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Effects of Promoters and Inhibitors of Ethylene and ABA on Flower Senescence of *Hibiscus rosa-sinensis* L.

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Abstract *Hibiscus rosa-sinensis* L. flowers (cv La France) senesce and die over a 12-h period after opening. The aim of this study was to examine the physiological mechanisms regulating the senescence process of ephemeral hibiscus flowers. Different flower stages and floral organs were used to determine whether any interaction existed during flower senescence between endogenous abscisic acid (ABA) and the predisposition of the tissue to ethylene synthesis. This was carried out on whole flowers treated with promoters and inhibitors of ethylene and ABA synthesis or a combination of them. Treatments with 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene biosynthesis, enhanced flower senescence, whereas aminooxyacetic acid (AOA) and fluridone, an ethylene and an ABA inhibitor, respectively, extended flower longevity. These effects were more significant when applied before anthesis. Ethylene evolution was substantially reduced in all organs from open and senescent flowers treated with fluridone and AOA. Similarly, endogenous ABA accumulation was negatively affected by AOA and fluridone treatments. Application of fluridone plus ACC reduced ethylene evolution and increased ABA content in a tissue-specific manner but did not overcome the inhibitor effect on flower longevity. AOA plus fluridone treatment slightly accelerated flower longevity compared to AOA-treated flowers. Application of ABA alone promoted senescence, suppressed ethylene production, and, when applied with fluridone, countered the fluridone-induced increase in flower longevity. Taken together, these results suggest that the senescence of hibiscus flowers is an endogenously regulated ethylene- and ABA-dependent process.

Keywords Abscisic acid · Fluridone · Ethylene · AOA · ACC · Flower organs · Petal · Style-stigma plus stamens · Ovary

Introduction

Flower senescence is an important and final event of flower development regulated by internal and external changes occurring from anthesis to senescence (Tripathi and Tuteja 2007). The complicated nature of the flower senescence process involves a tightly controlled program that is coordinated by interorgan signals to achieve the maximum efficiency for fertilization (O'Neill and others 1993; Verlinden 2006). Senescence occurs in floral tissues at different stages of development and can be characterized by distinct morphological changes and triggered by independent hormonal or other endogenous signals (Rogers 2006). Plant hormones and other signal molecules have long been implicated in the regulation of flower senescence. During senescence, there is a complex reciprocal action of different hormones; for example, ethylene and abscisic acid (ABA) play a major role in the initiation of

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senescence, whereas cytokinins prevent it (Chang and others 2003). The mechanisms through which the hormones or their precursors perceive and trigger senescence signals, thus determining changes in different tissues such as petals, style-stigma plus stamens, and ovary, are still not well known.

The involvement of ethylene during flower senescence and its specific role have both been clearly demonstrated (Borochov and Woodson 1989). This hormone is the main regulator of flower senescence, in species where petal wilting is accompanied by a burst of ethylene production and accelerated by exogenous ethylene, and in species where petals abscise without wilting (Reid and Chen 2007). Ethylene biosynthesis can be inhibited by using specific molecules such as amino-ethoxyvinylglycine (AVG) and amino-oxyacetic acid (AOA) (Reid and Wu 1992), which affect the 1-aminocyclopropane-1-carboxylic acid synthase (ACS) enzyme. The ACS catalyzes the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC), which is directly converted into ethylene by the 1-aminocyclopropane-1-carboxylic oxidase (ACO) (Wang and others 2002). Another approach to prevent the effects of this hormone on an ethylene-sensitive flower is the application of 1-methylcyclopropene (1-MCP), which binds to the ethylene receptor and regulates tissue responses to ethylene (Serek and others 1994). New data suggest that ABA is also a natural regulator of flower senescence (Reid and Chen 2007). In flowers where the senescence process does not respond to ethylene, ABA has been envisaged as being the key factor regulating flower senescence through an early and continuous accumulation in various flower tissues. Exogenous ABA in daylily accelerated senescence-associated events, such as a loss of membrane permeability, and lipid peroxidation (Panavas and others 1998). In narcissus, exogenous ABA has been shown to lead to an early premature accumulation of senescence-associated transcripts in the tepals (Hunter and others 2004). Furthermore, ABA seems to be involved in the regulation of the senescence of ethylene-sensitive flowers. In rose and carnation, exogenously applied ABA modulates an early increase in ethylene production, enhancing the sensitivity to ethylene (Mayak and Halevy 1972; Mayak and Dilley 1976).

In hibiscus ephemeral flowers, the senescence of isolated petals has been associated with increased ethylene evolution, and the onset of flower senescence was found to be much faster under exposure to ethylene (Woodson and others 1985). In the same way, the flower longevity of ephemeral and long-lived hibiscus cultivars was found to be negatively correlated with ethylene evolution (Trivellini and others 2007a) and, interestingly, ABA seemed also to be tightly linked with hibiscus flower life, showing the same trend as ethylene production. ABA may act in concert with or parallel to an ethylene signaling pathway during flower senescence. In petunia, Ferrante and others (2006) found that AOA treatment prevented ABA accumulation during flower senescence. Treatment with silver thiosulfate (STS), an inhibitor of ethylene action, prevented the ABA-induced acceleration of senescence in the carnation flower (Onoue and others 2000). In the rose, the expression of some genes encoding for ethylene receptors is induced under exogenous ABA treatments (Müller and others 2000). Moreover, the application of fluridone, an inhibitor of ABA biosynthesis, reduced ABA levels and extended the longevity of cocoa flowers (Aneja and others 1999).

