



Reduced ethylene synthesis of mangoes under high CO₂ atmosphere storage

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ABSTRACT. High CO₂ atmospheres have been reported to be accountable for slower ripening processes of many fruit species. In modified or controlled atmosphere storage of mangoes (*Mangifera indica* L.) delayed ripening is attributed to the effects of CO₂ on ethylene biosynthesis, which is reduced under CO₂ concentrations beyond 10%. In the present work the objective was to determine if those elevated CO₂ atmospheres on ethylene synthesis could be attributed to the action of CO₂ upon ACC oxidase. Mature green or tree ripe 'Tommy Atkins' mangoes were, in four experiments, held in a flow through system of either 10 or 25% CO₂ mixed to 5% O₂ or only air for 14 or 21 days at 5, 8 or 12°C. Mangoes in the 25% CO₂ atmosphere did not produce detectable levels of ethylene, whereas under 10% CO₂ the production rates were significantly suppressed at 5 or 8°C. However, 1-aminocyclopropane-1-carboxylic acid (ACC) concentrations in mango mesocarp tissue at retrieval from storage were similar to the air controls and ACC synthase activity was not completely inhibited. The direct effects of CO₂ concentrations on ACC oxidase activity is to be considered the most important factor in inhibiting ethylene biosynthesis of mangoes under 25% CO₂ atmospheres.

Keywords: 1-aminocyclopropane-1-carboxylic acid; ACC oxidase; ACC synthase; *Mangifera indica*.

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Introduction

In the literature there are innumerable papers on the effects of CO₂ on ripening processes credited to the condition of reduced ethylene production rates under elevated CO₂ atmospheres occurring in conjunction with hindrance in ripening. Less chlorophyll breakdown (Imahori et al., 2007), maintenance of higher flesh firmness (Choi, 2013) and reduced oxidation of organic acids (Liu, Wang, Qin, & Tian, 2016) are some of the involved metabolic routes interrupted because of diminished ethylene synthesis (Yang, Song, Campbell-Palmer, Fillmore, & Zhang, 2013).

Reduced ACC synthase activity could be accountable, at least in part, for the inhibition of ripening (Bufler, 1984). The author observed that ACC synthase activity in apple tissues was severely reduced in atmospheres of up to 10% CO₂. However, De Wild, Otma, and Peppenlenbos (2003) indicate that CO₂ is an essential cofactor for ACC oxidase. Zacarías and Alférez (2007) presented evidences that low levels of CO₂ are required for ethylene synthesis. Smith and John (1993) demonstrated that ACC oxidase has a requirement for CO₂ for its maximum activity, although, with an increase in the apparent *K_m* for both ACC and O₂. Fernandez-Maculet, Dong, and Yang (1993) reported that CO₂ exerts its action on ethylene synthesis by activating the enzyme ACC oxidase rather than the substrate ACC.

Ethylene production in mangoes rapidly dropped to undetectable levels after exposure to atmospheres of 25% CO₂ and above (Bender, Brecht, Sargent, & Huber, 2000), in contrast to the observations of Rothan and Nicolas (1994) who found that CO₂ concentrations up to 28% only slightly reduced the ethylene production of kiwi fruit tissues. The inhibition of ethylene production in mango fruit by 25% CO₂ could not be attributed to tissue damage since ethylene production and other ripening processes recovered to control levels after transfer to air (Bender et al., 2000). Considering that the very rapid inhibition of ethylene production (≤ 2 hours) in response to the elevated CO₂ atmosphere probably rules out depletion of ACC as a mechanism for CO₂-induced inhibition of ethylene production and because of the requirement for CO₂ as an activator of ACC

oxidase (Sun et al., 2017), the objective in the present experiments was to determine if the effects of elevated CO₂ atmospheres on ethylene synthesis could be ascribed to the action of CO₂ upon ACC oxidase.

Material and methods

'Tommy Atkins' mangoes harvested from a grove of Brooks Tropicals in Homestead, Florida were stored for 21 days at 12°C plus 5 days in air at 20°C. The mangoes were hand graded for uniform size and ripeness stage and freedom from defects, and visually categorized as mature green or tree ripe based on ground color and fruit shape as described in Medlicott, Reynolds, New, and Thompson (1988). The hue angles determined by means of a Konica/Minolta CR300 (Ramsey, NJ) chromameter at the beginning of the experiment for the epidermal ground color of mature green and tree ripe mangoes were: 107.96 ± 1.90 and 83.68 ± 6.54 , respectively. Three replicates of four fruit per treatment and ripeness stage were placed in 10.05-L glass jars fitted with screw-top lids and inlet and outlet tubes. The treatments, 10 or 25% CO₂ combined with 5% O₂ and an air control in a flow-through system, were applied to both ripeness stages.

