# Ethylene Biosynthetic Responses of Mango (*Mangifera indica* L. cv. 'Carabao') Fruits to 1-methylcyclopropene (1-MCP) Preharvest Aqueous Spray Applications

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**ABSTRACT---** ***The ethylene antagonist 1-methylcyclopropene (1-MCP) was used as a tool to investigate the mechanism of ethylene feedback regulation in mango (Mangifera indica L. cv. 'Carabao') fruits. The ethylene biosynthetic responses of mango fruits to 1-MCP applied prior the upsurge in ethylene production on-tree which was around 100 days after flower induction (DAFI) were studied. Aqueous 1-MCP spray formulation with 10 ppm concentration was applied to on-tree mango fruits at 100 DAFI. From these sprayed fruits, four (4) sets of fruits were tagged for a second round of 10 ppm aqueous 1-MCP spraying. One set was sprayed the second time at 105 DAFI, while the remaining sets at 110, 115 and 120 DAFI respectively. The positive control was sprayed with 0 ppm 1-MCP while the negative controls were the unsprayed fruits. Representative fruit samples from each set were harvested 2 d after spraying and every 2 d thereafter until reaching 120 DAFI. Right after each harvest, samples were analyzed for internal ethylene, ethylene production, ACC levels and ACC oxidase activities. The negative control exhibited two internal ethylene peaks before harvest maturity. However, in 1-MCP treated fruits, low levels of ethylene were detected and the second peak was not observed. A single application of 1-MCP at 100 DAFI resulted in lower ethylene production compared with controls. However, a second application proved to be more effective as the lowering of ethylene production was carried over until the day of harvest. The levels of 1-aminocyclopropane-1-carboxylate (ACC) increased after 110 DAFI for the controls. Reapplication of 1-MCP at 110 DAFI resulted to lower ACC levels, which was not observed with the other treatments with 1-MCP reapplication. Possibly, new ethylene receptors are synthesized at 110 DAFI, and 1-MCP reapplication at this time effectively blocks ethylene binding to the new receptors. ACC oxidase activity was lowered by 1-MCP applied at 100 DAFI compared with the controls. However, trends in ACC oxidase activities were almost the same for all the treatments and controls after 105 DAFI. Thus, ACC oxidase proceeds with its normal activity without being inhibited by 1-MCP. This suggests that ethylene biosynthesis in 'Carabao' mango fruit is regulated at the level of ACC synthase and not ACC oxidase. Thus, autocatalytic ethylene biosynthesis in 'Carabao' mango can be controlled by regulating ACC synthase activity, which proves that ACC synthesis is the rate limiting step in ethylene feedback mechanism in 'Carabao' mango.***

**Keywords—**1-methylcyclopropene, ACC, ACC synthase, ACC oxidase, 'Carabao' mango, ethylene

## 1. INTRODUCTION

Ethylene is one of several plant growth regulators that affect growth and developmental processes including ripening and senescence [1]. Once the autocatalytic ethylene production starts, a wide range of both physical and chemical changes occur such as tissue softening, pigment degradation and biosynthesis of new ones, and changes in sugar and organic acids [2]. Thus, any factor that affects the synthesis and/or the action of ethylene will invariably affect the course of ripening.

The biosynthesis of ethylene involves three steps (Figure 1). The reaction catalyzed by ACC synthase forms the cyclic intermediate 1-aminocyclopropane-1-carboxylate (ACC) and 5'-methylthioadenosine (MTA), which is recycled to L-methionine via the Yang cycle. ACC is then converted to ethylene by the enzyme ACC oxidase or the ethylene-forming enzyme. Autocatalytic ethylene biosynthesis is controlled by either or both ACC synthase and ACC oxidase depending on the commodity. In mango (*Mangifera indica* L. cv. 'Carabao') fruits, determination of the mechanism of

regulation of autocatalytic ethylene biosynthesis is important in postharvest studies on delaying ethylene ripening effects.

