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# *IN VIVO* AND *IN VITRO* ACTIVITY OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE IN GERMINATING SEEDS OF CHINA ASTER (*CALLISTEPHUS CHINENSIS* NEES)

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ABSTRACT

The activity of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO; EC 1.4.3.3) in germinating seeds of *Callistephus chinensis* was studied. For maximum recovery of ACO activity *in vitro*, the presence of 10% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and 30% of glycerol in the extraction medium was necessary. The optimum pH for this activity was 7.0. Ethylene production by whole achenes or enzymatic extract increased due to increasing 1-aminocyclopropane-1-carboxylic acid (ACC) concentrations. Saturation level of ACC for *in vivo* ACO activity was $10^{-1}$ M and $V_{max}$ was 10.89 nL $C_2H_4 \cdot$mg protein$^{-1} \cdot h^{-1}$. For *in vitro* ACO activity, the saturation level of ACC was $10^{-3}$ M and $V_{max}$ was 2.299 nL $C_2H_4 \cdot$mg protein$^{-1} \cdot h^{-1}$. Both, *in vivo* and *in vitro* ACO activities did not follow Michaelis-Menten kinetics. The Hill coefficients (*h*) were estimated on the basis of non-linear estimation. Their values were 0.63 for *in vivo* ACO activity and 1.73 for *in vitro* ACO activity. The experimental data show that ACO from *C. chinensis* seeds is an oligomeric enzyme with at least two active sites. During seed germination, *in vitro* ACO activity was detectable after 12 hours of imbibition, while *in vivo* ACC conversion to ethylene was observed after 24 h, i.e. – after radicle protrusion. The activity of ACO in *C. chinensis* seeds is associated with germination *sensu stricto*, and might be a good marker of this process.

**Keywords**: ACC oxidase, ACO, *Callistephus chinensis*, germination *sensu stricto*

## INTRODUCTION

Ethylene has a crucial function in breaking dormancy and stimulating germination of seeds (Kępczyński & Kępczyńska 1997; Matilla 2000, Corbineau et al. 2014). However, the quantities of endogenous ethylene released from seeds are barely detectable before seed coat rupture by the radicle. The evolution of ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) conversion into ethylene is mainly located in the embryonic axes (Petruzzelli et al. 2000) and can be regulated by positive feedback. Close relationships between ethylene production by germinating seeds of some species and their vigor have been found (Samimy & Taylor 1983; Fu et al. 1988; Gorecki et al. 1991; Chojnowski et al. 1997, Siriwitayavan et al. 2003). For some vegetable seeds, measurement of ACC-dependent ethylene production was proposed as a rapid vigor test (Khan 1994). Incubation of seeds in the solutions of ACC, an immediate precursor of ethylene synthesis, significantly increased sensitivity of this test (Khan 1994). Seeds of *Callistephus chinensis*, differing in vigor due to their position on the mother plants show variability in their ability to convert ACC to ethylene (Grzesik et al. 1997) corresponding to seed germinability.

Since the complete recovery of ethylene forming activity *in vitro* was described by Ververidis and John (1991), 1-aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase, ACO; EC 1.4.3.3) (Pech et al. 2010) has been isolated and characterized in many plants.

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The essential cofactors necessary for its activity are ascorbate and iron (Bouzayen et al. 1991; Ververidis & John 1991) as well as $CO_2$ (Smith & John 1993; Yang & Dong 1993; Mathooko 1996). ACC-oxidase is a bi-substrate enzyme and oxygen is necessary as a second substrate (McGarvey & Christoffersen 1992; Bailly et al. 1995; Mirica & Klinman 2008).

There are many isoforms of ACO described in plant tissues. The biochemical properties, genes encoding ACO as well as the transcriptional, post-transcriptional, and post-translational control of ACO were reviewed by Ruduś et al. (2013) and Houben and Van de Poel (2019). In germinating seeds, ACO was isolated and studied for many species, but it was characterized only for some of them, for example, for chickpea (Muñoz de Rueda et al. 1995), *Amaranthus caudatus* (Kępczyński et al. 1999), and *Stylosanthes humilis* (Barros et al. 2013).

The aim of this study on regulating ACO activity in germinating seeds of *C. chinensis* was: (I) to optimize the extraction and incubation methods for measuring ACO *in vitro* activity, (II) to study the effects of ACC concentration on *in vivo* and *in vitro* activity of ACO, and (III) to evaluate this activity in dry and germinating seeds.

