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Antagonistic Interrelation Between Abscisic Acid and Gibberellic Acid in the Regulation of Senescence in Ray Florets of *Calendula officinalis* L.

Mohammad Lateef Lone[1] · Sumira Farooq[1] · Aehsan ul Haq[1] · Shazia Parveen[1] · Foziya Altaf[1] · Inayatullah Tahir[1]

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Abstract
Petal senescence represents an extraordinary phase of flower development, involving precisely regulated biochemical and physiological reprogramming. Plant growth regulators (PGRs) stand as the chief regulatory switches to elicit such reprogramming causing programmed cell death (PCD) of petals. Ethylene is recognized as the key hormone that regulates senescence in ethylene-sensitive flowers. In contrast, there has been a constant pursuit to dispense the same role to a hormone other than ethylene in the ethylene-independent class of flowers. Pertinently, abscisic acid (ABA) is presumed to play a decisive role in the petal senescence of ethylene-insensitive flowers. Additionally, oxidative stress characterized by the accumulation of reactive oxygen species (ROS) is assumed to be the hallmark of PCD and senescence in petals. Consistent with this idea, the current investigation ascertains the role of PGRs viz., ethylene, ABA, and gibberellic acid (GA), besides ROS in regulating the senescence in ray florets of *Calendula officinalis*; a least documented ornamental of Asteraceae. The ray florets were analyzed for the transient biochemical changes from juvenility through maturity to senescence. Based on the current findings, it was ascertained that ABA plays a significant role in instigating senescence in ray florets of *C. officinalis*. Furthermore, postharvest treatment with ABA antagonists such as GA and sodium tungstate (ST) combatively delayed the senescence of this flower. GA and ST significantly reduced the hydrogen peroxide (H_2O_2) accretion and protein degradation, besides accentuating the cell membrane integrity and antioxidant system in the detached flowers of *C. officinalis.*

Keywords Senescence · Ethylene · Abscisic acid · Gibberellic acid · Calendula · Sodium tungstate

Introduction

The remarkable plasticity of plant ontogeny is shaped by hormone signaling pathways. PGRs (phytohormones) interact antagonistically or synergistically with one another, demonstrating multifaceted crosstalk across different signaling pathways to precisely determine the specific plant responses (Ma et al. 2018; Aftab and Roychoudhury 2021). Petal senescence, a genetically regulated mechanism of PCD is coordinated by an intricate crosstalk between phytohormones (Sun et al. 2021). This crosstalk acts as a regulatory mechanism to trigger physiological, biochemical, and genetic reprogramming in petals, inevitably leading to the cellular dismantling and subsequent mortality. It is this molecularly and biochemically optimized program that accomplishes a regulated salvage of essential nutrients from dying tissue to the developing ovary well before petal death (Rogers and Munné-Bosch 2016). The lifespan of petals is genetically determined, as maintaining the flower structure requires a significant outlay in terms of energy metabolism and osmotic balance. Therefore, petals undergo programmed death after the cessation of stigma receptivity or after pollination. Petal senescence proceeds along a typically slower, predetermined developmental pattern, accompanied by a pronounced increase of ROS followed by PCD (Rogers and Munné-Bosch 2016). Although balanced levels of ROS are crucial for nearly every facet of plant metabolism, their excessive levels pose a significant threat that could result in cellular damage and eventual death (Xie

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✉ Inayatullah Tahir
tahir.inayatullah@gmail.com

[1] Plant Physiology and Biochemistry Research Laboratory, Department of Botany, University of Kashmir, Srinagar 190006, India

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et al. 2014; Oracz and Karpiński 2016). Excessive ROS induces the oxidation of lipoproteins, decomposition of bio-membranes, inactivation of enzymes, and alterations in gene expression that eventually leads to cellular death (Hossain et al. 2006; Raza et al. 2020).

