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Promoting effect of oxygen on the synthesis of different enzymes in squash cotyledons in the early phase of germination

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Fisiologia vegetale. — *Promoting effect of oxygen on the synthesis of different enzymes in squash cotyledons in the early phase of germination.* Nota di RAFFAELLA CERANA, ROBERTA COLOMBO e PIERA LADO (*), presentata (**) dal Corrisp. E. MARRÈ.

RIASSUNTO. — La maturazione del seme è accompagnata dalla diminuzione dei livelli enzimatici che nel seme secco sono bassissimi. La biosintesi di nuovi enzimi nelle prime fasi della germinazione si svolge in condizioni di $\not p O_2$ limitante sia il metabolismo energetico che quello anabolico. In questo lavoro si è studiato l'effetto di tensioni di O_2 crescenti sulla sintesi di alcuni enzimi (isocitrato liasi, glucosio-6-fosfato deidrogenasi, isocitrato deidrogenasi NADP-dipendente e malico deidrogenasi) in cotiledoni di semi di zucca, il cui sviluppo fisiologico è particolarmente sensibile a diverse disponibilità di ossigeno. La sintesi di tutti gli enzimi viene accelerata aumentando la tensione di ossigeno dal 3% al 20% e questo effetto non dipende dagli effetti dell'ossigeno sullo stato di idratazione del seme. Lo stimolo dell'ossigeno sulla velocità di sintesi enzimatica è quantitativamente diverso per i vari enzimi e risulta molto più intenso sulla sintesi della isocitrato liasi (l'aumento della velocità di sintesi è di *ca.* 30%-100% per l'isocitrico deidrogenasi NAPD-dipendente e per la glucosio-6-fosfato deidrogenasi, e del 380% per l'isocitrato liasi). L'interpretazione di questo effetto suggerisce alcune interessanti ipotesi.

INTRODUCTION

The synthesis of new enzymes is one of the fundamental events in the resumption of metabolic activity at the onset of germination. Both external (water, oxygen, temperature) and endogenous (hormones, metabolites) factors appear to control the synthesis of new enzymes [11, 12].

The investigation of the mode of action of these factors is difficult because of reciprocal interference which makes it impossible to single each factor out for detailed analysis. A single controlling factor can however be studied more easily when this is also a limiting factor in a given material.

Squash seeds are a suitable material for the study of the effect of oxygen, as the metabolic development at the onset of germination (respiration, water uptake, synthesis of RNA and protein, lipid → glucid conversion) is in these seeds strongly retarded by the presence of a thin membrane enveloping the seed. The reduced diffusion of oxygen through the seed tissues is the main factor involved in the inhibition caused by the presence of the membrane [16].

In this material, the development of the enzymatic pattern which characterizes germination depends upon the concentration of oxygen. It had been found in previous experiments that the rate of synthesis of total protein, as

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Abbreviations: CHI, cycloheximide; GA_3, gibberellic acid.

measured *in vivo* by the incorporation of leucine, is closely dependent upon the availability of oxygen at the time of measurement [15]. The effect of oxygen on the increase of enzymatic activities might therefore only depend upon a generalised effect on protein synthesis. The effect of oxygen, however, was found to be more conspicous on the increase of the enzyme isocitrate lyase than on the increase of other enzymatic activities in the cytoplasm, suggesting a preferential effect of oxygen on the synthesis of this enzyme [16]. The interpretation of these results was however complicated by the following facts: *a*) the increase in the concentration of oxygen stimulates water uptake, and the level of hydration of the seed also affects the synthesis of enzymes [1, 6, 17]: *b*) reactivation of existing enzymatic activities overlaps with synthesis of new enzymes during the early stages of germination [2, 4, 12].

In this work we have investigated in squash cotyledons the effect of oxygen on the synthesis of isocitrate lyase, glucose-6–phosphate dehydrogenase, NADP-dependent isocitrate dehydrogenase and malate dehydrogenase, controlling as accurately as possible both enzyme reactivation and the condition of hydration of the seed.

The results obtained show that the synthesis of isocitrate lyase is more deeply affected by the availability of oxygen than the other enzyme activities examined.