## MATERIALS AND METHODS

### Seed germination

Seeds of *C. chinensis* Nees 'Hölderin' were obtained from PlantiCo Gołębiew (Poland). The seeds were stored in paper bags in a cold room, at a temperature of 5 °C and at 40% relative humidity. Seeds (whole achenes) were placed in 60 mm Petri dishes on one layer of filter paper (Whatman, no. 17) moistened with 3 mL of distilled water. Petri dishes were tightly closed with Parafilm. Germination was carried out in darkness at 25 °C, i.e. – optimal temperature for this cultivar (Chojnowski 2000). To estimate the enzyme activity, seeds not germinated and germinated for 12, 24, 36 or 48 hours (depending on the experiment) were used.

### ACC oxidase extraction

For extraction of ACC oxidase the whole achenes or seeds without pericarps were frozen at −70 °C and ground with a mortar and pestle. The powder was mixed with an extraction medium that consisted of 0.1 M Tricine buffer (pH 6.5, 7.0, 7.5 or 8.0), 30% (v/v) glycerol, 5 or 10% insoluble polyvinylpolypyrrolidone (PVPP), 30 mM sodium ascorbate, 0.1 % (v/v) Triton X-100 and 5 mM dithiothreitol, using 1 g of seed material and 2 mL of extraction medium. The mixture was centrifuged for 30 min at 28,000 × g. The extraction procedure was conducted at 4 °C.

### *In vitro* enzyme assay

The activity of ACO was estimated according to a modified method of Bailly et al. (1995). *In vitro* activity of ACO was assayed in a 1-mL reaction mixture including 0.2 mL of seed crude extract, 0.1 M Tricine buffer, 30% glycerol, 30 mM sodium ascorbate, 0.1 mM $FeSO_4$, 25 mM $NaHCO_3$ and ACC at different concentrations. Ethylene produced at 25 °C in 10 mL flasks tightly closed with serum caps was collected for various periods (15 minutes to 5 hours depending on the experiment) and measured using gas chromatography.

### *In vivo* enzyme activity and ethylene measurements

The *in vivo* conversion of ACC to ethylene was assayed in whole achenes. Five replications of 10 seeds each were placed in 10 mL flasks with 0.5 mL water or various ACC solutions. After 1 h pre-incubation at 25 °C, flasks were tightly closed with serum caps for an additional 1 h. For ethylene measurement, 1 mL headspace gas samples were withdrawn from sealed flasks and injected into a gas chromatograph (HP 5890) equipped with an activated alumina column (90 cm long, 0.4 cm i.d.) and a flame ionization detector. Argon was used as a carrier gas at a flow rate of 20 $mL \cdot min^{-1}$, and the oven temperature was 100 °C. Results are expressed as nL $C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$.

### Protein determination

Determination of protein content in germinating seeds was conducted according to the method of Bradford (1976), and BSA was used as a standard.

### Statistical analysis

$K_m$ defined as the concentration of the substrate, which results in half-maximal velocity for the enzymatic reaction (Copeland 2000), for *in vivo* and *in vitro* ACO activity was calculated by non-linear

estimation using Levenberg-Marquardt method on the basis of the Henri-Michaelis-Menten equation:

$$V = (V_{max}[S])/(K_m + [S]).$$

The Hill coefficient (*h*) was calculated by non-linear estimation using Levenberg-Marquardt method on the basis of the Hill equation:

$$V = (V_{max}[S]^h)/(K' + [S]^h),$$

where V is the *in vivo* or *in vitro* ethylene production rate at a given concentration of substrate [S] and $V_{max}$ is the ethylene production rate at a saturating substrate concentration, K' is a constant, however, it is not a value that relates to the concentration of substrate, which is necessary to obtain half of maximum velocity of the enzyme activity (Copeland 2000).

All the data were statistically analyzed and presented graphically in Statistica 13 data analysis software system (Dell 2016). Data were presented as a mean of 10 replications (two independent experiments of 5 replications each) ± standard error (SE) or compared by Duncan's test.

## RESULTS AND DISCUSSION

### Extraction and incubation conditions

The highest *in vitro* activity of ACC oxidase from *C. chinensis* seeds was obtained after extraction and incubation at pH 6.5 and 7.0 (Fig. 1). Increasing pH to 7.5 decreased activity by 30%, and at pH 8.0, it was almost completely inhibited. A higher amount of PVPP (10% compared with 5%) in the extraction medium and addition of $NaHCO_3$ (25 mM) to the incubation buffer significantly increased the ACC activity – by 77% (Table 1). Applied alone, the higher level of PVPP or the addition of $NaHCO_3$ increased the activity of ACC oxidase by 16.4% and 6.6%, respectively.