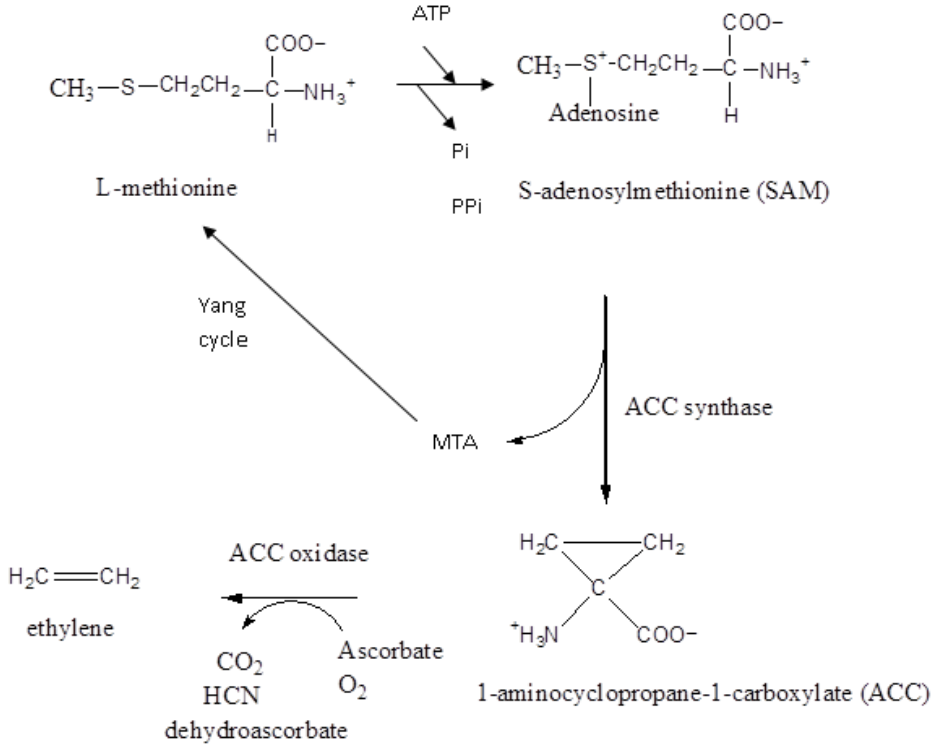


**Figure 1.** Ethylene biosynthetic pathway. Shown are the reactions catalyzed by the two key enzymes: ACC synthase and ACC oxidase

Perception of ethylene is accomplished by a small family of integral membrane receptors called ethylene receptors. The ethylene antagonist 1-methylcyclopropene (1-MCP) effectively blocks the hormone action of ethylene, by irreversible binding to the ethylene receptor. The affinity of 1-MCP for the receptor is approximately 10 times greater than that of ethylene. Compared with ethylene, 1-MCP is active at much lower concentrations [3].

Aside from its commercial use in prolonging shelf-life of agricultural produce, 1-MCP is also used as a research tool to further understand plant ethylene responses. Ethylene biosynthesis being regulated by itself, can be investigated by using an ethylene antagonist such as 1-MCP to verify whether induction of ethylene biosynthesis is mediated by the action of ethylene. Thus, the feedback mechanism in ethylene biosynthesis can be elucidated.

1-MCP was found to reduce the normal increase in activities of ACC synthase (ACS) and ACC oxidase (ACO) enzymes during ripening of 'Tegan blue' plum [4]. Reduced activities of ACS and ACO enzymes have been reported in 1-MCP treated plum during fruit ripening [5]. 1-MCP treatment of ripening kiwifruit resulted in the suppression of ethylene biosynthesis and AC-ACS1 gene expression, suggesting that in ripening kiwifruit, ethylene biosynthesis is regulated by a positive feedback mechanism [6]. In apple and peach, inhibition of ethylene production was also accompanied by reduced expression of ACS and ACO transcripts [7] which suggests that the reduction in ethylene production by 1-MCP is regulated at the gene level. The same was observed in broccoli [8], wherein 1-MCP treatment delayed the senescence of broccoli by inhibiting the activities of enzymes involved in ethylene biosynthesis (ACS and ACO). Inhibitory effects of 1-MCP on ethylene biosynthesis with reduced ACS and ACO enzyme activities and their respective gene transcription have been reported in banana [9] and tomatoes [10].

In 'Carabao' mango, events associated with ripening have been shown to initiate in the mesocarp prior to full maturation and with ethylene production showing a peak at about 10 days before harvest maturity [11]. This is evidenced by ripening signs in the inner mesocarp prior to full maturation. Studying the effects of a preharvest application of 1-MCP before the upsurge in ethylene production could prove to be useful in elucidating the regulatory feedback mechanism of ethylene biosynthesis in 'Carabao' mango fruits.

