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ORIGINAL PAPER

Effect of Exogenous Ethylene on ACC Content and ACC Oxidase Activity During Ripening of Manila Mangoes Subjected to Hot Water Treatment

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Abstract Mangoes (*Mangifera indica* L.) 'Manila' were subjected to the USDA-approved hot water treatment and then exposed to synthetic air mixtures containing 0.5, 0.75 or 1 ml l^{-1} of ethylene for 6, 12 or 18 h at 25 °C, to induce accelerated ripening. After treatment the mangoes were allowed to ripen in air at 24–25 °C. The content of 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase (ACO) activity increased in fruit treated with 0.5 and 0.75 ml l^{-1} of ethylene for 6 or 12 h. Ethylene production was reduced in fruit treated with 1 ml l^{-1} of ethylene. This was due to the decreased of ACC synthesis rather than to lower ACC oxidase activity. Treatment with 0.5 ml l^{-1} of ethylene for 12 h was found best for accelerate ripening; fruits were fully ripened and edible 3 days after treatment, compared to 6–7 days for untreated mangoes.

Keywords Accelerated ripening · ACC oxidase activity · 1-aminocyclopropane-1-carboxylic acid · Ethylene · *Mangifera indica*

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
EPR	ethylene production rate
RH	relative humidity
TSS	total soluble solids

Introduction

Mango (*Mangifera indica* L.) is a climacteric fruit in which the rate of ethylene production varies widely depending on the cultivar [1]. The two key enzymes in the pathway of ethylene biosynthesis are those catalyzing the conversion of *S*-adenosyl methyonine to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC to ethylene, called ACC synthase (ACS) and ACC oxidase (ACO), respectively [2]. The cDNA encoding for ACO has been isolated and characterized from mango [3]. Ethylene is perceived by a family of five membrane-bound receptors (ETR1, ETR2, ERS1, ERS2, EIN4) that have similarity to two-components regulators from bacteria [4]. The ethylene receptors activate the kinase activity of CTR1 (negative regulator of ethylene signalling) in the air (absence of ethylene). CTR1 then actively suppresses the downstream responses, such that EIN2 (positive regulator of the pathway) and the EIN3/EIL transcription factors remain inactive. Upon binding ethylene, the receptors no longer activate CTR1, and so CTR1 no longer suppresses the pathway. This relief of suppression allows for activation of EIN2, induction of the transcriptional cascade and the establishment of ethylene responses [4]. Ethylene receptors have been identified in climacteric fruit,

including six genes in tomato [5]; three in pears [6]; two in peach [7] and one gene in mango [8].

Ethylene signalling, from biosynthesis to response, is highly regulated at both the transcriptional and post-transcriptional levels. Gene families encoding ACS and ACO are under tight transcriptional control, being induced by multiple developmental and stress cues [9]. Immature fruits produce low levels of ethylene and exogenous ethylene treatment does not stimulate further synthesis (System 1). In contrast, the ethylene produced by ripening fruit is autocatalytic, stimulating its own synthesis (System 2) [10]. The differences between the two systems can be explained at a molecular level by the lack of induction of ACS transcription, which is rate-limiting, during System 1 versus induction during System 2. Immature fruits do recognize and respond to ethylene, as indicated by increased expression of certain ethylene-inducible genes. However, ethylene does not initiate ripening in immature fruits. Only mature fruits respond to ethylene by induction of a larger set of ripening-associated genes, indicating a developmental component to ethylene regulation. Thus, those combinations of transcription factors, both developmental and ethylene-regulated, control expression of some genes [11]. The progression of softening in mango fruit pulp was associated with the expression of an α-expansin gene, *MiExpA1*. Expression of this gene is associated with ripening in mango both at the transcriptional and translational level. Regulation of the gene is under dual control, being governed by ethylene as well as a ripening related components [12].