The method of Bailly et al. (1995) originally used for sunflower hypocotyl segments, applied to *C. chinensis* seeds without any modifications, resulted in very limited level of ACO recovery *in vitro* (data not shown). Optimizing both buffers and pH levels was necessary. ACO from *C. chinensis* seeds shows the highest activity in Tricine buffer (data not shown). Our work showed that ACO from *C. chinensis* has a pH optimum close to 7.0, similar to the enzyme obtained from carnation petals (Nijenhuis-de Vries et al. 1994), corn and sunflower (Finlayson et al. 1997), and ACO from seedlings of *Pinus nigra* (Reynolds & John 2000). Other reports showed that optimum pH for *in vitro* activity of ACO isolated from apple fruit was 7.2 and 7.6 depending on the extraction buffers (Kuai & Dilley 1992). These differences may result from a buffer applied for extraction and/or incubation procedure. For example, to obtain maximum *in vitro* activity of ACO from carnation petals, different pH for extraction and incubation was necessary (Nijenhuis-de Vries et al. 1994; Wawrzyńczak 2002). On the other hand, the most important factor might be the plant and organ dependent characteristics of the enzyme. Gong and McManus (2000) showed that ACC oxidases expressed during the ontogeny of white clover leaves differ in their characteristics, and two different isoforms of the enzyme showed optima of activity at pH 7.5 and 8.5.

Contrary to the method described by Bailly et al. (1995), our results showed that higher levels of PVPP and the addition of $NaHCO_3$ were necessary for optimal ACO activity (Table 1). The higher glycerol concentration (30% compared with 10%) improved the recovery of ACC oxidase activity *in vitro* (data not presented) and such concentration of glycerol was used in further experiments. It had been previously shown (Fernández-Maculet & Yang 1992) for apple fruits that PVPP greatly improved the recovery of EFE activity from a pellet fraction, and this recovery was enhanced by the presence of ascorbate. In our experiments, increasing the amount of PVPP from 5 to 10% followed by incubation of the enzyme extract with 25 mM of $NaHCO_3$ gave a significant increase of *in vitro* ACC oxidase activity (Table 1). The addition of $HCO_3^-/CO_2$ to the incubation medium also stimulated *in vitro* ACC oxidase activity in melon fruits (Smith & John 1993), carnation petals (Nijenhuis-de Vries et al. 1994), chickpea embryonic axes (Muñoz de Rueda et al. 1995) and corn seedlings (Finlayson et al. 1997). ACC oxidase, similar to the RuBisCO, is not only activated by $CO_2$, but $CO_2$ is also involved in the enzymatic reaction (Smith & John 1993; Yang & Dong 1993).

**Concentration of ACC**

The rate of ethylene production *in vivo* increased with increasing ACC concentration and the highest rate (10.89 nL $C_2H_4$·mg protein$^{-1}$·h$^{-1}$) was detected at $10^{-1}$ M ACC (Fig. 2). The *in vitro* activity of ACC oxidase was also dependent on substrate concentration, and the saturation level (2.299 nL $C_2H_4$·mg protein$^{-1}$·h$^{-1}$) was reached at a concentration of $10^{-3}$ M ACC (Fig. 3).

Saturation level of ACC for *in vitro* ACC oxidase highly depends on the experimental material. The amount of ACC ($10^{-3}$ M), which was necessary to reach saturation level for ACC oxidase from *C. chinensis* seeds (Fig. 3) was similar to that observed for sunflower hypocotyls (Bailly et al. 1995) and chickpea seeds (Muñoz De Rueda et al. 1995). Bailly et al. (1995) reported that increasing the amount of ACC to $10^{-2}$ M lowered the activity of ACC oxidase from sunflower hypocotyl segments. For other materials like corn roots and leaves (Finlayson et al. 1997) or leaves of white clover (Gong & McManus 2000), two to a five-fold higher amounts of ACC were necessary to saturate the enzyme.