A second experiment was conducted with 'Tommy Atkins' mangoes imported from Mexico by Brooks Tropicals in Homestead, Florida. These mangoes were handled under normal commercial conditions, treated with 46°C water for 60 minutes for insect disinfestation, and coated with a commercial formulation of carnauba wax prior to shipment to Florida by refrigerated truck. The mangoes were stored for 14 days at 5°C or 8°C in a flow-through system with atmospheres of 10 or 25% CO₂ plus 5% O₂. Control fruit were held in air also in the flow-through system.

Short term experiments were conducted also with 'Tommy Atkins' mangoes from the same lot of the previous experiment. Individual fruit were placed in 1.75-L glass jars at 8°C and exposed to 25% CO₂ plus 5% O₂ for 4 hours in a flow-through system. Control fruit were held in air in also the flow-through system.

Besides individual fruits, mango mesocarp tissue was used to reduce variability between replicates, which is considerable among individual fruits. Ten cylindrical plugs, 8 x 5 mm, prepared from the median portion of mango mesocarp were exposed in 65-mL glass vials at 12°C to the same atmospheres as the individual fruits.

During the CO₂ treatments, 0.5-mL headspace gas samples were taken for ethylene measurements on a photoionization GC, Photovac 10A10 (Thornhill, Ontario, Canada) equipped with a 76 x 3.18 mm 60/80 mesh activated alumina column. ACC concentrations were determined after storage using mesocarp tissue from individual fruits or from the tissue plugs exposed to CO₂. ACC concentrations were determined as described in Concepcion, Lizada, and Yang (1979) and modified by Hoffman and Yang (1980), but using 80% methanol (v/v) as the extractant (Nieder, Yip, & Yang, 1986), and with no prior purification by ion exchange resin.

ACC oxidase activity *in vivo* was determined as described in Hoffman and Yang (1982) from mesocarp plugs vacuum infiltrated for 1 min. in a solution containing 1 mM ACC plus 2% KCl (w/v). ACC oxidase activity *in vitro* was determined as described in Fernandez-Maculet and Yang (1992) with slight modifications. The extraction buffer was 0.4 M 3-(N-morpholino) propane-sulfonic acid (MOPS) instead of TRIS (Tris[hydroxymethyl]aminomethane), and the assay buffer had the following composition: 1.64 mL of 50 mM MOPS (pH 7.2), 10% glycerol (v/v) and 20 mM sodium bicarbonate buffer to which a 0.2-mL aliquot of crude ACC oxidase extract was added. Just prior to the 1-h incubation at 30°C, 40 µL of each of the following reagents were added to the test tube: 250 mM sodium ascorbate, 1 mM FeSO₄·7H₂O, 50 mM dithiothreitol (DTT) and 50 mM ACC.

ACC synthase was extracted as described by Su, McKeon, Grierson, Cantwell, and Yang (1984) with the exception that the crude extract was dialyzed for 18 hours against 300 volumes of dialysis buffer. After dialysis, 400 µL of the extract were added to 60 µL 600 mM N-[2-hydroxyethyl]piperazine-N'-[3 propanesulfonic acid] (EPPS), 0.1 mM DTT and 5 µM pyridoxal phosphate buffer at pH 8.5 plus 90 µL of deionized water. The reaction was started by adding 60 µL of 0.5 mM S-adenosylmethionine. ACC concentrations were determined as described above, from one fruit of every replicate, after 3 hours of incubation at 30°C.

Results and discussion

Mangoes in the 25% CO₂ treatment at both temperature regimes had no detectable ethylene during the 14 days of CA storage. Only the control fruit at 8°C showed increased ethylene production during storage. The 10% CO₂ treatment at both temperatures presented reduced but did not completely suppress the ethylene production rates (Figure 1).

The ethylene production rates of the control mangoes at 8°C was comparable to the ethylene production rates of tree ripe 'Haden' and 'Tommy Atkins' fruit stored at 12°C (Bender, Brecht, & Sargent, 1995). Examining the ACC concentrations and the activity of ACC oxidase (Figure 2) a possible conclusion is that the reduction in ethylene production during CA storage may be predominantly attributed to the effects of CO₂ on ACC oxidase. The mangoes stored in air at 8°C had significantly higher ACC oxidase activity compared to the other treatments, which indicates that at that temperature ethylene synthesis was not hindered.

