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Analyses of Accumulation Pattern of 1-Aminocyclopropane-1-Carboxylate Oxidase (*ACO*) and Ethylene Response Sensor (*ERS*) Transcripts in Fully-Opened Flower and Emasculation Response in Flower Development of *Vanda* Miss Joaquim

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ABSTRACT

This research studied the transcripts of *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*) and *ethylene response sensor* (*ERS*) genes from non- and emasculated ethylene-sensitive *Vanda* Miss Joaquim flowers and then examined the ethylene production of flower-stages I, II, III and of the whole inflorescence (5 flowers/inflorescence). The results showed that their expressions were increased in the lip, decreased in the perianth, and remained unchanged in the column for 24 h. In particular, the accumulation of the *ACO* transcripts in the column, perianth, and lip of the emasculated flower compared with the non-emasculated flower were 2.18-fold, 2.31-fold, and 24.13-fold, respectively. In addition, the *ERS* transcripts in the perianth and lip of the emasculated flower compared with the non-emasculated flower were 2.10-fold and 55.90-fold, respectively. The high ethylene production levels of flower-stages I, II, and III were 0.51 $nl \cdot g^{-1} \cdot h^{-1}$, 0.42 $nl \cdot g^{-1} \cdot h^{-1}$, and 0.49 $nl \cdot g^{-1} \cdot h^{-1}$, respectively. In addition, the whole inflorescence produced a high level of ethylene (0.89 $nl \cdot g^{-1} \cdot h^{-1}$). These results revealed there was differential expression of *ACO* and *ERS* in different organs of flower. In particular, the *ACO* transcript of the column was the signal to induce greater expression in the perianth followed by the lip, resulting in the whole flower being sensitive to

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ethylene as fading flower color. In addition, the amount of ethylene production was correlated with the aging of tissue and the period after emasculation. Interestingly, the ethylene was produced after accumulation of *ACO* and *ERS* transcripts. Thus, the accumulation of their transcripts induced endogenous ethylene biosynthesis in the *V.* Miss Joaquim flower.

Keywords: 1-Aminocyclopropane-1-carboxylate oxidase; Ethylene response sensor; Vanda; Emasculation; Ethylene

1. Introduction

Orchids belong to the Orchidaceae family and consist of about 800 genera with 25,000 species [1]. *Vanda* Miss Joaquim (*Papilionanthe hookeriana* × *P. teres*) is the natural hybrid and most sensitive orchid which has the highest ethylene production [2-4]. Thus, it is a suitable orchid model to determine the genes involved in ethylene biosynthesis and ethylene response. Among the higher plants, ethylene is synthesized from methionine via S-adenosyl-L-methionine (AdoMet) and then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). The final step requires oxygen and ACC oxidase (ACO) called the ethylene-forming enzyme (EFE) to convert the ACC to ethylene [5]. The ethylene biosynthetic pathway is also highly regulated with the increases in ACO activity seen during fruit ripening [6], flower senescence [7], and in response to wounding or elicitor treatments [8]. Therefore, ACO is a major molecular marker for both ethylene formation and ethylene responsiveness [9]. Nagtong et al. [10] reported that the *Den-ACO* expression of the *Dendrobium* orchid could be detected in the petals, sepals, pedicels, lip, and stigma as well as in vegetative tissues (leaves and roots), while the *ACO* gene of *V.* Miss Joaquim was more highly expressed in flower tissue (column, lip, and perianth) at the fully-opened stage compared with vegetative tissues [11]. Moreover, ethylene perception in plant tissue requires specific receptors and a signal transduction pathway to regulate transcription of the ethylene response genes at the nucleus [12-13]. In higher plants, five *ethylene receptor* genes have been cloned from *Arabidopsis thaliana—ethylene response1* (*ETR1*) and correspondingly *ETR*2, *ethylene response sensor1* (*ERS1*) and correspondingly *ERS2*, and *ethylene insensitive4* (*EIN4*)—and all showed similarity to the bacterial two-component histidine kinases [14-17]. The mutation of *ERS1* caused the ethylene insensitive phenotype, suggesting its role involved ethylene perception [15]. In addition, there was higher *ERS* transcript accumulation in the column and the lip followed by the perianth tissues in the *V.* Miss Joaquim flower than in vegetative tissues [18].

