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Synthesis and Applications of 1-Methylcyclopropene in the Storage of Harvested Tomato Fruit

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Abstract: The present study used a simple method to generate 1-Methylcyclopropene (1-MCP) by reaction between diisopropylamine and 3-chloro-2-methylpropene in the presence of lithium and tetrahydrofuran. The active concentration of 1-MCP was 12.76±0.72 μL L^{-1} and applied in harvested tomatoes during ripening. Results showed that application of the synthesized product to the fruit was accompanied by significantly decreased respiration rate and ethylene production relative to controls during storage. Color development was also delayed. ACC synthase, ACC oxidase activity and relative expression of the *ACO* and *ACS* genes were inhibited in treated fruit. These results indicated that 1-MCP might be a simple and effective means of preserving the quality of mass-produced tomatoes in storage.

Key words: 1-MCP, synthesis, apply, quality, tomato, production

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is a climacteric fruit. At the onset of ripening, there is a sharp increase in ethylene production. This is considered the key regulatory process responsible for causing changes in the fruit's physiological and biochemical attributes. The ripening process in tomato fruit as in other climacteric fruits is highly dependent on the action of ethylene (Alexander and Grierson, 2002). The limitation of tomato storage is increased by ripening after harvesting which leads to significant losses with regard to quality. The use of simple treatments that reduce the synthesis of ethylene or inhibit its effects would lengthen tomato shelf life so that the fruit would remain acceptable for consumption after longer storage periods.

1-Methylcyclopropene (1-MCP) which has been found to inhibit the effects of ethylene, binds irreversibly to ethylene receptors. It has shown potential for delaying ripening, maintaining quality and extending the shelf life of fruit, vegetables and ornamental crops (Sisler and Serek, 1997; Watkins, 2008). The use of 1-MCP has been reported with a diverse range of horticultural crops but it has been most successfully employed with climacteric fruits (Huber, 2008; Hurr *et al*., 2005; Liu *et al*., 2010; Manenoi *et al*., 2007; Watkins, 2006). At present, 1-MCP can be produced on a scale sufficient to the needs of the fruit industry to control ripening in both gaseous and liquid-solution form. However, little synthetic production of 1-MCP is currently under way. This study describes an investigation of the effects of 1-MCP synthesized from diisopropylamine and 3-chloro-2-methylpropene on the quality of harvested tomatoes during ripening.

MATERIALS AND METHODS

Synthesis of 1-methylcyclopropene: 1-MCP was synthesized as described by Williard and Salvino (1993) with modifications. A round-bottomed flask purged with argon was charged with Li metal (659 mg, 94 mmol), anhydrous ether (50 mL), diisopropylamine (13 mL, 94 mmol) and freshly distilled styrene (5.4 mL, 47 mmol). The reaction mixture was refluxed until all of the Li metal disappeared. About 5 mL of 3-chloro-2-methylpropene were then added slowly to the flask and the production gas was absorbed by β-cyclodextrin solution. The absorption liquid was centrifuged and dried, producing white powder. The reaction formula shows in Fig. 1.

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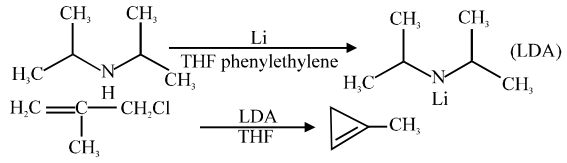

Fig. 1: 1-MCP concentration

1-MCP concentration generated by diisopropylamine and 3-chloro-2-methylpropene: The active 1-MCP concentration generated by diisopropylamine and 3-chloro-2-methylpropene was measured 3 h after 2 g powder was mixed with water in a container. Space gas was removed and injected into a GC2014/G-3900 gas chromatograph (Shimadzu/Hitachi Co., Ltd. Japan). The temperatures of the column, injector and flame ionization detector were 100, 120 and 260°C, respectively and the helium carrier flow rate was 50 mL min^{-1}. The 1-butylene was used as a standard to calculate 1-MCP concentration.