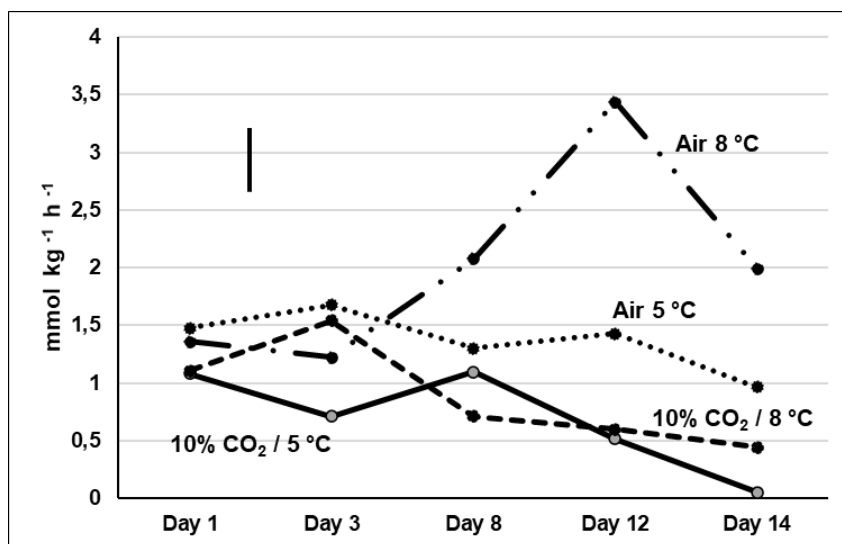


Figure 1. Ethylene production rates of cv. Tommy Atkins mangoes stored for 14 days in air, 10% CO₂ plus 5% O₂ at 5 or 8°C (Vertical bar = LSD at $p < 0.05 = 0.627 \text{ mmol kg}^{-1} \text{ h}^{-1}$).

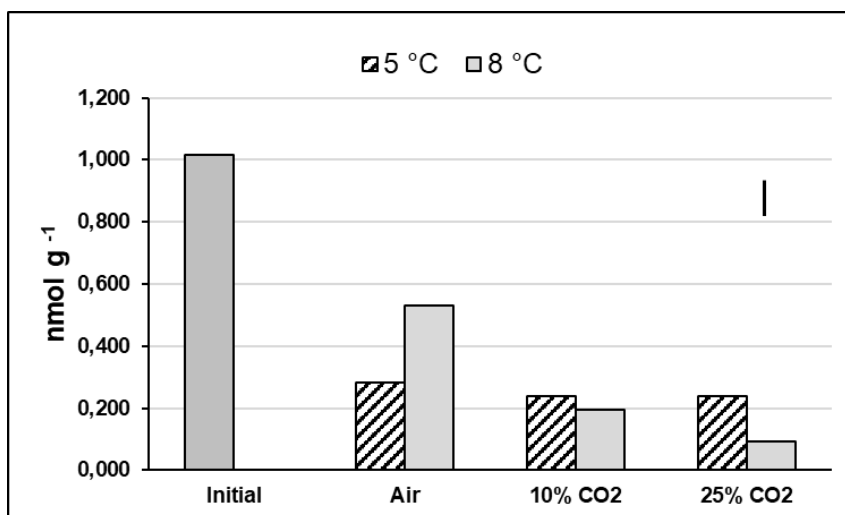


Figure 2. ACC oxidase activity in mesocarp tissue of cv. Tommy Atkins mangoes at beginning of storage and after 14 days in air, 10% CO₂ or 25% CO₂ plus 5% O₂ in a flow through system at 5 or 8°C (Vertical bar = LSD at $p < 0.05 = 0.104 \text{ nmol g}^{-1}$).

The other treatments at 5 and 8°C did not differ in ACC oxidase activity levels, which was unexpected considering that in the 25% CO₂ treatment no ethylene was detected during the CA period. One consideration is that the presence of high CO₂ may be required during the extraction and determination of the activity of ACC oxidase so that the inhibition is retained.

The effects of CO₂ could also inhibit ACC synthase since that enzyme is the rate-limiting step in the ethylene biosynthetic pathway (Yu, Adams, & Yang, 1979). Moreover, Bufler (1984) had already determined that, in apples, CO₂ concentrations up to 10% are, at least in part, responsible for the reduced ethylene production via inhibition of the activity ACC synthase. But with mangoes, it does not appear that the activity of ACC synthase is the major limiting factor, because ACC concentrations in CA did not differ from the controls (Figure 3), even though ethylene production was low in 10% CO₂ and not detectable in the 25% CO₂ treatment.

