

Detection of 1-aminocyclopropane-1-carboxylate oxidase activity in seeds of *Stylosanthes humilis* H.B.K

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ABSTRACT: The activity of 1-aminocyclopropane-1-carboxylate oxidase (ACO) was characterized in seeds of the tropical legume Townsville stylo (*Stylosanthes humilis*) both *in vitro* (desalted extract of non-dormant seeds) and *in vivo* (entire dormant seeds). Optimum conditions for maximum *in vitro* ACO activity in a Trizma-HCl 100 mM buffered medium were: pH 7.0, temperature 32°C and cofactors and co-substrate at the following concentrations: NaHCO₃ 30 mM, sodium ascorbate 30 mM and FeSO₄ 50 μM. Rates of *in vitro* reaction catalyzed by ACO were shown to be constant within the interval 15-150 minutes from the onset of the reaction. The apparent K_m for *in vitro* ACO, as determined from the non-linear curve fitting to the Michaelis-Menten equation, was 156±8.3 μM ACC with a V_{max} 5.4±0.08 mmol (ET) g⁻¹ h⁻¹ on a fresh matter (FM) basis. *In vivo* (control basal medium: HCl-KOH 10 mM pH 7.0, 30°C, reaction time 15 hours) apparent K_m was 230±27 μM ACC and V_{max} 11.9±0.38 mmol (ET) g⁻¹.h⁻¹ on a FM basis. These data suggest that the enzyme exhibits a relatively low affinity for the substrate. The well-known inhibitors of ACO activity, α-aminoisobutyric acid, salicylic and acetylsalicylic acids, *n*-propylgallate and cobaltous ions, were highly effective in inhibiting ACO activity of Townsville stylo seeds.

KEYWORDS: dioxygenase, enzymatic kinetics, ethylene, inhibition, ACO.

ABBREVIATIONS: ACC: 1-aminocyclopropane-1-carboxylic acid; ACO: ACC oxidase; ACS: ACC synthase; AMIB: α-aminoisobutyric acid; ASA: acetylsalicylic acid; FM: fresh mass; *n*PG: *n*-propylgallate; SA: salicylic acid.

INTRODUCTION

Seeds of the tropical annual forage legume Townsville stylo (*Stylosanthes humilis* H.B.K.) exhibit a relatively hard integument, which can be weakened with chemical or mechanical scarification. Up to six to eight months post-harvest age they also show a physiological dormancy which is then gradually released so that at 12 to 15 months they germinate completely. Physiological dormancy is also broken by any chemical or stressing conditions leading to ethylene production. Ethylene has been shown to be an absolute requirement for dormancy-breakage and germination of Townsville stylo seeds (Ribeiro and Barros 2006).

In most higher plants ethylene is synthesized from 1-aminocyclopropane-1-carboxylic acid (ACC), a

non-proteinic amino acid, whose formation is catalyzed by the ACC synthase (ACS; EC 4.4.1.14). This compound is further oxidized to ethylene by the enzyme ACC oxidase (ACO; EC 1.4.3.3) (Pech et al. 2010). ACO seems to have arisen from a common ancestor of the extant groups Cycads, Gymnosperms, Gnetales and Angiosperms (John 1997). Their ancestors seemed to have synthesized ACC which was employed in defense mechanisms. It seems that ethylene as a signaling compound became more useful at the end of the wet Devonian and Permian eras, when plants acquired the property of transforming ACC to ethylene. Thus the release of ethylene from plants or plant organs when provided with ACC constitutes a good assay to ascertain ACO activity, especially

under *in vivo* conditions as suggested by Osborne et al. (1996) and Kępczyński and Kępczyńska (1997). Homology analysis of ACO genes reveals that sequence evolution in this enzyme is likely to have started before the diversion between mono- and dicotyledonous species (Ruduś et al. 2013). ACO is encoded by a small multigene family composed of three to four members. This dioxygenase utilizes ascorbate as a reductant instead of α -ketoglutarate like the other members of the family (John 1997, Pech et al. 2010). Ferrous ions and CO_2 (replaced by HCO_3^- in several assays) are cofactors absolutely required by the enzyme; CO_2 seems to protect the enzyme active site by complexing with Fe^{2+} . Therefore determining the amounts of co-substrate and cofactors for the optimum activity of ACO is a matter of great concern, and constitutes one of the objectives of the present work.