The response of harvested fruit to applied exogenous ethylene depends on various factors, including cultivar, tissue sensitivity and stage of maturation, as well as on ethylene concentration and time of exposure, with an overall optimum temperature range of 20–25 °C [13]. Exposure of 'Tommy Atkins' mangoes to atmospheres containing either 0.00121 or 0.01 ml l^{-1} of ethylene for 24 h at 25 °C was sufficient to initiate ripening, while these mangoes reached complete ripening after exposure to 1.0 ml l^{-1} of ethylene for same time and temperature [14]. Mohamed et al. [15] employed 250, 500, or 1,000 µl l^{-1} of ethylene produced from ethrel on 'Dr Knight', 'Kitchner' and 'Abu-Samaka' mangoes, and found that 1,000 µl l^{-1} of ethylene for 24 h at 20 °C and 90–95% relative humidity (RH) achieved accelerated ripening of the fruit by 6, 8 and 6 days, respectively. The production of most of monoterpenes, esters and aldehydes in 'Kensington Pride' mango is strongly dependent on ethylene production and action [16]. Zamora et al. [17] applied exogenous ethylene on 'Kent' mangoes that were previously treated with hot water and kept refrigerated for 4 days at 13 °C and further ripened at 27 °C. The authors found that exposure to 100 and 500 µl l^{-1} of ethylene for 18 h was able to induce development of peel colour and better homogeneity in the ripened fruit. Montalvo et al. [18] found that ACC content and ACO activity were dependent on exposure time, producing greater values at longer exposure periods. Additionally, the ripening process was inhibited when 1,000 µl l^{-1} of ethylene were used for 12 h on 'Ataulfo' mangoes that were treated with hot water and maintained at 13 °C for 4 days.

Mango fruit in México can be infested with several insects, the most important of which are the fruit flies *Anastrepha ludens* and *A. obliqua* [19]. Mangoes must be subjected to quarantine treatments before shipment to certain domestic and export markets. Mango fruit exported to the US and Japanese markets are treated with hot water at 46.1 °C for 65, 75 or 90 min (depending on fruit weight). Manila is the most cultivated variety of Mango in Veracruz, México. Local handling practices include empirical usage of calcium carbide to release acetylene and produce accelerated ripening. Fruit treated with calcium carbide do not ripen homogeneously and colour and flavour development are often uncoupled from the normal ripening patterns. The purpose of the present work was to evaluate the effect of application of exogenous ethylene on ACC content and ACC oxidase activity during ripening of 'Manila' mangoes subjected to the USDA-approved hot water treatment.

Materials and Methods

Fruits and Treatments

Open pollinated mangoes (105 days post-anthesis) were obtained from a commercial grower in Actopan (state of Veracruz, México; latitude N19°30′, W96°37′, altitude 260 m) and transported to the laboratory in open crates for ca. 1 h after collection. Fruits (60 count for each treatment) with a firm texture and deep green colour, ranging in weight from 215–260 g were selected. The USDA-approved hot water treatment was applied, by immersing the fruit in hot water so that they were exposed to 46.1 °C for 65 min using a pilot scale unit. After treatment, the fruit was cooled by immersion in tap water for 35 min, air dried at room temperature and transferred to specially constructed chambers. The chambers (22.1 l) were constructed of PVC tubes that can be hermetically sealed, creating a static system. Each chamber holds up to 20 kg of fruit (ca. 60 mangoes). A gas inlet and outlet, a fan, a manometer and a septum for gas sampling were attached to each chamber. Certified synthetic air mixtures (AGA gas, México) containing 0.5, 0.75 or 1 ml l^{-1} of ethylene in air were circulated through each chamber until the ethylene concentrations of the outgoing streams matched that of the certified mixtures. Both inlet and outlet valves were then closed. The gas mixtures were

twice bubbled through water at room temperature before entering the chambers, producing a RH of 98%. The fruit was held at room temperature (24–25 °C) for 6, 12 or 18 h, at which times one chamber was opened, and the fruit allowed to ripen in air at room temperature for 5 days. Samples were withdrawn daily for analyses. Control mangoes were not exposed to ethylene and handled in open air and room temperature.