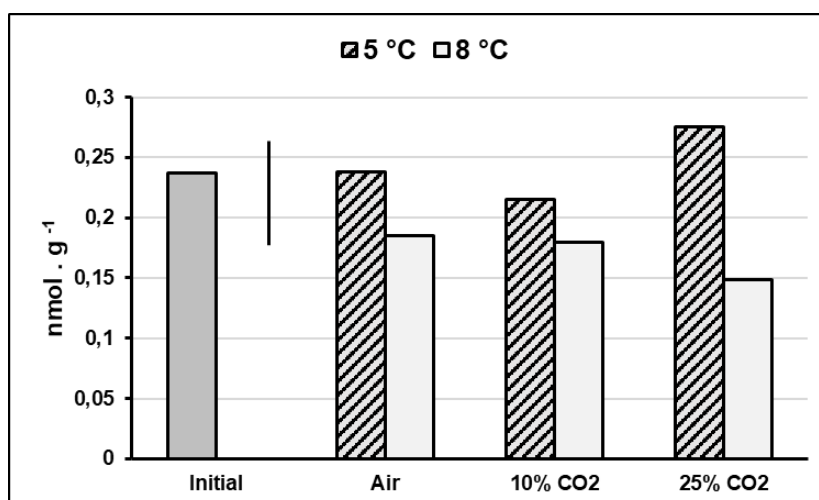


Figure 3. ACC concentrations in mesocarp tissue of cv. Tommy Atkins mangoes at the beginning of storage and after 14 days in air, 10% CO₂ or 25% CO₂ mixed to 5% O₂ in a flow through system at 5 or 8 °C (Vertical bar = LSD at $p < 0.05 = 0.089 \text{ nmol g}^{-1}$).

Examining the activity *in vitro* of ACC synthase (Figure 4) there is further indication that elevated CO₂ atmospheres reduce the activity of ACC oxidase leading to an accumulation of ACC. The significantly lower activity of ACC synthase in the 25% CO₂ treatment compared to control fruit at 8 °C resulted in slightly lower ACC concentrations, which is in accordance with the results of Bufler (1984). The results suggest that both ACC synthase and ACC oxidase were inhibited by 25% CO₂ at 8 °C and, because of the presence of ACC in concentrations similar to control fruit at 8 °C, but with no ethylene production, the predominant effect of the CO₂ atmosphere may be on ACC oxidase. The 10% CO₂ atmosphere did not completely suppress ethylene production, indicating that neither ACC synthase nor ACC oxidase of mangoes under that atmosphere were completely inhibited, which was confirmed by the results showing inhibition of ACC synthase equal to that of the 25% CO₂ treatment while ACC concentrations were almost twofold the concentrations in the 25% CO₂ fruit.

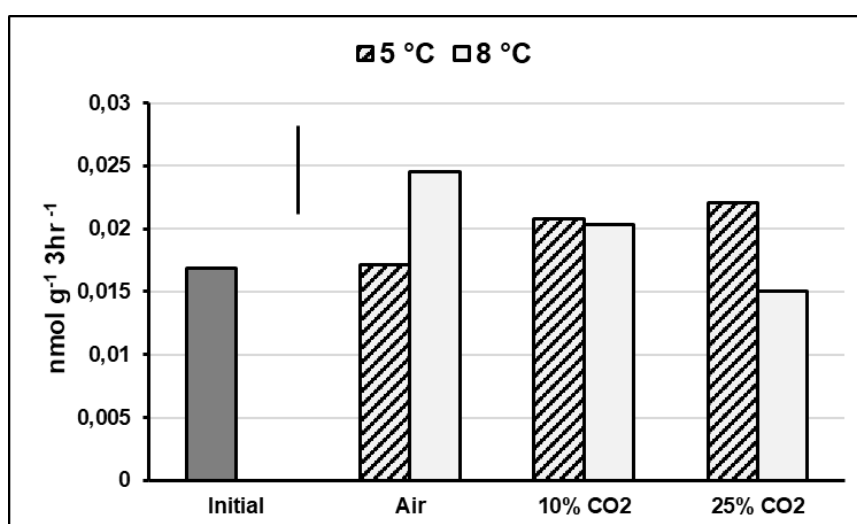


Figure 4. *In vitro* ACC synthase activity in mesocarp tissue of cv. Tommy Atkins mangoes at the beginning of storage and after 14 days in air, 10% CO₂ or 25% CO₂ mixed to 5% O₂ at 5 or 8 °C (Vertical bar = LSD at $p < 0.05 = 0.00615 \text{ nmol g}^{-1} 3\text{h}^{-1}$).

