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The effect of oxygen on the development of enzyme activities in germinating seeds of Cucurbita maxima

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SEZIONE III

(Botanica, zoologia, fisiologia e patologia)

Fisiologia vegetale. — *The effect of oxygen on the development of enzyme activities in germinating seeds of* Cucurbita maxima (*). Nota (**) di FRANCO ROLLO, ROBERTA COLOMBO, PIERA LADO e FRANCA RASI-CALDOGNO, presentata dal Corrisp. E. MARRÈ.

RIASSUNTO. — I semi di *Cucurbita maxima*, liberati dai tegumenti esterni, sono ancora avvolti da una sottile membrana (costituita da residui della nucella e dell'endosperma) che nelle prime 24 ore di germinazione limita fortemente lo sviluppo del seme e cioè l'assunzione di acqua, lo sviluppo della respirazione, l'aumento di attività enzimatiche solubili (glucosio-6-P-deidrogenasi, NADP-dipendente isocitrato-deidrogenasi) e la comparsa della isocitrato liasi. Fra i possibili meccanismi dell'effetto inibente esercitato dalla membrana, i dati ottenuti indicano che la limitazione della diffusione dell'ossigeno nel seme è il principale fattore implicato nel rallentamento dello sviluppo metabolico.

Successivi esperimenti in cui i semi sono stati fatti germinare senza pellicola ma in diverse concentrazioni di O_2 (dal 3% al 21%), hanno ulteriormente dimostrato che gli aumenti delle tre attività enzimatiche solubili (glucosio-6-P deidrogenasi, isocitrato-deidrogenasi, isocitrato liasi) come quelli di cinque attività enzimatiche mitocondriali (citocromo *c* ossidasi, fumarasi, succinico-deidrogenasi, malato-deidrogenasi, glutammato-ossalacetato transaminasi) sono stimolati da un aumento di concentrazione di O_2.

Questi risultati portano alla conclusione che sia l'evoluzione del quadro enzimatico tipico della germinazione (isocitrato liasi in particolare), sia lo sviluppo del sistema mitocondriale sono sotto il controllo della concentrazione di O_2. Si prospettano alcune ipotesi sui passaggi metabolici che potrebbero mediare l'effetto dell'ossigeno.

INTRODUCTION

In mature seeds the level of almost all enzymes is very low, owing to the inactivation of cytoplasmic as well as mitochondrial enzymes during the last phase of maturation [2, 14, 16].

Seed germination is therefore constantly characterized by a rapid increase of a number of enzyme activities [3, 16]. Obviously, this almost general rise of catalytic activities depends on the availability of some generally important environmental factors such as water, oxygen, temperature and in several instances, light.

The mode of action of these factors and of their interplay in controlling the development of the enzyme pattern characteristic of germination is still far from being satisfactorily understood. A serious difficulty in this connection arises from the close interconnection between the available level of every one of these factors and the capacity of utilising the other ones. For instance,

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water availability, by controlling enzyme activation and synthesis, limits the development of oxidative metabolism, while the limitation of oxygen deeply depresses the capacity of the seed to take up water from the medium.

On the other hand, a series of external factors obviously influence the appearance and the physiology of internal stimula, such as substrate and hormone level, deeply involved in the regulation of both the overall protein synthesis and the control of the activity of specific genes. The resolution of this intricate network of interactions requires a detailed investigation of the mode of action of each single factor.

In the present study we report some preliminary observations on the role of oxygen on the development of some soluble and mitochondrial enzyme activities in germinating seeds of squash (*Cucurbita maxima*).

Materials and Methods

Plant material: Squash seeds (*Cucurbita maxima*) were decoated and, when specified in the single experiments, deprived of the inner membrane after one hour of imbibition at 30° C. Then the seeds were germinated in Petri dishes on wet filter paper, in the dark at 30° C and in atmosphere containing 21 %, 12 % or 3 % of oxygen, for the time indicated in the single experiments. In the experiments where the mitochondrial enzyme activities were assayed, the seeds were preincubated at 0° C for 22 hours on wet filter paper.