This study aimed to investigate the ethylene biosynthetic responses of 'Carabao' mango fruits to preharvest 1-MCP spray applications in terms of activity of ACC oxidase, levels of ACC, ethylene and $CO_2$ production and elucidate the feedback regulatory mechanism of ethylene.

## 2. MATERIALS AND METHODS

The experiments were conducted in a mango farm in Barangay. Siranglupa, Calamba City, Laguna, Philippines. To determine the maturity stage at which 1-MCP application will be carried out, ethylene production at each maturation stage was first determined as a preliminary experiment. Fruits were harvested at 90, 100, 110 and 120 days after flower induction (DAFI) and analyzed for internal ethylene and ethylene production. The maturity stage at which an ethylene peak was observed was considered as the spraying time for 1-MCP and this was found out to be at 100 DAFI.

From the result of the preliminary experiment, another experiment was conducted wherein 1-MCP was applied as an aqueous spray to 'Carabao'mango fruits on-tree. The 1-MCP used was a gift from Dr. Xiang Chun Meng from Guangdong Academy of Agricultural Sciences, China. The formulation contains 0.43% 1-MCP. Triton-X which served as surfactant, was added to the amount of 0.25 mL/L. Spraying was done within 30 mins after the preparation of the spray solution.

Mango fruits on the tree at 100 DAFI were unbagged then sprayed with 10 ppm 1-MCP solution. The fruits were then enclosed in plastic bags for 2h to allow time for binding of 1-MCP. The plastic bags were then removed and the fruits were again bagged with paper. Color tags were placed on each paper bag to facilitate identification of treatments (Figure 2). From these sprayed fruits, four (4) sets of fruits consisting of sixty (60) fruits per set were tagged for a second round of 10ppm aqueous 1-MCP spraying. Thus, one set was sprayed the second time at 105 DAFI, while the remaining sets at 110, 115 and 120 DAFI respectively. Re-spraying with 1-MCP was done in order to determine approximately when new ethylene receptors are being synthesized. The positive control was sprayed with 0 ppm 1-MCP while the negative controls were the unsprayed fruits. Representative fruit samples from each set were harvested 2 d after spraying and every 2 d thereafter until reaching 120 DAFI. Right after each harvest, samples were analyzed for percent internal ethylene, ethylene production, ACC levels and ACC oxidase activities.


**Figure 2.** Preharvest application of aqueous 1-MCP spray. (a) Mango fruits were unbagged (b) sprayed with aqueous 1-MCP (c) enclosed in plastic bags for 2 h (d) bagged again with tagged paper bags.

### *2.1 Ethylene production*

The rate of ethylene biosynthesis was measured by measuring the ethylene production. Fruits were weighed and then enclosed in respiration jars for 1h after which, 1 mL gas samples were collected. The gas samples were injected into a Shimadzu model 80A gas chromatograph with flame ionization detector (GC-FID). Peak heights for ethylene and the standard were measured after injection. Ethylene production was calculated as nL $C_2H_4$ $g^{-1}h^{-1}$ .

### *2.2 Internal ethylene measurement*

Samples of internal gas from the whole fruit were obtained by vacuum evacuation method [12]. A pressure of 60 cm Hg was regulated. One mL of the evacuated gas sample was obtained. The amount of ethylene gas was determined using

Shimadzu model 80A gas chromatograph equipped with a flame ionization detector (GC-FID). Results were expressed relative to the concentration of ethylene standard which is 1ppm.

### *2.3 1-Aminocyclopropane-1-carboxylate (ACC) levels*

***ACC extraction and assay***. About 20 g tissue was homogenized with 40 mL 95% ethanol for 2.5 minutes. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4$^{o}$C. The total volume of the supernatant was determined. An aliquot of the supernatant was obtained in rubber serum cap covered tubes equivalent to 0.5 g tissue. This was then dried using a stream of air. After which, 1 drop (0.05 mL) 0.1M $HgCl_2$ was added and the tubes were sealed with rubber serum caps. An oxidizing agent (0.1 mL) mixture of 5% NaOCl and saturated NaOH (2:1 v/v) were added dropwise into the sealed tubes immersed in an ice bath, shaken in a vortex mixer and a 1.0 mL headspace gas was analyzed for ethylene using GC-FID. The method was standardized using pure ACC [13].