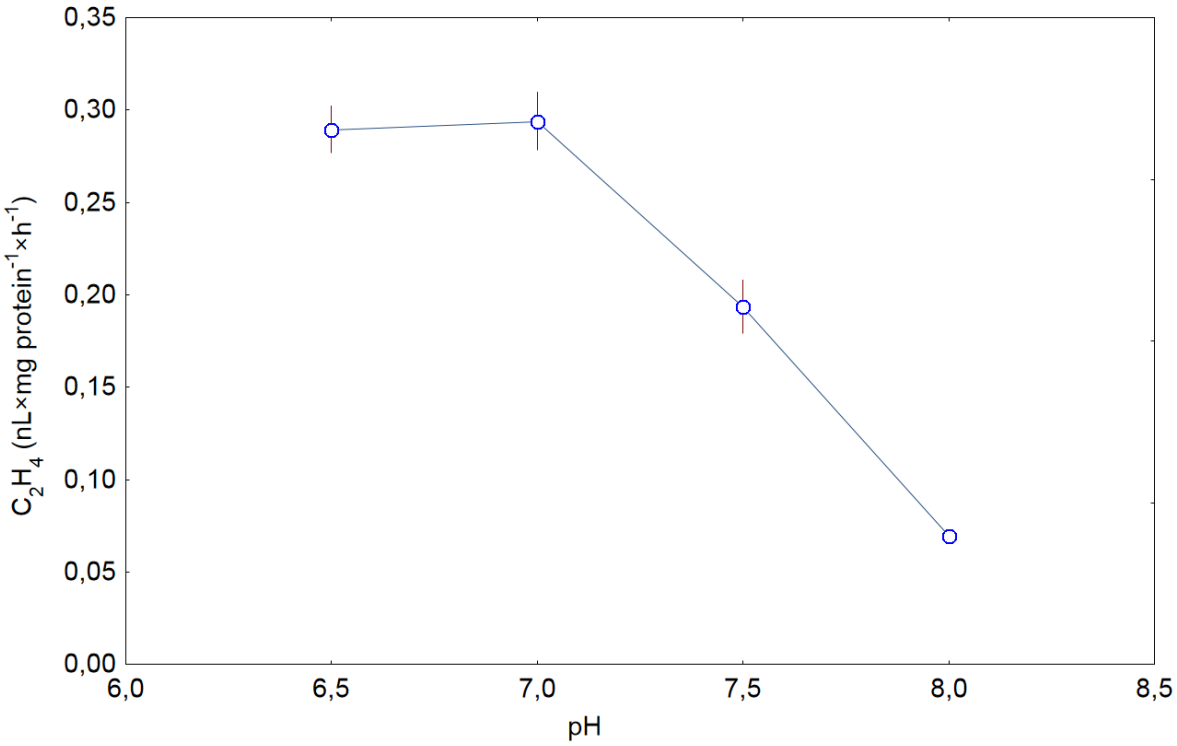


Figure 1. Effect of pH on *in vitro* ACC oxidase activity from *Callistephus chinensis* seeds germinated for 24 h; vertical bars denote ±SE

Table 1. Effect of PVPP and $NaHCO_3$ on *in vitro* activity of ACC oxidase from *Callistephus chinensis* seeds germinated for 48 h. Means followed with the same letter are not significantly different at p = 0.05 according to Duncan's test; mean ± SE

| PVPP | $NaHCO_3$ | Ethylene (nL·mg protein$^{-1}$·h$^{-1}$) | Increment (%) |
|---|---|---|---|
| 5% | - | 1.086 a ± 0.042 | - |
| 5% | 25 mM | 1.158 a ± 0.154 | 6.6 |
| 10% | - | 1.246 a ± 0.073 | 16.4 |
| 10% | 25 mM | 1.925 b ± 0.217 | 77.0 |