Until recently, it was believed that the key step to the biosynthesis of ethylene depended exclusively on the ACS activity. Although this may hold true for most systems affected by ethylene biosynthesis, currently it is known that ACO activity may also constitute a limiting step. For instance ethylene production by ACO orthologs *Lepidium ACO2* and *Arabidopsis ACO2* isoforms seems to be a key regulatory step (Linkies et al. 2009). Furthermore in sugar beet (*Beta vulgaris* L.) seeds, ACO transcript accumulation was promoted by abscisic acid and by pericarp removal but not by ACC. In the latter system ACC treatment of seeds caused a great increase in ethylene release but does not affect ACO transcript levels in seeds and fruits. This increase in ethylene release seems to be a result of supplying a saturating ACC concentration that is required for optimum ACO activity (Hermann et al. 2007). In summary the importance of ACO as a key point in regulating ethylene biosynthesis is being stepwise established.

Activity of ACO both *in vitro* and *in vivo* has been described for several tissues and plant organs especially in climacteric fruits, such as tomato (*Lycopersicon esculentum* Mill.) and apple (*Malus domestica* Borkh). Characterization of genes encoding for several ACO isoenzymes has been described and their diversity has been grouped in phylogenetic trees (John 1997, Ruduś et al. 2013). Meaningful studies on the genic characterization of ACO in seeds have been produced in embryonic axes of chick-pea (*Cicer arietinum* L.) (Gómez-Jiménez et al. 1998), and in seeds of pea (*Pisum sativum* L.) (Petruzelli et al. 2003), turnip tops (*Brassica rapa* L.) (Rodríguez-Gacio et al. 2004) and beechnut (*Fagus sylvatica* L.) (Calvo et al. 2004) and in a few others (Ruduś et al. 2013). Biochemical characterization of ACO activity of tropical seed species is not particularly abundant and hence in the present study some physical and chemical properties and the kinetics of the ACO activity *in vitro*

(enzymatic extract) and *in vivo* (entire seeds) of Townsville stylo are described.

MATERIAL AND METHODS

Plant material and general conditions: Seeds were harvested from Townsville stylo plants cultivated in 3 L plastic pots in a greenhouse in Viçosa (30° 45' S, 42° 15' W), Minas Gerais state, Brazil. Seeds were kept dry under laboratory conditions until assays were conducted, when they were scarified with fine sand paper and sterilized with a 0.5% NaOCl solution for 10 min, and thoroughly washed with distilled water. They were afterwards vacuum-infiltrated with the several test solutions and placed in sealed 25 mL Erlenmeyer flasks (2 mL solution, 25 seeds, for ethylene determination) with two Whatman number 1 filter paper layers lining their bottoms. Erlenmeyer flasks with filter paper were previously heated at 105°C for 4 h, in order to effect a partial sterilization of that material. Flasks were taken to a growth chamber (Forma Scientific Inc., Ohio, USA) and maintained in the dark at 30 °C. All test solutions were prepared by dissolving chemicals (Sigma-Aldrich, USA) in water at pH 7.0, HCl-KOH 10 mM, containing 0.05% Tween 80 (control basal medium).

Ethylene quantitation: Air samples were taken from the sealed Erlenmeyer flask atmospheres with a gas tight syringe and ethylene levels were determined by the technique of Saltveit and Yang (1987). Air samples of 1 mL were injected in a Hewlett-Packard 5890 series II chromatograph, equipped with a flame-ionization detector and a stainless steel column (1 m x 6 mm) packed with Porapak-N 80-100 mesh. Nitrogen carrier gas and hydrogen flow rates were 30 mL min⁻¹ and that of air 320 mL min⁻¹. Column, injector and detector temperatures were kept at 60, 100 and 150°C, respectively. Ethylene peaks were recorded with an HP 3395 A integrator coupled to the chromatograph and measured by comparison with authentic ethylene standards.

Determination of *in vitro* ACC oxidase activity: Non-dormant seeds (older than 12 months post-harvest age) were employed for determination of *in vitro* ACO activity as enzyme activity was very low or undetectable in dormant seeds. Furthermore several previous experiments defined the optimum conditions for analysis (Figure 1). Petri dishes (150 mm diameter) containing 150 seeds on two layers of filter paper and 16 mL of control basal medium were incubated in the growth chamber at 30°C for 18 h, when germination was just starting. Afterwards, seeds were paper-dried, had the fresh mass (FM) determined, frozen in liquid nitrogen and kept

in a freezer at -80°C until analysis. The activity of ACO was determined as described by Fernández-Maculet and Yang (1992) with slight modifications. Seeds were ground with a mortar and pestle with 4 mL of the extraction buffer solution containing 100 mM Trizma-HCl pH 7.0, 10% (w/v) glycerol, 30 mM sodium ascorbate, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100 and 5% (w/v) polyvinylpyrrolidone (PVPP). The slurry was centrifuged at $28,000 \times g$ for 20 min at 4°C , and the supernatant (1 mL) was desalted by passing through a Sephadex G-25 column (Pharmacia PD-10) and eluted with reaction buffer solution (without ACC). Desalted enzymatic extract was used for the assay of ACO activity. The first 0.5 mL of the eluate was discarded and the next 4 mL (active portion) were collected for analysis. From that desalted extract an aliquot (0.2 mL) was dispensed into the reaction buffer medium containing 1.8 mL of extraction buffer (without PVPP), $50 \mu\text{M FeSO}_4$, 30 mM NaHCO_3 and 1 mM ACC in a 6 mL test tube. Tubes were stoppered with rubber caps and incubated in a water bath at 32°C for 2 h. Ethylene produced was determined as described above. No ACO activity was observed with boiled extract or when extract was omitted from the incubation medium. Protein concentrations in the various extracts were measured according to the technique described by Bradford (1976).

