Methyl Jasmonate plays a role in fruit ripening of ‘Pajaro’ strawberry through stimulation of ethylene biosynthesis

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Abstract

The role of methyl jasmonate (MJ) in strawberry (Fragaria x anassa Duch. Cv Pajaro) fruit ripening was investigated by monitoring its endogenous concentrations in fruit at various stages of development and the effects of exogenously applied MJ at these stages on ethylene biosynthesis. The concentration of endogenous trans-MJ was significantly higher in the white fruit (31.7 – 162.2 ng·g⁻¹) and decreased sharply in half and fully ripe fruit. Higher concentrations of endogenous trans-MJ at the white stage of strawberry fruit development followed by a decline during fruit ripening indicate that MJ may play an important role in modulating fruit ripening. Significantly increased ethylene production was measured in the fruit when MJ was applied at white, half ripe and at fully ripe stage. The application of MJ (50 µM) resulted in significantly highest ethylene production and increased activities of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase as compared to all other treatments. The effect of exogenously applied MJ on ethylene production, ACC synthase and ACC oxidase activities was dependent on concentration of MJ applied and on fruit developmental stage. In conclusion, MJ in strawberry modulates fruit ripening, as its concentration is higher in white fruit and is declined with the progression of ripening and exogenous application of MJ increases ethylene production, activities of ACC oxidase and ACC synthase depending upon the concentration of MJ applied and fruit developmental stage.

Keywords: Fragaria x anassa Duch., MJ, ethylene, ACC synthase, ACC oxidase
1. Introduction

Jasmonic acid (JA) and its methyl ester (methyl jasmonate), are cyclopentanone compounds and are regarded as naturally occurring plant growth regulators (Sembner and Parthier, 1993 and Fan, et al., 1998). Jasmonic acid and MJ are present in low concentration in various plant parts including buds, shoots, leaves, flowers, fruits, and seeds (Meyer et al., 1984) and largest amount in fruits. MJ has been reported to modulate chlorophyll degradation and anthocyanin formation (Creelman and Mullet, 1997 and Perez et al., 1997), aroma development (Olias et al., 1992), and ethylene production (Lalel et al., 2003; Khan and Singh 2007; Kondo et al., 2007). In apples [Malus sylvestris (L.) Mill. var. domestica (Borkh.) Mansf.], the concentration of endogenous MJ has been reported to be low at the initial stages of fruit development followed by general increase toward harvest (Kondo et al., 2000). Likewise, (Lalel et al., 2003) reported that the concentration of trans-MJ in the pulp of mango (Mangifera indica L.) fruit was higher at harvest and decreased as the ripening progressed. But endogenous MJ in non-climacteric fruits has been reported to be higher at the immature stage and steadily decreasing during fruit development such as strawberry (Gansser et al., 1997), sweet cherries (Prunus avium L.) (Kondo et al., 2000) and grape (Vitis vinifera L.) berries (Kondo and Fukuda 2001). Moreover, in vitro application of MJ to immature green strawberries has increased respiration, ethylene production, and transitory induction of anthocyanin biosynthesis and degradation of chlorophyll, suggesting a role of MJ in ripening of this fruit (Perez et al., 1997). It is surmised that endogenous MJ may act as inducer of fruit ripening in strawberry. Some sporadic and inconclusive research reports are available on changes in endogenous level of MJ in strawberry at various stage of fruit development (Perez et al., 1997 and Gansser et al., 1997).
Ethylene is thought to play an essential role in regulation of ripening of climacteric fruits. But it has only a minor effect on non-climacteric fruit such as strawberry (Given et al., 1988 and Abeles and Takeda, 1990). At present, hormonal regulation of strawberry ripening is not fully understood. Auxins produced by achenes are probably the key hormone in strawberry development and ripening (Given et al., 1988). GA₃ has been reported to inhibit strawberry fruit ripening (Martinez et al., 1994). Abscisic acid has been reported to accelerate sucrose uptake and advance colour development in tissue-cultured strawberry fruit and cortex discs (Archbold, 1988 and Kano and Asahira, 1981). The role of key ripening hormone ethylene in strawberry fruit ripening remains unclear and inconclusive with contradictory results from various investigations (Perez et al., 1997; Abeles and Takeda, 1990; Basiuomy, 1989; Atta-Aly et al., 2000).