Preparation of extracts and mitochondrial fraction for the determination of enzyme activities: cotyledons from five seedlings (ca. 750 mg.) were homogenized in a mortar with 8 ml of 10^{-1} M potassium phosphate buffer (pH 7.6), containing 10^{-2} M $MgCl_2$.

The supernatant of 27,000 × g was used for the determination of enzyme activities. Glucose–6–P dehydrogenase, NADP–dependent isocitrate-dehydrogenase activities were measured spectrophotometrically [12, 18], isocitrate-lyase activity was measured according to the method of Dixon and Kornberg, as described previously [15].

For the preparation of the mitochondrial fraction the cotyledons from sixteen seedlings (ca. 2 g.) were gently homogenized in 22 ml of a grinding medium containing 0.56 M mannitol, 62.5 mM potassium phosphate buffer (pH 7.8), 0.16 % bovine seroalbumine, 1.25 mM EDTA. After centrifugation for 15 minutes at 500 × g, the sediment was washed and discarded. The two combined supernatants were centrifuged at 15,000 × g, for 20 minutes; the sediment, resuspended in 0.49 M mannitol in 10 mM potassium phosphate buffer (pH 7.2), was centrifuged at 4,000 × g, for 20 minutes, then the mitochondrial pellet, resuspended in 8 ml of the same medium, was used for enzyme activity measurements.

Succinic dehydrogenase activity was measured by the method of Hiatt [11]. Cytochrome oxidase activity was determined as described by Smith [21] by measuring in 60 mM potassium phosphate buffer, (pH 7.2), the oxidation rate of cytochrome *c* (1 mg./3 ml), reduced by sodium dithionite.

Malatedehydrogenase activity was determined by the method of Ochoa [17]. Glutamate-oxalacetate transaminase activity was measured as described by Schwartz [20]. Fumarase activity was determined according to Racker [19], employing 45 mM Tris buffer (pH 8) instead of phosphate buffer.

Measurement of the respiration: oxygen uptake was measured at 30° C according to the usual techniques with a Warburg microrespirometer [22].

EXPERIMENTS AND RESULTS

Squash seeds, deprived of the seed coat, are still closely enveloped in a thin transparent membrane (inner membrane) constituted by a few layers of different kinds of tissues (remains of the nucellus and the endosperm) which under normal conditions break down after one or two days of germination.

The main apparent physiological meaning of this structure is the protection of the young seed from physical and biological injuries; in fact the seeds put in the soil after the removal of the inner membrane fail to germinate and undergo rapid rotting.

On the contrary, when the seeds are germinated under controlled sterile conditions on wet paper in Petri dishes, the removal of the inner membrane results, as shown in Table I, in a marked speeding up of the early germination pattern in the cotyledons, including water uptake, the development of respiration, the increase of the activities of the soluble glucose 6P–dehydrogenase and NADP–dependent isocitrate–dehydrogenase and the appearance of the isocitrate-lyase which is the key enzyme of the lipid to sugar interconversion in fatty seed.

TABLE I.

Variations of fresh weight, Q_{O_2} and enzyme activities in cotyledons from squash seeds germinated under different conditions of oxygen availability, for 24 hours at 30° C, in the dark.

Conditions of germination	% Increase in fresh weight	Q_{O_2} (µl/h/seed)		Isocitrate–lyase	Glucose 6PDH	Isocitrate DH
		(1)	(2)	(mµmoles/min/seed) *		
Inner membrane present, 21 % O_2	32.2	40	83.5	24.9	151	242
Inner membrane removed, 21 % O_2	48	—	171	147	260	295
Inner membrane removed, 3 % O_2	29	—	89	22.8	141	230
Inner membrane present, 98 % O_2	33.8	39	117	101	193	234

Oxygen uptake was measured in 21 % oxygen, (1) with inner membrane present, (2) after the removal of the inner membrane.