The rate of ethylene response in plant cells is influenced by biosynthesis and sensitivity. Therefore, cell aging of flower tissue also plays a role in ethylene sensitivity [19-20]. Moreover, pollination induced flower senescence by enhancement of ethylene production [21-22]. The phenomena of flower senescence are yellowing, drooping, epinasty, and venation of florets [23]. Thus, the emasculation of the *V.* Miss Joaquim flower led to the elevated production of ethylene because the flower changed to a pale color within 24 h [11]. Moreover, the *ACO* and *ERS* genes involved ethylene after emasculation at 4 h according to the gene expression in the *V.* Miss Joaquim fully-opened flower [11,18]. *V.* Miss Joaquim is the most ethylene-sensitive orchid; thus, it is difficult to use it as a cut flower orchid. However, it can be used as a profitable model of orchid plants to study genes involving ethylene biosynthesis and ethylene response. The expression of genes

involving ethylene and the correlation of flower aging with ethylene production supported that the flowers of *V.* Miss Joaquim orchid are sensitive to endogenous ethylene. Therefore, this research studied the evaluation of *ACO* and *ERS* transcript patterns in the emasculated *V.* Miss Joaquim flower using the relative real time-polymerase chain reaction (PCR) method. Moreover, the ethylene production was measured from individual flowers to examine how the tissue aging of flowers was correlated with ethylene sensitivity.

2. Materials and Methods

2.1 Plant material

Fully-opened stage flowers of *Vanda* Miss Joaquim were purchased from Chao Praya Orchids Nursery, Pathum Thani, Thailand and were extracted using the lithium chloride precipitation method [24]. These flowers were cut, followed with or without pollinia cap dislodgment for (0 h) and placed in distilled water for the next 6, 12, 18, and 24 h and then were separated into lip, perianth and column and subsequently were frozen in liquid nitrogen and stored at -80℃ until use.

2.2 RNA isolation and evaluation of *ACO* and *ERS* transcript patterns in *V.* Miss Joaquim flower

A sample of flower tissues (200 mg) was ground in liquid nitrogen and then homogenized in extraction buffer (0.2 M Tris-HCl (pH 7.5), 0.1 M LiCl, 5 mM EDTA and 1% (w/v) SDS) and further homogenized twice with the addition of phenol:chloroform:isoamyl (25:24:1, v/v/v). The RNA was precipitated overnight in 3M LiCl at 4℃. The RNA was collected using centrifugation at 3,000×*g* for 20 min at 4℃ and then the pellet was resuspended in diethylpyrocarbonate (DEPC)-treated water and precipitated again with the addition of ethanol and resuspended in a small volume of DEPC-treated water. RNA samples (each 1 μg) of lip, perianth and column tissues were reverse transcribed to cDNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, USA) with three primers, namely RT−ACO(R) (5′−ATGGCGGAGGAAGAAGGTGCT−3′), RT−ERS(R) (5′−GCATCCATAAAGCACATTCTG−3′), and 5.8S rRNA(R) (5′−GCTTGAAGCCCAGGCAGACG−3′). Using KAPATM SYBR® FAST qPCR kits (Kapa Biosystems, USA), the 207 bp of *ACO*, 380 bp of *ERS*, and 198 bp of 5.8*S rRNA* were together generated using two pairs of primers: RT−ACO(F) (5′−GACGCCTGTGAGAACTGGGG−3′) and RT−ACO(R) for *ACO*; RT−ERS(F) (5′−GTTTGGTGCCTTCATTGTTCTT−3′) and RT−ERS(R) for *ERS*, and 5.8S rRNA(F)(5′−ATGACTCTCGACAATGGATTT−3′) and 5.8S rRNA(R) for 5.8*S rRNA*. Each orchid sample reaction containing 0.3 μl of cDNA template along with 7.5 μM of each primer in a final reaction volume of 10 μl was set up in triplicate to ensure the reproducibility of the results. Relative real-time PCR was accomplished using the following conditions: denaturizing for 2 min at 95℃ followed by 35 cycles of amplification with 3 sec of denaturizing at 95℃, 30 sec of annealing at 62℃, and 8 sec of extension at 72℃ with Eppendorf Mastercycle® ep realplex real-time PCR (Eppendorf, USA). At the end of each PCR run, a melting curve was generated and analyzed using the following conditions: denaturizing for 15 sec at 95℃, 15 sec at 60℃, and 15 sec at 95℃. For each sample, the reactions were set up in triplicate to ensure the reproducibility of the results. The target-to-reference ratio in each sample was normalized by the target-to-reference ratio in the calibrator. The result was expressed as a fold ratio of the normalized target amount. The threshold cycle (C_T) value of gene expression at 0 h ($(\Delta C_T)_0$) was used to calibrate each ΔC_T value of the dislodged pollinia flowers at times 6, 12, 18, and 24 h.