For studies of ACO inhibition the following well-known inhibitors were added to the reaction medium: α -amino-isobutyric acid (AMIB, 0–100 mM range), salicylic (SA) and acetylsalicylic (ASA) acids (both at 0–10 mM) and *n*-propylgallate (*n*PG, 0–1 mM). All attempts to inhibit ACO activity with $\text{Co}(\text{NO}_3)_2$ supplied in the reaction medium failed whether or not the extract was added to the medium; ethylene production was high, indicating that the gas was non-enzymatically formed. This problem was circumvented in a side experiment by previously imbibing seeds in a $\text{Co}(\text{NO}_3)_2$ 2 mM solution for 48 h, and then extracting as described above. Ethylene could thus be measured showing much less than in the extract of control seeds. This suggests that Co^{2+} was removed from the extract whilst desalting through the Sephadex column.

Determination of *in vivo* ACC oxidase activity. *In vivo* ACO activity was determined as postulated by Osborne et al. (1996) and Kępczyński and Kępczyńska (1997), and according to De Rueda et al. (1995) for chick-pea embryonic axes, and Mohamed et al. (2001) for *Striga asiatica* seeds, with a few modifications. For determination of *in vivo* ACO activity dormant seeds (younger than six months post-harvest age) were employed as they respond promptly to ACC by promoting seed germination (Barros and Delatorre 1998). Twenty-five seeds

were incubated in sealed 25 mL Erlenmeyer flasks containing two filter paper layers and 2 mL of control basal medium or ACC 1 mM solution. Flasks were maintained in the dark in the growth chamber at 30°C , for 15 h. Flasks air samples of 1 mL were taken for ethylene measurement as described before. Seed FM was assessed by taking the means of the mass determined at the beginning and at the end of the experiments.

In order to examine the action of inhibitors of *in vivo* ACO activity 25 dormant seeds were incubated in 25 mL Erlenmeyer flasks with 2 mL solutions of $\text{Co}(\text{NO}_3)_2$ or *n*PG at concentrations 0.5 and 1 mM. Flasks were kept opened in the growth chamber under conditions previously described for 24 h. Afterwards, inhibitor solutions were replaced by control basal medium, ACC only or ACC plus the inhibitor solutions, and flasks were sealed with rubber caps. Fifteen hours later 1 mL air samples were taken from the flask headspace for ethylene determination.

Apparent K_m determination: For kinetic studies of *in vitro* ACO activity, ACC (0–2 mM range) was added to the reaction medium and ethylene measured 2 h later as described above. At this time ethylene production was occurring within the linear period range of reaction (Figure 1). The same ACC concentration range was also taken for the apparent K_m and V_{max} *in vivo* determinations. Twenty-five dormant seeds were taken in sealed 25 mL Erlenmeyer flasks with two filter paper layers and 2 mL of ACC solutions for 15 h, in the dark, at 30°C , in the growth chamber. Fifteen hours later 1 mL air samples were collected from the flasks for ethylene determination.

In each case data points were enough to follow a normal distribution producing a good fitting between reaction velocity (rate of ethylene release over time) as a function of ACC concentration. To the resulting Michaelis-Menten equations (Figure 2) the non-linear least squares fit was applied (Ritchie and Prvan 1996, Schnell 2003, Sjögren et al. 2009) by using the Software – GraphPad Prism (Prism 6 for Windows; Version 6.01) – San Diego, California, USA. In this way apparent K_m and V_{max} were calculated.

Statistical design: For all assays conducted with entire seeds (*in vivo* assays) the statistical design was based on a completely randomized distribution with 10 replicates of an Erlenmeyer flask with 25 seeds each. For determination of *in vitro* ACO activities five replicates were taken into account. All experiments were analyzed through Tukey test at 5% probability level. All mean comparisons were performed with SPSS (Statistical Package for the Social Sciences) 11.0 for Windows Statistical Software Package.