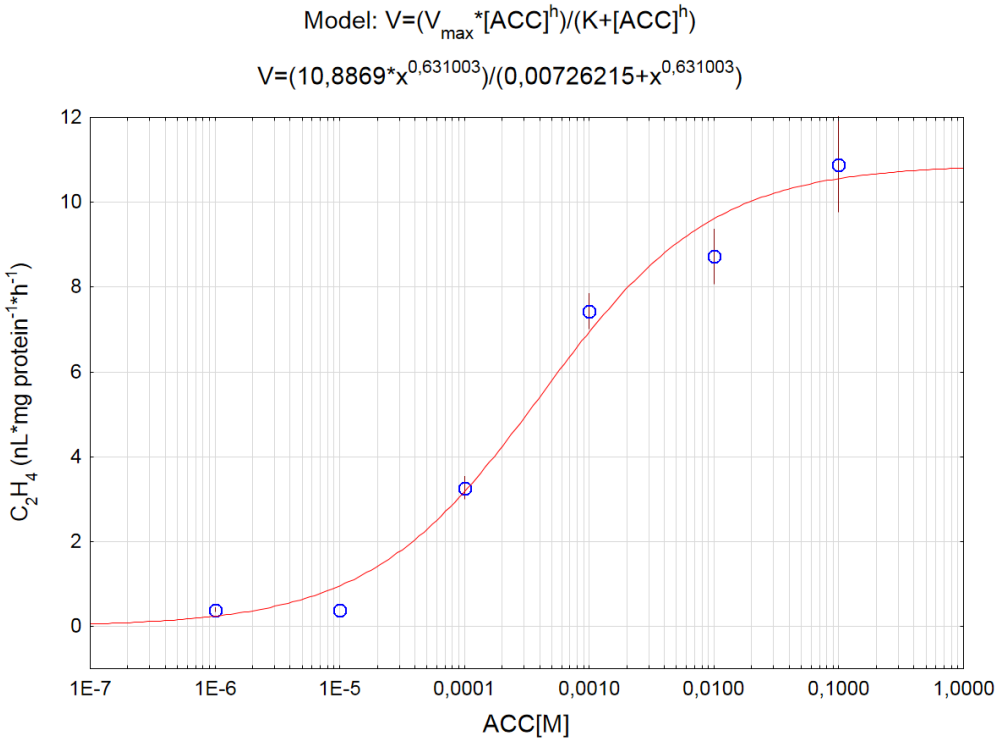


Figure 2. Effect of ACC concentration on *in vivo* ACC oxidase activity at 25 °C in *Callistephus chinensis* seeds germinated for 48 h; vertical bars denote ±SE

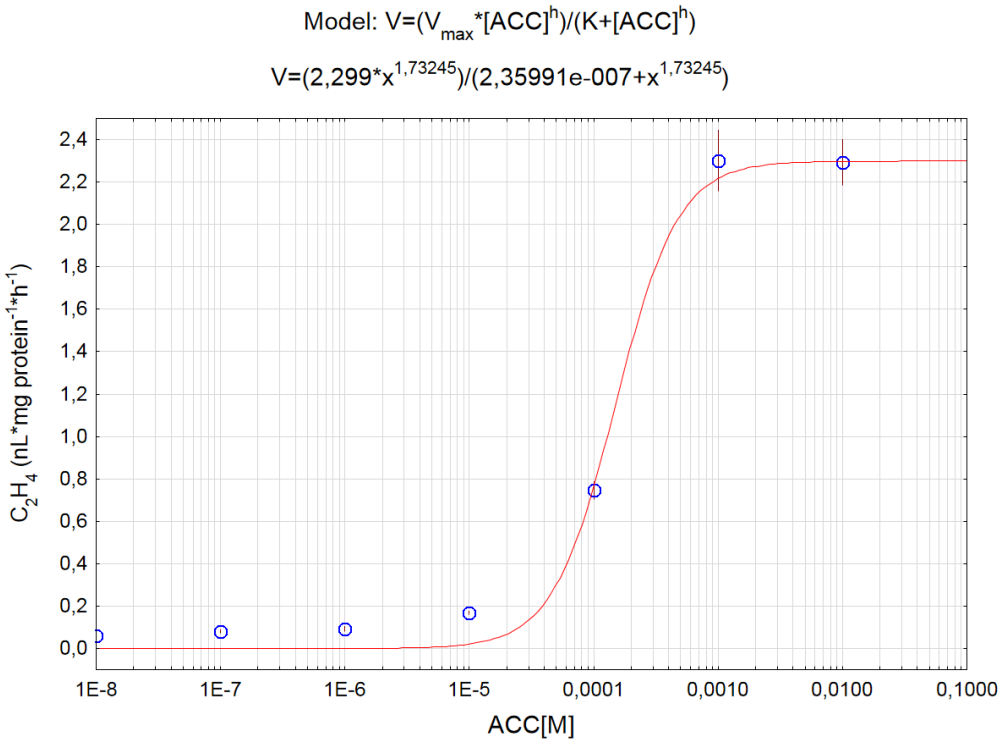


Figure 3. Effect of ACC concentration on *in vitro* ACC oxidase activity at 25 °C in *Callistephus chinensis* seeds germinated for 48 h; vertical bars denote ±SE

