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Production of ethylene, its precursors and implied enzyme activities in isolated chickpea embryonic axes during the onset of growth

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Abstract

The embryonic axes of chickpea (*Cicer arietinum*) seeds were used to quantify 1-(malonyl)aminocyclopropane-1-carboxylic acid (MACC), 1-aminocyclopropane-1-carboxylic acid (ACC), ethylene and some related enzymes during the initial 18 h imbibition period (anaerobic growth phase). Longer cold storage (stratification) of seeds produced higher levels of MACC and ACC. Maximum accumulation of MACC and malonyl-transferase activity occurred after 5 h of growth but MACC levels later became insignificant. ACC-synthase activity and endogenous ACC seem to reach a maximum 2 h after MACC accumulation. MACC-hydrolase activity was measured "*in-vitro*" and reached a maximum after 5.5 h of growth. These results suggest that endogenous MACC did not seem to be an end product; it may be involved in ACC production and the regulation of ethylene production before the emergence of the radicle. Ethylene-forming enzyme (EFE) activity reached a maximum after 12 h and ethylene production after 18 h of growth. The physiological implications of this temporal separation of MACC, ACC, ethylene and related enzymes is discussed.

Introduction

During the earliest stages of germination, chickpea seeds undergo a period of natural anaerobiosis due to the poor penetration of oxygen through the seed coat.

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Abbreviations: ACC - 1-aminocyclo-propane-1-carboxylic acid; AOA - amino-oxyacetic acid; EFE - ethylene-forming enzyme; MACC - 1-(malonylamino)-cyclopropane-1-carboxylic acid; SAM - S-adenosyl-methionine.

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During this anaerobic phase, increases in the energy charge, fermentative and glycolytic pathways and dark CO_2 fixation have been observed in the embryonic axis (Aldasoro and Nicolas 1979, 1980). More recently we have demonstrated that ethylene production is related to cell elongation before radicle emergence (Sánchez-Calle *et al.* 1989), requiring a low-level synthesis of ethylene (Gallardo *et al.* 1993).

Ethylene plays a regulatory role in seed germination (Esashi 1991) and in higher plants it is derived from an immediate precursor ACC (Adams and Yang 1979, Yang and Hoffman 1984). ACC has been found in both its free and conjugated forms. A presumably inactive ACC conjugate has been identified as MACC (Amrhein *et al.* 1982, Hoffman *et al.* 1982, Hoffman *et al.* 1983). The cytosolic conversion of ACC into MACC is catalyzed by an ACC-N-malonyl-transferase (Kionka and Amrheim 1984) which has been purified recently (Guo *et al.* 1992) and this is thought to participate in the regulation of ethylene biosynthesis by removing unwanted ACC (Yang and Hoffman 1984, Mansour *et al.* 1986). Since the ethylene production rate is generally controlled by ACC levels and these levels are regulated in turn by the malonylation of ACC, malonylation should play an important role in regulating ethylene production (Hyodo 1991). Because MACC is a poor ethylene precursor and the conjugation of ACC to MACC is essentially irreversible, it used to be assumed that MACC was biologically inactive end product of ACC rather than a storage pool for ACC and hence for ethylene (Amrhein *et al.* 1982, Hoffman *et al.* 1983). ACC conjugation has been found to be induced when ACC-synthesis rates are high (Yang and Hoffman 1984).

Nevertheless, the conversion of MACC to ACC is possible. The hydrolysis of exogenous MACC to ACC, leading subsequently to ethylene production, has been shown (Jiao *et al.* 1986, Hanley *et al.* 1989), with MACC-hydrolase being involved in this process. The physiological importance of the MACC-hydrolase reaction is not clear because endogenous MACC levels are normally far below the apparent Km for MACC-hydrolase. Nevertheless, the conditions required to obtain significant MACC hydrolysis from plant tissues are an incubation time of several hours together with a high MACC concentration to induce the enzyme and maintain its activity.

