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LACK OF EMBRYONIC AXIS CONTROL IN *PISUM* COTYLEDON MITOCHONDRIA

IRITH MARBACH and ALFRED M. MAYER

Botany Department, The Hebrew University of Jerusalem, Jerusalem, Israel

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Key Word Index—*Pisum elatius*; *Pisum sativum*; Leguminosae; cotyledons; respiratory control ratio; ADP:O ratio; mitochondria.

Abstract—Respiratory control ratio (RCR), ADP:O and oxygen uptake by isolated mitochondria from cotyledons of the genus *Pisum* were studied. It is shown that in *P. sativum* the embryonic axis has a slight effect on the behaviour of the mitochondria in the cotyledons, accelerating their degeneration. The inducing factor is transferred within 1 hr from the onset of imbibition from the axis to the cotyledon. In *P. elatius* the embryonic axis completely lacked an effect on the mitochondria in the cotyledons. Mitochondria in *P. elatius* seemed to be highly organized and not leaky.

INTRODUCTION

In a previous paper[1] we showed that mitochondria of cotyledons from *P. elatius* remained fully functional for 96 hr of germination while those from *P. sativum* began to degenerate between 48 and 72 hr of germination. The behaviour of mitochondria from cotyledons of *P. sativum* has been investigated in considerable detail[2–9]. Most of this work indicated that the embryonic axis was responsible for inducing degenerative changes in the cotyledons. It was possible to prevent such changes by excision of the axis.

The interaction of the embryonic axis with storage organs such as the cotyledons has been studied in many seeds[10]. However, as far as control of respiration is concerned, there are relatively few data except those relating to peas. Since there was a large difference in the behaviour of *P. sativum* and *P. elatius*, we decided to reinvestigate axis–cotyledon interactions in the respiration of *P. sativum* and to extend the study to *P. elatius*. This paper reports some of our results.

RESULTS AND DISCUSSION

Mitochondria isolated from the cotyledons of the cultivated pea *P. sativum* are uncoupled after 72 hr of seed germination while those of the wild pea, *P. elatius*, remain efficient and coupled even after 96 hr of seed germination[1]. The present paper tries to investigate the cause of the difference between the two species. The cotyledons of the wild pea after 72 hr of germination were ground together with those of the cultivated pea of the same age. Mitochondria from *P. elatius*, in the presence of those from *P. sativum*, behaved as if they had been examined alone. The rates of O_2 uptake were simply additive. The respiratory control ratio (RCR), calculated after correction for the O_2 uptake of the *P. sativum* mitochondria, was the same as that of mitochondria of *P. elatius* alone. This indicated that the cotyledons from the cultivated peas did not contain an uncoupling factor and that the uncoupling did not occur during preparation of the mitochondria.

Other workers have also reported that mitochondria from the cotyledons of germinated *P. sativum* are uncoupled after 72 hr[6, 7], but that this uncoupling could be prevented. Thus, Nawa *et al.*[6] showed that if the embryonic axis of *P. sativum* is excised, the mitochondria in the cotyledons remain fully functional for 144 hr. They suggested that the embryonic axis exerts a direct influence on the behaviour of mitochondria in the cotyledons. The attached cotyledons of *P. elatius*, appeared to behave like the detached cotyledons of *P. sativum* described in ref. [6]. A detailed comparison of the two species might lead to a better understanding of the control of mitochondrial behaviour in the cotyledons.

We measured the respiratory activity, O_2 uptake, RCR and ADP:O ratios of mitochondria isolated from attached and detached cotledons of wild and cultivated peas during seed germination. There are differences in the behaviour of mitochondria from attached and detached cotyledons of *P. sativum*, (Fig. 1 and Table 1). In the first period, up to 48 hr, degeneration of the mitochondria began, and was more rapid in the mitochondria of attached cotyledons, which were uncoupled after 72 hr of seed germination. Those of the detached ones still responded to ADP, although with poor efficiency, after 72 hr, but were uncoupled after 96 hr of seed germination. These results differ from those of Nawa *et al.*[6] who found that in mitochondria of the detached cotyledons the O_2 uptake continued to rise until day 6 of seed germination and that they remained highly efficient. In our experiments the only differences between the excised and attached cotyledons is in the time of onset of mitochondrial degeneration. Mitochondria, both of the attached and the detached cotyledons, degenerate within the first 96 hr of seed

Table 1. Respiratory control ratio (RCR) and ADP:O ratio in mitochondria of attached and detached cotyledons of *P. sativum* during seed germination

Germination (hr)	RCR		ADP:O	
	Attached	Detached	Attached	Detached
24	1.89	1.85	1.63	1.56
48	1.73	1.76	1.50	1.37
72	1.0	1.51	—	0.90
96	1.0	1.0	—	—

The cotyledons were detached before onset of imbibition. Succinate was used as a substrate.—, No response to ADP (uncoupled).