In the present study, changes in the hormonal levels of ethylene and ABA among different flower stages and floral organs after exogenous application of promoters and inhibitors of ethylene and ABA synthesis were investigated to elucidate the relationship between these two hormones.

Materials and Methods

Plant Materials and Growth Conditions

Hibiscus rosa-sinensis L. cv. 'La France' plants were used in all experiments. The experiments were performed using flowers harvested from flowering potted plants 2–3 years old that were grown in a greenhouse under natural conditions. The plants were grown in 45-cm-diameter pots with substrate containing peat and perlite. Mineral nutrition was achieved using slow-release fertilizers (osmocote) and fertigations containing 1 g/l nutrients concentration.

Flowers cut from the plants were used as experimental material and were harvested at the following stages: buds 24 h before full bloom, fully opened flowers, and petals in rolling senescent flowers. All experiments were performed between May 15 and September 30. Flowers were harvested between 7:30 and 8:00 a.m. either on the morning of flower opening [open flowers (OF), day 0] or on the morning of the day before opening [bud flowers (B), day −1] or on the morning of the day after opening [senescent flowers (SF), day +1]. The stages of flower development used in this study are shown in Fig. 1. The cut flowers were

Fig. 1 *Hibiscus rosa-sinensis* flower development stages. *B* bud stage, *OF* open flower stage, *SF* senescent flower stage. The pattern presented is representative of at least ten replicates

immediately placed into the controlled growth chamber under the following environmental conditions: 16-h photoperiod, cool white fluorescent light (100 μmol m^{-2} s^{-1}) at 22 ± 2°C, and approximately 60% RH.

Treatments of Flowers

Hibiscus flowers were used at their bud (day −1), open (day 0), and senescence (day +1) stages. Flowers were trimmed to 4 cm in stem length and treated by placing the cut end in 20 ml deionized water with or without chemicals in all experiments. The chemicals used were 0.1 mM aminocyclopropane-1-carboxylic acid (ACC, Sigma Italy), 2 mM amino-oxyacetic acid (AOA, Sigma Italy), 10 μM fluridone (Elanco Products Canada Ltd), and 100 μM abscisic acid (ABA, Sigma Italy).

The relationship between ethylene and ABA with respect to *H. rosa-sinensis* flower longevity was examined in different ways. The main experiment was conducted on whole detached flowers derived from the previously described three independent developmental stages (bud, open, and senescent flower). The effects of exogenously applied ethylene precursor (ACC) and ABA and ethylene inhibitors (fluridone and AOA, respectively) on the rate of visual senescence and on the hormonal content of ABA and ethylene were evaluated by placing the flowers in each of the treatments for 6 h.

The ability of ABA to influence ethylene levels and vice versa was examined in detached intact flowers using bud and open flower stages. In the first experiment, the rate of visual senescence symptoms was tested on whole flower buds by placing their cut ends into each of the following treatments: water (control), 0.1 mM ACC, 2 mM AOA, 10 μM fluridone, 100 μM ABA, 2 mM AOA + 100 μM ABA, 10 μM fluridone + 0.1 mM ACC, and 10 μM fluridone + 100 μM ABA. Ten flowers were tested and the experiment was repeated twice. In the second experiment, the cut ends of ten intact whole open flowers were placed in water (control), 100 μM ABA, 2 mM AOA + 100 μM ABA, 10 μM fluridone + 0.1 mM ACC, and 10 μM fluridone + 100 μM ABA. Five open flowers were treated for 6 h and the hormonal levels of ethylene and ABA were determined. The rate of visual senescence symptoms of the various chemical combinations on hibiscus flower longevity was examined on the remaining set of flowers ($n = 5$). The experiment was repeated twice.

Water Uptake

The water uptake by the cut end of whole detached buds, open, and senescent flowers that were trimmed to 4 cm in stem length and placed in 20 ml deionized water was measured. The mount of the tube was plugged with nonabsorbent cotton, which effectively prevents direct water loss. The amount of water taken up by the flower stem was recorded after 6 h for the three independent flower stages and after flower opening, which occurs 24 h after sampling, for the bud flowers, and was expressed as volume taken up (μl) per 6 and 24 h, respectively.

Determination of Endogenous ABA and Ethylene Production

Ethylene production and ABA content were investigated on the three independent flower stages 6 h after treatments were applied. Petals, style-stigma plus stamens, and ovaries were carefully isolated from flowers after the treatments and used for hormonal level determination assays. The ethylene and ABA levels were also evaluated after cutting; the data are reported in Supplementary Table 1.