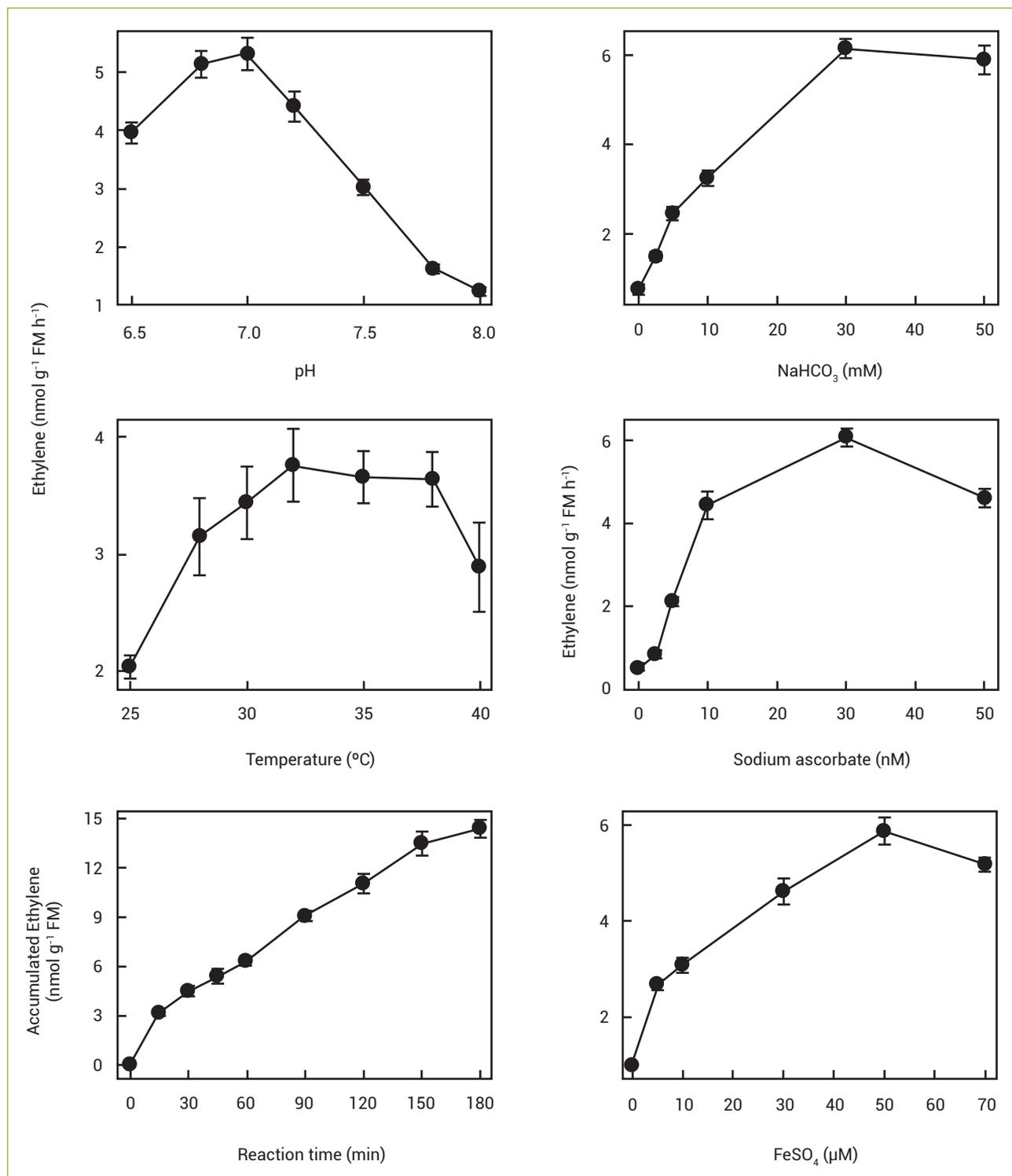


Figure 1. Effects of pH, temperature and reaction time (left) and cosubstrate and cofactors (right) on the activity of desalted extracts of non-dormant Townsville stylo seeds. Note that as for the reaction time total ethylene accumulation is shown in the y-axis. For the other sections the rate of ethylene formed was expressed in relation to time unity (h). Means of five replications \pm standard errors.

RESULTS

Establishment of optimum conditions for ACC oxidase activity.