* mµ moles substrate consumed/min/seed.

In regard to the mechanisms of this inhibitory effect of the inner membrane, the following possibilities have been considered: *i*) the presence of inhibiting substances in this tissue; *ii*) the prevention of the loss by diffusion of inhibitors present in the seed; *iii*) the reduction of water uptake, due to the poor permeability of the membrane; *iv*) the reduction of the rate of oxygen diffusion to the seed.

Possibilities *i*) and *ii*) appear unlikely in view of the observation that similar rises of both respiration and enzyme activities are induced by either removing the membrane or by cutting it around the seed and divaricating the cotyledons in such a way to expose their inner surface to air. The third possibility is difficult to eliminate and it is quite probable that the more rapid hydration following the removal of the membrane plays some role in increasing the rise of the metabolic activities in cotyledons (for such an effect in castor bean endosperm cf. [22]).

The fourth possibility is supported by qualitative measurements by means of an oxygen electrode, which clearly indicate a very low permeability of the membrane to oxygen, thus confirming the similar results obtained by Brown on the inner membrane of a closely related species (*Cucurbita pepo*) [6]. Morever, the experiments in Table I indicate that a limitation of oxygen diffusion is the main factor involved in the effect of the inner membrane in retarding the development of the metabolic activity in the cotyledons.

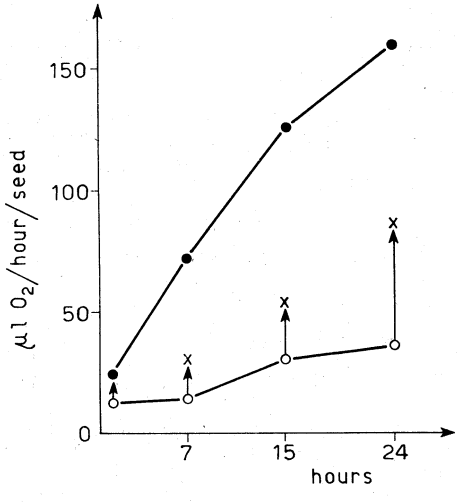

Fig. 1. – Time course of respiration of cotyledons from squash seeds germinated for 24 hours at 30° C in the dark: ●—● seeds germinated after the removal of the inner membrane; ○—○ seeds germinated with the inner membrane; X seeds germinated with the inner membrane, which was removed before the measurement of the respiration.

In fact the data of Table I show that a significant speeding up of the capacity of oxygen uptake and of the development of enzyme activities is obtained when the seeds are germinated whithout removing the membrane, under a condition of high O_2 partial pressure (98 % O_2). It is interesting that the % increase of fresh weight (corresponding to water uptake) remained unchanged compared to the controls germinated with the membrane and in 21 % oxygen. On the other hand in the seeds germinated under the membrane-removed condition, lowering O_2 partial pressure to 3 % strongly inhibited both enzyme development, and water uptake. The conclusion appears therefore legitimate that at least a large fraction of the retarding effect of the inner membrane on the development of the enzyme activities is due to its capacity to limit the rate of oxygen uptake.

The data of fig. 1 confirm this conclusion and show that indeed the removal of the inner membrane from seeds incubated under conditions appropriate

for germination (unlimited water availability, temperature of 30° C) induces an immediate rise of oxygen uptake and strongly enhances the rate of development of respiration. As shown by the figure, in the seeds with the membrane intact, the Q_{O_2} after 24 hours of germination has little more than doubled in respect to the initial value. The removal of the membrane at this moment induces an immediate increase of the respiration (ca. 120 %), indicating that oxygen availability rather than enzyme development is limiting oxygen uptake. On the other hand, in the seeds deprived of the inner membrane since the beginning of the germination, the capacity of taking up oxygen has developed at a much faster rate, reaching at 24th hour a value ca. 7 times higher than the initial rate, ca. 4 times than that of the seeds germinated for the same time with the membrane and tested for O_2 uptake with the membrane, and 2 times higher when tested without the membrane.