Analyses

During ripening, the following analyses were performed daily in quadruplicate: ACC content and ACO activity were analysed during ripening according to the methods reported by Cua and Lizada [20]. The results are reported in nmol g^{-1} fruit and nl ethylene g^{-1} h^{-1}, respectively. Ethylene production rate (EPR) was determined on four fruit (replicates) per treatment by individual sealing each in a 1-l glass jar, and letting the sample stand at room temperature for 5 h. Gas samples were withdrawn through a septum and injected into a Hewlett-Packard HP5890 Series II gas chromatograph (Avondale, PA, USA) fitted with a 25 m Poraplot-Q column (Chrompack) fitted with Thermal Conductivity and Flame Ionization Detectors connected in series. Total soluble solids (TSS) were measured with an Abbe refractometer (Leica Mark II). Titratable acidity was determined by titration with 0.1 N NaOH and expressed as percentage of citric acid [21]. Firmness was measured on both sides of the whole fruit with an IRC force gauge (Norfolk, VA, USA) using an 8 mm conical probe.

Statistical Analysis

Data were analyzed by ANOVA (GLM-ANOVA) using the statistical package SAS for Windows™ version 6.11 (SAS Institute, Cary, NC, USA). Mean comparison was made by the least significant difference (LSD). All tests were made at the 0.05 significance level.

Results

ACC Content, ACC Oxidase Activity and Ethylene Production Rate

ACC content in control mangoes at day 0 was 0.4 nmol g^{-1} and then decreased to less than 0.2 nmol g^{-1} from days 1 to 4 (Fig. 1a). ACO activity of the same fruit remained constant for the first 3 days (Fig. 1d) but increased after day 3, reaching 9 nl $g^{-1}h^{-1}$ by day 5. EPR (Fig. 1g) increased gradually from 13.8 to 23.5 μg $kg^{-1}h^{-1}$ in control mangoes.

ACC content was higher in fruit treated with 0.5 ml l^{-1} of ethylene compared to control mangoes (Fig. 1a). However, there were significant differences in the time needed to reach the peak ACC concentration when fruit were treated for 6, 12 or 18 h. Peak activity for ACO occurred at day 3 in mangoes treated for 12 h, and at day 4 on fruit treated for 6 or 18 h. Similar behaviour was not observed for the control fruit. ACO activity in treated fruit for 12 h decreased at day 4 (Fig. 1d), and thus increased levels of ACC (Fig. 1a). As a consequence EPR reached a peak at day 3 for mangoes treated for 12 h (52 μg $kg^{-1}h^{-1}$) but not until day 4 for mangoes treated for 6 h (47.2 μg $kg^{-1}h^{-1}$). Peaks were not observed for either the fruit treated for 18 h (25.9 μg $kg^{-1}h^{-1}$ at day 4) or the control (23.5 μg $kg^{-1}h^{-1}$ at day 5; Fig. 1g).

Mangoes exposed to 0.75 ml l^{-1} of ethylene for either 6 or 12 h at day 1 (Fig. 1b) exhibited a greater initial response in ACC synthesis relative to those treated with 0.5 ml l^{-1} (Fig. 1a, day 1). After the day 1, ACC content decreased to a low level on day 3 (Fig. 1b) and this trend coincided with the peak of ACO activity and EPR for the same day (Fig. 1e,h, respectively). Changes in ACC content in fruit treated for 18 h with 0.5 and 0.75 ml l^{-1} of ethylene were similar with the highest values in ACC content observed on days 4 and 2, respectively (Fig. 1a,b). A peak for ACO activity was observed on day 4, but not a maximum EPR was observed (Fig. 1e,h) in fruit treated by 18 h.

Treatment with 1 ml l^{-1} of ethylene induced smaller increases in ACC content and also delayed the increase in ACO activity compared to treatments with lower ethylene concentrations (Fig. 1c,f). Ethylene production rate by fruit treated with ethylene for 12 and 18 h remained similar to the rates measured in control fruit (Fig. 1i).

Total Soluble Solids, Titratable Acidity and Firmness

Ethylene treatments increased TSS (Fig. 2a,b,c) relative to control fruit. This increase was observed for all treatments and was statistically different from the control 2 days post-treatment; except for mangoes exposed to 1 ml l^{-1} of ethylene for 18 h, which produced TSS at concentrations similar to the control fruit.