### *2.4 ACC Oxidase (ACO, EC 1.14.17.4) Activity*

***Extraction.*** Twenty g of fruit tissues were weighed and dipped in liquid nitrogen. While being thawed, 5 times the volume of 0.1M Tris-HCl buffer, pH 7.2 containing 10% (w/v) glycerol and 10 mM sodium ascorbate was added and homogenized in a mortar and pestle at 4$^{o}$C. The homogenate was filtered through a layer of miracloth and centrifuged at 10,000 rpm for 15 minutes at 0$^{o}$C. The supernatant was saved as crude extract and this was used to determine the enzyme activity [14].

***Assay.*** The standard assay medium consisted of 70 mM Tris-HCl pH 7.2, 7% glycerol, 1mM ACC, 30 mM sodium ascorbate and 100 µM Fe $SO_4$ in a total volume of 2 mL including the enzyme. The reaction was carried out in a 16 mL test tube which was sealed with a rubber serum cap and shaken in a vortex mixer. The reaction started by the addition of the enzyme with a glass hypodermic syringe through the stopper. After 30 min incubation at 30$^{o}$C, a 1-mL gas sample was withdrawn from the headspace of the tube and analyzed for ethylene using GC-FID. The ACC oxidase activity was expressed as nL ethylene produced per h per g fresh weight of mesocarp tissue [14].

## 3. RESULTS AND DISCUSSION

### *3.1 Ethylene Production at Different Stages of Maturity of 'Carabao' mango fruits*

Control fruits were harvested at 90, 100, 110 and 120 DAFI and analyzed for internal $C_2H_4$ and ethylene production (Figure 3A and 3B). Upsurge in internal ethylene and ethylene production were observed at 100 DAFI. This was the basis for applying 1-MCP preharvest spray at 100 DAFI. At around this time, the upsurge in ethylene production will occur, thus applying 1-MCP will prevent ethylene binding to receptors, and the mechanism of autocatalytic ethylene regulation can be elucidated.

### *3.2 Internal Ethylene and Ethylene Production as influenced by Preharvest 1-MCP Spray*

Lower internal ethylene level was observed in fruits sprayed at 100 DAFI and evaluated 2 d right after spraying compared with the controls (Figure 4). However, ethylene surged for this treatment at about 107 DAFI, which was higher than the controls. Re-spraying at 105 DAFI did not result in lower internal ethylene levels even until 120 DAFI. Re-spraying at 110 DAFI resulted in lower internal ethylene levels until about 117 DAFI. It can be noted that two ethylene peaks were obtained in the negative control, the first at about 103 DAFI and the second at about 117 DAFI. The author [11] also observed onset of ethylene production occurring about 2 weeks before harvest maturity (106 DAFI), and exhibiting a peak at 111 DAFI. For the 1-MCP treatments however, the second peak was absent. This is an evidence of possible suppression of ethylene biosynthesis caused by 1-MCP. The absence of the second peak in 1-MCP treatments could be due to inhibition of ethylene biosynthesis caused by 1-MCP which inhibits autocatalysis of ethylene. Autocatalytic ethylene production may have been hampered due to the low concentration of ethylene being retained in tissues. Ethylene cannot bind to receptors due to the presence of 1-MCP which is an ethylene antagonist, therefore, it is possible that they just diffuse out of the tissues and only a small amount is retained which is below the threshold level for autocatalytic ethylene production.

Lower ethylene production was also observed in the 100 DAFI treatment (Figure 5) 3 d after spraying compared with the positive and negative controls. Ethylene peaks occurred at the same time at about 107 DAFI for 100 DAFI treatment and the controls. However, it can be noted that a much lower peak was obtained in the 100 DAFI treatment. A second ethylene peak at about 117 DAFI was observed in the positive and negative controls. The 100, 105 DAFI treatment also exhibited a second peak which was smaller and showed earlier onset at about 113 DAFI. The rest of the treatments did not exhibit a second ethylene peak and ethylene levels were almost the same.