The exogenous application of MJ affects ripening parameters including ethylene production in various fruits such as apple (Fan, et al., 1998); mango (Lalel et al., 2003); Japanese plum (Prunus salicina Lindl.), (Khan and Singh 2007); pear (Pyrus communis L.) (Kondo et al., 2007) and aroma development (Olias et al., 1992; Lalel et al., 2003; Fan et al., 1997), and pigment changes (Lalel et al., 2003; Perez et al., 1993). For immature strawberries, some preliminary research work on the effect of MJ has indicated increased respiration, ethylene production and transitory induction of anthocyanin biosynthesis and chlorophyll degradation (Perez et al., 1997). Recently, (Yilmaz et al., 2007) reported that response of ‘Tufts’ and ‘Cruz’ strawberries fruit ripening to jasmonic acid is concentration dependant. Postharvest exogenous application of MJ has also been reported to suppress fruit decay caused by Botrytis cinerea during storage at 5°C (Zhang et al. 2006). No research work has been reported on the role of exogenously applied MJ on enzymes involved in ethylene
biosynthesis, including ACC synthase, and ACC oxidase, in strawberry during fruit ripening. We hypothesized that externally applied MJ might affect ACC synthase, ACC oxidase and ethylene biosynthesis leading to enhanced ripening. We therefore investigated the dynamics of endogenous MJ concentrations in strawberry fruit at various developmental and ripening stages and the effects of exogenously applied MJ at these stages on ethylene production including activities of ACC synthase and ACC oxidase.

2. Material and methods

In experiment 1 we investigated the dynamics of endogenous methyl jasmonate in fruit at various developmental stages and in experiment 2 we studied the effects of exogenously applied methyl jasmonate (Sigma-Aldrich, Castle Hill, NWS, Australia) on strawberry fruit discs at various maturity stages in relation to ethylene biosynthesis and activities of ACC synthase and ACC oxidase.

2.1. Expt. 1 Endogenous methyl jasmonate in fruit at various developmental stages

Strawberry fruit (Fragaria x anassa Duch. cv Pajaro) fruit at fully ripe, half ripe and white stage were harvested from a commercial farm in Wanneroo (31° 42'S, 115° 46'E), Western Australia. Fruit were put into punnets and kept at 20 ± 1 °C for 6 d. Each punnet contained 250 ± 10 g fruit and it was considered as an experimental unit and replicated three times. Concentrations of endogenous MJ were determined at 0, 3 and 6 days after harvest.
Estimation of endogenous methyl jasmonate

MJ was analysed using the method described by Fan et al. (1998) and Kondo et al. (2000). Fruit (50 g) were homogenised with a 50-mL saturated NaCl solution, 2.5-mL of 1M citric acid, and 50 mL of diethyl ether containing 10 mgL⁻¹ butylated hydroxytoluene (BHT) as an antioxidant and 4.8 µg of 9,10 dihydro methyl jasmonate as the internal standard. The ether phase was removed after centrifugation for 10 min at 2000 g, and the aqueous layer was extracted with 150 mL diethyl ether containing 10 mgL⁻¹ BHT. The extracts resulted from ether phase were dried under N₂. The dried residue was dissolved in 5 mL n-Hexane and passed through a silica gel column (5 mm i.d. x 140 mm) (250 mg of silica gel 60 Fluka, Steinheim, Germany). The pooled sample was then eluted with 7 mL of n-hexane/ether (2:1, v/v), and dried under N₂. Dried samples were redissolved in 50 µL n-hexane/ether, (2:1, v/v), and 1-µL samples were injected into a GC (Hewlett Packard 5890 series, Walnut Creek, Calif.) fitted with flame ionisation detector (FID) and DB5MS capillary column (50 m x 0.2 mm i.d., 0.33 µm film thickness; J&W Scientific, Folsom, Calif.). The injector temperature was 250°C. The column temperature was maintained at 100°C for 1 min, increased to 190°C at the rate of 5°C per minutes. The temperature then increased to 200°C at the rate of 2°C per min, held for 2 min and increased again to 280°C at the rate of 15°C per min. It was then maintained for 5 min. The detector temperature was maintained at 290°C. Hydrogen was used as the carrier gas. MJ was identified using MJ standard by comparing their retention time (RT). To reconfirm MJ, a GC (Hewlett Packard 5890 series II, Walnut Creek, Calif.) coupled to a mass detector (MS, Hewlett Packard 5971 series, Walnut Creek, Calif.) was used. The ultra performance capillary column, Hewlett Packard model 19091B-105 (30 m x 0.2 mm; 0.33 µm film thickness), was coupled directly to the ion source (70 eV) of the MS detector. The
inject port temperature of GC-MS was 240°C. The temperature of column was held at 10°C for 3 min, increased to 120°C (at 8°C/min), then increased to 290°C at the rate of 10°C/min and kept for 3 min. MJ was identified by matching its mass spectra with the spectra of MJ standard and WILEY275.L Library. The concentration of MJ was calculated as ng·g⁻¹ using internal standard.