A series of preliminary experiments were carried out to determine the best physical and chemical conditions for the optimum activity of ACO in seed extracts. Trizma-HCl 100 mM was proved as an excellent buffer system for ACO activity. The optimum pH for ACO activity was found to be 7.0, with no significant difference between its effects and those of pH 6.8; pH 7.0 was employed thereafter and also for determination of *in vivo* activity. An optimum temperature plateau was attained between 32 and 38°C, the first being chosen for further work for its effects being slightly higher. Due to our experience with Townsville stylo seeds 30°C was employed with experiments *in vivo*. As for the

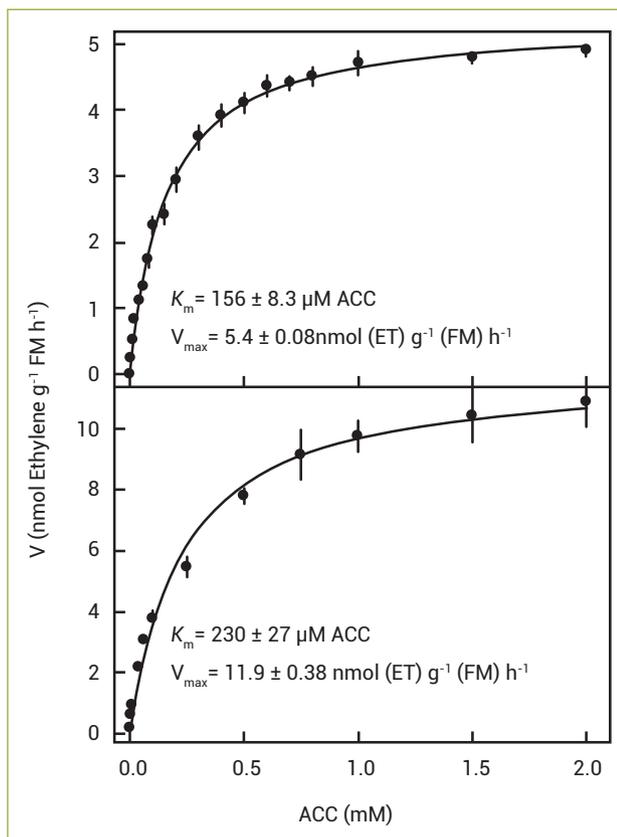


Figure 2. Effects of ACC concentration on ACO activity *in vitro* (enzymatic extract of non-dormant seeds, upper section) and *in vivo* (entire dormant seeds, lower section) on the velocity of reaction (ethylene emanation). For K_m and V_{max} determinations see Material and Methods. For *in vitro* reaction V_{max} (s) were 177 ± 2.6 pmol (ET) mg^{-1} (protein) h^{-1} and 37.9 ± 0.55 pmol (ET) $seed^{-1} h^{-1}$. K_m for *in vivo* was 265 ± 32.5 μM ACC on a seed unity basis; V_{max} was 86 ± 3.0 pmol (ET) $seed^{-1} h^{-1}$. Note that whatever the basis considered - FM, protein or seed unity - *in vitro* K_m is identical. Means of five replications \pm standard errors.

concentrations of the enzyme co-substrate and cofactors, the optimum one for sodium ascorbate was 30 mM with no difference with the effects of 50 mM. An upper saturating limit for ACO activity was observed at 30 mM $NaHCO_3$, this being chosen for further experiments. The optimal $FeSO_4$ concentration for ACO activity *in vitro* was observed to be 50 μM , with similar effects at 70 μM . A linear increase in ethylene production (constant ACO activity rate) was observed within the time interval 15 to 150 min following addition of enzymatic extract to the reaction medium. Therefore, 120 min was chosen as the reaction time for further work. Optimum conditions are summarized in Figure 1.

Kinetic studies on ACC oxidase activity. The curves from which the kinetic parameters were derived on a FM basis are shown in Figure 2. Both *in vitro* (156 ± 8.3) and *in vivo* (230 ± 27 μM ACC) apparent K_m (s) were relatively high. On the same basis, the respective V_{max} (s) are inserted in Figure 2. On protein and seed unity bases, the K_m (s) and V_{max} (s) of ACO are shown in the legends to Figure 2.

Inhibition of ACC oxidase activity. The well-known inhibitors of *in vitro* ACO activity worked efficiently as shown in Figure 3. α -Aminoisobutyric acid (AMIB) 75 mM inhibited activity by 62%. Salicylic (SA) and acetylsalicylic (ASA) acids, both at 7.5 mM, inhibited activity to a similar extent of AMIB. *n*-Propylgallate (*n*PG) 0.5 mM almost completely inhibited the *in vitro* activity. Cobaltous ions supplied to entire seeds before enzyme extraction (see Material and Methods) led to the following results: germination $17 \pm 1.0\%$ and ACO activity 88 ± 15 pmol mg^{-1} FM h^{-1} as compared to the control untreated seeds: germination $56 \pm 1.6\%$, activity 267 ± 20 pmol mg^{-1} FM h^{-1} , i.e., enzyme activity was reduced by two thirds. Both inhibitors, Co^{2+} and *n*PG were highly effective in inhibiting *in vivo* ACO activity in dormant seeds of Townsville stylo (Table 1).