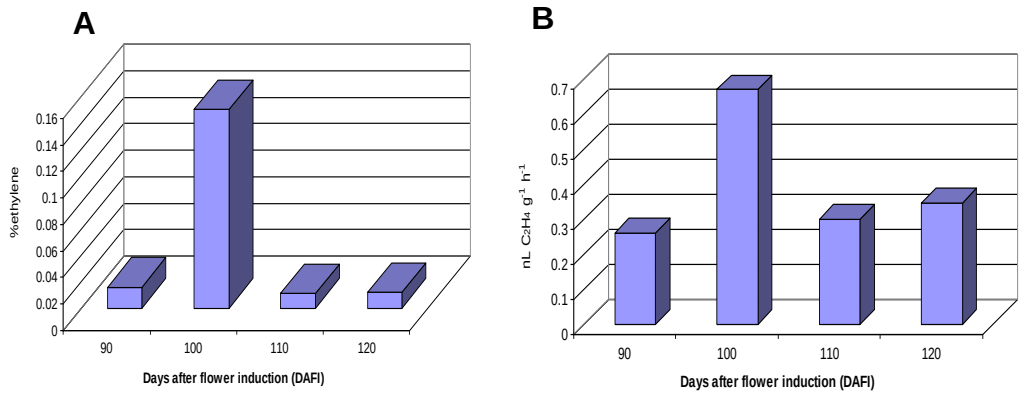


**Figure 3.** Internal ethylene (A) and ethylene production (B) in untreated (control) mango fruits harvested at different days after flower induction (DAFI). Each value is a mean of three (3) replicates, each replicate consists of two (2) fruits

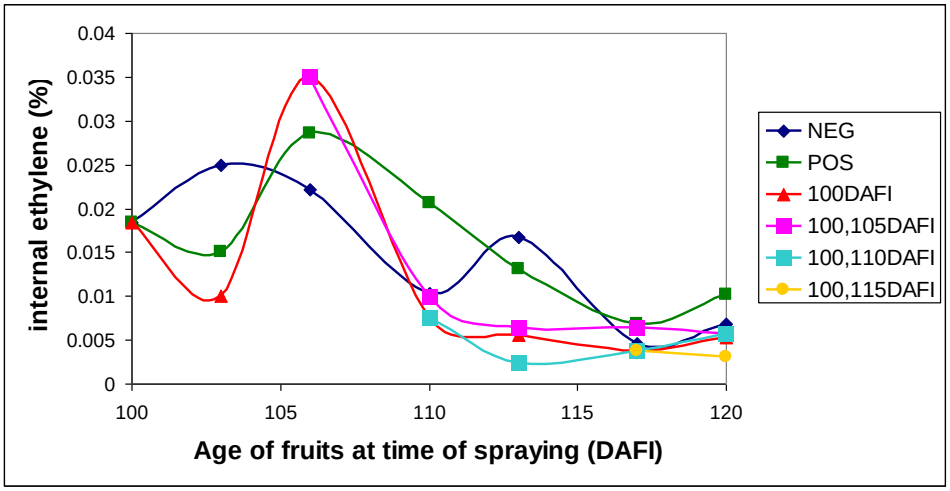


**Figure 4**. Internal ethylene levels in mango fruits preharvest treated with 10 ppm 1-MCP at 100 DAFI with reapplication at 105, 110, 115 and 120 DAFI. Samples were analyzed 2d after each spray and every 2d thereafter until harvest at 120 DAFI. Each value is a mean of three replicates, each replicate consisting of two fruits.

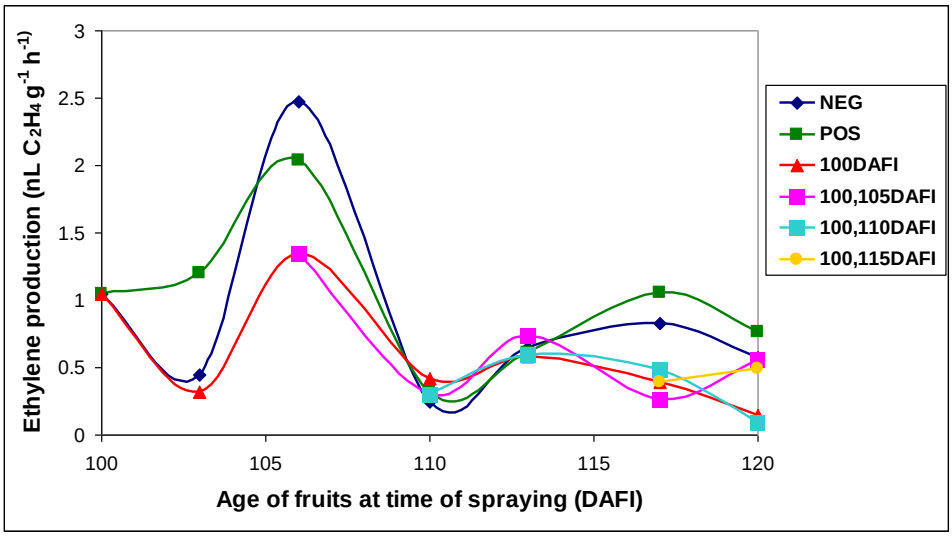


**Figure 5.** Ethylene production in mango fruits preharvest treated with 10 ppm 1-MCP at 100 DAFI with reapplication at 105,110,115 and 120 DAFI. Samples were analyzed 2d after each spray and every 2d thereafter until harvest at 120 DAFI. Each value is a mean of three replicates, each replicate consisting of two fruits.