Ethylene while involving complicated crosstalk with other PGRs is recognized as the chief hormone to coordinate senescence in ethylene-sensitive flowers (Dar et al. 2021). In such flowers, treatments such as silver thiosulfate (STS), aminooxyacetic acid (AOA), cyclic olefin norbornadiene, 1-methyl cyclopropene, and amino ethoxy vinyl glycine inhibit the synthesis and functioning of ethylene thereby delaying their senescence (Trivellini et al. 2011; Dar et al. 2021). In contrast, the other class of flowers exhibit ethylene-independent senescence (Ma et al. 2018) and are not affected by the administration of ethylene inhibitors. This suggests that senescence in these flowers is regulated by a hormone other than ethylene. Pertinently, studies advocate that ABA is a principal component of the signal transduction chain that causes PCD and senescence in these flowers (Panavas and Rubinstein 1998; Hunter et al. 2004a, b; Kumar et al. 2014).

ABA is a monocyclic sesquiterpenoid compound shown to act as a positive regulator of not only flower senescence but also leaf senescence (Lim et al. 2007; Jan et al. 2019). The efficacy of various substances like fluoridone and norflurazon in inhibiting ABA synthesis has been evaluated, and notably, these compounds have markedly delayed flower senescence (Hunter et al. 2004a, b). On the other hand, gibberellins, structurally diterpenes, have been classically implicated in several vital physiological processes such as seed dormancy, plant stature, flowering, and petal senescence (Mattoo and Sobieszczuk-Nowicka 2019; Fan et al. 2021). Gibberellins have been demonstrated to delay flower senescence by serving as ABA action blockers (Kumar et al. 2014). Recent findings infer the direct relations between ABA and GA signaling mechanisms, which offer novel clues about their antagonistic regulation (Liu et al. 2016; Shu et al. 2018; Liu and Hou 2018; Jan et al. 2019).

Understanding the hormonal crosstalk, particularly the involvement of ethylene, ABA, and GA in the petal senescence of Asteraceae flowers is elusive. The question of whether *Calendula officinalis* is ethylene-sensitive remains uncertain, and it is plausible that the existing techniques aimed at enhancing vase life may not yield sufficient commercial viability in this beautiful ornamental. In light of this, the current study was undertaken to gain insights into the role of PGRs viz., ethylene, ABA, and GA and their crosstalk in regulating the senescence in the ray florets of *Calendula officinalis* (pot marigold); a prolific and vibrant flower of Asteraceae. Additionally, this study explores the role of ABA antagonists, specifically GA and ST in delaying the senescence of detached flowers of *Calendula officinalis*.

Materials and Methods

Experimental Setup

The plant material for the present study was grown in Kashmir University Botanic Garden (KUBG). The flower development was constantly monitored and categorized into five stages viz., closed bud (I), mature bud (II), partially open (III), fully open (IV), and senescent (V) stage, based on the opening and diameter of ray florets (Fig. 1). The petal tissues of selected developmental stages were harvested and brought to the laboratory for hormonal analysis such as ABA and ethylene. Additionally, the H_2O_2 content was quantified at these selected stages. For the postharvest study, the fresh buds of *C. officinalis* were harvested one day before the anthesis stage and transferred to the laboratory. The flower stems were re-cut to a length of roughly 5 cm and categorized into various sets. Two sets were supplied separately with two growth regulators viz., ABA and GA (in the form of GA_3), at 100 µM, and 150 µM concentrations, respectively, in glass vials. Another set of flowers was supplemented with ST(Na_2WO_4) at 50 µM concentration. Two other sets were supplied with the combination of these treatments, i.e., ABA (100 µM) + ST (50 µM) and ABA (100 µM) + GA (150 µM). A set of flower buds placed in distilled water (DW) represented the control. Each set along the control had ten flowers, ensuring ample availability of material for conducting analyses during the investigation. However, the various parameters were analyzed by taking