Relative calculation was performed according to Eqs. (1)-(3) [25]:

$$(\Delta C_T) = (C_T)_t - (C_T)_{5.8S\ rRNA}, \quad (1)$$

$$\Delta\Delta C_t = (\Delta C_T)_t - (\Delta C_T)_0, \quad (2)$$

$$\text{Relative expression ratio} = 2^{-\Delta\Delta CT}. \quad (3)$$

2.3 Measurement of ethylene production

Individual flowers from the inflorescence (first flower, stage I; second flower, stage II; and third flower, stage III) with or without dislodged pollinia were collected and placed in vials containing distilled water and then placed into 350 ml plastic bottles. Floral inflorescence samples containing 2 bud flowers and 3 fully-opened flowers were also determined. At various time intervals (0, 2.5, 8, 10, 12, 16, 20, and 24 h), the bottles were sealed for ethylene determination and 1 ml gas samples of head space gas were withdrawn using a gas-tight hypodermic syringe, and then injected into a gas chromatograph (GC 17A, Shimadzu, Japan) for ethylene concentration measurement. The gas chromatograph was equipped with a flame ionization detector and an activated alumina column. After each determination, the bottles were opened for aeration. Three flowers and 3 flower inflorescence samples were used for independent measurements and the average values were presented as mean ± SD of ethylene production for the replications and expressed per gram fresh weight of the plant material and calculated as $nl \cdot g^{-1} \cdot h^{-1}$ of ethylene concentration.

3. Results and Discussion

3.1 Transcript accumulation pattern of *ACO* and *ERS* in *V.* Miss Joaquim

The emasculation stimulated principally the transcription of *ACO* and *ERS* genes. The transcript level was the highest in the lip of the emasculated flower at 6 h, medium in the perianth/petal at 12 h, and the lowest in the column. However, we found that the *ERS* transcript in the column at 6 and 12 h of the non-emasculated flower was higher than in the dislodged pollinia (Fig. 1a-f). Shibuya et al. [26] revealed that *DC-ERS2* of carnation was responsible for ethylene perception during flower senescence, and that gene expression was regulated in a tissue-specific manner. During flower senescence, they found *DC-ERS2* decreased in petals, increased slightly in the ovaries, and remained unchanged in styles.