Plant materials and treatment: Mature green tomatoes were harvested at commercial maturity from an orchard in Guangzhou. The samples were transported to the laboratory immediately after harvesting. They were washed with tap water and dried at room temperature. Fruits of uniform size and maturity, free from visual blemishes and disease were used for the experiments. Tomatoes were randomly divided into two groups of 180 fruits each. One group was treated with 1-MCP for 12 h in a sealed container while the control fruits were sealed for 12 h a container without 1-MCP. Each treatment was performed three times with 30 fruits in each replicate. The experiments were carried out at room temperature (22°C).

Evaluation of fruit color: Changes in fruit color were recorded using a colorimeter (Minolta TC; P11 G Minolta China Ltd. Shanghai, China). Three fruits were assessed per treatment per replicate and three readings were recorded per fruit on the blossom end and on the two opposite sides of the fruit. The same fruits were analyzed throughout the period of the experiment. Color assessment was based on (L) Lightness, (a) a green to red scale (negative to positive) and (b) a blue to yellow (negative to positive) scale. The formula 2000a/L $(a^2+b^2)^{1/2}$ was used to produce a Tomato Color Index (TCI) (Hoeberichts *et al*., 2002).

Determination of respiration rates and ethylene production: Five fruits per treatment were weighed and placed in separate plastic chambers sealed with plastic lids. After 2 h, triplicate samples of 1 mL of head space gas were removed and injected into a GC2014/G-3900 gas chromatograph (Shimadzu/Hitachi Co., Ltd. Japan) fitted with a thermal conductivity detector for CO_2 production. The temperature of the column was 80°C and that of the detector and injector was 150°C. The velocity of the carrier gas (He) was 30 mL min^{-1}. The results were expressed as mg/kg/h.

An additional triplicate sample of 1 mL of headspace gas was withdrawn and injected into a gas chromatograph (GC-2014C, Shimadzu Co., Ltd., Kyoto, Japan) for the determination of ethylene production. It was fitted with a stainless-steel column and the temperatures of the column, injector and flame ionization detector were 80, 140 and 150°C, respectively. The helium carrier flow rate was 50 mL min^{-1}. After the headspace gas samples were taken the chambers were aerated and the fruit was kept at room temperature until the next measurement. The results are expressed as μL/kg/h.

Extraction and assay of ACC synthase: The 1-Aminocyclopropane-l-Carboxylate (ACC) Synthase (ACS) was isolated from tomato fruit using the extraction method described by Kato *et al*. (2000). ACC synthase activity was assayed in a reaction mixture consisting of 50 mM EPPS-KOH buffer, pH 8.5, 50 μM SAM. The enzyme was prepared in a total volume of 1 mL. The reaction mixture was incubated for 30 min at 30°C and then the reaction was stopped by the addition 0.1 mL of 40 mM $HgCl_2$. The amount of ACC synthase formed in the reaction was assayed by the method described by Lizada and Yang (1979). ACC synthase activity was expressed as nmol ethylene formed per hour per g FW.

Extraction and assay of ACC oxidase: The extraction and assay of 1-Aminocyclopropane-l-Carboxylate (ACC) Oxidase (ACO) was isolated from tomato fruit using the extraction method described by Moya-Leon and John (1994). ACC oxidase activity is expressed as nmol ethylene formed per hour per g FW.

RNA extraction and reverse transcription-polymerase chain reaction analysis: Total RNA from tomato fruit was extracted using the hot borate method (Wan and Wilkins, 1994). The isolated total RNA was treated with RNase-free DNase I (Promega, Madison, WI, US) for 20-30 min at 37°C and used for Reverse Transcription (RT). Purified total RNA (1.5 and 2 μg) was used for first-strand cDNA synthesis using M-MLV® reverse transcriptase (Invitrogen, Carlsbad, CA, US) and oligo $(dT)_{18}$ primer. The cDNA fragments were used as templates for Polymerase Chain Reaction (PCR) with specific primer sets for ACS and ACO (Table 1). The conditions for RT-PCR

Table 1: Primer sequence for ACO and ACS

Primers	Sequence (5'-3')
LE-ACS1-F	GGATGATGGAACGGTTGATA
LE-ACS1-R	GTCTTAACGAACTAATGGTGAG
LE-ACO1-F	TTACAATCCAGGAAGTGATG
LE-ACO1-R	ACTTGAGTCCAGCATATAAC
LE-ACTIN2-F	CTGGTATTGCTGATAGGATGA
LE-ACTIN2-R	GCTGGAATGTGCTGAGAG

amplification were as follows: initial denaturation at 95°C for 3 min, 40 cycles at 95°C for 5 sec, 55°C for 10 sec and 47 cycles at 72°C for 10 sec.