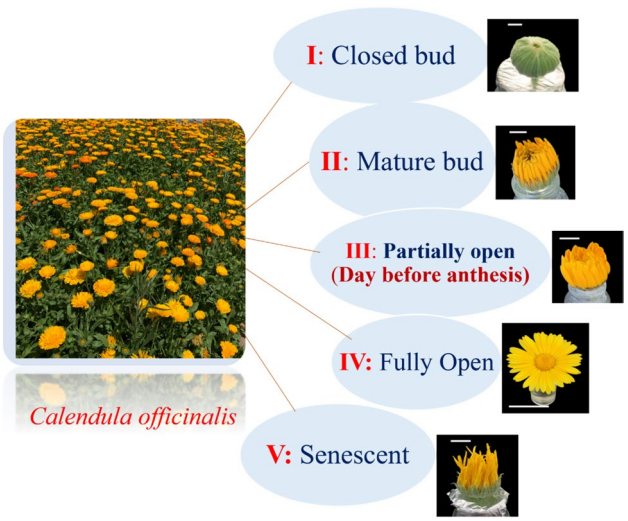


Fig. 1 Various arbitrarily selected developmental stages of *Calendula officinalis* viz., I—closed bud (scale bar = 5 mm), II—mature bud (scale bar = 6 mm), III—partially open (scale bar = 7 mm), IV—fully open (scale bar = 25 mm), and V—senescent stage (scale bar = 7 mm)

three biological replicates, each comprising the ray florets (petals) collected from different flowers. The chemicals, i.e., ABA, GA_3, and ST were procured from Sigma-Aldrich with purity > 99%. The day of administration of various treatments was interpreted as day 0. The concentrations of ethylene antagonists (STS and AOA) tested in the current study ranged from 0.1 to 1 mM and it was observed that none of these treatments affected the life of flowers. Results of ethylene antagonists (STS and AOA) at 0.50 mM concentration are presented in this study. The study was completed under appropriate conditions with a mean temperature of 21 ± 2 °C, 12-h light period/day, and relative humidity of 60 ± 12%. Changes associated with the flower development and senescence were documented on day 2 (D2) and day 4 (D4) of transferring flowers to the respective treatments by assessing various parameters such as flower life, ABA and ethylene content, membrane integrity index (MII), oxidative stress (H_2O_2 content), antioxidant enzyme activities (SOD, CAT, and APX), lipoxygenase (LOX) activity, specific protease activity (SPA), solution uptake, soluble proteins and reducing sugars. The flowers treated with ABA exhibited survival for 3 days. By the fourth day (D4), the ray florets had undergone complete senescence. The data recorded on D4 had values that were too diminished for meaningful analysis; consequently, the ABA-related data were collected only on the second day (D2) of the application of different treatments.

Standardization of Treatments

Before starting the experiment, standardization was carried out using a spectrum of concentrations of GA and ST viz., 25, 50, 100, 150, and 200 µM. ABA and GA were initially dissolved in a few drops of ethanol to facilitate their solubility and the solution was then adjusted to the desired final volume using DW. However, ST was dissolved directly in the DW. The threshold concentrations selected based on the improved life and postharvest characteristics of flowers were GA 150 µM and ST 50 µM.

Quantification of Abscisic Acid (ABA) and Ethylene

ABA was quantified by High-Performance Liquid Chromatography (HPLC) following the protocol as described by Almeida Trapp et al. (2014) with some modifications. The analysis was done in an Agilent HPLC system 1260 infinity series, connected to a quaternary pump, an injection port, and a photodiode array detection (DAD) detector. The petal tissue was macerated in a pre-cooled mortar and pestle in liquid nitrogen (N). 200 mg powder of petal tissue was thoroughly mixed in 10 mL of methanol (HPLC grade). The samples were shaken in a vortex shaker for 3 h, followed by double centrifugation at 12,000*g* for 15 min. The supernatant was taken and the pellet was discarded. Finally, the supernatant was injected into the HPLC system and ABA was quantified by using ABA (purity > 99%) procured from Sigma-Aldrich as standard.