However, our previous research revealed that the first detection of *ACO* and *ERS* transcripts was in emasculated *V.* Miss Joaquim flowers at 4 h using a quantitative PCR (qPCR) method to examine high accumulation of gene expression in the column and lip and the accumulation in the perianth [11,18]. The relative real-time PCR method detected gene expression, (afterward up to 24 h) being the highest in the lip followed by the perianth and was the lowest in the column. These results suggested that the column of the orchid is the initiator central organ for the production of high ACO and ACS enzymes and then signals are translocated to the perianth followed by the lip/labellum of the orchid flower [27] resulting in low accumulation of transcripts in the column and high accumulation in the perianth and then in the lip during flower senescence for 24 h.

We found that the *ACO* expression of *V.* Miss Joaquim was greater than the *ERS* expression followed by a transcript accumulation pattern. Therefore, the emasculation increased the *ACO* transcript more than the *ERS* transcript for *V.* Miss Joaquim (Fig. 1a-f). This result supported reports that pollination increased the ACO activity in pollinated orchid flowers [28-29]. The *ACO* genes were expressed constitutively in all vegetative and reproductive tissues of plants such as the five *ACO* paralogs in tulip [30]. Moreover, the various abiotic processes also stimulated the accumulation of specific *ACO* transcripts [31]. This could also explain why

the results of the levels of the *ACO* transcript in the column, perianth and, lip of the emasculated flower compared with the non-emasculated flower were 2.18-fold, 2.31-fold, and 24.13-fold, respectively (Fig. 1a-c).

However, *ERS* expression was also correlated with flower emasculation according to the levels of the *ERS* transcript in the perianth and lip (2.10-fold and 55.90-fold, respectively) of the emasculated flower compared with the non-emasculated flower (Fig. 1d-f). Furthermore, the *ERS* transcript in the column did not alter in emasculated and non-emasculated flowers (Fig. 1d). Similarly, the overall expression in *Dendrobium* showed accumulation of the *Den-ERS1* transcript during the flower senescence process as a result of ethylene production increasing during flower senescence [31].

The *ACO* and *ERS* transcripts in the perianth and lip of emasculated flowers expressed at the lowest level in the beginning and afterward increased to their highest levels before decreasing up until 24 h (Fig. 1b-c and e-f). In addition, the transcripts in the column tissue had lower levels of expression than in the perianth and lip tissues (Fig. 1a-f). These results supported a report that the increase in *ERS* transcripts was associated with the beginning of flower senescence and there was a decline in *ERS* expression during the senescence period [32]. In addition, we determined that *ACO* and *ERS* expression levels were slightly detected in the column, perianth, and lip of the non-emasculated *V.* Miss Joaquim flower due to the probable relation of the maturation of the column/pistil itself and the tissue-temporal-specific-manner during flower development [33-34].