Materials and methods

Squash (*Cucurbita maxima*) cotyledons were isolated from the seeds decoated and deprived of the inner membrane after one hour of imbibition at 30 °C. The isolated cotyledons, with their external surface towards the atmosphere, were incubated in the dark at 30 °C in Petri dishes on wet filter paper or directly on the dry dishes put in large containers where the relative humidity was mantained close to 100%. When specified in the single experiments, Petri dishes were kept in atmosphere containing 3%, 12%, 20%, 50% or 100% of oxygen.

Preparation of extracts for the determination of enzyme activities.

10 cotyledons (ca. 600 mg) were homogenized in a mortar with 5,5 ml of 10^{-1} M phosphate potassium buffer (pH 7,6), containing 10^{-2} M $MgCl_2$. The extracts were centrifuged at 27,000 xg and the pellet was washed with 2 ml of phophate buffer. The two supernatants mixed were used for the determination of enzyme activities.

Isocitrate lyase (E.C. 4.1.3.1) activity was measured according to the method of Dixon and Kornberg, as described previously [9]. Glucose–6–phosphate dehydrogenase (E.C. 1.1.1.49), NADP–dependent isocitrate dehydrogenase (E.C. 1.1.1.42), malate dehydrogenase (E.C. 1.1.1.37) activities were determined by standard spectrophotometric measurement [13].

RESULTS

1) *Reactivation of enzyme activities.*

Before considering the effects of different availability of oxygen on the *de novo* synthesis of enzymes, we investigated the condition under which enzymes, already present in the dry seed, would undergo complete reactivation. We therefore compared the increase in the activity of some enzymes (glucose–6–phosphate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase) in cotyledons germinated in 20 % oxygen with and without cycloheximide.

As shown in fig. 1, the three enzymes considered were found to be present in the dry seed and their activity increased during the first 15 hours of germination in the presence of CHI as well, the rate of increase being the same or slower than in the controls. In plant materials, 100 μg/ml CHI is known to completely inhibit protein synthesis: the increase in activity detected in the presence of the drug can therefore be considered as depending largely on

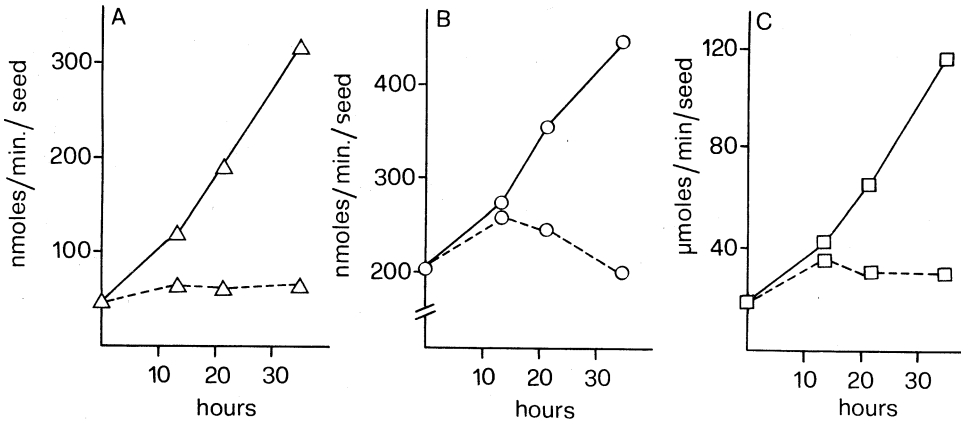

Fig. 1. – The effect of cycloheximide (100 μg/ml) on the development of glucose–6–phosphate dehydrogenase (A), NADP–dependent isocitrate dehydrogenase (B) and malate dehydrogenase (C) activities in isolated squash cotyledons, incubated at 30° C in the dark.
(———) control in water; (– – –) CHI–treated samples.

reactivation protein synthesis-independent. Reactivation processes are over within the first 15 hours of germination: the activity of both glucose–6–phosphate dehydrogenase and malate dehydrogenase remains more or less constant up to 40 hours, whereas isocitrate dehydrogenase activity rapidly decreases, suggesting a fairly short half-life for this enzyme.

No isocitrate lyase activity is detectable in the dry seed: a variety of data in both squash seeds and other seeds are in agreement with the conclusion that this enzyme is synthesized *de novo* at the beginning of germination [5, 9, 10].