ABA was determined by an indirect ELISA based on the use of a DBPA1 monoclonal antibody raised against S(+)-ABA (Vernieri and others 1989). The ELISA was performed according to the method described by Walker-Simmons (1987), with minor modifications. Petals, style-stigma plus stamen, and ovary samples (approximately 100 mg FW) were collected, weighed, frozen in liquid nitrogen, and then stored at −80°C until the analysis. ABA was measured after extraction in distilled water (water:tissue ratio = 10:1 v/w) overnight at 4°C. Plates were coated with ABA-4′-BSA conjugate at 200 μl per well and incubated overnight at 4°C, then washed three times with 75 mM PBS buffer, pH 7.0, containing 1 g l^{-1} BSA and 1 ml l^{-1} Tween 20, keeping the third washing solution for 30 min at 37°C. Next, 100 μl ABA standard solution or sample and, afterwards, 100 μl DBPA1 solution (lyophilized cell culture medium diluted in a PBS buffer containing 10 g l^{-1} BSA and 0.5 ml l^{-1} Tween 20, at a final concentration of 50 μg/ml) were added to each well and competition was allowed to occur at 37°C for 30 min. Plates were then washed again as described above and 200 μl per well of a secondary antibody (alkaline phosphatase-conjugated rabbit anti-mouse (Sigma Italy) in a PBS buffer containing 10 g l^{-1} BSA and 0.5 ml l^{-1} Tween 20, at a final dilution of 1:2000) was added and incubated for 30 min at 37°C. Plates were washed again and *p*-nitrophenyl phosphate at 200 μl per well was added and incubated for 30 min at 37°C. Absorbance readings at 415 nm were obtained using a MDL 680 PerkinElmer microplate reader. For each treatment, five independent samples were assayed in triplicate.

Ethylene production was measured by enclosing either petals or style-stigma with stamen in 30-ml air-tight containers, whereas the ovaries, given their small size, were enclosed in 3-ml air-tight containers. Two-milliliter gas samples were taken from the headspace of the containers

after 1 h of incubation at 22 ± 2°C for petals and style-stigma plus stamens and after 30 min for the ovary tissue. The ethylene concentration in the sample was measured using a gas chromatograph (HP5890, Hewlett-Packard, Menlo Park, CA) with a flame ionization detector (FID), a stainless-steel column (150 × 0.4 cm ø packed with Hysep T) (column and detector temperatures were 70 and 350°C, respectively), and nitrogen carrier gas at a flow rate of 30 ml min^{-1}. Quantification was performed against an external standard and results were expressed on a fresh-weight basis (nl h^{-1} g^{-1} FW). For each treatment, five independent samples were assayed in duplicate.

Statistical Evaluation

For each experiment, the means from at least five independent biological samples (ABA and ethylene measurements) analyzed with two technical replicates were calculated. Statistical differences among mean values ($P < 0.05$) were determined using two-way ANOVA followed by Bonferroni's multiple comparison test to compare the effects of each treatment with the relative hormonal level of the control. A one-way ANOVA was used to assess the ethylene and ABA level distribution among various chemical treatment combinations and differences between means were determined using Tukey's test. The statistical analyses were carried out using GraphPad Prism 5 for Windows v8.0 (GraphPad Software, San Diego, CA, USA).

Results

Description of Flower Senescence in *Hibiscus rosa-sinensis*

The time and the features of ephemeral *H. rosa-sinensis* flower senescence were similar to those previously described by Woodson and others (1985); the flowers senesce and die over a 12-h period after opening. The first visible sign of the senescence of *H. rosa-sinensis* flowers was an in-rolling of the corolla followed by corolla wilting and subsequent abscission. The application of treatment for 6 h did not cause any visible changes compared to the control flowers at the different developmental flower stages, with the exception of the ACC treatment at the open flower stage, which slightly accelerated the wilting process localized on the apical portion of the petals (Fig. 2). When the flowers were held for 10 h in the treatment solutions, the corolla from control flowers started to wilt and 12 h later was completely in-rolled. ACC-treated open flowers showed an acceleration of the wilting process, and after 10 h the corolla was almost in-rolled. With the AOA and fluridone treatments, the corolla wilting and petal in-rolling were partially impeded after 10 and 12 h compared to the controls.

Fig. 2 Effects of ACC, AOA, and fluridone on open flower stage. Flowers were treated with 0.1 mM ACC, 1 mM AOA, and 10 μM fluridone for 12 h. The experiment was repeated five times and the pattern presented is representative of at least 10 replicates

