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FERMENTATIVE PRODUCTS AND DARK CO_2 FIXATION DURING GERMINATION OF SEEDS OF *CICER ARIETINUM*

J. Aldasoro and G. Nicolás

Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Salamanca, Spain

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Key Word Index—*Cicer arietinum*; Leguminosae; chick pea; germination; fermentation; dark CO_2 fixation.

Abstract—During the germination of *Cicer arietinum* L. the amounts of ethanol, lactate and malate reached their highest values at 24 hr, the concentration of ethanol being about 4 times that of lactate and twice that of malate. The activities of phosphoenolpyruvate carboxylase and malic enzyme seem to be correlated with the ability of cotyledons to fix CO_2 from $NaH^{14}CO_3$ into malate and with the further decrease in this metabolite from 36 hr onwards.

INTRODUCTION

It is well known that during the first hours of germination many seeds undergo a period of anoxia after imbibition and before the rupture of the testa, which coincides with an increase in the activity of glycolytic and fermentative enzymes [1–3] and with the accumulation of ethanol as well as lactate [3]. Considerable activity of phosphoenolpyruvate (PEP) carboxylase activity has also been observed in several seeds [4–7], in relationship with seed vigor [7] or with the production of malate and dormancy [5].

The main object of this paper was to study the relationship between the glycolytic and fermentative activities with the PEP-carboxylase activity and the accumulation of malate during the first hours of germination of *Cicer arietinum* seeds.

RESULTS AND DISCUSSION

Figure 1 shows the levels of ethanol, lactate and malate during the germination of chick pea seeds. Their concentration increased during the initial hours of germination, reaching a maximum value at 24 hr, then decreased. The concentration of ethanol was *ca* 4 times higher than that of lactate and twice that of malate. This is in agreement with our previous results [1] in which we found a very active alcohol dehydrogenase during the same period of germination, after which a decrease in both enzyme and product was observed, which also coincides with the shift from anaerobic to aerobic respiration in chick pea [1]. Similar results have been found in other seeds [2, 3].

Figure 2 shows the activity of PEP-carboxylase and malic enzyme during germination. PEP-carboxylase activity increased during the first hours of germination reaching the maximum value at 18 hr. A decrease followed by a further but lower increase from 36 hr onwards was also observed. The NADP-dependent malic enzyme activity was kept very low during the first hours of germination. Its activity increased from

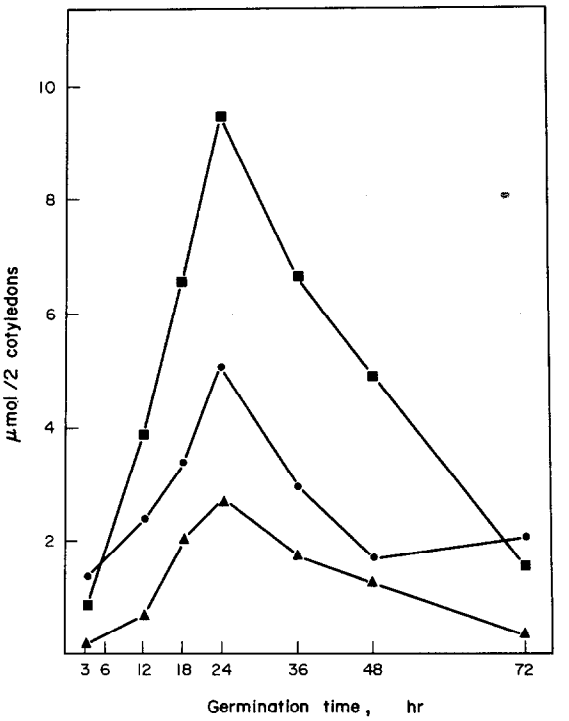

Fig. 1. Changes in the concentration of ethanol (■), malate (●) and lactate (▲) during germination of *Cicer arietinum* seeds. Each point is the average of at least 4 determinations. The variability was less than 10%.

36 hr onwards, although it was always lower than that of PEP-carboxylase. It is interesting to note that the *in vivo* CO_2 fixation into malic acid (Table 1) during the first 36 hr followed the same pattern as the *in vitro* rate of PEP-carboxylase activity measured under optimal conditions. It should also be considered, however, that the levels of malate are considerably lower than those theoretically expected from the values of PEP-carboxylase activity obtained *in vitro*. This apparent

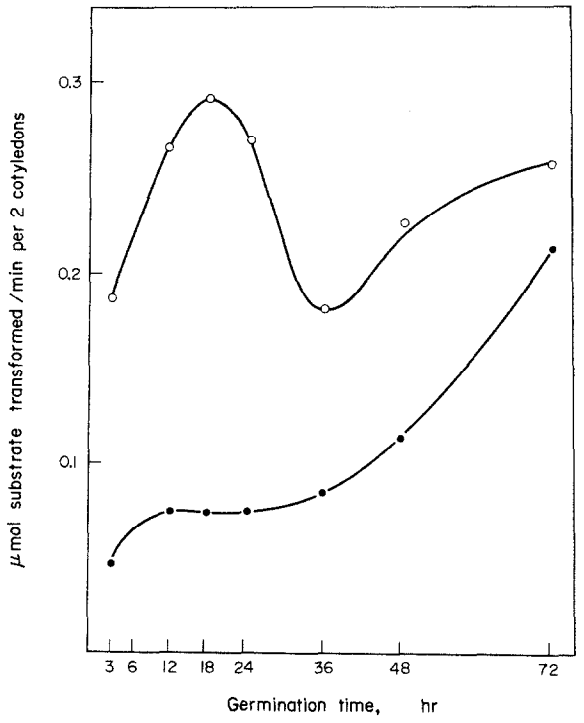

Fig. 2. Changes in the activities of PEP-carboxylase (○) and malic enzyme (●) during germination of *Cicer arietinum* seeds. Each point is the average of at least 3 determinations. The variability was less than 10%.