Even though the activity of ACC synthase at 8 °C decreased with increasing CO₂ concentrations in the storage atmosphere, the presence of ACC in the mango mesocarp tissues after 14 days in CA indicates that either ACC synthase was not completely inhibited or that ACC oxidase was inhibited during CA exposure but recovered during extraction. At 5 °C, on the other hand, ACC synthase activity increased with increasing CO₂ concentrations. This increase is probably a stress response to CO₂ enhanced by the chilling temperature, and not an effect of CO₂ inducing *de novo* synthesis of ACC synthase as determined by Mathooko, Kubo, Inaba, and Nakamura (1995) in cucumbers treated with 60% CO₂ plus 20% O₂ at 25 °C for 12 hours leading to higher ACC concentrations and higher ethylene production compared to controls held in air at 25 °C.

Ethylene production was lower in mangoes at 5°C than in mangoes stored at 8°C, while the activity of ACC oxidase was similar. This situation would be expected to indicate similar ACC synthase activity at the two lower temperatures, but, on the contrary, there was higher ACC synthase activity in the mangoes in the 25% CO₂ atmosphere at 5°C, which, again, leads to the conclusion that the ACC accumulation was predominantly derived from the effects of the CO₂ treatment on ACC oxidase activity (Cheverry, Sy, Pouliqueen, & Marcellin, 1988).

Both mature green and tree ripe fruit stored at 12°C showed decreasing *in vivo* activity of ACC oxidase with increasing CO₂ concentrations (Figure 5), causing ACC to accumulate (Figure 6). The effects of CO₂ on ACC oxidase activity during storage seem to be more pronounced in tree ripe fruit than in mature green fruit. The accumulation of ACC after 21 days of CA storage to levels of about 250 $\mu\text{mol kg}^{-1}$ fresh weight for mature green fruit and between 600 to 900 $\mu\text{mol kg}^{-1}$ FW in tree ripe mangoes indicates that ACC synthase activity was neither significantly enhanced by CO₂ atmospheres as reported by Mathooko et al. (1995) in cucumbers, nor was inhibited as observed by Bufler (1984) and Levin, Sonogo, Zutki, and Ben-Arie (1993) in apples. But the activity rather reflected the differences in ripeness stage of the mangoes. Mature green fruit had lower ACC concentrations than the tree ripe fruit because the former were at a less advanced ripeness stage.

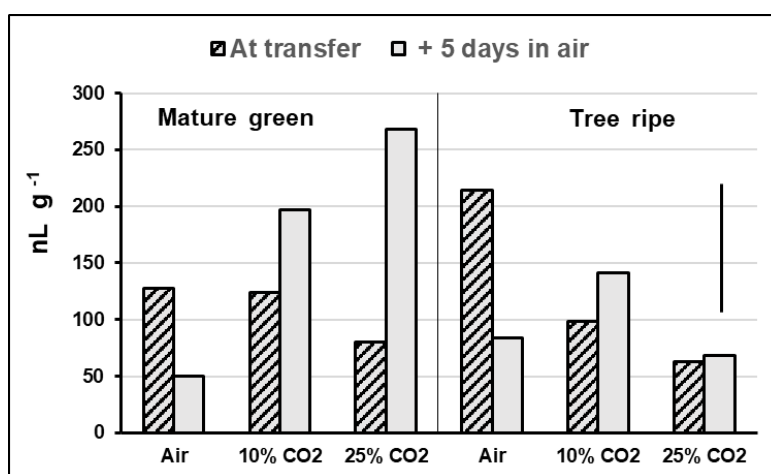


Figure 5. *In vivo* ACC oxidase activity in mesocarp tissue of mature green and tree ripe cv. Tommy Atkins mangoes at transfer from a 21-day storage period in air, 10% CO₂ or 25% CO₂ mixed to 5% O₂ in a flow through system at 12°C and after 5 more days in air (Vertical bar = LSD at $p < 0.05 = 138 \text{ nL g}^{-1}$).