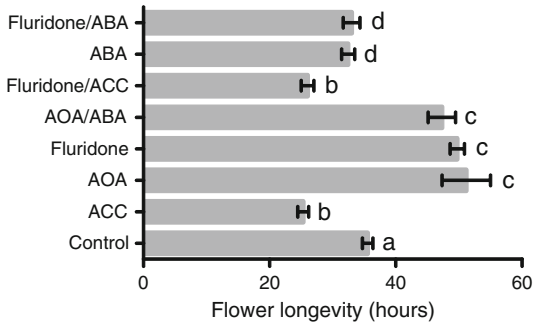

Fig. 3 Effect of the timing of the treatment with 0.1 mM ACC, 2 mM AOA, 10 μM fluridone, 2 mM AOA/100 μM ABA, 10 μM fluridone/0.1 mM ACC, 100 μM ABA, and 10 μM fluridone/100 μM ABA on longevity of *H. rosa-sinensis* L. flowers treated as bud flowers. The flowers were detached and subsequently treated. Flower longevity was evaluated as the time from −24 h before anthesis to full corolla in-rolling. Data were subjected to one-way analysis of variance (ANOVA), and differences between developmental stages were analyzed using the Tukey test. Different letters denote significant differences at $P \leq 0.05$. Values are means of at least ten independent biological samples. Error bars represent standard deviations; the experiment was repeated twice showing the same results

The longevity of *H. rosa-sinensis* flowers was substantially extended when they were treated in the bud stage with the putative inhibitors of ethylene and ABA biosynthesis, AOA and fluridone, respectively, in a similar manner for both treatments (Fig. 3). On the other hand, the ACC treatment solution applied on hibiscus buds was particularly deleterious, causing the premature in-rolling corolla symptom within 28 h (Fig. 3). Although the rate of senescence symptoms of hibiscus flower was largely decreased by AOA and fluridone treatment, or dramatically

increased by ACC treatment, when they were applied on bud flowers rather than on open flowers, the effects observed ignoring the time scale were the same. However, it must be reported that when ACC treatment was applied to flower buds, flower opening was impeded by 40%.

Placing intact whole bud flowers in 2 mM AOA/100 μM ABA (AOA/ABA) or 10 μM fluridone/0.1 mM ACC (fluridone/ACC) did alter their rate of senescence from that seen in the detached flowers in water. AOA/ABA treatment was effective and delayed the visual symptoms of hibiscus flower senescence, although the AOA/ABA-treated flowers senesced more quickly than those held in AOA alone (Fig. 3). ABA applied alone accelerated slightly the flower senescence time course (at the onset of petal wilting), and the effect was less pronounced compared to that of ACC application on both the bud and open flower stages (Figs. 2, 3). When the bud flowers were treated with fluridone/ACC, the senescence symptoms were accelerated similarly to that observed in ACC-treated buds (Fig. 3). ABA applied in combination with fluridone had the same effect as exogenous ABA alone. AOA/ABA and fluridone/ACC had no effect on the rate of visual senescence when they were applied to whole open detached flowers.

Water Uptake

After 6 h there were no significant changes in water uptake among the three independent flower stages (B, OF, and SF) (Table 1). However, when the flower buds were held continuously in water for 24 h, thus covering the entire flower opening process (B-OF as reported in Table 1), water uptake was four times higher compared to the other independent stages.

Endogenous Ethylene and ABA Content During Hibiscus Flower Development and Senescence

The ethylene production and endogenous ABA content in petals, style-stigma plus stamens, and ovaries of detached control hibiscus flowers increased as the flowers senesced (Figs. 4, 5). In bud flowers, the ethylene production was higher in the ovary, with a mean value of 28.8 nl g^{-1} h^{-1}. The style-stigma plus stamens showed intermediate values, whereas petals produced the lowest ethylene amount of 3.7 nl g^{-1} h^{-1}. In the fully open flowers, on the other hand, ethylene production was higher in all organs compared with the bud stage but had the same ratio. In the senescent flowers, the highest ethylene production was in the ovaries at 69.8 nl g^{-1} h^{-1}, intermediate values were observed in petals, and the lowest levels were found in style-stigma plus stamens with a mean value of 9.1 nl g^{-1} h^{-1}. These organs were the only ones that showed a reduction in ethylene during flower development. Throughout the entire development and senescence process of the hibiscus flowers, ovaries showed the most significant ethylene production, with the highest values at the senescent stage.

Table 1 Water uptake among the three independent flower stages and during flower opening

	Flower stages			
	B	OF	SF	B-OF
Water uptake	0.104 ± 0.013a	0.122 ± 0.021a	0.087 ± 0.070a	0.391 ± 0.043b

Values are means of at least 10 independent biological samples. Data were subjected to one-way analysis of variance (ANOVA), and differences between developmental stages were analyzed using the Tukey test. Different letters denote significant differences at $P \leq 0.05$