Table 1. CO_2 fixation into organic acids during germination of seeds of *Cicer arietinum*

Organic acid	Time of germination (hr) 3	12	18	24	36	48	72
Succinic acid	0.26*	0.36	0.41	0.49	0.18	0.05	0.03
Malic acid	0.58	0.71	0.84	1.91	1.61	1.12	0.68
Citric acid	—	—	—	0.03	0.06	0.10	0.09

* Dark fixation was estimated as μmol of CO_2 fixed/15 min/24 cotyledons in the conditions described in Experimental. The values are means of 3 experiments, the variability of results of different experiments was less than 10%.

disagreement could be due to the poor penetration of ^{14}C-bicarbonate into the cotyledons or to a lower activity of the PEP-carboxylase *in vivo* than *in vitro*.

According to Crawford [8], germinating seeds and plant roots may be compared in their response to anoxia. In intolerant seeds and plant roots, ethanol is the major end-product of glycolysis, while malic acid does not show any significant accumulation. On the other hand, McManmon and Crawford [9] claim that, in tolerant roots, a diversion from ethanol to malate accumulation is observed. On the basis of these observations, chick pea seeds seem to show an intermediate position between intolerance or tolerance to anoxia, since the accumulation of ethanol and also that of malate is observed during the anaerobic phase of germination, both metabolites reaching their maximum concentration after 24 hr. This is in good agreement with the pattern of changes showed by PEP-carboxylase and malic enzyme. PEP-carboxylase seems to be responsible for the carboxylation of phosphoenolpyruvate and malate accumulation; this is not toxic, and may remain in the cotyledons until aerobic respiration is restored after which the activity of malic enzyme increases and malate is transformed into CO_2 and pyruvate in the cytoplasm and metabolized through the TCA cycle. Similar results have also been found by Cameron and Cossins [10] in pea cotyledons. It should be noted, however, that in chick pea cotyledons, the drop in the malate level takes place prior to the rise in malic enzyme activity. This could be due to the increase in the TCA cycle activity and the utilization of malate through this pathway.

The enzyme PEP-carboxylase has been studied in seeds of several species [4, 7]. Perl [7] suggests that this enzyme may be the malate-providing system at the early stage of germination. On the other hand, Hedley *et al.* [6] suggest that the main function of PEP-carboxylase in cotyledons may be the recycling of CO_2 during germination. Both suggestions are in agreement with our data and give a satisfactory explanation of the function of dark CO_2 fixation during seed germination.

EXPERIMENTAL

Chick pea (*Cicer arietinum* L.) seeds harvested in 1976 and of a uniform size were soaked for 5 min in H_2O and their surface sterilized with Na hypochlorite after which they were germinated in a dark room at 25° and 70% relative humidity on moistened filter paper in a 15 × 20 cm plastic box from 0 to 72 hr, and then separated into cotyledons and embryos after removal of the seed coat. Eighty cotyledons were immediately submerged in liquid N_2 and reduced to a fine powder. The frozen powder (25 g) was homogenized with 50 ml 7% (w/v) $HClO_4$ at 0° and the homogenate was centrifuged at 1° at 22 000 *g* for 15 min. The supernatant was neutralized (pH 7.4) with 5 M K_2CO_3 in an ice bath. The $KClO_4$ was separated by two centrifugations and filtration, and the supernatant used immediately for the determination of EtOH, lactate and malate. EtOH was determined by the method of ref. [11], lactate by the method of ref. [12] and malate according to ref. [13].

PEP-carboxylase and malic enzyme were extracted by homogenization of 16 g of cotyledons in 40 ml 50 mM Tris–HCl buffer pH 7.5 containing 10 mM 2-mercaptoethanol and 4% insoluble PVP in an ice bath. The homogenate was centrifuged at 22 000 *g* for 15 min and then passed through a Sephadex G-15 column. PEP-carboxylase was assayed by the method of ref. [4] and malic enzyme according to ref. [14].

The incorporation of CO_2 into organic acids was followed by incubation for 15 min at 27° in the dark and constant shaking of 24 cotyledons with 4 ml 10 mM of NaPi buffer pH 6; 5 mM $NaHCO_3$ and 40 μCi (0.67 μmol) of $NaH^{14}CO_3$. The reaction was stopped by submerging the cotyledons in boiling 80% EtOH, after which they were homogenized in 80% EtOH, centrifuged and the supernatant was fractionated by ion exchange chromatography on Dowex 1 × 8 (200–400 mesh) in formate form, and eluted with a linear gradient of 4 N H_2O–HCO_2H. The organic acids were identified by co-chromatography of known samples. Malic acid was determined according to ref. [13]. The samples, dried at 45° under a stream of air and redissolved in H_2O, were assayed for

radioactivity in a scintillation counter. A scintillation soln of 3 g PPO, 0.2 g POPOP, 60 g naphthalene, 20 ml ethylene glycol, 100 ml MeOH and 880 ml dioxane was used.

$NaH^{14}CO_3$ was obtained from Radiochemical Center, Amersham, U.K. All other chemicals were from Sigma Chemical Co., St. Louis, Mo, U.S.A.

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