DISCUSSION

Once conditions were defined as optimum for ACO activity, they were afterwards employed for the establishment of the factors. The best pH for maximum *in vitro* ACO activity in enzymatic extract was 7.0, its effects being slightly higher than those of neighboring pH(s) (Figure 1). These pHs are within the optimum pH range exhibited for all plant extracts shown in the literature, as for example 6.7 for pear (*Pyrus malus* L.) fruit (Vioque and Castellano 1994) and 7.8 for apple leaves (Binnie and McManus 2009).

A broad range (32 to 38°C) of optimum temperature was observed for the highest *in vitro* ACO extract activity, with 32°C producing a slightly higher activity. Optimum

Table 1. Effects of Co^{2+} and *n*PG on the inhibition of *in vivo* ACO activity of Townsville stylo seeds. Dormant seeds were exposed to the inhibitors for 24 hours (shown anteceding the arrows); incubation media were then replaced by a solution containing the inhibitors plus ACC (following the arrows, flasks now sealed) for another 15 hours when 1 mL air samples was collected from the flasks for ethylene measurements. Concentrations of compounds (mM) are shown parenthetically. Inhibition is presented as percentage of control of the respective treatment. Data represent means of 10 replicates \pm SE

Treatment	Ethylene ($\text{nmol g}^{-1} \text{ FM h}^{-1}$)	Inhibition (%)
HCl-KOH→HCl-KOH	0.26±0.03	-
HCl-KOH→ACC (0.1)	0.84±0.04	-
HCl-KOH→ACC (1.0)	4.08±0.22	-
Co^{2+} (1.0)→ Co^{2+} (1.0)	0.01±0.00	94.5
Co^{2+} (1.0)→ACC (0.1)+ Co^{2+} (1.0)	0.02±0.00	97.7
Co^{2+} (2.0)→ Co^{2+} (2.0)	0.01±0.00	94.2
Co^{2+} (2.0)→ACC (1.0)+ Co^{2+} (2.0)	0.12±0.01	97.0
<i>n</i> PG (0.5)→ <i>n</i> PG (0.5)	0.02±0.00	91.8
<i>n</i> PG (0.5)→ACC (0.1)+ <i>n</i> PG (0.5)	0.09±0.01	89.1
<i>n</i> PG (1.0)→ <i>n</i> PG (1.0)	0.03±0.00	89.6
<i>n</i> PG (1.0)→ACC (1.0)+ <i>n</i> PG (1.0)	0.04±0.00	99.0

temperature as low as 20°C and as high as 38°C have been described respectively for avocado (*Persea americana* Mill.) fruit (McGarvey and Christoffersen 1992) and pear fruit extract (Vioque and Castellano 1994). In some cases two peaks of maximum activity — 27.5 and 35°C (chick-pea embryonic axes, De Rueda et al. 1995), and 30 and 45°C (*Citrus reticulata* Blanco peel extracts, Dupille and Zacarías 1996) — were observed. This might be accounted for by the occurrence of different ACO(s) isoforms in the same tissue or organ (Hermann et al. 2007, Binnie and McManus 2009, Linkies et al. 2009, Ruduś et al. 2013).

The maintenance of the stability of ACO is an important issue since this oxidase loses its activity very rapidly in about 20 min (Pech et al. 2010) or even less (Binnie and McManus 2009). Thus, assays with enzymatic extracts must be accomplished over a time range when the reaction rate may be kept constant. In Figure 1 it is observed that ethylene production catalyzed by Townsville stylo seed enzymatic extract occurred with a linear increase in an interval between 15 and 150 min from the start