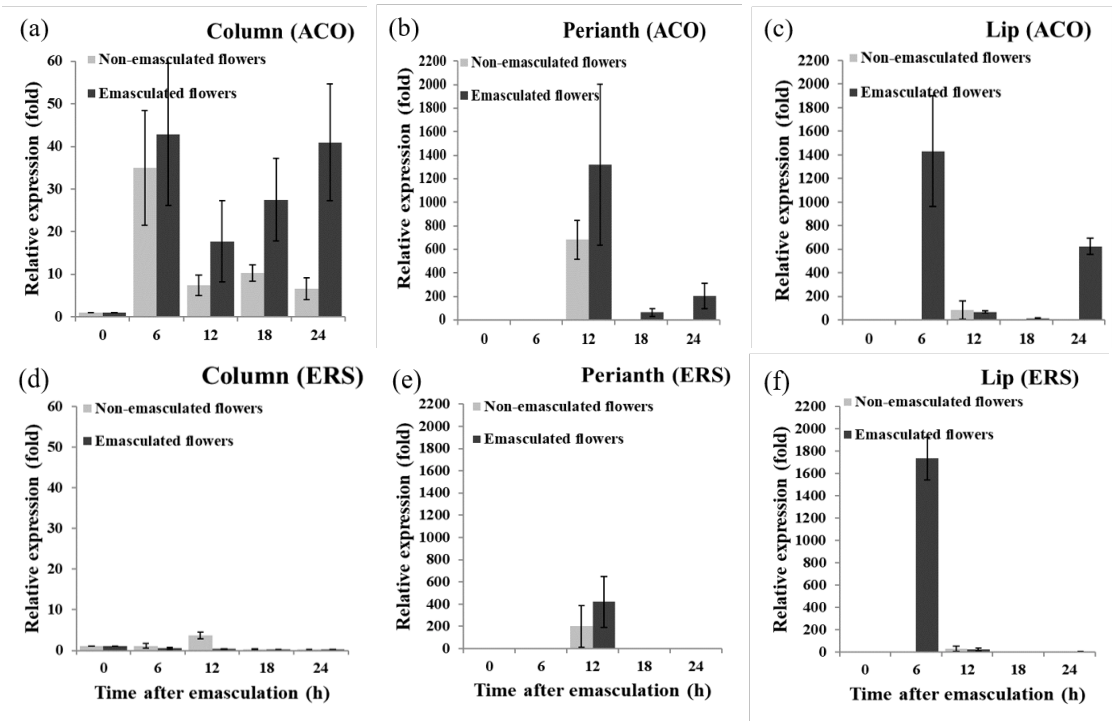

Fig. 1. Expression analysis of *ACO* and *ERS* transcripts in various organs of fully-opened *V.* Miss Joaquim flower after emasculation evaluated using relative real-time PCR with 5.8S *rRNA* as an

internal control (a-c); expression of *ACO* transcripts of column, perianth and lip, respectively (d-f); expression of *ERS* transcripts of column, perianth and lip, respectively.

3.2 Effect of emasculation on ethylene production in *V.* Miss Joaquim flower

The level of ethylene production was determined in the first-stage flower (stage I), second-stage flower (stage II), and third-stage flower (stage III) using ethylene measurement. The flowers of *V.* Miss Joaquim were individually classified into the following stages: I, a young fully-opened flower; II, a mature fully-opened flower; and III, a flower in early senescence with faded and twisted petals and lip (Fig. 2a1, 2b1 and 2c1). At 24 h after emasculation, the flower color changed from purple to a paler color in all flower stages (Fig. 2a2, 2b2 and 2c2). The removal of pollinia caused anthocyanin destruction (fading or bleaching) which was related to endogenous ethylene production [35,4]. Moreover, the removal of the pollinia caused the onset of several postpollination phenomena in *Cymbidium* [4], and *Phalaenopsis* [36]. Our results supported reports that pollination-induced senescence of orchid flowers was mediated by changes in ethylene biosynthesis and action [28,37]. The available evidence suggested that the effect was ethylene-mediated according to its action being based on two types of response: (a) responses to a change in the concentration of cellular ethylene (such as an increase in ethylene production caused by various environmental stresses and developmental processes, such as pollination) and (b) responses to a change in the sensitivity of tissue to ethylene (such as in fruit ripening, organ senescence, and abscission) [38].

The results showed that both ethylene production patterns of flowers in stages I and II started 2.5-8 h after emasculation with a trend towards a gradational increase at 10-20 h and a subsequent decrease, while the ethylene production of flowers in stage III increased greatly at 16 h and then subsequently decreased. Furthermore, the whole inflorescence produced a high level of ethylene at 12 h and this then decreased at 16 h with a trend toward a gradational increase until 24 h. Ethylene production commenced (Fig. 3a-d) at about 8-10 h after *ACO* and *ERS* expression (Fig. 1a-f). This result revealed that transcripts accumulated during flower development and then declined by the time the flowers had been in senescence for several hours. Thongkum et al. [39] indicated that *Den-ERS1* in the petals of *Dendrobium* orchid flower increased ethylene production and produced a lower level of *Den-ERS1* transcript after pollination.