### *3.3 ACC Levels as Influenced by Preharvest 1-MCP Spray*

1-aminocyclopropane-1-carboxylate (ACC) is an intermediate of the pathway for ethylene biosynthesis. ACC synthase catalyzes the reaction for its synthesis from S-adenosylmethionine (SAM). The step catalyzed by ACC synthase is considered the rate-determining step of the ethylene biosynthetic pathway [15]. Thus, ACC levels within tissues reflect the extent of ACC synthase activity.

Three days after spraying at 100 DAFI, which is considered an immature stage of mango, a distinct decrease in ACC levels was observed compared with both negative and positive control (Figure 6). Re-spraying at 105 DAFI did not maintain low ACC levels but instead resulted in an increase in ACC. The single-sprayed treatment at 100 DAFI however, still maintained lower ACC levels until sampling at 113 DAFI. Re-spraying at 110 DAFI and 115 DAFI also caused increased ACC levels after spraying. Peak in ACC production for the negative control was observed at 110 DAFI while the positive control at 113 DAFI. For the single-sprayed treatment however, the occurrence of the peak was delayed. The peak which was lower than those of the controls was observed at 117 DAFI. It can be noted though, that the re-sprayed treatment at 110 DAFI seemed to have prevented the onset of this peak at 117 DAFI. Based on these results in terms of ACC production, spraying with 10ppm 1-MCP at 100 DAFI then followed by a re-spray at 110 DAFI seemed to be effective in controlling ethylene biosynthesis. Spraying at 100 DAFI effectively controlled ACC production until 113 DAFI. A reapplication at 110 DAFI was able to carry over the suppression of ACC production until 120 DAFI. This suggests that possibly at about 110 DAFI, new receptors have already been synthesized, thus the increase in ACC after 110 DAFI. Reapplication at 110 DAFI provided a new batch of 1-MCP molecules to bind with the newly synthesized ethylene receptors. Thus, the upsurge in ACC at 117 DAFI was prevented.

Ripening processes depends on the synthesis of new ethylene receptors based on findings that exogenous ethylene cannot induce 1-MCP treated fruit ripening [16]. Further suppression of ripening by 1-MCP reapplication was also observed in tomato [17] and 'Sunrise' summer apples [18]. It was proposed though, that secondary applications could serve to occupy pre-existing receptors from which 1-MCP has dissociated [19]. The re-application of 1-MCP at 105 DAFI was just timely presumably because it is the stage at which synthesis of new ethylene receptors is high.

The reapplication at 115 DAFI was not able to prevent increase in ACC. It can be noted from Figure 5 that a short ethylene peak was observed at about 117 DAFI. This means that ethylene production at this stage has already started to take an upsurge. Thus, reapplication at 115 DAFI was not able to take effect because the receptors have already been saturated with ethylene. An earlier reapplication was therefore proven to be effective in blocking ethylene. The dependence of 1-MCP effects on fruit maturity was also demonstrated in studies on goldenberries [20]. The effect of ethylene receptor blockage strongly depends on the fruit stage. Goldenberries in the orange stage showed less responsiveness to 1-MCP than the mature green or yellow stages.

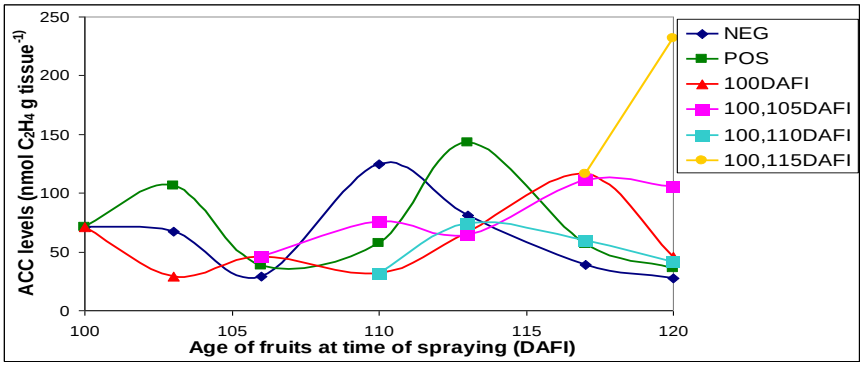


**Figure 6.** ACC levels in mango fruits preharvest treated with 10 ppm 1-MCP at 100 DAFI with reapplication at 105,110,115 and 120 DAFI. Samples were analyzed 2d after each spray and every 2d thereafter until harvest at 120 DAFI. Each value is a mean of three replicates, each replicate consisting of two fruits.