The aim of this work is to present a quantitative analysis of ACC, MACC, ethylene and related enzymes in the embryonic axes of chickpea seeds during the onset of the anaerobic germination phase and to discuss their contribution to the hormonal control of radicle emergence.

Materials and methods

Plant material and seed germination: Seeds of chickpea (*Cicer arietinum* L. cv. Castellana) were bought commercially and stored in the dark at 4 °C until required. For each experiment seeds of uniform size were washed three times in sterile distilled water, soaked for 4 h in distilled water and the seeds then germinated in plastic trays (50 seeds and 150 cm^3 distilled water). After indicated periods the embryonic axes were excised in order to measure MACC, ACC, ethylene and related enzymes.

Quantification of ACC and ACC conjugate: Isolated embryonic axes were homogenized in a mortar with 30 cm^3 of cold ethanol 70 % (v/v) and then extracted under reflux with boiling ethanol for 20 min. The homogenate was subsequently filtered through four layers of fine gauze and evaporated under a vacuum at 45 °C, as described elsewhere (Philosoph-Hadas *et al.* 1985). The residue was resuspended in 2 cm^3 re-distilled water and then centrifuged for 20 min at 27 000 *g*. The ACC content in the aqueous extract was determined by its chemical conversion to ethylene upon the addition of NaOCl (Lizada and Yang 1979), with an efficiency of 80 %. The MACC was quantified by hydrolyzing a 0.6 cm^3 aliquot of ACC extract in 12 M NaOH at 100 °C for 3 h as described elsewhere (Hoffman *et al.* 1982). In all the determinations corrections were made for the efficiency of the conversion of ACC to ethylene by using authentic MACC synthesized by us according to Satoh and Esashi 1984) as an internal standard. The efficiency was of 80 - 84 %. Similar results in MACC quantification were obtained with acid hydrolysis (1 M HCl). The difference in ACC contents before and after basic or acid-hydrolysis was taken to be the amount of MACC in the extract (Hoffman *et al.* 1982).

Ethylene measurements: At different growth stages, isolated embryonic axes were aseptically transferred to 25 cm^3 flasks containing 0.25 cm^3 of distilled water. The flasks were sealed with silicone-rubber stoppers and incubated in darkness at 25 °C. After 30 min, 1 cm^3 of head-gas was taken from flasks with a hypodermic syringe and ethylene was quantified by gas chromatography with a *Perkin-Elmer MOD 8600*, equipped with 2 m × 3 mm glass columm packed with 80 - 100 mesh *Porapack-R*. Other details are described in Sánchez-Calle *et al.* (1989). Ethylene production was expressed in pmol g^{-1}(f.m.) h^{-1}.

***In vivo* EFE activity:** 1 g of embryonic axes was placed in darkness in a vial with 0.25 cm^3 of either 200 µM ACC or distilled water at 25 °C. To assay EFE the vials were left open for 60 min and the ethylene production rate was measured as described above. Controls were vials with either ACC or distilled water, but without plant material. EFE activity is expressed as the increase in embryonic-axis ethylene production due to added ACC.

***In vivo* ACC-synthase and ACC-N-malonyl-transferase activities:** ACC-synthase activity in homogenates of some plant tissues is often either much lower than expected or not even measurable. This is also the case with ACC-synthase from the embryonic axes of chickpea seeds. We were unable to quantify ACC-synthase *in vitro* as has also been the case with other plants (*e.g.* Boller *et al.* 1979, Mansour *et al.* 1986, Tsai *et al.* 1988) possibly due to their labile nature and its loss during homogenization. Conversion of ACC to ethylene is blocked in a nitrogen atmosphere and thus ACC accumulates under these conditions (Adams and Yang 1979). Therefore, the accumulation of ACC under nitrogen can serve to measure *in vivo* ACC-synthase activity (Cohen and Kende 1987, Sarquis *et al.* 1992). We have measured *in vivo* ACC synthase basically as described in Gallardo *et al.* (1992). Embryonic axes (2 g) sampled at certain time interval were divided into two batches to quantify the ACC and MACC contents: before and after incubation for 4 h under

nitrogen. Preliminary experiments indicated that the accumulation of ACC was linear for at least 6 h. The oxygen content in the nitrogen gas phase was less than 0.1 %. At the end of the anaerobic incubation period the tissue was immediately frozen in liquid nitrogen. ACC-synthase and malonyl-transferase activities were expressed as an increase in ACC or MACC content (before and after nitrogen treatment) in nmol g^{-1} (f.m.) h^{-1}.