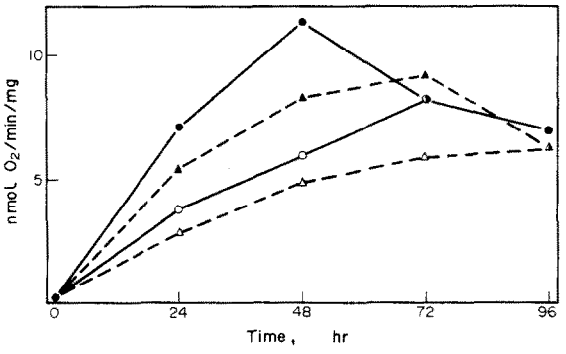

Fig. 1. O_2 uptake by mitochondria prepared from attached or detached cotyledons of *P. sativum* after varying periods of germination. Results as nmol O_2/min/mg protein. Substrate succinate. (○—○) attached −ADP; (●—●) attached +ADP; (△- - - -△) detached −ADP; (▲- - - -▲) detached +ADP.

germination and removal of the embryonic axis prior to germination only delays the degeneration process by 24 hr but does not prevent it.

When the cotyledons were detached after 1 or 5 hr of contact with the embryonic axis at 26° they behaved like cotyledons which were excised before and were completely uncoupled after 72 hr. When the cotyledons were excised after 5 hr at 4° they behaved like cotyledons which were excised before the onset of imbibition (Table 2), with delayed uncoupling, which occurred only after 96 hr of seed germination. Apparently whatever induced the accelerated degeneration of the mitochondria is transferred from the axis to the cotyledons within 1 hr, and its transfer or its action is temperature dependent.

In the wild pea the mitochondria from both the attached and detached cotyledons remained coupled even after 96 hr of seed germination (Fig. 2, Table 3). The difference between the attached and detached cotyledons are in the level of O_2 uptake (especially state 3 respiration) and ADP:O ratios which are slightly lower in the case of the detached cotyledons, especially after 72 and 96 hr of seed germination. The mitochondria of the detached cotyledons are slightly less efficient than those of the attached ones. They still respond to ADP and show no signs of degeneration. The wild pea therefore behaves quite differently from the cultivated pea.

The changes in mitochondrial activity during the first 4 days of seed germination are controlled by the cotyledons themselves. In *P. sativum* some factor is transferred from the embryonic axis in the first hour of seed imbibition which partially regulates the rate of change of mitochondrial activity. During the first 2 days of seed germination, respiratory activity of mitochondria is stimulated by this factor, and this is

Table 2. Respiratory activity of mitochondria isolated from cotyledons of *P. sativum* germinated for 72 hr attached or excised at different times of imbibition

Treatment of cotyledon	O_2 uptake nmol O_2/min/mg protein		RCR	ADP:O
	State 4	State 3		
Attached	8.2	8.2	1.0	—
Detached	5.9	9.2	1.5	0.90
Excised after:				
1 hr at 26°	8.4	8.4	1.0	—
5 hr at 26°	8.6	8.6	1.0	—
1 hr at 4°	6.3	10.1	1.5	0.88
3 hr at 4°	6.6	9.8	1.4	0.86
5 hr at 4°	5.6	8.5	1.5	0.83

Succinate was used as the substrate.—, No response to ADP (uncoupled).

Table 3. Respiratory control ratio (RCR) and ADP:O ratio in mitochondria of attached and detached cotyledons of *P. elatius* during seed germination

	RCR		ADP:O	
Germination (hr)	Attached	Detached	Attached	Detached
24	2.07	1.96	1.97	1.84
48	2.01	1.84	1.75	1.64
72	2.03	1.91	1.78	1.40
96	1.91	1.89	1.90	1.29

Succinate was used as the substrate.