Ethylene was quantified by employing the Williams et al (1995) protocol. Briefly, 1 g of petal tissue collected from various flowers of the same stage was sealed in a 100 mL glass vessel for two hours to release possible ethylene. Subsequently, 1 mL of the gaseous mixture from each vessel was aspirated from the vessels and ethylene was analyzed using a gas chromatograph (Shimadzu Gas Chromatograph) with flame ionization detection. The quantification was accomplished based on a standard curve derived from an external standard gas calibration with certified ethylene gas.

Flower Life and Solution Uptake

Flower life was determined from day 1 of the experiment till flowers displayed senescence symptoms and lost their ornamental value (Lone et al. 2021). The observable symptoms, such as the decline in petal turgidity followed by lateral rolling and upward clustering of petals (ray florets), served as the criteria marking the conclusion of the vase life or ornamental quality of the flowers. Each vial contained 25 mL of solution. The solution uptake was determined by comparing the total volume of the solution with the amount that remained in the vial after the complete senescence of flowers.

Determination of Hydrogen Peroxide (H_2O_2) Content and Membrane Integrity Index (MII)

The H_2O_2 content was assessed by employing the protocol of Alexieva et al. (2001). 500 mg of petal tissue was macerated in 0.1% (w/v) TCA buffer, followed by centrifugation of homogenate at 12,000*g* for 15 min. 0.5 mL supernatant was taken and added with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0), followed by the addition of 1 mL of 1 M KI. Finally, the absorbance of the mixture was detected at 390 nm. The H_2O_2 concentration was determined by a standard curve prepared by known H_2O_2 concentration.

MII was evaluated by observing the electrical conductivity based on electrolyte leakage in the petal tissues (Sairam 1994). 500 mg of petal tissue was incubated in 25 mL of DW at 25 and 100 °C for 30 and 15 min, respectively. The conductivity was detected on the conductivity meter (Elico CM180). The MII was computed by using the formulae:

$$\mathrm{MII} = (1 - C1/C2) \times 100$$

The expressions $C1$ and $C2$ imply the sample conductivities at 25 and 100 °C, respectively.

Determination of Enzyme Activities

Superoxide Dismutase (SOD)

The SOD (EC 1.15.1.1) activity was assessed by following Dhindsa et al. (1981) method. The activity was evaluated by determining the inhibition of enzymatic photochemical reduction of nitroblue tetrazolium (NBT). One unit of SOD activity was defined as the concentration of the enzyme that lowers the absorbance of the reaction mixture by 50% as compared to the reaction mixture without the enzyme. The reaction mixture included 50 mM sodium carbonate, 75 µM NBT, 0.1 M ethylenediamine tetraacetic acid (EDTA), and 13 mM methionine in 50 mM phosphate buffer (pH 7.8). The absorbance of the reaction mixture was recorded at 560 nm.

Catalase (CAT)

Catalase (EC 1.11.1.6) was assayed by measuring the disappearance of H_2O_2, employing the Aebi (1984) protocol. The reaction mixture included potassium phosphate buffer (50 mM) of pH 7.0, 50 µL enzyme extract, and DW making a final volume of 3 mL. Finally, the absorbance of the reaction mixture was monitored at 240 nm.

Ascorbate Peroxidase (APX)

APX (EC 1.11.1.1) activity was assayed by the protocol of Chen and Asada (1989) with slight modifications. The procedure is based on a decrease in absorbance of the reaction mixture at 290 nm brought on by the oxidation of 0.1 mM ascorbate. Besides ascorbate, the reaction mixture comprised 50 mM potassium phosphate buffer and 0.3 mM H_2O_2 maintained at pH 7.0. The absorbance of the reaction mixture was recorded at 290 nm.

Lipoxygenase Activity (LOX)

LOX (EC 1.13.11) activity was assessed by Axelrod et al. (1981) method. 10 µL of petal extract was added to the mixture containing 50 mM Tris–Hydrochloric acid buffer of pH 6.5 and 0.4 mM linoleic acid to commence the reaction. The absorbance of the reaction mixture was observed at 234 nm.