Extraction and assay of MACC-hydrolase activity *in vitro*: The method described by Jiao *et al.* (1986) was used with some modifications. Embryonic axes (6 g) were homogenized in a mortar with 25 cm^3 of 25 mM potassium-phosphate buffer, pH 7.0. The homogenate was filtered through four layers of miracloth and centrifuged at 27 000 *g* for 12 min. The supernatant was fractionated with $(NH_4)_2SO_4$. The precipitate obtained at 35 % to 80 % saturation was redissolved in 2 cm^3 of 10 mM potassium-phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer. The dialyzed extract was employed as the crude enzyme solution. Protein concentration was determined according to Bradford (1976). The assay of MACC-hydrolase activity was carried out in a total volume of 1 cm^3 with a reaction mixture containing: 4 mM MACC [synthesized by us according to Satoh and Esashi (1984) from methyl malonyl chloride and ACC], 10 mM potassium-phosphate buffer, pH 8.0, 1 mM $MnSO_4$, and enzyme solution. After incubation at 37 °C for 1 h the reaction was stopped by transferring the tubes to 0 °C and the ACC formed was quantified according to Lizada and Yang (1979). Control assays were carried out in the same way with heat-inactivated enzyme. Enzyme activity was expressed in nmol ACC formed above that of the control.

Results and discussion

It has previously been found that during the anaerobic phase of chickpea seeds germination a rapid increase in ATP and glycolytic intermediates occurs in the embryonic axis (Aldasoro and Nicolás 1979). We have demonstrated that elongation, ethylene production and the sequential appearance of RNA species take place during the onset of growth of the axis (Rodriguez *et al.* 1982, Bueno and Matilla 1992). A relationship between elongation and ethylene production has recently been found (Sánchez-Calle *et al.* 1989). To gain further insight into the ethylene pathway in relation to the earliest stages of germination we determined the levels of MACC, malonyl-transferase activity, MACC-hydrolase activity, ACC, ACC-synthase activity and EFE activity during the first hours of growth of the embryonic axes of chickpea seeds.

The first intermediate detected was MACC, which reached a maximum at 5 h germination (Fig.1). No significant amounts of MACC were detected before 4 h and after 6 h germination. In a previous paper (Gallardo *et al.* 1991), we have demonstrated that transport of MACC and ACC from cotyledons to embryonic axis is possible in chickpea seeds. Two hours after MACC accumulation, which is most probably synthesized in the cytosol and stored in the vacuole (Bouzayen *et al.*

1990), an ACC peak appeared (Fig. 2). MACC and ACC in chickpea seeds axes were identified and confirmed using the combined techniques of mass spectrometry and gas chromatography of their dimethyl ester, and by a comparison between synthetic MACC (Satoh and Esashi 1984, Pech, personal communication) and commercial ACC.