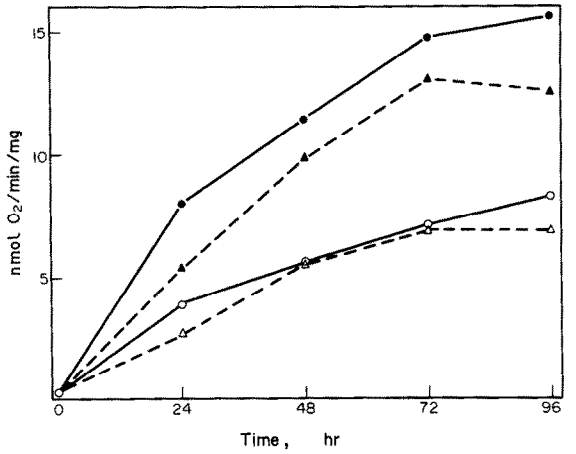

Fig. 2. O_2 uptake by mitochondria prepared from attached or detached cotyledons of *P. elatius* after varying periods of germination. Results as nmol O_2/min/mg protein. Substrate succinate. (●—●) attached +ADP; (○—○) attached −ADP; (▲---▲) detached +ADP; (△--△) detached −ADP.

followed by accelerated mitochondrial degeneration. No such factor exists in *P. elatius*. Morohashi[8] also assigned a role to the embryonic axis of *P. sativum* in controlling mitochondrial behaviour of the cotyledons, the effect being greater when malate was used as substrate, instead of succinate. We compared the effects described above when using malate as substrate instead of succinate. Similar results were obtained with the two substrates, in contrast to the results of Morohashi[8]. Our results if calculated per cotyledon have the same order of magnitude for O_2 uptake as those of refs. [7, 8].

According to Nawa *et al.*[6] and Morohashi[8] the decrease in mitochondrial activity in the later stages is due to mitochondrial degeneration. Both cytochrome oxidase and malate dehydrogenase appeared in post mitochondrial fractions. We therefore looked at the location of cytochrome oxidase activity in our extracts. Cytochrome oxidase activity in the mitochondrial and post mitochondrial fraction in the wild and cultivated pea was studied in the presence or absence of Triton X-100 to determine whether the loss of functionality of the cultivated mitochondria is accompanied by loss of cytochrome oxidase activity in the mitochondrial fraction (Fig. 3). In the cultivated pea (Fig. 3) the specific activity of cytochrome oxidase in the mitochondrial fraction both in the absence and presence of Triton X-100 increased until 72 hr of seed germination and then began to decline. The decline was accompanied by an increase in the specific activity of the enzyme in the post-mitochondrial fraction, but at 96 hr of seed germination the activity in the mitochondrial fraction still remained high. After 72 hr when the mitochondria were fully uncoupled (Tables 1, 2) cytochrome oxidase activity was at its peak. Similar results were obtained by Nakayama *et al.*[9] and Morohashi[8].

In the wild pea the activity of cytochrome oxidase in the mitochondrial fraction in the presence of Triton X-100 (Fig. 3) increased continuously until 96 hr of seed germination but there was no increase in the post-mitochondrial fraction. Cytochrome oxidase is located on the mitochondrial inner membrane and therefore its retention in the organelle gives no information on the integrity of the mitochondria[11].

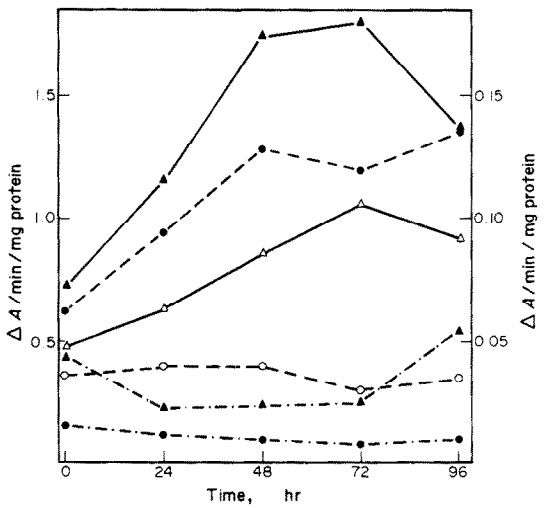

Fig. 3. Cytochrome oxidase activity in mitochondrial and post-mitochondrial fraction of the cotyledons of peas. Results as ΔA/min/mg protein. Left hand scale: mitochondrial fraction; right hand scale: post-mitochondrial fraction. (△—△) *P. sativum* mitochondrial fraction; (▲—▲) *P. sativum* mitochondrial fraction + Triton X-100; (○—○) *P. elatius* mitochondrial fraction; (●--●) *P. elatius* mitochondrial fraction + Triton X-100; (▲- - - - -▲) *P. sativum* post-mitochondrial fraction + Triton X-100; (●—·—·—●) *P. elatius* post-mitochondrial fraction + Triton X-100.