Assessment of Reducing Sugars

For the quantification of reducing sugars, Nelson's (1944) method was employed. An appropriate volume of an aliquot from the alcohol-soluble fraction of the tissue extract was made up to 5 mL with the DW, to which 1 mL of copper (Cu) reagent, (mixture of Cu reagent A and B in the ratio 50:1) was added. The mixture was heated at 100 °C for 20 min. The samples were removed and allowed to cool down. This was followed by adding 1 mL of arsenomolybdate. The volume was increased to 25 mL by adding DW. Finally the absorbance was read at 520 nm.

Assessment of Specific Protease Activity (SPA) and Soluble Proteins

For assessment of SPA, 1 g of chilled petal tissue was homogenized in 15 mL of pre-chilled phosphate buffer (0.1 M) of pH 6.5 in a chilled pestle and mortar. The mixture was squeezed through a fourfold muslin cloth and centrifuged at 5000*g* for 15 min in a refrigerated centrifuge at 5 °C. The supernatant was collected to assess the protease activity by following the protocol of Tayyab and Qamar (1992) with slight modifications. 1 mL of enzyme extract was mixed with 1 mL reaction mixture (0.1% bovine serum albumin in 0.1 M phosphate buffer, pH 6.5). To initiate the reaction, the mixture was incubated at 37 °C for 2 h and was terminated by adding 2 mL of pre-cooled TCA solution with a concentration of 20%. Blanks in which TCA was added before adding the enzyme extract were processed along with each mixture sample. The reaction mixture was centrifuged and subsequently supernatant was taken. Finally, Lowry et al. (1951) protocol using tyrosine as the standard was employed to estimate the free amino acids (as tyrosine equivalents) by utilizing a suitable volume of the supernatant.

For quantification of soluble proteins, 1 g petal tissue was homogenized in 100 mM phosphate buffer (pH 7.2) comprising 150 mM sodium chloride, 1 mM EDTA, 1% triton X-100, 10% glycerol, 10% PVP, and 1 mM dithiothreitol. The mixture was centrifuged at 12,000*g* at 4 °C for 15 min in a pre-cooled refrigerated centrifuge and the supernatant was collected. Following the Lowry et al. (1951) method, an appropriate volume of aliquot was used for the quantification of soluble proteins.

Experiment Design and Data Analysis

During the current study, a completely randomized experimental design was employed. The data were subjected to analysis of variance (ANOVA) to compare treatment means using SPSS (SPSS; version 25). To analyze the changes incurred in various parameters with the progression of flower development, treatment comparisons were computed across days, i.e., between D2 and D4 by two-way ANOVA. The significance between the individual treatments was computed through Duncan's Multi Range Test (DMRT $P < 0.05$). Three biological replicates, each consisting of ray florets (petals) collected from various flowers were employed to analyze each parameter. The study was repeated to test the reproducibility of the experiment by examining the commencement of senescence symptoms and flower life.

Results

Senescence Description

The visible signs of senescence revealed the loss of petal turgidity followed by an in-rolling and upward assemblage of ray florets. The senescent ray florets remained adhered to the capitulum ring without showing abscission from the pedicel. The mean lifespan of the flower after it opens fully was found to be 5 days.

Phytohormonal Analysis (ABA and Ethylene Content)

The ethylene content increased marginally throughout the flower development without exhibiting a significant rise towards the senescent (V) stage. While the petal tissues showed a slight increase in ethylene production on progressing from the fully open (IV) to the senescent (V) stage, this upsurge was, however, observed to be insignificant. On the contrary, the petal tissues showed a significant increase in ABA content with the advancement of flower development from the closed bud (I) to the senescent (V) stage. During the earlier phases, the ABA content remained more or less constant from the closed bud (I) stage to the mature bud (II) stage, subsequently demonstrating a significant increase towards the partially open (III) stage and remaining constant up to the fully open (IV) stage. However, as the flowers progressed from the fully open (IV) stage to the senescent (V) stage, the petal tissues exhibited a significant increase in ABA content, as maximum ABA content was quantified in the senescent petal tissue (Fig. 2).