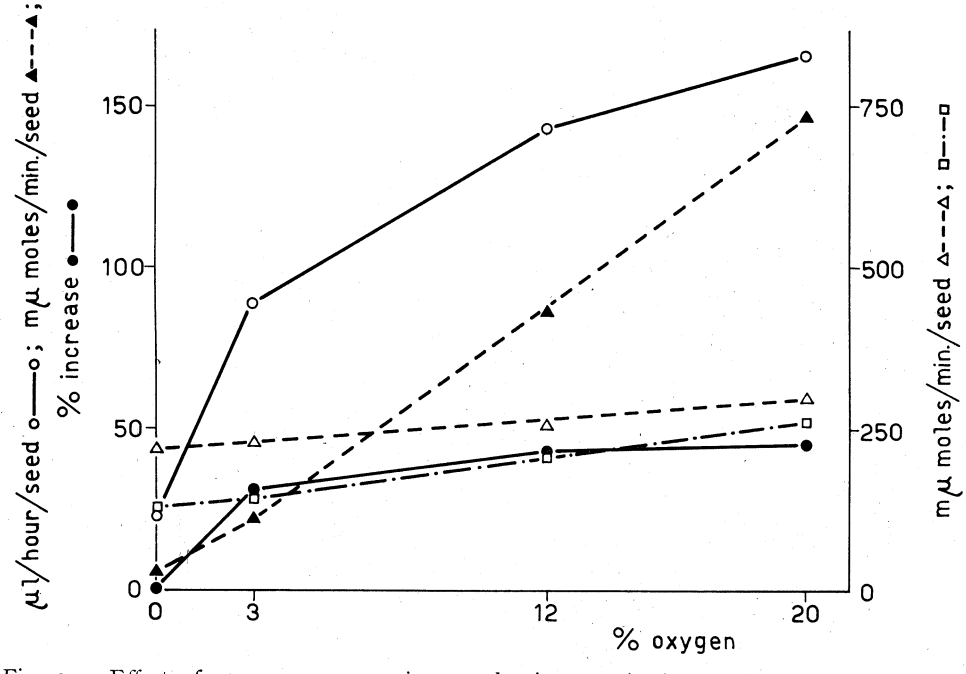

Fig. 2. – Effect of oxygen concentration on the increase in fresh weight (●—●), on the development of oxygen uptake (○—○) and enzyme activities [isocitrate-lyase (▲—▲), isocitrate-dehydrogenase (△—△), glucose 6P-dehydrogenase (□—□)] in cotyledons from squash seeds germinated for 24 hours at 30° C, in the dark. The values of enzyme activities are expressed as mμmoles substrate consumed/minute/seed.

In the further experiments the study of the effects of O_2 availability on the development of the respiratory activity of germinating seeds was approached by determining the rate of the increase of some soluble and some mitochondrial enzyme activities during the early phase of germination of *Cucurbita maxima* seeds, put to germinate after having removed the inner membrane. The oxygen concentrations tested ranged from 21 % to 3 %. The lowest concentration had been shown in previous experiments to repress the Q_{O_2}

of the seeds without membrane to a value similar to that of the seeds germinated with the membrane in an atmosphere of 21 % O_2. The data of fig. 2 show that the rate of the development of all three soluble enzyme activities tested (glucose 6P dehydrogenase, NADP–dependent isocitrate dehydrogenase and isocitrate lyase) is increased by the increase of O_2 concentration in the atmosphere; however, the increases of glucose 6P dehydrogenase and isoci-