B bud flower, *OF* open flowers, *SF* senescent flowers, *B-OF* flower opening

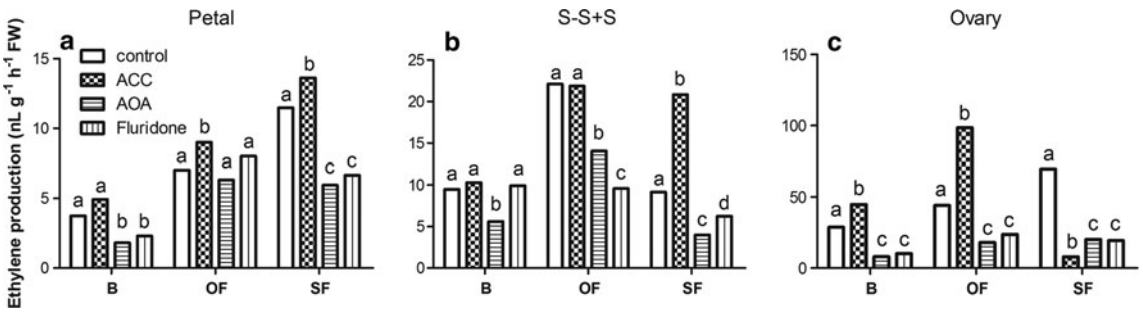

Fig. 4 Ethylene production in three floral tissues of *H. rosa-sinensis* at three development stages (*B* bud flower, *OF* open flowers, *SF* senescent flowers). The flowers were detached and subsequently treated for 6 h with 0.1 mM ACC, 2 mM AOA, and 10 μM fluridone and the controls were treated in water. Data were subjected to a two-way analysis of variance. Different letters within developmental flower stages denote significant differences (Bonferroni post test, $P < 0.05$) of ethylene production upon ACC, AOA, and fluridone treatments. Values are means of at least five independent biological samples with two technical replications for each sample

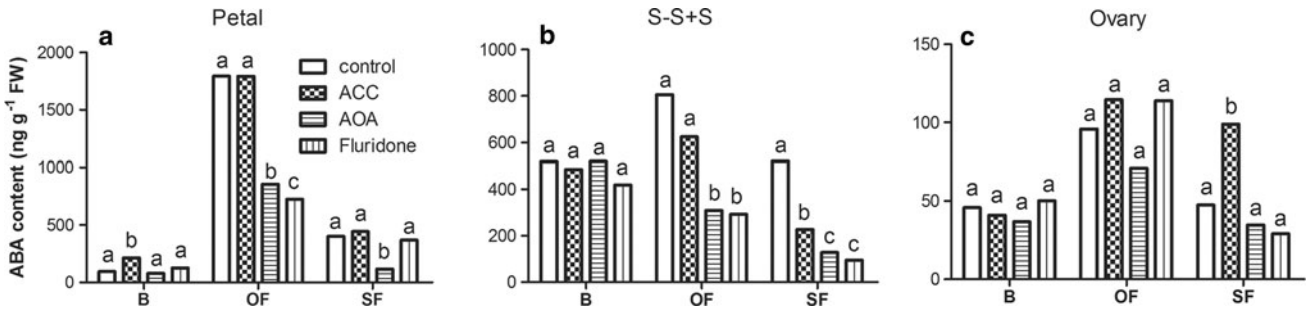

Fig. 5 Endogenous ABA content in three floral tissues of *H. rosa-sinensis* at three development stages (*B* bud flower, *OF* open flowers, *SF* senescent flowers). The flowers were detached and subsequently treated for 6 h with 0.1 mM ACC, 2 mM AOA, and 10 μM fluridone and the controls were treated in water. Data were subjected to a two-way analysis of variance. Different letters within developmental flower stages denote significant differences (Bonferroni post test, $P < 0.05$) of ABA content upon ACC, AOA, and fluridone treatments. Values are means of at least five independent biological samples with two technical replications for each sample

The ABA content in petals was low at the bud stage, had the highest value at the opened stage, and decreased during flower senescence. The ABA changes during petal development and senescence showed the same trend in the style-stigma plus stamens. No obvious change in the endogenous ABA content was detected in the ovary tissues during the development and aging processes. The ABA content in the bud stage was higher in style-stigma plus stamens (517.6 ng g^{-1}) compared with petals and ovaries. In the fully open flower development stage, the endogenous ABA increased in all flower organs, but the highest content was observed in petals with a value of 1793 ng g^{-1}, followed by style-stigma plus stamens with 805 ng g^{-1} and 95.7 ng g^{-1} in the ovary tissues. The ABA content in senescent flowers showed intermediate values compared with bud and fully open flowers. The concentration ratios in the organs were analogous to those observed in the buds.

Effects of ACC, AOA, and Fluridone on Ethylene Production

The trend of ethylene production in the petals of bud flowers treated with 0.1 mM ACC was similar to that in the control flowers held in water; whereas in open and senescence flowers production of this hormone increased by approximately 28 and 19%, respectively (Fig. 4a). When flowers were maintained in 2 mM AOA, petal ethylene evolution compared with controls decreased in bud and senescent flowers, whereas no difference was observed in the open flower stage. To investigate whether ethylene production could be affected by ABA accumulation, whole flowers were tested on 10 μM fluridone solution. The pattern of ethylene evolution in petals treated with fluridone (Fig. 4a) was similar to that of petals placed in 2 mM AOA, showing a significant reduction in bud and senescent flowers by approximately 40%. In ACC-treated style-stigma plus stamens, ethylene production was similar to the control in bud and open flowers (Fig. 4b), except in senescent flowers where the ethylene production did not decline as observed for the controls and other treatments.