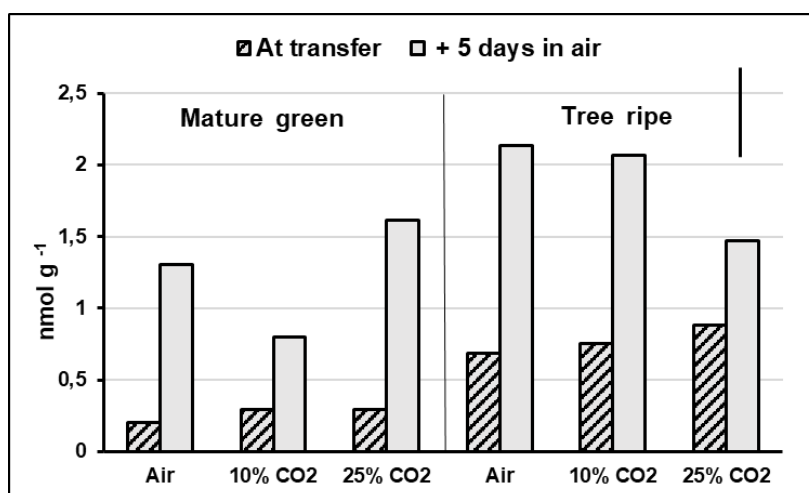


Figure 6. ACC concentrations in mesocarp tissue of mature green and tree ripe cv. Tommy Atkins mangoes at transfer from a 21-day storage period in air, 10% CO₂ or 25% CO₂ mixed to 5% O₂ in a flow through system at 12°C and five more days in air (Vertical bar = LSD at $p < 0.05 = 0.767 \text{ nmol g}^{-1}$).

After transfer from CA at 12°C to air at 20°C, the mature green and tree ripe fruit had significant increases in ACC oxidase activity and ACC concentrations because of enhanced ripening at the higher temperature. The air control fruit had lower ACC oxidase activity and higher ACC levels 5 days after transfer to 20°C, reflecting their overripe state.

Neither immersion of mangoes in 46°C water for 60 min. for insect disinfestation nor immersion in 53°C water for 3 min. for postharvest anthracnose control significantly affected ACC oxidase activity. In a preliminary experiment (data not shown), the *in vivo* activity of ACC oxidase of 'Keitt' mangoes from either hot water treatment had returned to the initial activity levels after 4 days of storage at 12°C. Significantly enhanced levels of ACC oxidase were determined only immediately after the fruit had equilibrated to room temperature following the hot water treatments. Thus, it does not appear likely that the hot water treatments received by the imported mangoes influenced their response to CA treatments.

If the half-life of mango ACC oxidase is in the range of 4 to 5 h, as estimated by Cheverry et al. (1988) for avocados, and the complete inhibition of ethylene in the 25% CO₂ atmosphere is due only to inhibition of ACC oxidase, then no enzyme should be detected after 2 w storage, which was not the case in these experiments. The recovery of ethylene production in mango mesocarp tissue that did not produce any ethylene after a 60 min exposure to 25% CO₂ was almost immediate upon return to air (Table 1), while tissue that was in air and then transferred to 25% CO₂ showed a significant drop in ethylene production after only 30 min. of exposure to the elevated CO₂ level. A similar decrease in ethylene production was observed by Chaves and Tomas (1984) with apples in a flow-through system of 20% CO₂ at 20°C for 2 hours, which was attributed by the authors to the effects of CO₂ on ACC oxidase.

Table 1. Ethylene production ($\mu\text{L kg}^{-1} \text{h}^{-1}$) of mesocarp tissue plugs from 'Tommy Atkins' mangoes stored in air or 25% CO₂ mixed to air at 12°C.

Time (min.)	10	20	33	60		70	90	105
Air	2.82	1.88	1.93	1.57	transferred to 25% CO ₂	1.37	0.61	0.92
25% CO ₂	0.56	0.98	0.92	0.00	transferred to air	1.92	1.68	1.83

Examining the ethylene production rates of the 'Tommy Atkins' mangoes for a few more hours (Figure 7) it is possible, again, to determine that the effect of CO₂ on ethylene production occurs by inhibiting the activity of ACC oxidase rather than restricting ACC synthase synthesis and activity as reported by Chaves and Tomas (1984). ACC levels at the end of the 6 hours CA storage period were 0.225 or 0.214 nmol g⁻¹ fresh weight for cv. Tommy Atkins mangoes in air or 10% CO₂, respectively.