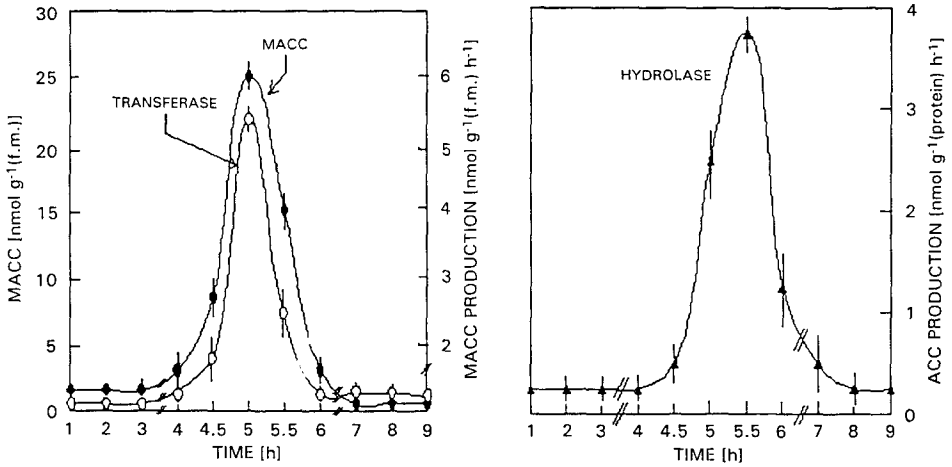

Fig. 1. MACC (*closed circles*) content, *in vivo* ACC-N-malonyl-transferase activity (*open circles*) and *in vitro* MACC-hydrolase activity (*closed triangles*) during the first nine hours of growth of chickpea embryonic axes. Each point is the mean of three different extractions ± SE (*vertical bars*).

Since ACC-synthase activity cannot be measured in homogenates of chickpea seeds we made an *in vivo* assay to determine the activity of this enzyme. This assay is based on the accumulation of ACC in tissues kept under N_2 (Cohen and Kende 1987, Gallardo *et al.* 1992). ACC accumulation was linear until 6 h of anaerobiosis and was not affected by 50 μM cycloheximide (Fig. 2, inset). The *in vivo* ACC-synthase activity was detected after 4 h of germination and reached its maximum at 7 h, after which the capacity of the axis to accumulate ACC in anaerobiosis decreased gradually until it could not be detected at all after 9 h. ACC-synthase activity during the first 4 h of germination was very slight (Fig. 2). These data may mean that either the enzyme is not synthesized during this period or that an already existing enzyme is inhibited. The absence of ACC-synthase activity before 4 h of growth is in agreement with: (*1*) not detectable ACC and MACC, (*2*) not quantifiable EFE activity, and (*3*) no production of ethylene. In wounded tomato fruit, Kim and Yang (1992) found that ACC-synthase has a rapid turnover *in vivo* and suggest that the ubiquitin-requiring pathway may be involved in the degradation of this enzymatic protein.

ACC malonylation may play a role in the regulation of endogenous ACC levels and hence ethylene production (Yang and Hoffman 1984). For this reason it can be concluded that MACC and ACC synthesis are synchronized. It can be supposed that some MACC detected at 5 h (Fig. 1) was produced from ACC newly synthesized after 4 h. This ACC must be rapidly transformed into MACC because both EFE activity and ethylene production were very low (Fig. 4). The malonyl-transferase

activity (measured as an anaerobic accumulation of MACC) after 5 h of growth (Fig. 1) prevents the accumulation of ACC, and consequently ACC-synthase activity (measured as an anaerobic accumulation of ACC) was very low at that time (Fig. 2). The *in vivo* activities of both malonyl-transferase and ACC-synthase were higher at low partial O_2 pressure than in aerobiosis (data obtained from aerobic controls of the *in vivo* ACC-synthase measurements). Similar conclusions have been arrived at with deep-water rice (Cohen and Kende 1987). Our results indicate that malonylation of ACC may participate in regulating ethylene production during the anaerobic phase of germinating chickpea seeds.