2) *Effects of different oxygen concentrations on the synthesis of enzymes.*

The results reported above indicate that a 15 hours incubation period in 20 % oxygen is sufficient to obtain complete reactivation of the enzymes studied. However, in view of the fact that the cotyledons were to be successively exposed to different oxygen concentrations, we carried out the preincubation treatment at the lowest concentration of oxygen that was going to be used in the experiments to follow. At 3 % oxygen we found that a 24-hour incubation period was necessary for the enzymes undergoing reactivation to reach a level of activity equal to the maximum level obtained in the absence of protein synthesis. The increase above this level could be considered as dependent on protein synthesis.

The exposure to different concentrations of oxygen could affect the level of hydration of the seeds [16] in such a way to influence the rate of protein synthesis. To avoid this possible interference, after the preincubation period the cotyledons were placed in dry Petri dishes at different oxygen concentration: 3 %–12 %–20 %–50 %–100 %. Under these conditions the fresh weight of the seeds did not show any change, allowing one to study the effect of different oxygen concentrations at a constant hydration level.

The changes in the activity of enzymes were measured after 15 hours of treatment at different concentrations of oxygen. The results are shown in fig. 2.

All enzyme activities increase above the level of activity shown at the end of the preincubation period, even at the lowest oxygen concentration (3 %). With increased availability of oxygen (12 % and 20 %), isocitrate dehydrogenase and glucose–6–phosphate dehydrogenase show barely significant increases. Malate dehydrogenase and isocitrate lyase show much more significant increases. Higher oxygen concentrations do not stimulate the development of the enzyme activities any further, under the conditions of hydration studied, with the exception of a slight increase in the isocitrate lyase activity at 50 % oxygen.

The " oxigen effect " on each enzymatic activity studied has been calculated as follows:

$$\frac{\Delta \text{ activity in } 20\% \, O_2 - \Delta \text{ activity in } 3\% \, O_2}{\Delta \text{ activity in } 3\% \, O_2} .$$

In other words, we have calculated how much higher is the increment of activity induced by 20 % oxygen as compared with that induced by 3 % oxygen. We have chosen this index to express the " oxygen effect " against the simple comparison of the percent increases on the initial activity, as initial activity levels are so widely different.

The data in Table I show that the stimulating effect of oxygen is different on the different enzymes. The effect on the development of the isocitrate lyase activity is very substantial. On the other hand, isocitrate dehydrogenase and glucose–6–phosphate dehydrogenase are very little affected by oxygen.

These enzymes, however, are already present at a considerable level of activity as a consequence of the process of reactivation: had oxygen had any effect, it could have been counteracted by regulatory factors tending to limit the synthesis of enzymes already present in amounts sufficient to fulfill the metabolic needs.