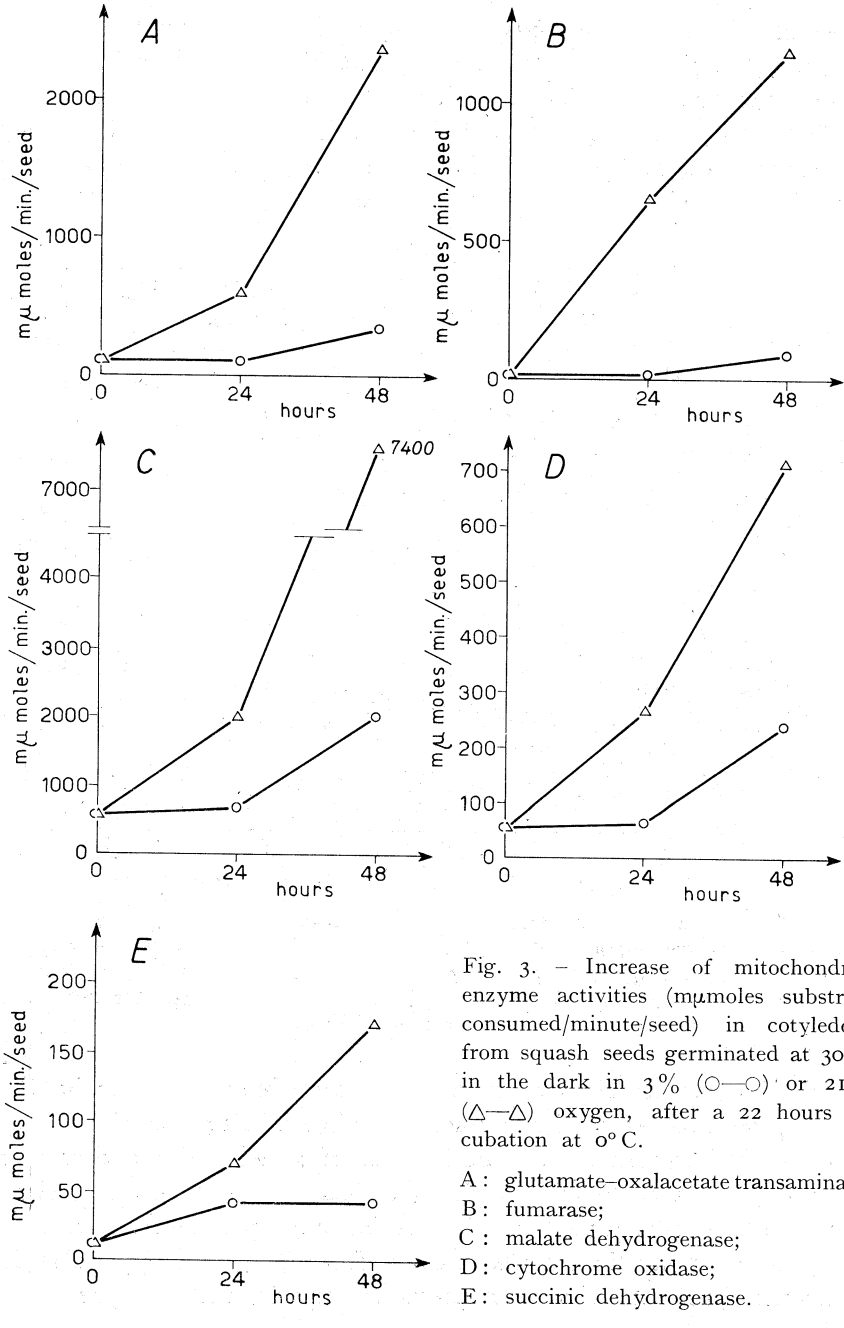

Fig. 3. – Increase of mitochondrial enzyme activities (mμmoles substrate consumed/minute/seed) in cotyledons from squash seeds germinated at 30° C in the dark in 3 % (○—○) or 21 % (△—△) oxygen, after a 22 hours incubation at 0° C.