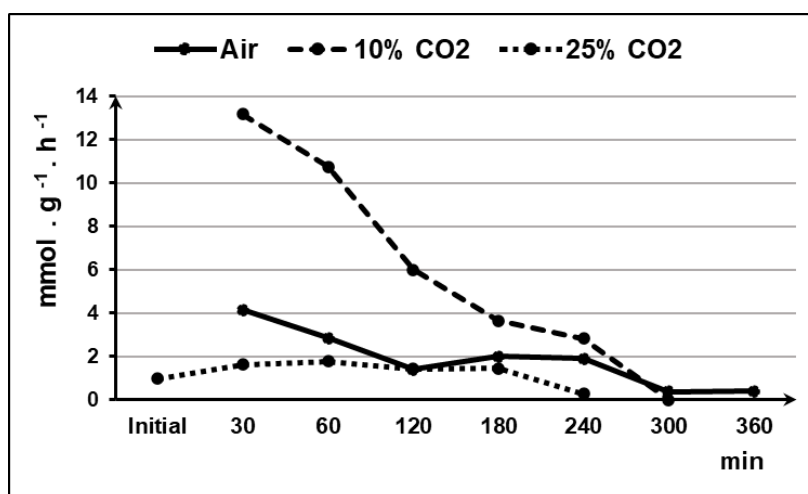


Figure 7. Ethylene production rates of cv. Tommy Atkins mangoes stored for 4 or 6 hours in air, 10% CO₂ or 25% CO₂ mixed to 5% O₂ at 8°C (LSD at $p < 0.05 = 0.841 \text{ mmol g}^{-1} \text{h}^{-1}$).

Breaking down this short period in segments and determining the ACC concentrations and the activity of ACC oxidase, it is apparent that a transient increase in ethylene biosynthesis in the high CO₂ treatments was accompanied by a significant drop in ACC concentration (Figure 8). The threefold increase in ethylene production rates after 30 min. in the 10% CO₂ atmosphere at 8°C, most probably represents a response of the fruit to an extreme environment that also could involve the activation of ACC oxidase by CO₂ as reported by Dong, Fernandez-Maculet, and Yang (1992), taking into consideration that CO₂ is required for the activity of ACC oxidase (Smith & John, 1993). Besides, this increase in ethylene is probably sustained by the existing ACC pools, since ACC synthase activity (Table 2) did not change significantly under the same conditions,

which is in contrast to the report of Chavez-Franco and Kader (1993), who found that pear discs had significantly reduced ACC synthase activity during the first 20 hours of exposure to 20% CO₂ in air at 20°C.

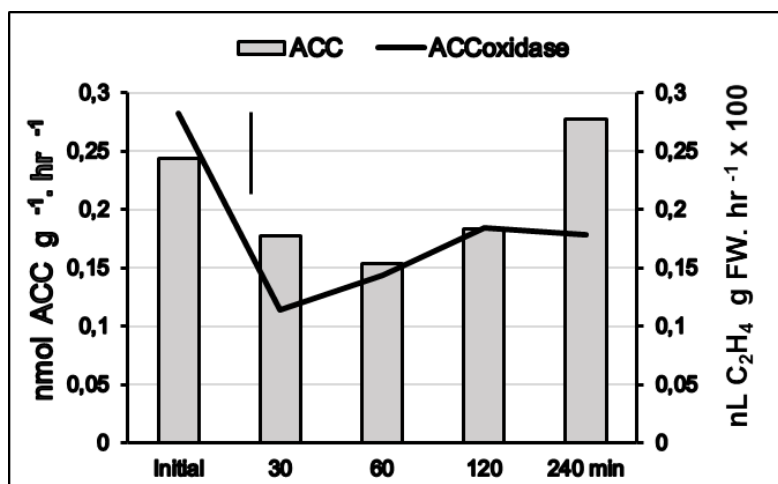


Figure 8. ACC concentrations and ACC oxidase activity *in vitro* in mesocarp tissue of cv. Tommy Atkins mangoes stored for 4 hours in air or 25% CO₂ mixed to 5% O₂ at 8°C (Vertical bar = LSD at $p < 0.05 = 0.056$ nmol ACC g⁻¹ FW).

Table 2. ACC synthase activity (nmol g⁻¹ h⁻¹) in mesocarp tissue of 'Tommy Atkins' mangoes stored for 4 hours at 8°C in a flow through system containing 25% CO₂ mixed to air.