The ACO from *C. chinensis* seeds show relatively low affinity to ACC. The $K_m$ value calculated on the basis of non-linear estimation was 368 μM. The *in vitro* estimated $K_m$ value (154 μM) showed that the affinity of the enzyme for ACC evaluated *in vitro*, was more than twice higher that for *in vivo* activity (Table 2). However, non-linear estimation of ACC enzyme kinetics showed that the *in vivo* and *in vitro* activities of the ACC oxidase did not follow Michaelis-Menten kinetics. However, many researchers reported Michaelis-Menten kinetics of ACO activity. For example, for ACO from sunflower hypocotyl segments (Bailly et al. 1995) – $K_m$ estimated *in vivo* was 219 μM, for ACO from *Stylosanthes humilis* seeds – $K_m$ was 230 μM (Barros et al. 2013), while for chickpea seeds – $K_m$ was only 120 μM (Muñoz de Rueda et al. 1995). For *in vitro* activity of ACO from *S. humilis* seeds, Barros et al. (2013) reported similar $K_m$ value to that obtained in our experiment (156 μM). On the other hand, $K_m$ for *in vitro* activity ACO from sunflower hypocotyl segments (Bailly et al. 1995) and chickpea seeds (Muñoz de Rueda et al. 1995) was as low as 20.6 μM and 28 μM.

ACC oxidase from *C. chinensis* does not show simple hyperbolic saturation curve, but a sigmoidal dependence of ethylene production as a function of ACC concentration (Fig. 2 & 3). The Hill constants (*h*) obtained on the basis of the non-linear estimation of Hill equation (Copeland 2000) were 0.63 for *in vivo* ACC oxidase activity and 1.73 for *in vitro* ACC oxidase activity (Table 2). The results obtained in our experiments suggest that ACO from *C. chinensis* seeds is an oligomeric enzyme with at least two substrate binding sites. The experimental data showed positive cooperativity between the subunits of ACO analyzed *in vitro*, while for *in vivo* ACO activity, this cooperation was negative. Hill coefficient $h = 1$ and the oligomeric enzyme activity follows the Michaelis-Menten kinetics only when there is no cooperativity between the active sites of the enzyme (Copeland 2000). Such a lack of cooperativity can explain the Michaelis-Menten kinetics of ACO activity obtained in previous experiments reported by other authors. The results of our experiments are in agreement with the results presented by Zhang et al. (2004), who showed in crystallographic studies the existence of dimeric and tetrameric forms of ACO from *Petunia hybrida*. An additional effect on the enzyme kinetics, both *in vivo* and *in vitro* can be the result of the fact that ACO is a bi-substrate enzyme, and additionally, the presence of iron, ascorbate and $CO_2$ are necessary for its activity (Bouzayen et al. 1991; Ververidis & John 1991; Smith & John 1993; Yang & Dong 1993; Mathooko 1996; Mirica & Klinman 2008).

**Time course of in vitro ACC oxidase activity**

The velocity (the rate of ethylene released over time) of the *in vitro* ACC oxidase activity at 25 °C reached the maximum value after 30 min (Fig. 4) and until 120 min velocity was maintained at about 2.3 nL ethylene·mg protein$^{-1}$·h$^{-1}$. Even after five hours, the velocity of the enzymatic reaction was maintained (2.1nL $C_2H_4$·mg protein$^{-1}$·h$^{-1}$). The time course of activity shows linearity in ACC ($10^{-3}$ M) conversion into ethylene in the crude extract at 25 °C for at least five hours (Fig. 4). However, non-linear time-course for *in vitro* ACO was previously reported in some cases – for partially purified enzyme from melon fruits (Smith et al. 1992), for tomato ACO purified from transformed *Escherichia coli* (Smith & John 1993) and for crude extract from carnation petals (Nijenhuis-de Vries et al. 1994).

***In vivo* and *in vitro* ACC oxidase activity in time-course of germination**

Figure 5 shows the activity of the ACC oxidase during seed germination. Conversion of ACC into ethylene *in vivo* was detectable after 24 hours of germination, i.e., after radicle protrusion through testa and pericarp. Neither *in vivo* nor *in vitro* activity was detectable in dry seeds. *In vitro* ACC oxidase activity was detectable in the crude extracts from whole achenes after 12 hours of seed germination (Fig. 5).