2.2. Expt. 2 Effect of methyl jasmonate on strawberry discs ethylene biosynthesis and activities of ACC synthase and ACC oxidase

Discs (20 mm diameter, 3 mm thickness) from strawberry fruit were placed into petri dishes containing 20 mL of 0.4 M mannitol with 0, 10 and 50 µM MJ and incubated for 24 and 48 h at 20 °C. The discs were transferred to MJ-free petri dishes containing a filter paper moistened with 2 mL of 0.4M mannitol. The discs from each strawberry were treated as a replicate and three strawberries were used. Ten fruit were randomly selected and used for preparing the discs in each replication. Ethylene production was measured at 0, 1, 2, and 3 d after MJ treatment. After ethylene determination, the discs were used to estimate the activities of ACC oxidase and ACC synthase.

2.2.1. Estimation of activities of ACC synthase and ACC oxidase

The ACC synthase and ACC oxidase activities were determined from fruit tissues according to the method described by Mathooko et al. (1993). ACC synthase activity was expressed as nmol ACC·gproetin⁻¹·h⁻¹. ACC oxidase activity was expressed as nmol C₂H₄·mg⁻¹·protein·h⁻¹.
2.2.2. Estimation of ethylene

Ethylene production was measured by sealing 5g fruit in 25-mL Erlenmeyer flasks for one hour. Ethylene in the headspace was measured using GC (Varian series Star 3400 CX, Walnut Creek, Calif.), fitted with flame ionisation detector and Porapak-Q column (2-m long, o.d. 3.175mm, 80/100mesh). The injector, column and detector temperatures were maintained at 100, 100 and 150°C, respectively. Nitrogen was used as the carrier gas. Ethylene was calculated and expressed as nmol·kg⁻¹·h⁻¹.

2.2.3. Estimation of protein

The protein content of the fruit was estimated using the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard and the concentration of protein in enzyme extract was determined from the standard curves. Protein was calculated and expressed as g·kg⁻¹ fruit.

2.3. Statistical analysis

The data were subjected to analysis of variance (ANOVA), using Genstat release 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, U.K.). Effects of different MJ concentrations, duration of treatment and fruit development stages and the interaction among these factors were assessed within ANOVA. Least significant differences (Fisher's protected LSD) were calculated, following significant F-test results \( P \leq 0.05 \), and all the assumptions of analysis of variance were checked to ensure validity of the statistical analysis. Unless otherwise specified, all the significant differences mentioned hereafter are for \( P \leq 0.05 \).
3. Results

3.1. Endogenous methyl jasmonate in fruit at various developmental stages

Trans-MJ was identified in strawberry fruit at different development and ripening stages using GC-MS (Fig. 1). The concentration of Trans-MJ was significantly higher in the white fruit (31.7 – 162.2 ng·g⁻¹) as compared to fully ripe (1.3 – 8.9 ng·g⁻¹) and at half ripe fruit (16.5 – 53.5 ng·g⁻¹) (Fig. 2). As the postharvest period progressed, the concentration of MJ decreased steadily at all development stages of the fruit, and the trend was more pronounced in white fruit compared to half ripe and fully ripe fruit.