	Initial	30	60	120	240 min.
ACC synthase activity	0.169	0.0179	0.0164	0.0192	0.0189
Standard deviation	0.0016	0.0029	0.0020	0.0089	0.0061

The subsequent recovery of ACC concentration in the 10% CO₂ treatment was most likely a consequence of inhibition of ACC oxidase activity, as ethylene production decreased over the same time period. Alternatively, the recovery of ACC levels could also derive from an upregulation of ACC synthase as a response to the stress of the high CO₂ environment. There was a slight increase in the activity of ACC synthase after 2 hours of exposure to 25% CO₂ though these differences were not statistically significant.

Slight changes in the activity of ACC synthase could result in significant changes in ACC concentrations, but, most probably, the effects of CO₂ on ACC oxidase are the most influential. Given that there was no ethylene being produced in the 25% CO₂ treatments despite maintenance of ACC at levels not significantly different from the controls, at either temperature, this indicates that ACC oxidase activity was reversibly inhibited *in vivo* by CO₂. In fact, the 25% CO₂ treatment induced *de novo* synthesis of ACC oxidase as observed by Philosophos-Hadas, Aharoni, and Yang (1986) with tobacco leaf discs.

Mizutani, Dong, and Yang (1995) showed with *in vitro* assays of ACC oxidase from apple tissue that the enzyme activity increased several times at a specific pH with increasing CO₂ concentrations. These authors also showed that lowering the pH also increased the CO₂ optima for ACC oxidase activity. At pH 7.5, maximum activity was observed at 4% CO₂, while at pH 6.0 the enzyme activity showed no sign of leveling off even at 30% CO₂. *In vitro* activity of ACC oxidase extracted from ripe 'Tommy Atkins' mangoes after a 30-min. exposure to 18% CO₂ in a static system was 1.87 ± 0.28 nL g⁻¹ fresh weight and, after 2 hours, activity had dropped to 0.87 ± 0.05 nL g⁻¹ fresh weight, which was comparable to the controls incubated under ambient CO₂ concentration (0.72 ± 0.08 nL g⁻¹ fresh weight). The drop in the ACC oxidase activity *in vitro* over time might be due to the oxidation of ascorbate in the assay medium, considering the requirement for ascorbate for enzyme activity *in vitro* (Ververidis & John, 1991) and based on information from the work of Finlayson and Reid (1994), who determined that ACC oxidase activity was strongly dependent on the presence of even small amounts of ascorbate.

The levels of ACC oxidase activity in the present experiments were comparable to the activity measured by Cua and Lizada (1990) in 'Carabao' mangoes, though almost tenfold lower than the activity of the enzyme from other species like apples (Mizutani et al., 1995) and tomatoes (Smith, Zhang, Schofield, John, & Baldwin, 1994). Mangoes produced relatively low levels of ethylene compared to other climacteric fruit species in all the experiments conducted, confirming the observations of Burdon, Zhang, Schofield, John, and Baldwin

(1996). What is intriguing, however, is the fact that ACC oxidase *in vivo* is inhibited by CO₂ concentrations of 25% but *in vitro* the behavior of ACC oxidase from mangoes is similar to what has been observed, for example, in apples (Mizutani et al., 1995) and in sunflower roots (Finlayson & Reid, 1994).

A possible mechanism of *in vivo* inhibition of ACC oxidase by CO₂ might be the presence of high levels of ethanol induced by the CO₂ atmospheres, as seen in Bender et al. (2000). Treatments with ethanol and/or acetaldehyde have been shown to affect ethylene biosynthesis in tomatoes (Suzuki & Nagata, 2019) and mangoes (Burdon et al., 1996). In cultivar 'Keitt' mangoes, Burdon et al. (1996) concluded that acetaldehyde either inhibited ACC oxidase activity directly or prevented new protein synthesis. While this conclusion would seem to be supported by the results reported here for the exposure of mangoes to CA at 5, 8 or 12°C, this hypothesis does not hold when examining the period following transfer to air at 20°C, in which increases in ethanol, and consequently acetaldehyde, occurred concurrently with increases in ethylene production rates.

Conclusion

The effects of CO₂ on ethylene biosynthesis are probably primarily a consequence of reversible inhibition of the activity of ACC oxidase. The mechanism of the inhibition of the *in vivo* activity of ACC oxidase can not be addressed based on the available data. The evidence, however, does not support the suggestion in the literature pointing towards metabolites from the anaerobic pathway, either acetaldehyde or ethanol or both, as inhibitors of ethylene synthesis. In mangoes, the effects of CO₂ on the activity of ACC synthase do not seem to be as evident as in other species.

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