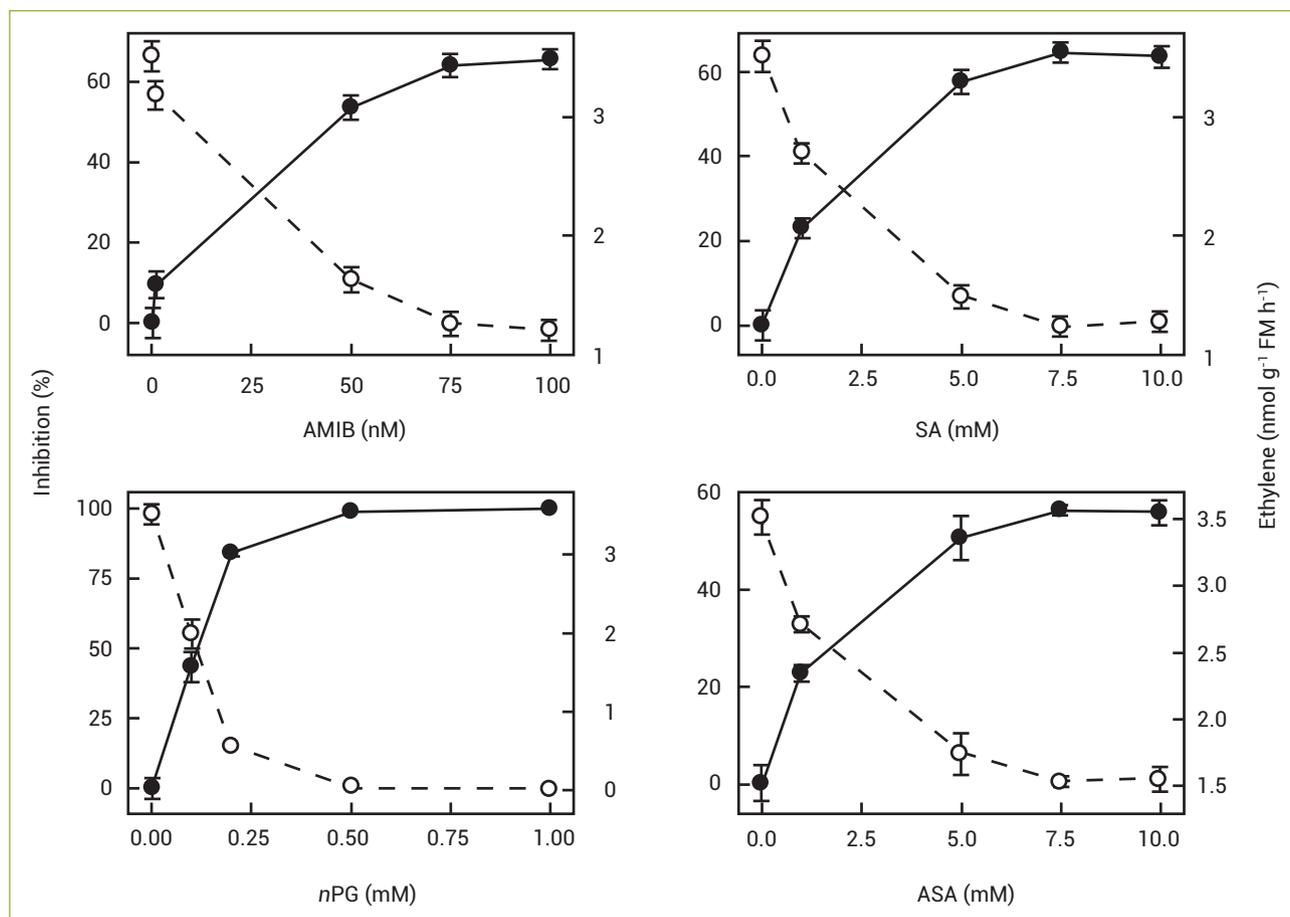


Figure 3. Effects of some inhibitors supplied in the reaction medium on the *in vitro* ACO activity (dotted lines). Inhibition percentage as related to the control is represented by full lines. Means of five replications \pm standard errors.

of the reaction. Hence a reaction time of 120 min was chosen for further work, although most workers employed a time of 60 min (Ververidis and John 1991, Smith and John 1993, Rodríguez-Gacio and Matilla 2001). Despite this low enzyme stability constant reaction rates larger than 120 min are not uncommon, as observed in papaya (*Carica papaya* L.) fruit by Dunkley and Golden (1998).

A saturating optimum level of 30 mM of NaHCO_3 was observed to stimulate ACO activity in Townsville stylo seeds and most tissues quoted from the literature (Ververidis and John 1991, McGarvey and Christoffersen 1992, Fernández-Maculet et al. 1993, Moya-Leon and John 1995, Kruznane and Ievinsh 1999, Binnie and McManus 2009). Lower values (20 mM) were obtained from extracts of barley (*Hordeum vulgare* L.) seedlings (Kruznane and Ievinsh 1999), banana fruit (Moya-Leon and John 1995) and breadfruit (*Artocarpus altilis* L.) (Williams and Golden 2002). Very low values were observed in chick-pea embryonic axes (6 mM) (De Rueda et al. 1995) and orange peel (10 mM) (Dupille and Zacarías 1996) and pear fruit extracts (Vioque and Castellano 1994). A much larger variation for optimum ACO activity was observed in the requirement for ferrous ion, varying from 4 μM in chick-pea embryonic axes (De Rueda et al. 1995) to 200 μM in pear fruit extracts (Vioque and Castellano 1994). For the optimum activity of ACO *in vitro* 50 μM was observed in Townsville stylo seed extract (Figure 1), apple fruit (Fernández-Maculet and Yang 1992), and carnation (*Dianthus caryophyllus* L.) petal extracts (Nijenhuis-De Vries et al. 1994).