Hydrogen Peroxide (H_2O_2) Content

The H_2O_2 content remained more or less constant during the bud stages of flower development, thereafter exhibiting a significant increase towards the fully open (IV) stage. However, as the flowers progressed from the fully open (IV) stage to the senescent (V) stage, the petal tissues exhibited a significant increase in H_2O_2 content, as maximum H_2O_2 content was recorded in the petal tissues of the senescent (V) stage (Fig. 3).

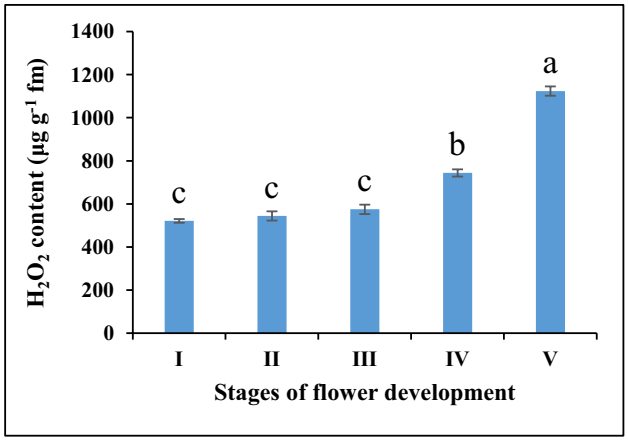
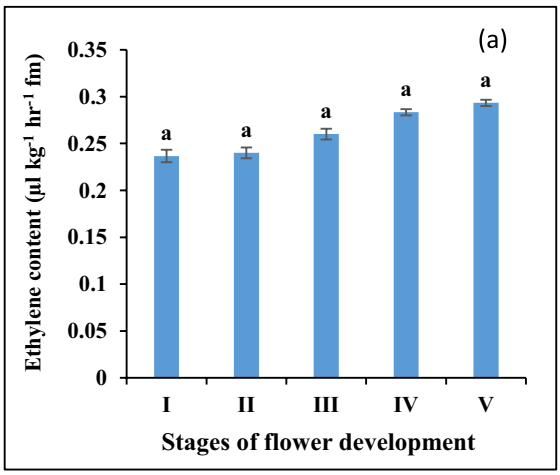


Fig. 3 H_2O_2 content at five different stages of *C. officinalis* viz., closed bud (I), mature bud (II), partially open (III), fully open (IV), and senescent (V) stage. The data infer the mean of three replicates ($n=3$), each repetition contained petals collected from multiple flowers of the same stage. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P<0.05$)



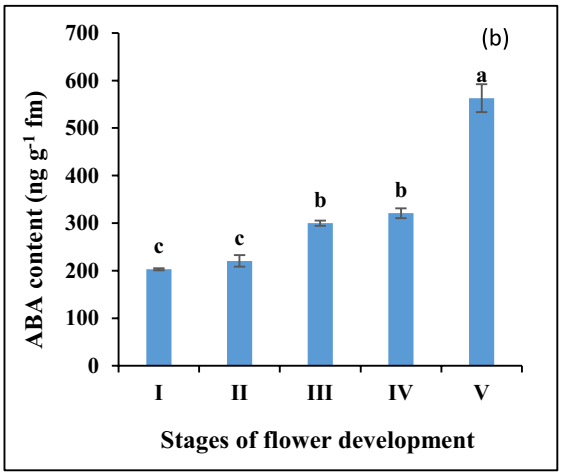


Fig. 2 Ethylene (**a**) and ABA (**b**) content at five different stages of *C. officinalis* viz., closed bud (I), mature bud (II), partially open (III), fully open (IV), and senescent (V) stage. The data infer the mean of three replicates ($n=3$), each repetition contained petals collected from multiple flowers of the same stage. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P<0.05$)

Abscisic Acid (ABA) Content

The ABA content was significantly reduced in the ray florets of flowers supplied with ST. On the contrary, the flowers held in GA registered similar ABA content as those of control flowers held in DW. The ABA content of flowers tested with different treatments including the control was also compared across the days, i.e., between D2 and D4. Interestingly, the ABA content increased significantly from D2 to D4 of the flower development. However, the flowers treated with ST showed a significantly lesser increase in ABA content as compared to those held in DW (control) and GA solution (Fig. 4).