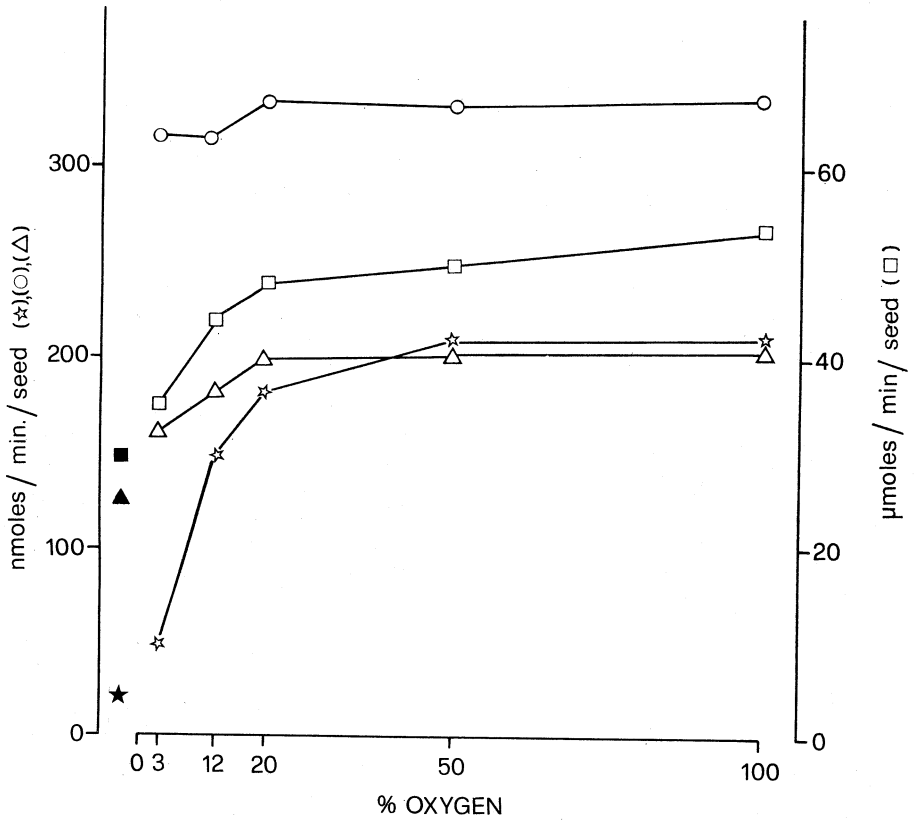

Fig. 2. – Effect of increasing concentrations of oxygen on the synthesis of enzymes in squash cotyledons. The isolated cotyledons were preincubated for 24 hours in Petri dishes on wet filter paper in atmosphere of 3% O_2 and then transferred on dry Petri dishes into a container where the relative humidity was close to 100% in an atmosphere containing 3, 12, 20, 50 or 100% O_2 for 15 hours, at 30 °C in the dark. In the figure the values of enzyme activities after 15 hours of treatment are reported. On the left side the closed symbols are the values of enzyme activities at the end of the preincubation (To). (☆) Isocitrate lyase; (△) Glucose–6–phosphate dehydrogenase; (□) Malate dehydrogenase; (○) NADP–dependent isocitrate dehydrogenase.

The stimulation by oxygen of the activity of malate dehydrogenase is consistent with even if lower than that of the activity of isocitrate lyase. The stimulation could be even higher if the effect of oxygen was directed only towards one of the three forms of the enzyme, which are found in different cell compartments [3] (cytoplasm, mitochondria, glyoxysomes), but our results do not allow such a distinction.

Further experiments were designed to find out whether the differential effect of oxygen on the enzymes studied was affected by a higher availability of water to the cotyledons, a condition closer to the physiological one.

TABLE I

Effect of treatment with 3% or 20% O_2 on the enzyme synthesis in squash cotyledons.

	T_0	Treatment with 3% O_2(15 hours)	Percent increase	Treatment with 20% O_2(15 hours)	Percent increase	Oxygen effect
Isocitrate lyase	13	48 (+35)	270%	181 (+168)	1290%	378
Glucose–6–phosphate dehydrogenase	125	162 (+37)	30%	200 (+75)	60%	100
Malate dehydrogenase . .	30	35 (+5)	17%	48 (+18)	60%	250
NADP–dependent isocitrate dehydrogenase	261	319 (+58)	22%	336 (+75)	29%	32

The conditions are the same as in fig. 2. The values in brackets are the increase of enzyme activities from the end of the preincubation (T_0). Enzyme activities are expressed as nmoles substrate consumed/min/ seed for all enzymes except for malate dehydrogenase (μmoles/ min/seeds). The oxygen effect was calculated as follows:

$$\frac{\Delta \text{ activity in } 20\%\ O_2 - \Delta \text{ activity in } 3\%\ O_2}{\Delta \text{ activity in } 3\%\ O_2}$$

After 26 hours preincubation on moist filter paper in 3 % oxygen, the cotyledons were transferred to either 3 % oxygen or 20 % oxygen, some in air at 100 % humidity, some in the presence of an unlimited supply of water. The changes in the fresh weight and the enzymatic activities after 15 hours of treatment are reported in Table II. The difference of water content between the two groups, placed in different conditions of water availability, are very small, especially in the cotyledons incubated in the presence of 3 % oxygen, but they are however significant. The different availability of water does not significantly affect the increase in glucose–6–phosphate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase activities neither in 3 % oxygen nor in 20 % oxygen. As far as these enzymes are concerned, therefore, the " oxygen effect " is not affected by a larger water uptake.

The increase in isocitrate lyase activity, on the contrary, is higher in cotyledons incubated at low availability of water, at both oxygen concentrations. The inhibition of the increase of isocitrate lyase detected at high availability of water could be explained by the fact that the cotyledons become covered by a film of water which impairs the gas exchanges between the air and the seed, thus limiting the availability of oxygen to the tissues. This result would provide further support for the conclusion that isocitrate lyase

is particularly sensitive to the concentration of oxygen. We cannot however exclude that the lower increase in the isocitrate lyase activity detected under these conditions does not depend upon a slower diffusion of a gaseous inhibitor present in the tissues.