Degeneration of the mitochondria probably begins with structural or conformational disorganization.

When cytochrome oxidase activity was measured in *P. elatius* mitochondria without the addition of Triton X-100 to the reaction mixture, activity remained at the same level for 96 hr of germination. This contrasts sharply with the behaviour of the mitochondria from *P. sativum*. The mitochondria of *P. elatius* are apparently very tightly organized and the degree of organization increases during germination. Their response to Triton X-100 increased markedly up to 72 hr. Preliminary studies using the electron microscope show that even after 96 hr of seed germination the mitochondria of the wild pea are well defined and organized, and rich in cristae, in contrast to the mitochondria of the cultivated pea which appear to have fewer cristae and are less well defined.

The embryonic axis of *P. sativum* exerts only very slight control over mitochondrial behaviour in the cotyledons, whereas there is no such effect in *P. elatius*. In contrast to the results of Nawa *et al.*[6], removal of the axis after 1 hr does not prevent the influence of the axis on the cotyledon. We are unable to account for the difference between our results and those of Nawa *et al.*[6]. The difference in behaviour of the 2 species is also evident in their mitochondrial cytochrome oxidase. In *P. sativum* the ratio of activity plus to minus Triton X-100 remains fairly constant during germination, i.e. the mitochondrial structure does not change markedly. In *P. elatius* this ratio changes from *ca* 1.8 in preparations from dry seeds to 4.0 after 72 hr of germination. This indicates that either marked changes occur in the structure of existing mitochondria or that the existing ones degenerate and new, tightly organized mitochondria are formed. It remains to determine what factors induce the rapid degeneration of mitochondria in the cotyledons of *P. sativum*, and why those of *P. elatius* retain their integrity.

EXPERIMENTAL

Plant material. Untreated seeds of the cultivated pea *P. sativum* L. cv Alaska were purchased from Ferry Morse Seed Company. Seeds of the wild pea *P. elatius* M.B. were grown in the garden of the University Botany Department in Jerusalem.

Cotyledons with (attached) or without (detached) embryonic axis were sterilized with 1% $HgCl_2$, washed with tap H_2O then imbibed in dist. H_2O containing: streptomycin sulfate 50 μg/ml, penicillin G 50 μg/ml and chloramphenicol 25 μg/ml at 26° for 5 hr and then transferred, under aseptic conditions, to 9 cm Petri dishes containing cotton wool moistened with H_2O. In all cases the testa was removed together with the embryonic axis. The period of germination was measured from the onset of imbibition.

Preparation of the mitochondrial fraction. The mitochondrial fraction of the cotyledons was prepared according to ref. [1] with some modifications. Cotyledons (6 g) were ground in 35 ml grinding buffer pH 7.2 containing: 50 mM KPi buffer pH 7.2, sucrose 0.4 M, EDTA 1 mM and cysteine 0.05%. The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant centrifuged again at 20 000 *g* for 20 min. The 20 000 *g* supernatant was used as the post-mitochondrial fraction. The ppt. was washed with 10 ml grinding buffer without cysteine and the washed pellet suspended in 2.5 ml of the washing buffer and 0.1 ml sample was taken for protein determination. To the remainder BSA dissolved in the same buffer was added to a final concn of 0.5% giving a final vol. of 2.7 ml.

Respiratory activity. O_2 uptake of isolated mitochondria RCR and ADP:O ratios were determined as previously described[1] using the Clark type O_2 electrode with 45 μmol succinate or 45 μmol malate + 3.3 μmol NAD as substrate in 3.3 ml reaction mixture.

Cytochrome oxidase activity of the mitochondrial and post mitochondrial fractions was determined according to ref. [12] in the presence or absence of Triton X-100 at a final concn of 0.065% in the reaction mixture. The change in A_{550} was recorded and the reaction rate determined in the linear part of the reaction and given as ΔA mg-protein/min.

Protein was determined by the method of ref. [13].

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