Ethylene Antagonists (AOA and STS)

During the current study, both AOA (ethylene synthesis blocker) and STS (ethylene action blocker) were ineffective in delaying the senescence of flowers. The flowers held in different concentrations of AOA and STS registered the same flower life as those of control (5 days). This suggests the non-involvement of ethylene in the petal senescence of *C. officinalis* (Fig. 5).

Flower Life

The flowers supplied with the ABA showed early senescence and lasted for 3 days. In contrast, the flowers treated with the different concentrations of GA and ST improved the flower longevity significantly. The maximum life was recorded in GA- and ST-treated flowers. The flowers supplied with the ABA + ST and ABA + GA in combination registered a similar life comparable to that of the control. This demonstrates that ABA inhibitors counteracted the ABA-induced onset of senescence in *C. officinalis* (Figs. 6, 7).

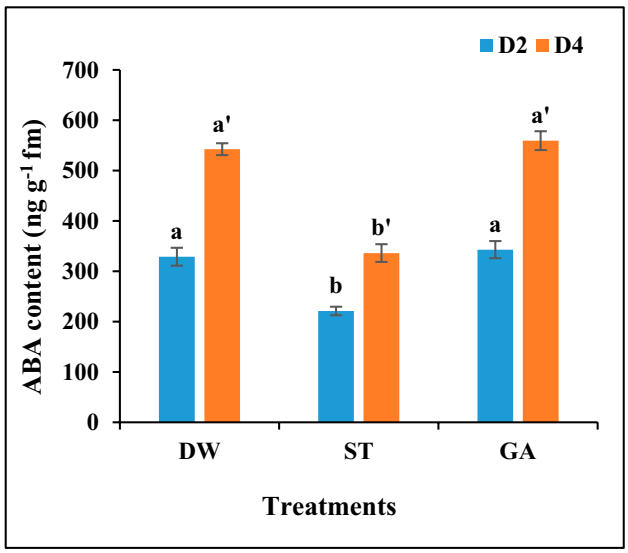


Fig. 4 Variations in ABA content in the ray florets of *C. officinalis* treated with ST (50 µM) and GA (150 µM). The data infer the mean of three replicates ($n = 3$), each repetition contained petals collected from multiple flowers of the same treatment. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

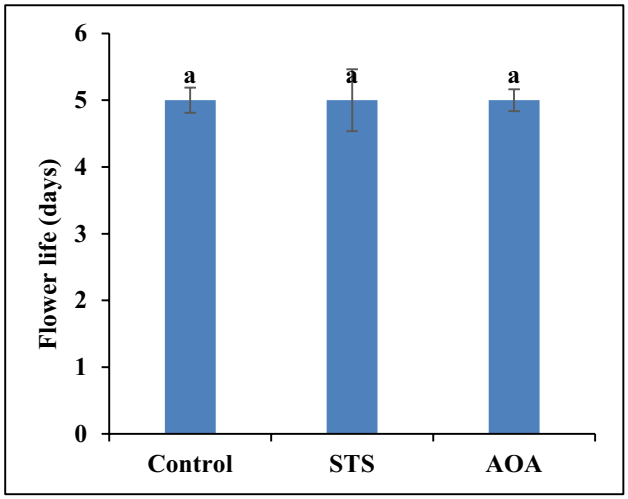


Fig. 5 Flower life of *C. officinalis* treated with STS (0.50 mM) and AOA (0.50 mM) at the partially open (III) stage. The data infer the mean of three replicates ($n = 3$). Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

Solution Uptake

The flowers supplemented with GA and ST registered significantly higher solution uptake as compared to other