In the emasculated flower, a noticeable increase in ethylene production was detected within 10 and 8 h after emasculation of flowers in stage I, and flowers in stages II and III, respectively. These results revealed that ethylene production was correlated with the period of emasculation of the flower. This was supported by reports of an increase in ethylene production followed by pollinia cap dislodgment in *Oncidium* detected after the sixth day [40] and that the ethylene production from the pollinia dislodgment of the cross-pollinated flowers from *Dendrobium* Pompadour at days 1-3 was higher than that of non-pollinated flowers but the differences thereafter were slight [41]. Moreover, ethylene production of pollinated *D.* Pompadour flowers changed slightly in the first 6 h after pollination, and then rapidly increased to a maximum rate (1.5-fold) at 9 h after pollination and declined thereafter [42].

The high ethylene production rates of flowers in stages I, II, and III were 0.51 $nl \cdot g^{-1} \cdot h^{-1}$, 0.42 $nl \cdot g^{-1} \cdot h^{-1}$ and 0.49 $nl \cdot g^{-1} \cdot h^{-1}$, respectively (Fig. 3a-c), revealing that ethylene production was correlated with aging tissue and supporting a report that the ethylene production of *Epidendrum ibaguense* at the bud stage had a rate of ethylene production that followed the same trend as the rate of respiration, namely remaining low up to the fully-opened flower

stage and then the rate of ethylene production increased 5-fold compared with the fully-opened flower stage in the early senescence stage [43]. During developmental stages, there was a higher rate of ethylene production in young (vegetative, floral, and fruit) tissues due to the high rate of cell division; afterward, the ethylene level declined during the elongation and expansion stages and finally it increased gradually until fruit ripening and leaf or floral senescence [44]. However, Trivellini et al. [34] reported that both the *ACS* and *ACO* genes involved ethylene biosynthesis at the early flower development stage of *Hibiscus rosa-sinensis*, but that solely the *ACO* gene regulated the ethylene level during flower senescence. Therefore, ethylene production was the highest in the early flower development stage and then decreased gradually, before finally increasing gradually until flower senescence.

In addition, we found that the whole inflorescence comprising 2 bud flowers and 3 fully-opened flowers produced a high level of ethylene (0.89 $nl \cdot g^{-1} \cdot h^{-1}$) at 12 h after emasculation (Fig. 3f). In many orchids, pollination and emasculation cause the inception of ethylene production, and this subsequently stimulates and then releases ethylene from floral organs [28]. Orchid varieties have different levels of ethylene production according to Goh et al. [4] who reported the *V.* Miss Joaquim flower produced an average level of ethylene at 0.26 $nl \cdot flower^{-1} \cdot h^{-1}$ or at a high level of 3,442 $nl \cdot g^{-1} \cdot h^{-1}$ after emasculation at 32 h; the *Dendrobium* flower produced 0.20 $nl \cdot flower^{-1} \cdot h^{-1}$; *Cymbidium* and mini-*Cymbidium* maintained a low rate of ethylene production at 0.60 $nl \cdot flower^{-1} \cdot h^{-1}$; the *Paphiopedilum* flower averaged 0.85 $nl \cdot flower^{-1} \cdot h^{-1}$; and the *Cattleya* Tearl Harbor flower also produced ethylene at 70 $nl \cdot flower^{-1} \cdot h^{-1}$.

In addition, we found that emasculation induced the highest *ACO* and *ERS* transcripts in the lip and perianth at 6 h and 12 h, respectively (Fig. 1c, 1b, 1e and 1f). This result revealed that the accumulation of a high level of transcripts resulted in the highest ethylene production at 12-16 h for all flower stages (Fig. 3a-d). Thus, the emasculation responded to ethylene sensitivity of *V.* Miss Joaquim flowers for 12-16 h. Although ACO has a low intrinsic catalytic power, a high level of this enzyme is probably necessary for high ethylene production [45], for example with the ethylene of *Phalaenopsis* orchids during senescence being associated with the accumulation of *ACO* transcript [28, 37]. Moreover, the highest level of *ERS* transcript was at the beginning *V.* Miss Joaquim flower senescence at 12-16 h due to the association of *ERS* transcript with the beginning of flower senescence [32] and then the flower faded within 24 h (Fig. 2a2, 2b2 and 2c2).