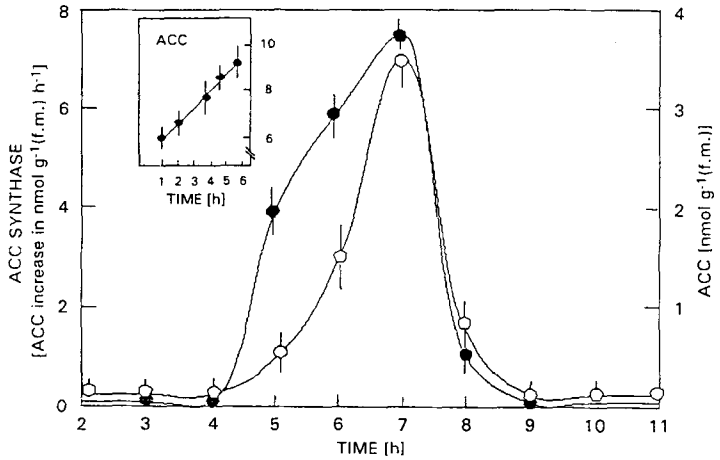

Fig. 2. ACC content (*closed circles*) and *in vivo* ACC-synthase activity (*open circles*) during the first 12 h of growth of chickpea embryonic axes. Inset: accumulation of ACC under anaerobic conditions both with and without cycloheximide. Each point is the mean of 3 or 4 different extractions ± SE (*vertical bars*).

Furthermore, the transformation of MACC into ACC, catalyzed by a MACC-hydrolase (Fig. 1) may also be involved in the appearance of ACC. Jiao *et al.* (1986) and Hanley *et al.* (1989) have demonstrated the presence of MACC-hydrolase activity in tobacco and carnation tissues, with MACC being transformed into ACC. Here it may be concluded that the fall in MACC levels after 5 h of growth is a consequence of this hydrolase activity. On assaying MACC-hydrolase activity in partially purified extracts of embryonic axes, the maximum quantity of ACC synthesized *in vitro* [3.75 nmol ACC mg^{-1}(protein) h^{-1}] was obtained in extracts of axes grown for 5.5 h (Fig. 1). Jiao *et al.* (1986) have reported that a significant conversion of MACC into ACC can only be induced by exogenous MACC since the endogenous MACC content is far below the Km of the enzyme. We measured an *in vitro* MACC-hydrolase activity without previous incubation of the tissue with exogenous MACC. The endogenous levels of MACC from 4.5 to 5.5 h (Fig. 1) seem to be capable of inducing this hydrolase activity. The addition to the germination medium of AOA, an inhibitor of ACC-synthase, was a further way of demonstrating that: (*1*) some ACC comes from MACC, and (*2*) actually this MACC must come

from cotyledons since ACC-synthase was inhibited. At 5 and 7 h, AOA (0.1 - 5 mM) induced an increase in ACC concomitantly with a decrease in MACC (Fig. 3). We did not test the effect of AOA on malonyl-transferase and MACC-hydrolase. Radicle emergence was not hindered by AOA (results not shown). The results shown in Fig. 1 seem to indicate that malonyl-transferase predominates over MACC-hydrolase before 5 h of germination and *vice versa* after 5 h.

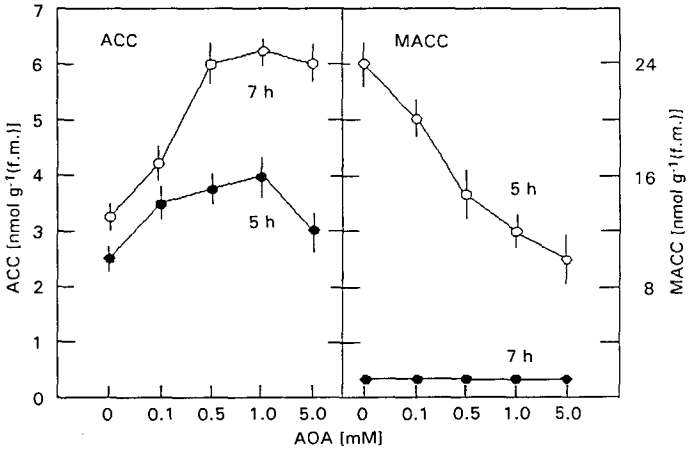

Fig. 3. Effect of AOA (0.1 - 5 mM) on both ACC and MACC content at 5 h (peak of MACC) and 7 h (peak of ACC). Each point is the mean of three different experiments ± SE (*vertical bars*).