3.2. Effect of MJ on ethylene biosynthesis

The discs of fully ripe fruit treated with MJ treatment (50 µM) incubated for 24 h significantly increased ethylene production at zero and two days after treatment as compared to the discs of untreated fruit (Fig. 3). At day 3, the effect of MJ incubation for 24 h on ethylene production in fully ripe fruit was not significant.

The discs of MJ-treated fully ripe fruit with 48 h incubation showed significantly higher ethylene production as compared to untreated fruit (Fig. 3). Ethylene production in strawberry fruit discs treated with 50 µM MJ after 48 h incubation period was significantly higher as compared to 10 µM MJ treatment and control on day 2 and 3 after treatment.

The discs of half ripe fruit treated with MJ after 24 h of incubation had significantly increased ethylene production at zero and one day after treatment as compared to control. However, the effect was not significant as the time after treatment progressed. Similar trend in ethylene production was recorded when MJ was applied to the discs of half ripe fruit and incubated for 48 h (Fig. 3).
The discs of white fruit treated with MJ also exhibited a significant increase in ethylene production one, two and three days after treatment as compared to untreated fruit (Fig. 3). MJ treated discs of white fruit after 24 h of incubation significantly increased ethylene production 1, 2 and 3 d after treatment. MJ (50 µM) applied to discs of white fruit after 24 h of incubation resulted in significantly higher ethylene production as compared to other treatments 1, 2 and 3 d after application. However, the increase in ethylene production in white fruit discs treated with MJ 48 h incubation was not significantly different as compared to control.

Mean ethylene production was significantly higher in the discs of fruit treated with MJ at white or half ripe stage than fully ripe stage irrespective of 24 h or 48 h incubation periods (data not shown). Fruit discs treated with MJ (50 µM) resulted in significantly increased mean ethylene production as compared to those treated with MJ (10 µM) and untreated fruit (data not shown). The interactions among MJ treatments, maturity stages and storage time for ethylene production was significant ($P \leq 0.05$) irrespective of incubation time 24 h or 48 h in all the MJ treatments.

### 3.3. ACC synthase activity

Fully ripe, half ripe and white fruit discs treated with 50 µM MJ after 24 h incubation period had increased ACC synthase activity. However, the increase was not significantly different compared to all other treatments at all days after treatment (Fig. 4). The activity of ACC synthase was significantly higher in the discs of fully ripe fruit treated with MJ (50 µM) with 48 h incubation as compared to all other treatments at day 0, 1 and 3 after treatment. Similarly in half ripe and white fruit, 50 µM MJ treatment after 48 h of incubation resulted in significantly higher ACC synthase activity as compared to other treatments at 0, 1, 2 and 3 days after treatment.
The discs of white fruit treated with MJ after 48 h of incubation showed significantly higher ACC synthase activity as compared to half ripe and fully ripe fruit (data not shown). MJ treatment (50 µM) after 48 h of incubation resulted in significantly higher ACC synthase compared to all other treatments (data not shown). The interaction between MJ treatments, maturity stage and time after treatment for ACC synthase activity was significant only when incubation period was 48 h.

3.3. ACC oxidase activity

Interaction among MJ treatments, fruit maturity stages and time after treatment significantly affected ACC oxidase activity irrespective of incubation periods. Discs of fully ripe fruit treated with 50 µM MJ with 24 h incubation period showed significantly higher ACC oxidase activity as compared to other treatments at zero day after treatment (Fig 5). As the time after treatment prolonged, the effect of MJ treatments on ACC oxidase activity in the discs of fully ripe fruit was not significant (Fig. 5). In the discs of half ripe fruit, the treatment of 10 and 50 µM MJ with 24 h or 48 h incubation periods resulted in significantly higher ethylene production as compared to control from day zero to three after treatment. The trend of ACC oxidase activity and ethylene production in the discs of half ripe fruit treated with MJ (Fig. 3) was similar. Similarly in the discs of white fruit, higher concentration of MJ (50 µM) resulted in significantly higher ACC oxidase activity as compared to untreated fruit at zero, one, two and three days after treatment (Fig 5).