Statistical analysis: The entire experiment was performed three times with three replicates each time. Data were plotted using Sigma Plot 10.0 Software and one-way Analysis of Variance (ANOVA) was performed using SPSS Version 16.0. Data are presented as mean±Standard Error (SE).

RESULTS AND DISCUSSION

Active 1-MCP concentration: Figure 2 shows that the retention time of 1-MCP was 1.187 min and that the peak area was 2353.1. The tested active 1-MCP concentration was 12.76±0.72 μL L^{-1}.

Changes in TCI during storage: Color development was delayed in treated fruit (Fig. 3). After 5 days of storage, the TCI of the control fruits began to turn positive, reaching 24.13 on day 7. The TCI of 1-MCP treated fruits did not begin to turn positive until day 7, reaching 6.99 that day. The 1-MCP was found to be significantly associated with delayed color development in fruit.

Respiration rate and ethylene production: Changes in respiratory rate were observed during 7 days of ripening (Fig. 4). The respiratory rates of the treated tomatoes changed slightly during days 1-3 while those of control fruits increased sharply, reaching 76.26 mg/kg/h on day 7. This showed that application of the synthesized product was associated with respiration rates significantly lower than those of controls.

Changes in ethylene production during storage are shown in Fig. 4. Ethylene production during storage increased in both control and treated fruit. Ethylene production in control fruit increased dramatically after day 3. Ethylene production in treated fruit also increased sharply but not until day 5. Treatment significantly reduced ethylene production during storage.

***LeACS1* gene expression and ACC synthase activity:** Semi-quantitative RT-PCR was performed to determine the transcription level of LeACS1 at the molecular level in both treated and control fruit. As shown in Fig. 5, the LeACS1 transcription rate was lower treated fruit than in controls during storage.

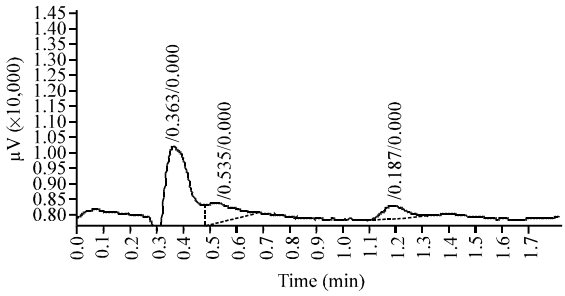

Fig. 2: 1-MCP product gas chromatogram

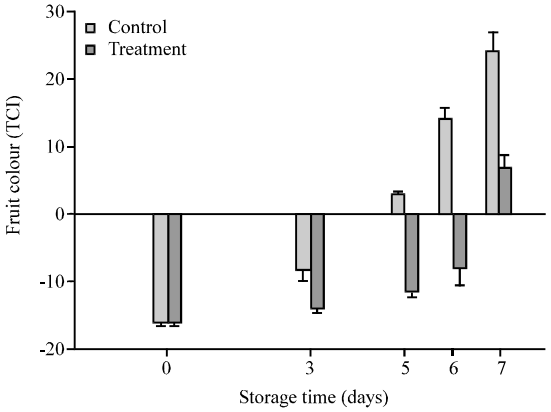

Fig. 3: Changes in TCI in tomatoes stored at 22°C. Each point represents the mean±SE of three replicates