The decrease in titratable acidity was generally greater in treated fruit (Fig. 2d,e,f). Exceptions were mangoes exposed to 1 ml l^{-1} of ethylene for 12 or 18 h. Greater differences were noted for treatments with 0.5 and 0.75 ml l^{-1} of ethylene.

Treatment with 0.5 and 0.75 ml l^{-1} of ethylene increased the rate of softening in control mangoes (Fig. 2g,h,i). Mangoes exposed to ethylene for 6 and 12 h were softer than control fruit. However, mangoes exposed to 1 ml l^{-1} of ethylene had firmness values similar to the control fruit after storage.

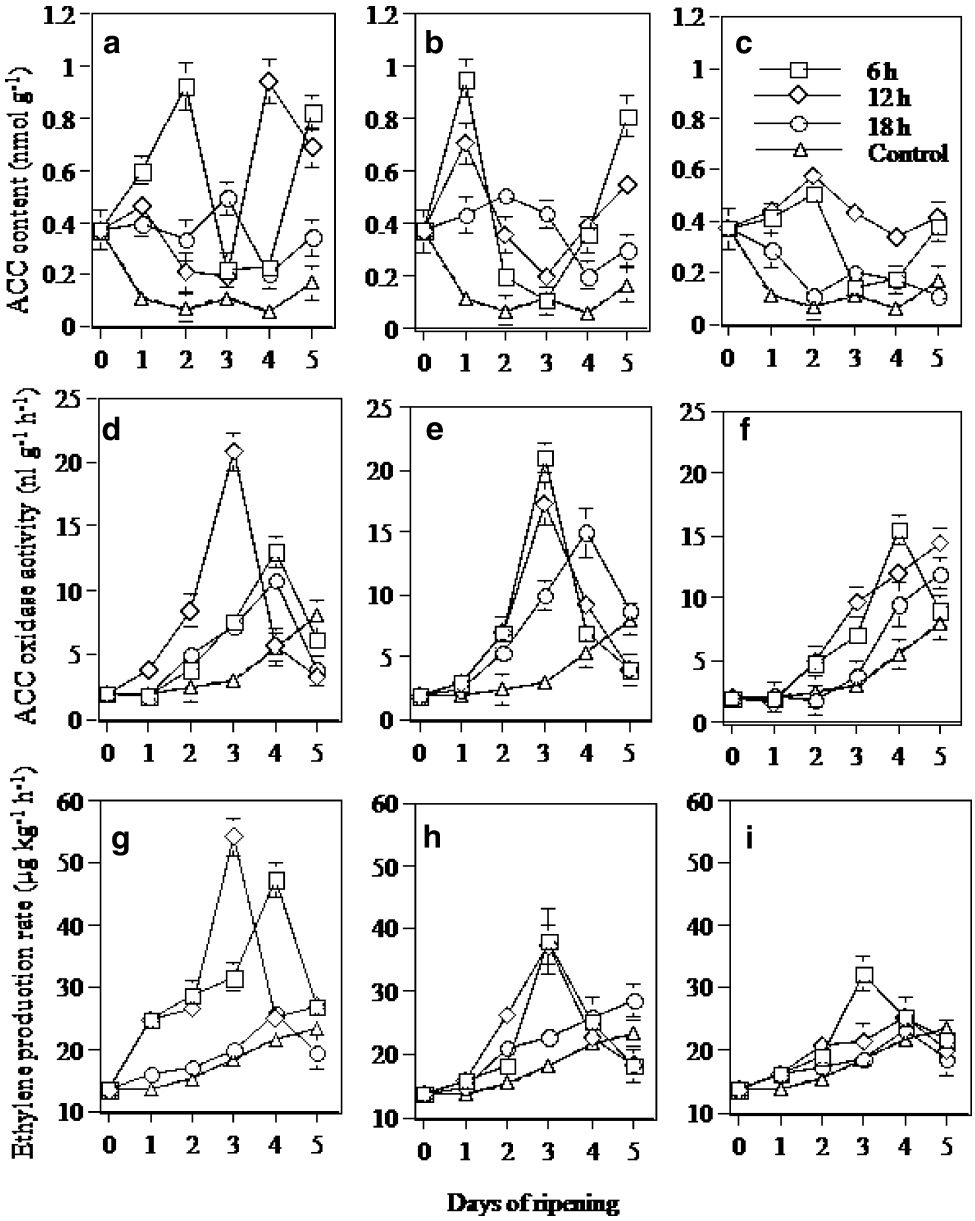

Fig. 1 Ripening of 'Manila' mangoes at 25 °C after exposure to **a**, **d**, **g** 0.5 ml l^{-1}; **b**, **e**, **h** 0.75 ml l^{-1} and **c**, **f**, **i** 1.0 ml l^{-1} of ethylene at different times, on 1-aminocyclopropane-1-carboxylic acid content, ACC oxidase activity, and ethylene production rate. Each value represents the mean of 4 fruits ± SD

Discussion

ACC contents decreased to low values in control mangoes from day 0 (Fig. 1a). This trend was correlated with low ACO activity and low ethylene synthesis (Fig. 1d,g) and implies that fruit has only a limited ability to convert *S*-adenosylmethionine to ACC and therefore ACS activity in fruit of this variety that has been subjected to hot water quarantine treatment is very low. Ketsa et al. [22] reported that ACS activity is lost in mangoes that have been exposed to temperatures greater than 40 °C for short periods. This activity is only partly regained when these fruits are returned to room temperature. This probably because the ACC content started to increase at day 5. ACO is apparently highly susceptible to thermal damage when the fruit is exposed to temperatures above 30 °C, but full recovery of ACO activity was noted within 3 days, after the mangoes had been restored to room temperature [22]. ACO activity increased after day 3 (Fig. 1d) and coincided in time with the EPR (Fig. 1g). This coincidence is characteristic of climacteric fruits. The increase in activity of this enzyme from day 2 confirmed that the hot water treatment did not prevent the activity of this enzyme. It is possible that on days 4 and 5 ACC content may have been the limiting factor for EPR. It has been reported that ACS and ACO expression are dependent on the developmental stage of the fruit and ethylene [11], which could be the cause of the small amount of ethylene produced.

Control 'Manila' mangoes displayed an elevated EPR (24 µg $kg^{-1}h^{-1}$ or 22 nl $g^{-1}h^{-1}$ at day 5), while it has been