Mean activity of ACC oxidase was significantly higher with MJ treatments irrespective of the incubation periods in white fruit as compared to fully ripe and half ripe fruit (data not shown). The activity of ACC oxidase was declined with MJ treatment when applied at half ripe and fully ripe stage and compared to white fruit.
Fruit discs treated with 50 µM MJ had significantly increased ACC oxidase activity as compared to those treated with 10 µM MJ after 24 h incubation and control (data not shown). The increased activity of ACC oxidase was less pronounced in 48 h incubation than 24 h with MJ treatment.

4. Discussion

Endogenous MJ detected in fully ripe, half ripe and white ‘Pajaro’ strawberry fruit was trans-MJ. Earlier, cis and trans isomers of MJ have been reported from strawberries by Gansser et al. (1997). MJ extracted from natural sources such as plants is mainly trans isomer, while cis isomer is presents in very small amount (Beale and Ward, 1998). Similar results have been reported in ‘Kensington Pride’ mangoes (Lalel et al., 2003). cis-MJ may be present in strawberry fruit but was not detected, since it is thermally unstable compound and readily epimerise at C-7 via the enol (Beale and Ward, 1998). The concentration of trans-MJ in strawberry was significantly higher at white stage (162 ng.g⁻¹), and declined up to 1.3 ng.g⁻¹ as the fruit developed to fully ripe stage. A concentration of MJ (280 ng.g⁻¹) in immature green strawberries, and it steadily decreased to 3.3 ng.g⁻¹ in over ripe fruit has been reported earlier by Gansser et al. (1997). A similar trend for non-climacteric fruits has been reported such as sweet cherries (Kondo et al., 2000) and grape berries (Kondo and Fukuda, 2001). Higher concentration of endogenous MJ in the white stage of strawberry fruit and it decline as the fruit ripen indicates that MJ may play an important role in modulating fruit ripening. Moreover, exogenous applications of MJ in strawberries have been associated with transitory induction of anthocyanin biosynthesis and chlorophyll degradation supports a role for MJ as inducer of ripening in strawberry (Perez et al., 1997). It has been reported that the decrease of MJ in
sweet cherries during fruit ripening decreased fruit firmness dramatically (Kondo et al., 2000). Although the possible role of MJ in non-climacteric fruit is still unknown, (Kondo and Fukuda., 2001) reported that endogenous MJ might stimulate abscisic acid (ABA) concentrations in grape berries since MJ activated lipoxygenase that is involved in ABA synthesis from carotenoids. It has been reported that ABA, rather than ethylene, plays a role in the onset of fruit maturation in non-climacteric fruit (Kondo and Inoue, 1997). In grape berries, endogenous ABA concentration increased toward ripening and decreased from ripening toward harvest (Kondo and Kawai, 1998).

Our experimental data support the hypothesis that MJ plays a role in the ripening of strawberry fruit through stimulation of ethylene biosynthesis. Exogenous application of MJ significantly increased ethylene production at fully ripe, half ripe and white fruit. Ethylene was significantly higher with MJ application especially at higher concentration (50 µM). The exogenous application of MJ in ‘Camarosa’ strawberries at white and pink stage significantly increased ethylene production and respiration rate (Perez et al., 1997). Similar effect of MJ on ethylene production in ‘Kensington Pride’ mango was observed in our pervious work (Lalel et al., 2003). A continuos low concentration of exogenous MJ stimulated ethylene production, while in high concentrations, the ethylene production decreased (Fan et al., 1998). Increased ethylene production in fruit treated with MJ may be due to the increase in activity of enzymes involved in ethylene biosynthesis. Our experimental results showed the increased ACC oxidase and ACC synthase activity in the fruit discs treated with MJ after 24 h as compared to untreated fruit. The application of MJ particularly in white and half ripe fruit increased ACC oxidase and ACC synthase. The increased ethylene production in fully ripe, half ripe and white strawberry fruit
treated with MJ is due to the increased activities of ACC synthase and ACC oxidase. Similarly, Kondo et al., (2007) reported that exogenous application of n-propyl dihydrojasmonate to pear fruit increased ethylene production in system 2, including ACC synthase and ACC oxidase. In apples, MJ treatment has also increased ACC oxidase and ACC synthase activity in preclimacteric stage (Fan et al., 1998). The effect of MJ on ethylene production, ACC oxidase and ACC synthase activity was greater in half ripe and white fruit as compared to fully ripe fruit. Higher concentration of MJ also resulted in greater increase in ethylene, ACC oxidase and ACC synthase. These results suggest that the responses to exogenous application of MJ to strawberry are dependent on concentration and developmental stage at which MJ was applied. Earlier it has been reported that MJ-stimulated ethylene production in apple is also stage dependent (Fan et al., 1997).