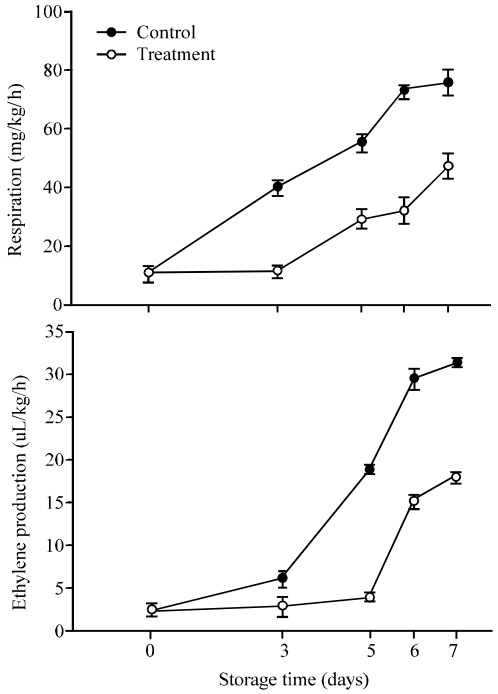

Fig. 4: Changes in respiration and ethylene production in tomatoes stored at 22°C. Each point represents the mean±SE of three replicates

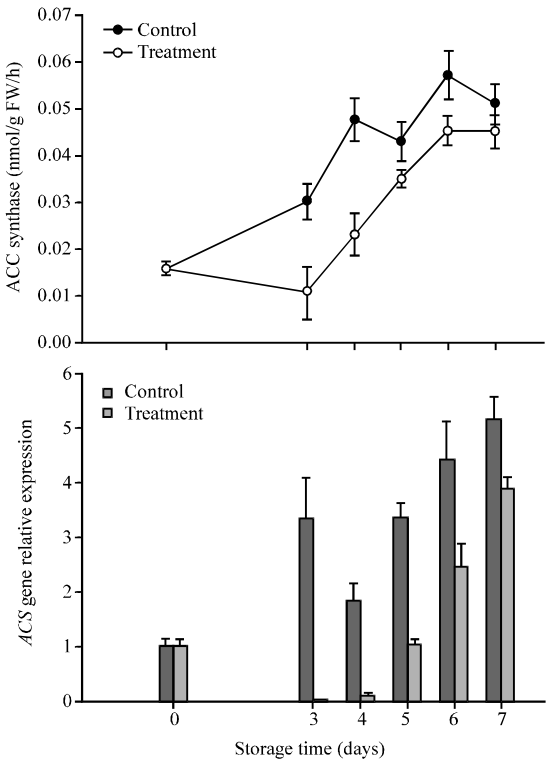

Fig. 5: Changes in relative expression of the *ACS* gene and ACC synthase (ACS) activity in tomatoes stored at 22°C. Each point represents the mean± SE of three replicates

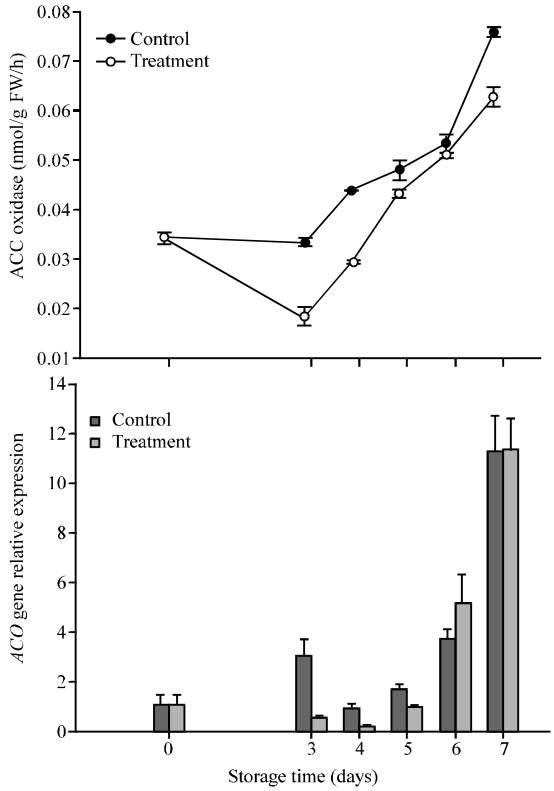

Fig. 6: Changes in *ACO* gene relative expression and ACC oxidase activity (ACO) in tomatoes stored at 22°C. Each point represents the mean±SE of three replicates

ACC synthase activity was also suppressed by treatment (Fig. 5). In both control and treated fruit ACC synthase activity increased dramatically during storage and reached its highest values on the 6th day but treated fruit had consistently lower levels than control fruit.