A : glutamate–oxalacetate transaminase;
B : fumarase;
C : malate dehydrogenase;
D : cytochrome oxidase;
E : succinic dehydrogenase.

trate dehydrogenase are modest when compared to the contemporaneous, very marked increase of the rate of respiration, which suggests that these enzymes are not apparently limiting the latter phenomenon.

In contrast, the effect of O_2 on the increase of activity of isocitrate lyase is very marked, a finding of considerable interest in wiev of the well known role of this enzyme in controlling the rate of the lipid to carbohydrate conversion, one of the major metabolic processes characteristic of germination.

The experiments of fig. 3, showing the effects of O_2 on five enzymes obtained from the mitochondrial fraction, indicate a good correlation between the oxygen induced rise of respiration and the development of mitochondrial activities.

In fact, all of the five enzymes investigated (cytochrome oxidase, succinic dehydrogenase, mitochondrial malate dehydrogenase, fumarase, glutamate-oxalacetate transaminase), starting from a very low initial value, increase quite rapidly in the seeds germinated in 21 % O_2, while this rise is much lower in the 3 % O_2 condition. Furthermore, in the seed germinated in 3 % O_2 a clear lag of 24 hours, during which no activity development is observed, is evident for all the enzyme activities, with the exception of succinic-dehydrogenase.

CONCLUSIONS

The results reported above indicated that the inner membrane of this kind of seeds strongly limits seed respiration during the early germination phase. This inhibition appears clearly dependent on the low permeability of this membrane to the diffusion of gases: oxygen and perhaps CO_2 in the first phase, but possibly, also other gases arising from seed metabolism or volatile substances, such as ethylene etc.. The inhibition of respiration by oxygen shortage appears to limit strongly the evolution of the metabolic pattern of germination as a whole (including processes such as water uptake and lipid conversion to sugars) and, in particular, the development of the respiratory system. Among the oxidative enzyme activities tested, very clear responses to changes of the availability of O_2 are observed for the mitochondrial malate and succinic dehydrogenases and cytochrome oxidase. The O_2 induced changes in the rate of increase of these enzymes are accompanied by similar changes of other non-oxidative mitochondrial enzymes such as fumarase and glutamate-oxalacetate transaminase. It seems therefore that the development of the mitochondrial system as a whole is under the control of oxygen concentration.

In regard to the mechanism of the oxygen induced acceleration of mitochondrial as well as other enzyme activities, the first question is how much the activity increases observed depend on " de novo " enzyme synthesis and how much on reactivation of inactive forms already present in the mature seed, as both processes have been shown to occur in germination [9, 1, 10]. However, in most cases, and in particular in the one of mitochondial enzymes and structures, " de novo synthesis " appears to play a predominant role,

and reactivation seems very limited in extent, and restricted to the very early phase (first 24 hours) of germination [3–13]. Moreover, this early enzyme reactivation, insensitive to protein synthesis inhibitors and relatively insensitive to temperature, appears to depend mainly on seed imbibition [8]. In our case the oxygen concentration dependent increases of mitochondrial enzyme activities have been observed in seeds previously imbibed with water at 0° C for 22 hours, so that most of the reactivation should have already occurred before the beginning of the treatment with the various O_2 concentrations. It seems therefore legitimate to conclude that the more rapid increase of mitochondrial activities induced by the higher O_2 concentration depends on the capacity of oxygen to accelerate the synthesis of mitochondrial enzymes and structures.

The problem of the intermediate metabolic steps mediating this effect of O_2 remains open. Several possibilities can be considered such as: *i*) a direct inductive effect; *ii*) an effect mediated by the state of phosphorylation of the high energy phosphate transfer system (energy charge of Atkinson) [4]; *iii*) the O_2 dependent breakdown of inhibitors; *iv*) the O_2 dependent synthesis of hormonal factors. Experimental evidence is lacking, at present, to allow a discrimination between these and other possibilities.

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