Whole achenes of *C. chinensis* do not produce detectable amounts of ethylene during the first hours of imbibition, similar to sunflower achenes (Corbineau et al. 1989; Chojnowski et al. 1997). Ethylene evolution *in vivo*, even in the presence of $10^{-3}$M ACC was observed when majority of seeds in the sample had already germinated, i.e., after 24 hours of imbibition (Fig. 5).

It is possible, similar to the sunflower seeds, that pericarp is the main barrier for ethylene release or can inhibit ACC conversion to ethylene, because it may be rich in phenolic compounds (Corbineau et al. 1989). However, if assayed *in vitro*, *C. chinensis* seeds did not show significant differences in ACO activity with or without pericarp (data not shown). Non-dormant seeds of sunflower, even after the removal of the pericarp, were not able to convert ACC to ethylene during the first four hours of imbibition (Corbineau et al. 1989; Chojnowski et al. 1997). Afterwards, seeds could convert ACC to ethylene and produce endogenous ethylene. The level of this production was increasing during the progress of germination and in many seeds was correlated with seed vigor (Fu et al. 1988; Gorecki et al. 1991; Khan 1994; Chojnowski et al. 1997, Siriwitayavan et al. 2003). Even though *in vitro* ACO activity in germinating seeds of *C. chinensis* was detectable earlier than *in vivo* (Fig. 5), there was no ACO activity in dry seeds. Both *in vivo* and *in vitro* ACO activity increased with progress in the germination process, contrary, for example, to the ACO from *A. caudatus* seeds assayed *in vitro*, which already increased after the germination was completed (Kępczyński et al. 1999).

Even if there is no radicle protrusion during germination in the water, germination *sensu stricto* (as defined by Côme & Thévenot 1982) takes place. In *C. chinensis*, similar to sunflower seeds, the ability to convert ACC to ethylene increases with increased imbibition time (Chojnowski et al. 1997). In sunflower seeds this effect can be maintained during storage of primed seeds (Chojnowski et al. 1997). Thus, the activity of ACO in *C. chinensis* seeds is associated with germination *sensu stricto*, and seems to be a good marker of this process, which takes place, for example, during seed osmoconditioning.

Table 2. Apparent $V_{max}$, $K_m$ and Hill coefficient *h* of ACC oxidase from *Callistephus chinensis* seeds analyzed *in vivo* and *in vitro* at 25 °C

| ACC oxidase activity | $V_{max}$ (nL $C_2H_4$·mg protein$^{-1}$·h$^{-1}$) ± SE | $K_m$ for ACC (μM) ± SE | Hill coefficient *h* ± SE |
|---|---|---|---|
| *In vivo* | 10.89 ± 1.13 | 368 ± 92 | 0.63 ± 0.097 |
| *In vitro* | 2.299 ± 0.14 | 154 ± 20 | 1.73 ± 0.39 |

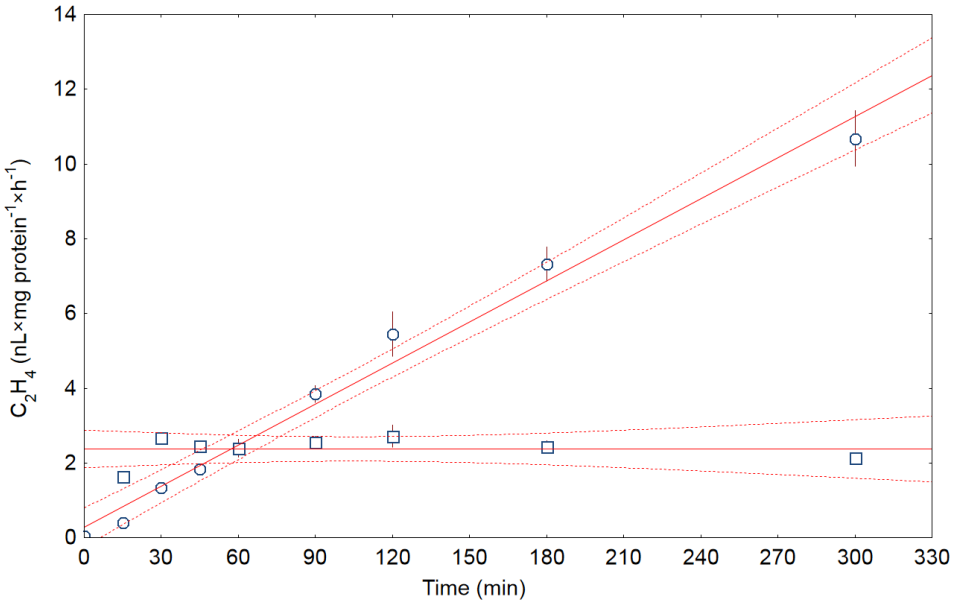


Figure 4. Time-course (total ethylene accumulation) (○) and velocity (amount of ethylene formed per hour) (□) of activity *in vitro* ACC oxidase from *Callistephus chinensis* seeds germinated for 48 h; vertical bars denote ±SE; dashed lines are 95% confidence intervals for linear data fitting