***LeACO1* gene expression and ACC oxidase activity:** Semi-quantitative RT-PCR was performed to determine the transcription level of LeACO1 at the molecular level in both treated and control fruit. As shown in Fig. 6, the LeACO1 transcription rate was lower in treated fruit than in controls from days 1-3. However, starting on day 6, LeACO1 expression levels began to increase sharply, matching that of control fruit on day 7.

ACC oxidase activity was also suppressed by treatment (Fig. 6). In control fruit, ACC oxidase activity tended to increase during storage. In treated fruit, it remained low.

The cyclopropenes play a very important role in the storage of plants and plant products. They can restrain the ethylene response very effectively and prevent the negative effects of ethylene on plants. They accomplish this by binding ethylene receptor sites. These compounds are safe and do not pollute the environment when they are used or disposed of. Recently, it has become popular to study the many applications of cyclopropenes. Cyclopropenes have small, unsaturated three member rings which are very strong. This makes these compounds unstable. There has also been a great deal of study of the methods by which these compounds may be synthesized.

Currently, there are three ways to perform cyclopropene synthesis: cycloaddition reaction of carbine and alkynes; elimination reaction with the existence of halogenated ring propane; rearrangement of carbine (Fisher and Applequist, 1965; Closs and Krantz, 1966; Magid *et al*., 1971). By far the most useful of these is sodium amide or phenyllithium-induced a elimination of 3-chloro-2-methylpropene. This method however has relatively low yields is unsafe and requires heat. The latter method has relatively high yields but its cost is prohibitive. The present study describes the use of diisopropylamine and 3-chloro-2-methylpropene to

generate 1-MCP. This process is simple and inexpensive. Ethylene production and respiration rate have an important role in physiological and genetic regulation in postharvest fruits. They are two of the most significant differences between post-harvest vegetables and fruits. Because the tomato is a climacteric fruit, ethylene production and respiration rate are especially important after postharvest. The present study showed that respiration rates and ethylene production were significantly inhibited in 1-MCP-treated tomatoes indicating that the tomato receptors had been blocked. This has been observed in other tomato cultivars (De Wild *et al*., 2005; Mir *et al*., 2004; Mostofi *et al*., 2003). Moreover, the absence of any sharp increase in ethylene production indicated that typical autocatalytic ethylene biosynthesis had also been inhibited. There is evidence that 1-MCP can reduce the respiration rate in other tomato cultivars (Wills and Ku, 2002). Similar results have been observed in apricots, bananas and plums (Fan *et al*., 2000; Harris *et al*., 2000; Valero *et al*., 2003). Results showed that treatment delayed color development (Fig. 3). This has also been shown in previous studies (Hoeberichts *et al*., 2002; De Wild *et al*., 2005; Mir *et al*., 2004; Valero *et al*., 2003).

ACS and ACO are the only two enzymes specific to the ethylene biosynthetic pathway. ACS converts S-adenosylmethionine to ACC and ACO converts ACC to ethylene (Choi *et al*., 2009; Gray *et al*., 1994). In particular, LeACS1 and LeACO1 which are members of the ACS and ACO families, respectively are responsible for the massive ethylene production that takes place during tomato ripening. Treatment with 1-MCP inhibited the softening of the fruit and reduced the rate of synthesis of endogenous ethylene by inhibiting the activities of ACS and ACO (Zhu *et al*., 2004). The 1-MCP was also found to influence ethylene biosynthesis through feedback inhibition of ACS and ACO enzyme expression (Blankenship and Dole, 2003).

CONCLUSION

The present study showed that *LeACS1*and *LeACO1* gene expression and ACS and ACO enzyme activity were inhibited by 1-MCP treatment. These results suggest that 1-MCP synthesized from diisopropylamine and 3-chloro-2-methylpropene treatment can restrain the expression of *ACS* and *ACO* genes, reduce their activities, control ethylene biosynthesis and delay tomato fruit ripening. This indicates that this simple and effective means of synthesis may have a great impact on the mass production of tomatoes.

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