The ethylene production in AOA-treated style-stigma plus stamens showed the same trend as the controls, except for being significantly decreased in the three different development flower stages, on average for all by 45% (Fig. 4b). Fluridone applied at the bud stage did not affect ethylene biosynthesis in style-stigma plus stamens. In open flowers and senescent flowers, it prevented the ethylene burst. Fluridone treatment lowered the ethylene levels compared with that of controls by approximately 56% in open flowers and 31% in senescent flowers (Fig. 4a).

The ovaries from flowers treated with ACC showed a significant increase in ethylene production compared to the controls (at the bud stage the increase was approximately 50%), whereas in open flowers it dramatically increased by approximately 2.5-fold (Fig. 4c). In senescent flowers, on the other hand, the ethylene production strongly decreased by approximately 88%. Similar to the style-stigma plus stamens from AOA-treated flowers, the AOA-treated ovaries showed a significant reduction of ethylene evolution in all three flower stages (Fig. 4c). Ovaries from flowers treated with fluridone yielded results similar to those previously observed for AOA-treated ovaries during flower development and senescence (Fig. 4c). Both AOA and fluridone treatments lowered ethylene production during flower development compared with controls but did not affect the trend.

Effects of ACC, AOA, and Fluridone on ABA Contents

The ABA content in petals treated with ACC was higher in the bud stage, whereas no statistical differences were observed in the other stages compared to controls (Fig. 5a). Treatments with AOA and fluridone did not influence the ABA concentration in petals of buds. On the other hand, in open flowers AOA and fluridone reduced the increase of ABA. In addition, AOA treatment also led to a strong

reduction (59%) in ABA concentrations in senescent petals (Fig. 5a). There was no difference in endogenous content of ABA in style-stigma plus stamens (Fig. 5b) between those treated with ACC and the controls in bud and open flower stages. ACC applied in senescent flowers reduced the ABA content in style-stigma plus stamens. On the other hand, ABA accumulation in style-stigma plus stamens from flowers treated with AOA and with fluridone dropped off dramatically at the open and senescent flower stages (Fig. 5b). In the ovaries from ACC-treated flowers, the ABA content did not decline in senescent flowers compared to the ABA content observed in the open flower stage. The ABA content in the ovaries of senescent flowers was almost twofold higher compared to that of flowers that had other treatments (Fig. 5c). No significant differences were observed in ovaries treated with AOA and fluridone during flower development and senescence (Fig. 5c).

Effect of the Inhibitors Plus the Related Hormone (or Precursor) on Hormone Content

When open flowers were treated with ABA, ethylene biosynthesis was reduced in all flower organs, but significant differences were observed only in petal tissues (Fig. 6). ABA applied in combination with fluridone substantially decreased ethylene evolution in petals and ovary tissue. The application of the ethylene inhibitor AOA with ABA (AOA/ABA) to open flowers significantly inhibited ethylene biosynthesis in all flower organs. Fluridone/ACC reduced ethylene production in all flower tissues, but it was

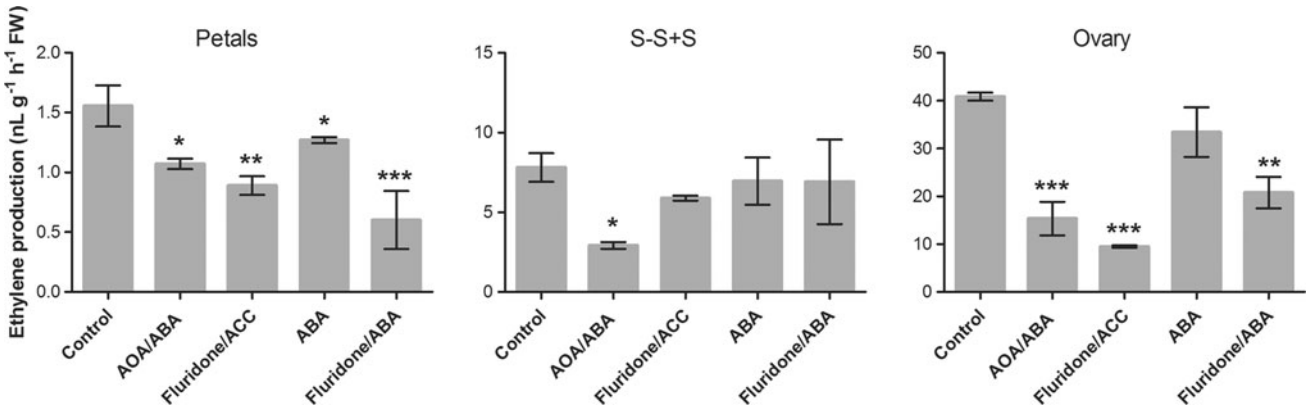

Fig. 6 Ethylene production in three floral tissues of *H. rosa-sinensis* at the open flower stage. The flowers were detached and subsequently treated for 6 h with 2 mM AOA/100 μM ABA, 10 μM fluridone/0.1 mM ACC, 100 μM ABA, and 10 μM fluridone/100 μM ABA and the controls were treated in water. Data were subjected to one-way analysis of variance (ANOVA), and differences among treatments were analyzed using the Tukey test. Significant differences are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$). Values are means of at least five independent biological samples with two technical replications for each sample. Error bars represent standard deviations; the experiment was repeated twice showing the same results