### *3.4 ACC Oxidase Activities as Influenced by Preharvest 1-MCP Spray*

ACC oxidase, also called the ethylene-forming enzyme (EFE) catalyzes the last step in the ethylene biosynthetic pathway where ACC is converted to ethylene. 1-MCP caused decreased ACC oxidase activity after spraying at 100 DAFI (Figure 7) compared with both positive and negative controls. This could be accounted for by the decreased levels of ACC at 100 DAFI as shown in Figure 6. ACC is the substrate of ACC oxidase to produce ethylene. However, trends in ACC oxidase activities were almost the same for all the treatments and controls after 105 DAFI. Only the 1-MCP treatment re-sprayed at 110 DAFI exhibited a drastic increase in ACC oxidase activity at 113 DAFI which coincides with the increase in ACC levels also at this time (Figure 6). Thus, ACC oxidase proceeds with its normal activity without being inhibited by 1-MCP.

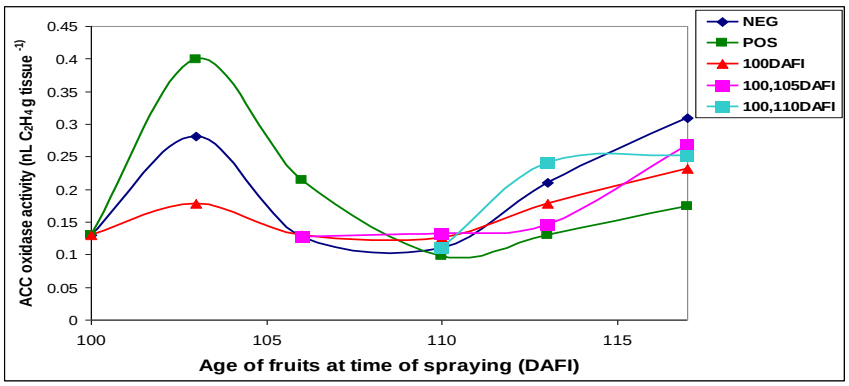


**Figure 7**. ACC oxidase activities in mango fruits preharvest treated with 10 ppm 1-MCP at 100 DAFI with reapplication at 105,110,115 and 120 DAFI. Samples were analyzed 2d after each spray and every 2d thereafter until harvest at 120 DAFI. Each value is a mean of three replicates, each replicate consisting of two fruits.

## 4. CONCLUSIONS

1-MCP was found to suppress ethylene biosynthesis as evidenced by the absence of a second ethylene peak in 1-MCP treated fruits. Hampering of autocatalytic ethylene production occurred due to the low concentration of ethylene being retained in tissues as 1-MCP binds to ethylene receptors ahead of ethylene. Possibly, ethylene just diffused out of the tissues and only a small amount was retained which is below the threshold level for autocatalytic ethylene production. ACC production was also affected by 1-MCP application when decreased ACC levels were observed in 1-MCP fruits sprayed at 100 then at 110 DAFI. Possibly at about 110 DAFI, new receptors have already been synthesized, thus the increase in ACC after 110 DAFI. But reapplication at 110 DAFI provided a new batch of 1-MCP molecules to bind with the newly synthesized ethylene receptors which prevented the normal increase in ACC. ACC oxidase activities on the other hand, showed almost the same trends for all re-sprayed treatments after the first spray at 100 DAFI. Decreased activities just coincided with the observed decrease in ACC levels, suggesting that ACC oxidase proceeds with its normal activity without being inhibited by 1-MCP. Therefore, autocatalytic ethylene biosynthesis in 'Carabao' mango fruit is regulated at the level of ACC synthase and not ACC oxidase and that ACC synthesis is the rate limiting step in ethylene feedback mechanism in 'Carabao' mango.

## 5. ACKNOWLEDGMENT

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