TABLE II

Effect of different availability of water on promoting effect of O_2 *on enzyme synthesis in squash cotyledons.*

	T_0	Treatment with 3% O_2 (15 hours)		Treatment with 20% O_2 (15 hours)	
		$+H_2O$	$-H_2O$	$+H_2O$	$-H_2O$
Isocitrate lyase	18.5±2	20.5±2.2	87.5±22.5	172.5± 9.3	230.0±27.5
Glucose–6–phosphate dehydrogenase . . .	132.6±3.6	170.2±4	174.6±15	193.4± 8.7	188.4± 7.7
Malate dehydrogenase	32.1±0.9	39.4±3.2	43.3± 0	62.0± 1.9	59.3± 3.7
NADP–dependent isocitrate dehydrogenase	268.1±7.5	306.5±6	313.0±10.2	355.0±12.7	342.0±14.4
Increase in fresh weight (%)	26.8	+2.2	— 3.4	+ 7.5	— 5.7

After 26 hours of preincubation in Petri dishes on wet filter paper in atmosphere of 3% O_2 (T_0), the cotyledons were transferred in dry Petri dishes into atmosphere with relative humidity close to 100% or on wet filter paper in atmosphere of 3% or 20% O_2 for 15 hours. The enzyme activities are expressed as nmoles substrate consumed/min/seed for all enzymes except for malate dehydrogenase (μmoles/min/seed).

In conclusion, the data as a whole show that oxygen stimulates the synthesis of a number of enzymes, the effect being more marked on the isocitrate lyase activity than on the other activities considered. Furthermore, this effect does not depend upon the degree of hydration of the tissues, at least under the conditions adopted.

CONCLUSIONS AND DISCUSSION

1. In isolated squash cotyledons, preincubated in 3 % oxygen in the presence of an unlimited supply of water for 24 hours and then transferred to 100 % humidity at different concentrations of oxygen (from 3 % to 100 %), isocitrate lyase, glucose–6–phosphate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase increase for at least 15 hours also without a supply of water. This increase is dependent upon protein synthesis.

2. The synthesis of all enzymes considered appears to be stimulated by increasing oxygen concentrations. The results obtained suggest that the

stimulating effect induced by oxygen is not mediated by an increased water uptake. This is in agreement with the fact that in the same material, increasing the concentration of oxygen from 3 % to 20 % results in a stimulation of the rate of protein synthesis (measured *in vivo* as incorporation of leucine) occurring in such a short time to exclude that changes in the level of hydration of the tissues can take place simultaneously [15].

3. The results obtained indicate that the increase in the concentration of oxygen not only affects protein synthesis as a whole, but also alters the rate of synthesis of different enzymes. The " oxygen effect " on the synthesis of isocitrate lyase appears to be more significant than that on the other enzymes.

The interpretation of this result suggests a number of considerations. As previously pointed out, the general effect of oxygen on protein synthesis could be selectively reduced through specific control mechanism operating at the level of the synthesis of some enzymes. Conversely, oxygen could, besides influencing protein synthesis as a whole, have more directly specific effects:

a) A direct action at the transcription level, affecting the amount of *m*-RNA for isocitrate lyase, remains purely theoretical.

b) Another possibility is that the synthesis of isocitrate lyase is induced by high levels of metabolic intermediates originating in the process of β–oxidation of lipids. In this case, the effect of oxygen could be explained by the more rapid re–oxidation of the coenzymes needed in the breakdown of lipids.

c) A third possibility is that oxygen could act by stimulating the synthesis of gibberellic acid, which in turn would promote the synthesis of the enzyme. This hypothesis is suggested by the fact that in other seeds, and in some instances in squash as well, this hormone induces an increase in the level of isocitrate lyase [8, 14]. Furthermore, one of the steps in the biosynthesis of GA_3 involves P_{450} which catalyses mixed oxidases and requires oxygen as a substrate [18]. Administration of GA_3 to the cotyledons incubated in 3% oxygen did not however induce any increase in the activity of isocitrate lyase as compared to the controls.

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