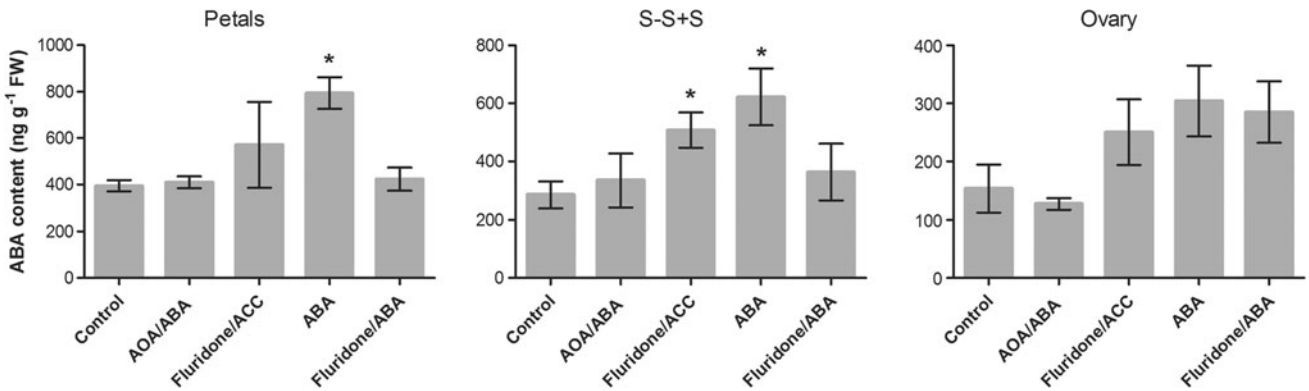

Fig. 7 ABA content in three floral tissues of *H. rosa-sinensis* at open flower stage. The flowers were detached and subsequently treated for 6 h with 2 mM AOA/100 μM ABA, 10 μM fluridone/0.1 mM ACC, 100 μM ABA, and 10 μM fluridone/100 μM ABA and the controls were treated in water. Data were subjected to one-way analysis of variance (ANOVA), and differences among treatments were analyzed using the Tukey test. Significant differences are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P = 0.001$). Values are means of at least five independent biological samples with two technical replications for each sample. Error bars represent standard deviations; the experiment was repeated twice showing the same results

significantly reduced only in petals and ovaries. These two treatments did not affect endogenous ABA accumulation in petals and ovaries, although higher values were observed in style-stigma plus stamens from fluridone/ACC-treated flowers in which the ABA content was doubled compared to control (Fig. 7). As expected, endogenous ABA content in ABA-treated open flowers increased, but when ABA was used together with fluridone, no differences were observed compared to control.

Discussion

Ethylene Production during Flower Development and Senescence and the Effects of ACC and AOA

In ephemeral flowers, no obvious pollination effect inducing the acceleration of flower senescence has been described (Lay-Yee and others 1992; Stead 1992). This suggests that only an endogenous internal clock is operating. The senescence process of these flowers is already so short that a further constraint does not provide any evident advantages (van Doorn 1997; Orzáez and others 1999).

Our results support the view that ethylene is the main regulator of hibiscus flower senescence, with a significant increase in production of ethylene at the advancing stages of senescence. Furthermore, the application of ACC, a precursor of ethylene biosynthesis, stimulated an increase in ethylene production, enhancing the rate of flower senescence, whereas AOA, which inhibits ACC synthesis, reduced its production which had a positive effect on flower longevity. Moreover, the sensitivity to ethylene was high in this species (Woodson and others 1985; Woltering 1987; Høyer 1996); in fact, the rate of visual senescence symptoms was dramatically enhanced or delayed in flowers treated with ACC or AOA as buds, respectively (Fig. 3), compared to those treated as open flowers (Fig. 2). This is reasonable because water and hence chemical uptake was substantially greater during flower opening (Table 1).

Furthermore, in our study the production of ethylene showed different responses from the floral organs considered. In all stages, the production of ethylene was higher in the ovaries, followed by the style-stigma plus stamens and finally the petals. In fact, it has been demonstrated that the activation of senescence, and the genes involved, is caused by the ovary, which activates the signals for aging (Shibuya and others 2000; Nukui and others 2004). This perspective also clarifies the finding that senescent ovaries from flowers treated with ACC showed a reduction in ethylene synthesis rather than an increase, and also that at the early stage of flower development, such as the bud stage, only the ovaries responded to exogenous ACC. In senescing tissues where the aging process has already been accelerated by ACC treatment, the activity of the ethylene-forming enzyme (EFE) may be low or even not present and ethylene production may be limited by the tissue's potential to oxidize ACC to ethylene. In style-stigma plus stamens, ethylene evolution peaked during the open flower stage and then decreased. However, the style-stigma plus stamen senescence tissue still maintained its ability to produce ethylene, as demonstrated by the fact that it responded to the ACC treatment. In hibiscus style-stigma plus stamens, ethylene evolution can therefore be considered as a pollination-independent process involved in triggering signals for corolla senescence (Nichols 1977).