To the knowledge of the authors most, if not all, K_m and V_{max} values for ACO so far described were derived from the Lineweaver-Burk double reciprocal equation which produces biased and imprecise figures (Ritchie and Prvan 1996, Schnell 2003, Sjögren et al. 2009) and thus are incomparable to data of Figure 2 obtained from the non-linear curve fitting to the Michaelis-Menten equation. An apparent exception to that was described by Binnie and McManus (2009) for the three protein isoforms of apple leaves, their kinetics constants although calculated by Lineweaver-Burk equation were allegedly similar to those estimated by Eadie-Hofstee regression. However, according to Ritchie and Prvan (1996), Schnell (2003) and Sjögren et al. (2009) this fact must constitute a fortuitous coincidence. On the Lineweaver-Burk basis *in vitro* ACO K_m (s) for several plant material varied from 6 (apple fruit, Dilley et al. 1993) to 401 μM ACC (MD-ACO2 protein from apple leaves, Binnie and McManus 2009). This diversity of K_m might reflect not only the several ACO isoforms occurring in plants but mainly the biases and inaccuracies brought about by Lineweaver-Burk estimations.

Figure 2 also shows the kinetic parameters for *in vivo* (entire dormant seeds) which were expectedly different from the *in vitro* non-dormant seeds. This might have resulted from the two different types of seed conditions and from the expression of the several ACO isoforms which can vary spatially and temporally (Hermann et al. 2007, Linkies et al. 2009, Ruduś et al. 2013). For a matter of comparison our data were also estimated with the Lineweaver-Burk fittings. On a FM basis the *in vitro* ACO K_m was halved as related to the one shown in Figure 2; *in vivo* K_m was about one fifth of that from Figure 2. In other words due to the inaccuracies produced by the Lineweaver-Burk determinations, the real enzyme affinity for the substrate which is relatively low should be incorrectly assigned as relatively high.

Studies with the traditional inhibitors of ACO activity produced strong additional evidence to further characterize the dioxygenase from Townsville stylo seeds. All inhibitors employed to inhibit ACO worked to a large extent (Figure 3). Cobaltous ions, a powerful inhibitor of *in vitro* activity, with a high affinity for the enzyme (Dilley et al. 1993), decreased activity by about two thirds although the inhibitor had to be previously supplied to entire seeds (see Material and Methods section). α -Aminoisobutyric acid, a competitive inhibitor (Satoh and Esashi 1983), decreased ACO activity by 65% at about 75 mM, a figure much higher than found by Fernández-Maculet and Yang (1992) in embryonic axes of chick-pea, and by McGarvey and Christoffersen (1992) in extract of avocado fruit. Salicylic acid and ASA inhibited about 60% of activity at a concentration 7.5 mM. A much reduced amount of SA, about one tenth of that value, was required to inhibit ACO activity in melon fruit (Smith et al. 1992), and ASA concentrations in the μM range of was required to inhibit *in vitro* ACO activity in apple fruits (Fan et al. 1996). Figure 3 also shows that of the inhibitors employed as to inhibit *in vitro* ACO activity in Townsville stylo seeds, the most effective was *n*PG, inhibiting almost 100% activity at 0.5 mM. Equally low amounts of *n*PG inhibiting more than 90% of *in vitro* activity was observed in melon fruits (Smith et al. 1992), embryonic axes of chick-pea (De Rueda et al. 1995) and pear fruits (Vioque and Castellano 1994). As for the *in vivo* (entire seeds, control basal medium, incubation time in ACC 15 h) inhibition by both Co^{2+} and *n*PG were highly effective; depending on the amounts employed inhibition varied between 90-100% of full activity (Table 1). Here detection of inhibition by Co^{2+} was not so problematic as in the case of *in vitro* inhibition (see above). These responses to cobalt (Yang and Hoffmann 1984) and to *n*PG (Dupille and Zacarías 1996) confirm that they are among the most powerful inhibitors of ACO activity.

In conclusion, it was demonstrated that both an enzymatic crude extract (non-dormant seeds) and entire seeds (dormant seeds) of Townsville stylo were able to release ethylene when provided with ACC, demonstrating that ACO was active (Osborne et al. 1996, John 1997, Kępczyński and Kępczyńska 1997). Although the amounts of cofactors and cosubstrate listed above stimulated optimum *in vitro* ACO activity in Townsville stylo seeds and several other plant materials it should be noted that they were present at levels far in excess of those found in a living plant cell (John 1997). Despite that, all data herein presented lend strong support for the characterization of the *in vitro* ACO activity. ACO from both systems studied (dormant and non-dormant seeds) exhibited a relatively low affinity for the substrate. Comparisons with ACO activities of other plant materials ought to await until their kinetics parameters

be produced freed from inaccuracies of Lineweaver-Burk calculations. Inhibition of ACO activity by some well-known inhibitors of the dioxygenase also provided some additional biochemical characterization of the enzymatic activity.

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