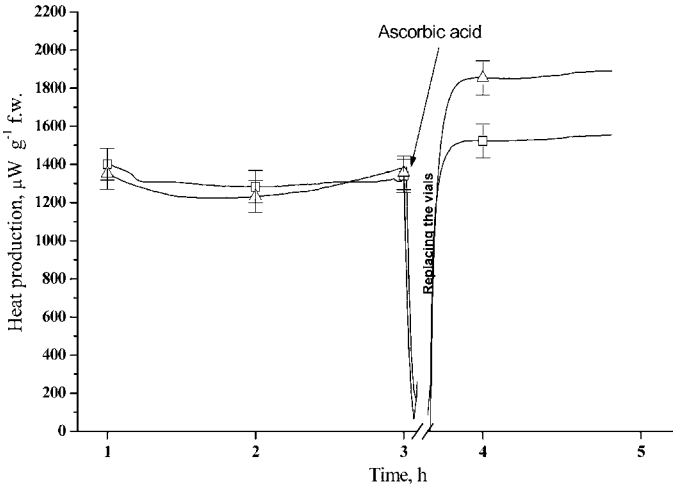

Fig. 7. The effects of rotenone, antimycin A and AsA on the heat production of wheat roots: □, 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A; △, 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A + AsA. After 3 h measuring the rates of heat production by roots in a solution of 0.25 mM $CaCl_2$ + 10 μM rotenone + 10 μM antimycin A, both vials were removed and AsA was added from stock solution to give a final concentration of 5 mM. The appropriate amount of 0.25 mM $CaCl_2$ was added to the control vial.

and antimycin A to $0.17 \pm .01$ μmol/g f.w. Under the same conditions, the amount of intracellular GTP increased from 0.18 ± 0.03 μmol/g f.w. with rotenone and antimycin A to 0.26 ± 0.01 μmol/g f.w.

4. Discussion

In our experiments, AsA greatly stimulated the oxygen consumption by roots during the 5 h treatment period. It is not clear yet which is the pathway for ascorbate oxidation in wheat root cells but there are two possible mechanisms for it. Firstly, it is due to the activity of ascorbate oxidase. This is found in the cytosol and also close to the cell wall [2]. To prove that ascorbate oxidase is involved in the metabolism of AsA, we used its specific inhibitor DEDTC. It was shown that the inhibition of this oxidase indeed caused a decrease in the rate of oxygen consumption (Fig. 1). But there is a second oxidizer in the cells, the mitochondrial cytochrome oxidase [3,13]. Using its inhibitor, cyanide, we made an attempt to estimate the contribution of this enzyme to AsA oxidation and found that it had a strong effect (see Fig. 1). However, cyanide not only can inhibit the activity of cytochrome oxidase but also of ascorbate oxidase. In addition, DEDTC has a weak inhibitory effect on the activity of cytochrome oxidase. Thus, it is hard to distinguish AsA oxidation by ascorbate oxidase from that by cytochrome oxidase. According to recent data in the literature [5,14], the mitochondrial synthesis of AsA is coupled with the electron transport and is sensitive to rotenone, antimycin A and cyanide. It suggests that the AsA oxidation can involve the mitochondrial electron transport chain. In our experiments the AsA caused the decrease of the respiratory coefficient at the same time as the intensification of oxygen consumption (see Fig. 5). Reduction of the respiration coefficient might suggest that AsA is incompletely oxidized by oxygen without the formation of

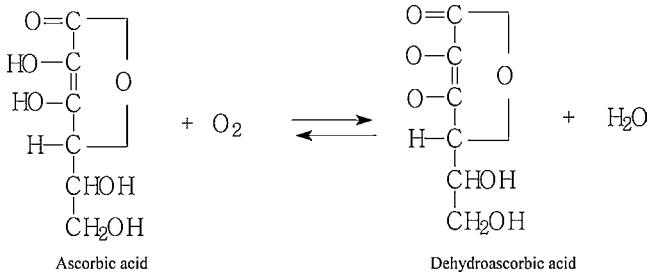

Fig. 8. Scheme for the partial oxidation of AsA without the formation of CO_2.

H_2O and CO_2. The chemical mechanism of AsA is presented in the scheme according to Davies et al. [2] which is shown in Fig. 8.

It is possible that AsA caused physical damage to the cells. However, the AsA-induced intensification of oxygen consumption did not result in the increase of the potassium loss by cells. This suggests that at least the AsA did not disrupt the integrity of plasma membrane.

When roots were treated with AsA, there was a great increase in heat production (see Fig. 2) but the cause of it is still an open question. On the other hand, a most interesting finding was that depicted in Fig. 5 showing that AsA oxidation occurred under stress conditions when the oxygen consumption was inhibited by the combination of the respiratory poisons, rotenone and antimycin A, respectively, of the I and III complexes of mitochondrial electron transport chain. However, the inhibition of respiration was only observed during the first few hours of incubation and was followed later by its stimulation. In an earlier paper, we showed that the intensification of root respiration caused by rotenone and antimycin A is due to the activation of electron transport via the outer mitochondrial membrane and cytochrome *c* to cytochrome oxidase [15]. Now we have shown that the AsA-induced increased oxygen consumption in root cells poisoned with rotenone and antimycin A was completely prevented by the application of KCN (see Fig. 1). These results allow us to suggest that the AsA in such conditions is oxidized via cytochrome oxidase and possibly coupled with ATP synthesis. Indirectly this assumption is confirmed by data showing that AsA is an energy substrate which under heat shock conditions can even prevent apoptosis [16]. In this respect, it should be recalled that, for humans suffering from myopathy caused by the deficiency of the middle part of the mitochondrial respiratory chain, the intake of high doses of ascorbate can ameliorate the energy expense of skeletal muscles [17,18].

Based on the results obtained we suppose that the AsA is a powerful energy substrate, which can be used in the conditions of inhibition of the ETC I and III complexes.

Acknowledgments

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