ABA Content during Flower Development and Senescence and the Effects of Fluridone

Flower senescence in hibiscus is accompanied by changes in the endogenous concentration of ABA and these changes were nearly all the same in all of the tissues examined. A relatively low level was found during early flower development, thus the endogenous concentration peaked at the open flower stage and then decreased during senescence. A similar pattern of ABA rise and fall was reported in cut roses during the senescence process under water limitations (Kumar and others 2008). Previous studies have reported that endogenous alteration in the ABA level is an important factor in flower senescence (Mayak and Halevy 1972; Swanson and others 1975).

Fluridone was used to inhibit ABA production in hibiscus flowers, and, as expected, the ABA content was strongly reduced in petals and style-stigma plus stamens during the open flower and senescence stages of treated flowers. These results suggest that the reduction in ABA content, particularly in the style, might be related to the role of the style in maximizing flower longevity (Lovell and others 1987), and ABA seems to be a key component because the longevity of both fluridone-treated buds and open flowers was prolonged. Fluridone did not suppress ABA biosynthesis in ovaries probably because ample substrate for ABA synthesis was provided by this storage-complex tissue.

Effects of AOA and Fluridone on ABA Accumulation and Ethylene Synthesis

We were able to show that AOA and fluridone inhibitors antagonize both ethylene (Fig. 4) and ABA synthesis (Fig. 5) with a positive effect on flower life (Fig. 2). Other researchers have also shown a reduction in ABA content using ethylene synthesis inhibitors (Onoue and others 2000; Ferrante and others 2006), and an extension of flower longevity using fluridone (Aneja and others 1999). Moreover, in ripening tomato fruits a direct effect of ABA on ethylene

synthesis and/or the perception signaling pathway has been recently reported by Zhang and others (2009). Although these results suggest that ethylene and ABA interact during flower senescence, there was no direct evidence that the effects were caused by ethylene and/or ABA because the actions of many inhibitors are not specific. For example, AOA is a general inhibitor of pyridoxal phosphate-dependent enzymes (John and others 1978; Jenkins and others 1983; Hirase and Molin 2001) and not only effective in inhibiting ACC synthase. The ABA signaling pathway is known to interact antagonistically or synergistically with ethylene in plant development and senescence (Beaudoin and others 2000; Cheng and others 2009). In hibiscus, ABA appears to be involved in flower senescence dependently on the ethylene pathway as the flowers following AOA/ABA and fluridone/ACC treatment did not overcome both inhibitor effects on flower longevity. However, in fluridone/ACC-treated flowers, fluridone and ACC independently countered the ACC-induced increase of ethylene evolution and the fluridone-induced inhibition of ABA synthesis; instead, a reduction of ethylene and an increase of ABA content were observed. These results suggest that ACC stimulates ABA biosynthesis directly, for example, through the mevalonate or terpenoid pathways (Milborrow 2001). In AOA/ABA-treated flowers, ethylene evolution was reduced but ABA was not increased by exogenously supplied ABA. Because the AOA/ABA treatment slightly reduced flower longevity compared to the effect of AOA alone, perhaps it is the lack of an ABA decrease that influences the longevity. In our previously work, higher ABA concentrations were found in short-lived hibiscus cultivars compared to long-lived cultivars (Trivellini and others 2007a), suggesting a direct role for ABA in hibiscus flower senescence. Unlike other ethylene-sensitive flowers, such as carnation and rose, in which ABA accelerated floral senescence by increasing ethylene production (Müller and others 1999; Onoue and others 2000), hibiscus senesces slightly early in response to ABA without evolving increased amounts of ethylene but by decreasing its level. Moreover, the application of ABA plus its inhibitor fluridone countered the fluridone-induced increase of flower longevity, showing a slight reduction as in ABA-treated flowers. These results indicate that the flower longevity of *H. rosa-sinensis* is mediated also by ABA that may act by increasing ethylene sensitivity through the regulation of ethylene receptors (Trivellini and others 2007b).

All these observations taken together suggest that the mechanism underlying the longevity of the hibiscus flower may be regulated by the combined action of ethylene and ABA through a tight coordination of the levels of both hormones which are dependent on endogenous and external stimuli. Plant developmental processes have been shown to be influenced by the interaction of several hormones (Davies 2005), so it is not surprising that their respective pathways have convergent points, function in parallel and with a synchronous behavior, ensuring the progression of the senescence process in hibiscus flowers under various external stimuli.

Further research will be performed to test the presence of a convergent point between ethylene and the ABA signaling pathway by evaluating the regulation of ethylene biosynthesis and receptor gene expression, because their synchronous (or not) behavior under exogenous stimuli in floral tissues has made it difficult to assess their role in the physiological event.

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