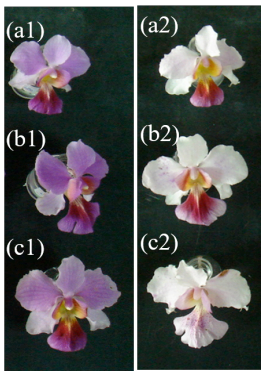

Fig. 2. Three stages of flower development in *V.* Miss Joaquim: (a1, b1, c1) non-emasculated flowers at stages I, II, and III, respectively; (a2, b2, c2) emasculated flowers (24 h) at stages I, II, and III, respectively.

4. Conclusion

Our results showed that the expression of *ACO* and *ERS* was differentially expressed in different organs of the *V.* Miss Joaquim fully-opened flower. There were low accumulation levels of transcripts in the column and high levels in the perianth

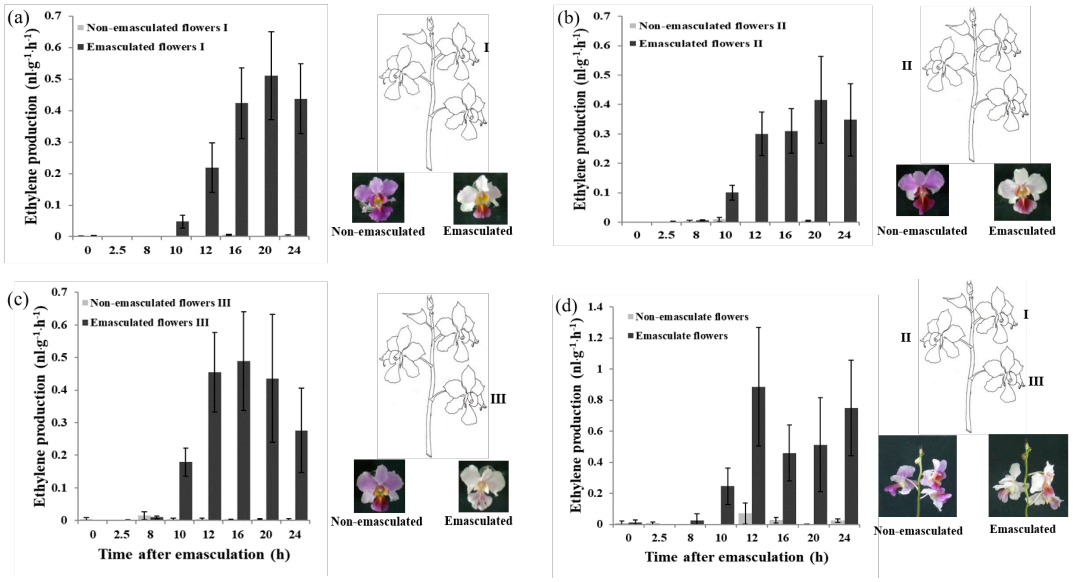

Fig. 3. Ethylene production in: (a) stage I; (b) stage II; (c) stage III; and (d) the whole inflorescence of *V.* Miss Joaquim flowers and comparison between emasculated and non-emasculated flowers.

followed by in the lip during flower senescence for 24 h.Moreover, ethylene was produced after *ACO* and *ERS* transcripts 8-10 h. In particular, the amount of ethylene production was correlated with the aging tissue of the fully-opened flower, with ethylene production in flower-stage I of flower development at a high level, then less in the mid-flower stage (stage II) and a high level in the fully mature flower (stage III) involving flower senescence. Moreover, both gene transcripts and ethylene production were correlated with the period after emasculation of the fully-opened flower with a low level of accumulation in the beginning, followed by a high level afterward and then a low level until 24 h.

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