Table 1. Effect of stratification (3, 7 or 11 months) of chickpea seeds on MACC and ACC contents [pmol g^{-1}(f.m.)]. Embryonic axes were isolated at 5 and 7 h and precursors quantified as described. Values represent the means of 3 - 4 different extractions ± SE.

Time [h]	3 months		7 months		11 months	
	ACC	MACC	ACC	MACC	ACC	MACC
5	8 ± 1	96 ± 5	5 ± 1	843 ± 21	1990 ± 100	24400 ± 1000
7	7 ± 1	60 ± 2	40 ± 6	14 ± 5	3300 ± 100	381 ± 40

All the results in this work were obtained using seeds stratified for 11 months at 4 °C, since shorter storage periods (3 and 7 months) resulted in lower levels of intermediates (Table 1). This indicates that an important induction of metabolic activities related to the ethylene pathway takes place during cold storage, as is also the case with other phytohormones (Julin-Tegelman and Pinfield 1982). Some pear cultivars require a period of cold storage for the initiation of ripening and ACC accumulation has been shown to precede ethylene production (Morin *et al.* 1985). MACC increases concomitantly with the storage period of chickpea seeds (Table 1). This endogenous MACC might be used to some extent by germinating stratified seeds as an ACC pool, and therefore for ethylene, thus conferring an important physiological role to MACC-hydrolase.

Finally, we measured *in vivo* EFE activity and ethylene production. From 4 h to

12 h EFE activity increased 8-fold and then decreased (Fig. 4). Ethylene production was detected after 6 h and increased until 16 h (Fig. 4). The present data, together with others published elsewhere (Esashi 1991), reveal that the embryonic axis of the

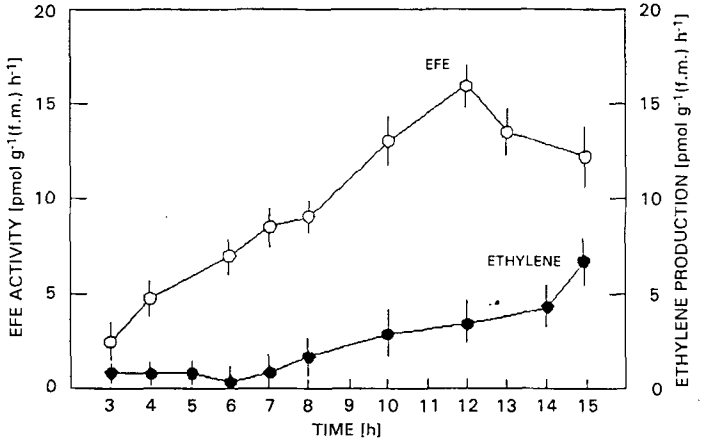

Fig. 4. Variations in EFE activity (*open circles*) and ethylene production (*closed circles*) during the first 16 h of growth of chickpea embryonic axes. Each point is the mean of 3 or 4 different experiments ± SE (*vertical bars*).

chickpea seeds produces ethylene before radicle emergence. This anaerobic production of ethylene is necessary for germination (Gallardo *et al.* 1993) and clearly indicates that ethylene is capable of acting on seed germination independently of O_2. The action of ethylene on germination was observed even in anoxia, where traces of the gas were produced (Esashi *et al.* 1976). Ethylene production during the anaerobic germination phase may result from a more active CO_2 evolution (Esashi *et al.* 1985, 1986) suggesting a synergistic relationship in the promotion of germination by CO_2 and ethylene. The nature of ethylene action in seed germination is not at present clear although it may promote radicle cell expansion (Abeles 1986). It is concluded that the metabolism of both ACC and MACC and subsequent anaerobic ethylene production in the embryonic axis play an important role in the emergence of the radicle axis in chickpea seeds.

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