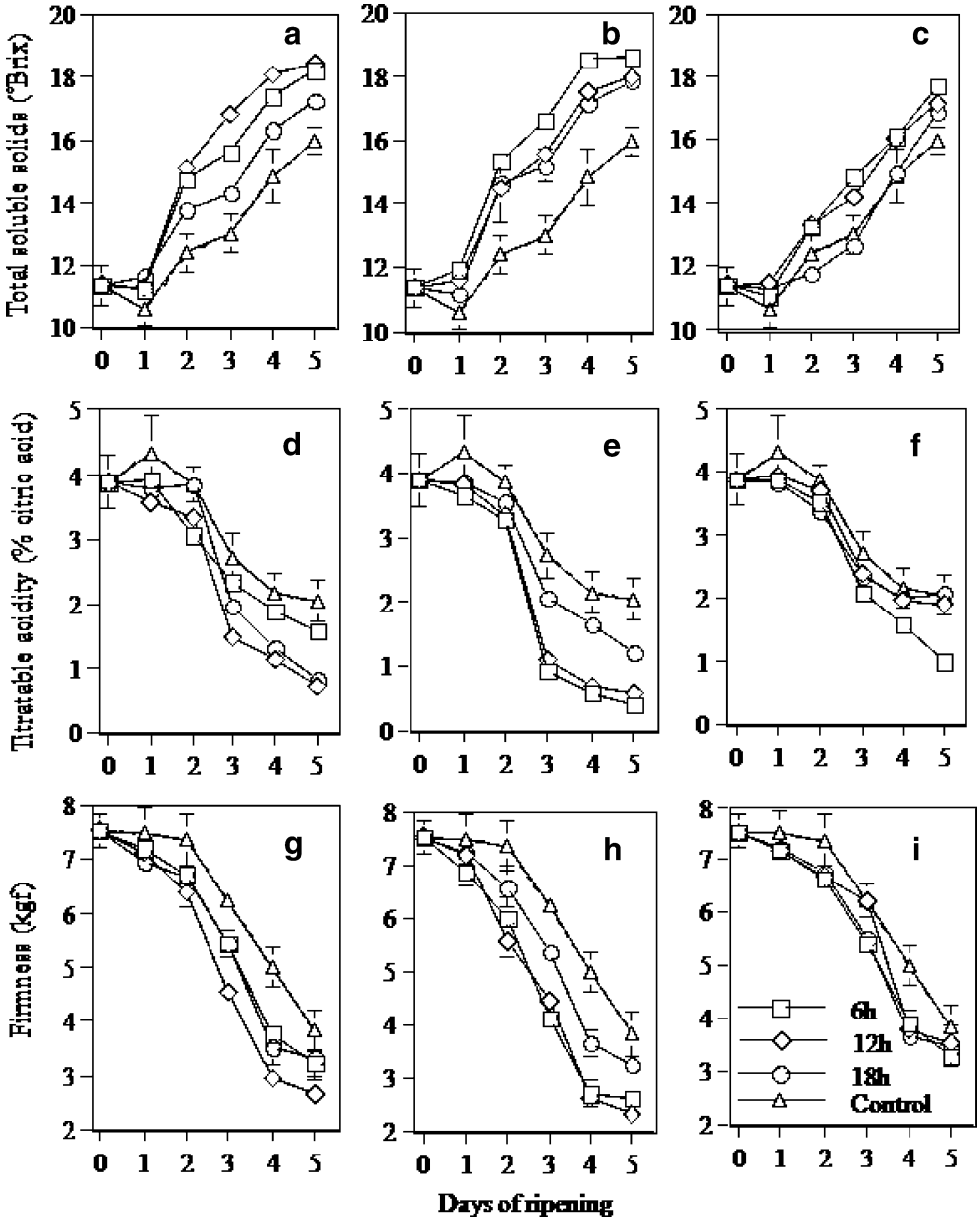

Fig. 2 Changes in total soluble solids, titratable acidity and firmness during ripening of 'Manila' mangoes at 25 °C after application of **a**, **d**, **g** 0.5 ml l^{-1}; **b**, **e**, **h** 0.75 ml l^{-1} and **c**, **f**, **i** 1.0 ml l^{-1} at different times. Each value represents the mean of 4 fruit ± SD

reported that 'Carabao' ('Manila' parental variety) and 'Ataulfo' (with hot water treatment) mangoes produced 0.7 and 2.1 nl $g^{-1}h^{-1}$ of ethylene after storage for 10 and 12 days at 25 °C, respectively [18, 20]. These contrasting results can be attributed to differences between varieties, since the ACO activity in 'Manila' mangoes is generally greater than in 'Carabao' mangoes [20]. Hot water treatment did not prevent ethylene synthesis during storage.

The higher ACC concentrations in samples treated with external ethylene are believed to be due to induced ACS activity [13]. Our data show that increased ACO activity was induced after day 1 (Fig. 1d,e,f). Both ACS and ACO belong to multigene families, the genes are under tight transcriptional control, being induced by multiple developmental and stress cues, as well as by ethylene on mature fruit [9, 11]. A short exposure to ethylene may induce ACO without a concomitant increase in ethylene production because induction of ACS requires a longer exposure [23]. However, in our experiments we noted a greater response for ACS than for ACO since ACC accumulated from day 1. It has been reported [10] that treatment of unripe immature fruit with exogenous ethylene is not sufficient to induce ACS activity; because the exogenous ethylene does not stimulate further synthesis (System 1), therefore, neither the stage of maturity of our mangoes nor the hot water treatment affected this response.