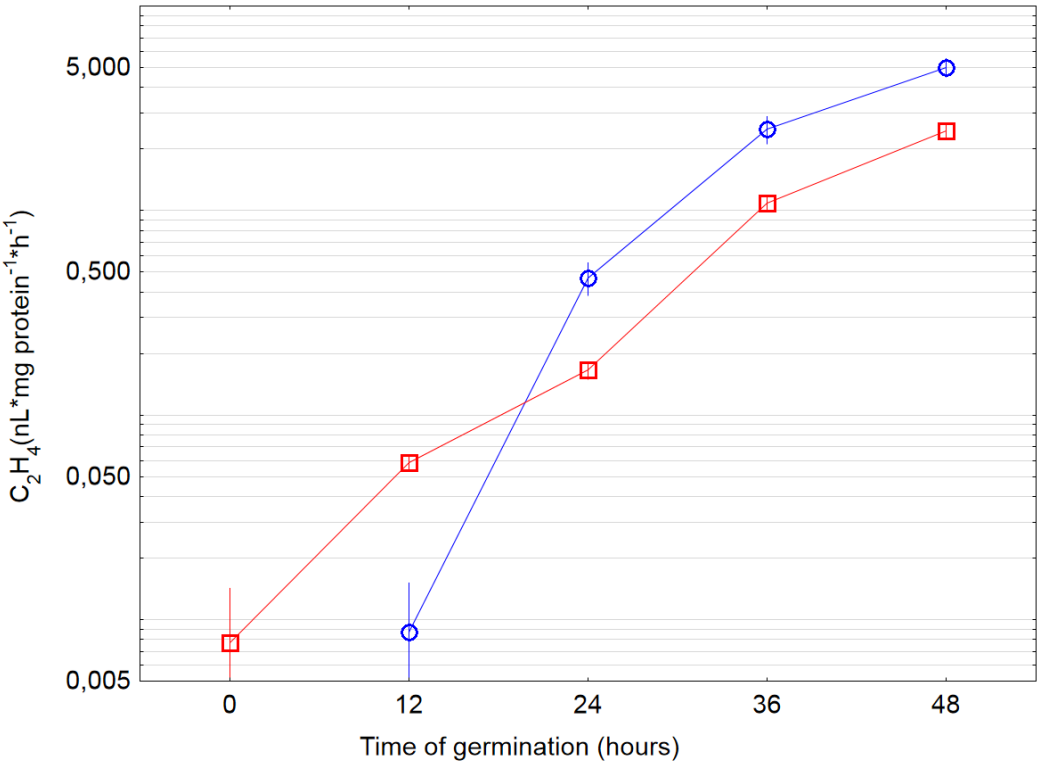


Figure 5. *In vivo* (○) and *in vitro* (□) ACC oxidase activity in seeds germinated for different period. Ethylene production shown in log-decimal scale; vertical bars denote ±SE

## CONCLUSIONS

The data obtained suggest that ACO in *Callistephus chinensis* seeds has relatively low affinity for the substrate. The results of our experiment suggest that ACO from *C. chinensis* seeds is an oligomeric enzyme with at least two substrate binding sites.

The activity of ACO in *C. chinensis* seeds is detectable *in vitro* after 12 h of germination and *in vivo* after 24 h of germination. Testing seed vigor by measuring ACO activity is relatively fast and easy by using gas chromatography. However, surrounding tissues – testa and/or pericarp are barriers for early detection of its activity *in vivo*.

High correlation of endogenous ethylene production and ACC conversion into ethylene with seed germination, particularly with their vigor, which is correlated with both advancement in germination *sensu stricto* and seed ageing, enable precise control of seed quality during storage. The method can be particularly useful in testing the vigor of the seeds stored in gene banks, where regular monitoring of the germplasm quality is necessary.

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