In conclusion, endogenous MJ in strawberry modulated fruit ripening, as its concentration was higher in white fruit and decreased with the progression of ripening and the exogenous application of MJ increased ethylene production, as well as activities of ACC oxidase and ACC synthase depending upon the concentration of applied MJ and fruit developmental stage.

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CAPTIONS TO FIGURES

Figure 1: Mass spectra of trans-MJ extracted from strawberries at half ripe stage
Figure 2: Postharvest changes in endogenous trans-MJ concentration in strawberries harvested at different maturity stages. Vertical bars represent the LSD at $P \leq 0.05$. LSD maturity stage x storage time = 22.78, LSD maturity stage = 13.15, LSD storage time = 13.15, n = three replications, 10 fruit per replication.

Figure 3: Effects of different concentrations of MJ applied to strawberry discs at different fruit maturity stages and for incubation times of 24 hours and 48 hours on ethylene production during postharvest phase. Vertical bars represent the LSD at $P \leq 0.05$. LSD treatment x maturity stage x storage time = 1.84 (24 hrs) and 2.18 (48 hrs), LSD treatment x storage time = 1.06 (24 hrs) and 1.26 (48 hrs), LSD stage x treatment = 0.92 (24 hrs) and 1.09 (48 hrs), LSD stage x storage time = 1.06 (24 hrs) and 1.26 (48 hrs), LSD maturity stage = 0.53 (24 hrs) and 0.63 (48 hrs), LSD storage time = 0.62 (24 hrs) and 0.73 (48 hrs), LSD treatment = 0.53 (24 hrs) and 0.63 (48 hrs), n = three replications, six discs per replication.

Figure 4: ACC synthase activity in strawberry discs treated with different concentrations of MJ at different maturity stages. Vertical bars represent least significant difference (LSD) at $P \leq 0.05$ for 48 hours incubation and non significant for 24 hours incubation, thus represent standard error (s.e). LSD treatment x maturity stage x storage time = 0.002, LSD treatment x storage time = 0.001, LSD stage x treatment = 0.001, LSD stage x storage time = 0.001, LSD maturity stage = 0.005, LSD storage time = 0.007, LSD treatment = 0.006, n = three replications.

Figure 5: ACC oxidase activity of the fruit discs incubated for 24 and 48 hours in different MJ concentrations at different maturity stages. Vertical bars represent least significant difference (LSD) at $P \leq 0.05$. LSD treatment x maturity stage x storage
time = 0.24 (24 hrs) and 0.16 (48 hrs), LSD treatment x storage time = 0.07 (24 hrs) and 0.09 (48 hrs), LSD stage x treatment = 0.12 (24 hrs) and 0.08 (48 hrs), LSD stage x storage time = 0.14, and 0.09 (48 hrs), LSD maturity stage = 0.07 (24 hrs) and 0.05 (48 hrs), LSD storage time = 0.08 (24 hrs) and 0.05 (48 hrs), LSD treatment = 0.07 (24 hrs) and 0.05 (48 hrs), n = three replications, six discs per treatment.
Mukkun and Singh (Figure. 1)
Mukkun and Singh (Figure 2)
Mukkun and Singh (Figure. 3)
Mukkun and Singh (Figure. 4)