Ethylene signalling, from biosynthesis to response, is highly regulated at both the transcriptional and post-transcriptional level [5]. Regulation of either hormone levels or that of key proteins in the signal transduction

pathway can regulate flux through the pathway, and thereby regulate the final level of ethylene response. Not surprisingly, ethylene itself is often an important regulator of expression. Thus the signalling pathway is able to feed back on itself to regulate its own sensitivity to ethylene [5]. In our work, there is evidence of suppression of ethylene production by application of exogenous ethylene. EPR was found to be similar in both control and fruit treated for 18 h at all three ethylene concentrations as well as for those exposed to 1 ml l^{-1} of ethylene for 12 h, although ACC content and ACO activity were not affected. Similar results were observed in 'Kent' and 'Ataulfo' mangoes [17, 18]. Ethylene concentrations and exposure times which could cause this effect are variable with respect to mango varieties. Mohamed et al. [15] did not find inhibition of ripening in three mango varieties after 1,000 μl l^{-1} of ethylene were applied. It is not yet clear how time of exposure and exogenous ethylene concentration affect at the molecular level this complex system of ethylene perception and biosynthesis.

Increased TSS were observed in 'Dr Knight', 'Kitchner' and 'Abu-Samaka' mangoes treated with ethylene released from ethrel at 500 and 1,000 μl l^{-1}; such values were higher than those of control fruit [15]. This response was also observed in 'Tommy Atkins', 'Kent' and 'Ataulfo' mangoes treated with gaseous ethylene [14, 17, 18]. In our experiments, application of all three ethylene concentrations for 6 and 12 h increased TSS in the fruit, but the treatment of 1 μl l^{-1} for 18 h caused an evident ripening inhibition of the mangoes.

Zamora et al. [17] and Montalvo et al. [18] reported a more pronounced drop in titratable acidity of 'Kent' and 'Ataulfo' mangoes; respectively, that were treated with ethylene; however, Kumar and Dhawan [24] found that titratable acidity values in treated 'Dashehari' mangoes were similar to those of the control fruit at the end of the storage period. Hence, because of the greater TSS and smaller titratable acidity, flavor changes from the typical sweet-sour notes of this fruit could be expected.

We believe that increased EPR affects fruit firmness (Fig. 2g,h,i). After day 2 of ripening, treated samples softened faster than control mangoes. This trend coincided with greater EPR. Furthermore, smaller values of EPR for samples subjected to ethylene treatment were associated with smaller changes in fruit firmness. This trend is consistent with the reported by Sane et al. [12] in which progression of softening in mango fruit pulp was associated with the expression of α-expansin gene, and its regulation was controlled by ethylene.

It is evident that based on most physiological indicators, treatment with 0.5 ml l^{-1} of ethylene for 12 h accelerates the ripening process. Exposure to the same concentration but for shorter 6 and 18 h periods produced a smaller response. At day 4, fruit treated with 0.5 ml l^{-1} of ethylene for 6 and 18 h was as soft as control fruit (5 days of normal ripening). Consequently, the 0.5 ml l^{-1} of ethylene treatment advanced ripening by 1 day. Treatments with 0.75 ml l^{-1} of ethylene for 6 or 12 h advanced ripening by 2 days, while the same ethylene concentration applied for 18 h gave a gain of 1 day.

Treated fruit contained higher TSS than controls, and although the titratable acidity trends did not follow the same trends as TSS, in general mangoes treated with the best conditions found in this study for faster ripening developed external qualities typical of ready-to-eat mangoes. Even though changes in pulp colour were not measured, we noted that a typical and homogenous yellow colour started developing in the treated mangoes on the second day after ethylene exposure.

Conclusions

Application of exogenous ethylene for 6 h increased ACC synthesis on days 1 and 2 of ripening and with reference to ACO activity increased by effect of the ethylene exposure for 12 h at day 3. Ethylene synthesis was suppressed due to diminished ACC synthesis. Treating mature green 'Manila' mangoes with 0.5 ml l^{-1} of ethylene for 12 h gave even-ripened mangoes with normal composition and appearance that were ready to eat in only 3 days.

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