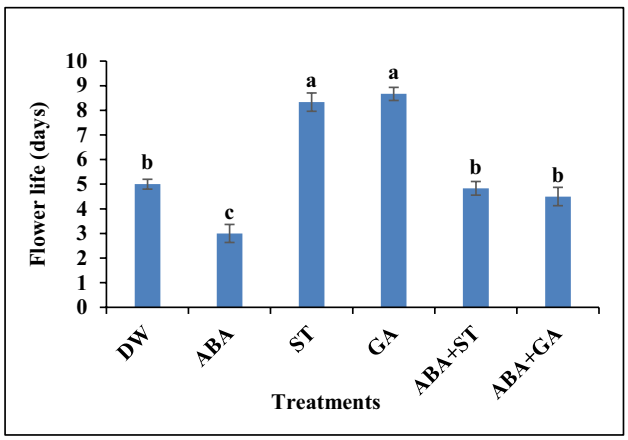


Fig. 6 Flower life of *C. officinalis* treated with ABA (100 µM), ST (50 µM), GA (150 µM), ABA (100 µM) + ST (50 µM), and ABA (100 µM) + GA (150 µM). The data infer the mean of three replicates ($n = 3$). Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

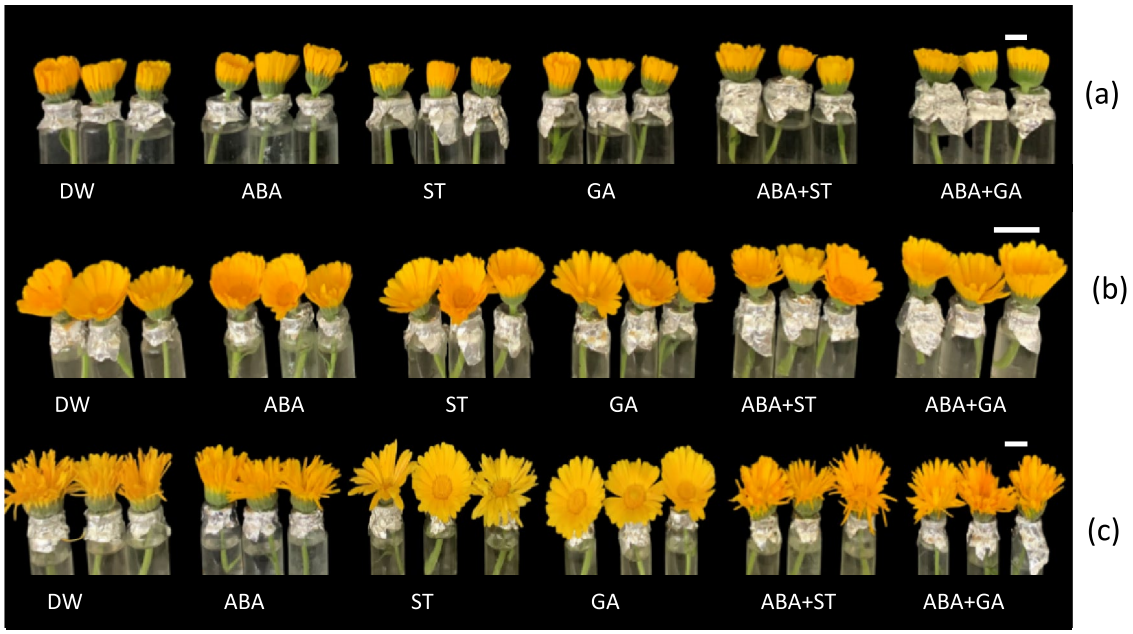


Fig. 7 Apparent changes in the ray florets (petals) of *C. officinalis* with the advancement of flower development from the partially open stage (scale bar = 7 mm) through the fully open stage (scale bar = 25 mm) to the senescent stage (scale bar = 7 mm) on day 0 (**a**), day 1 (**b**), and day 7 (**c**), respectively, after the application of ABA (100 µM), ST (50 µM), and GA (150 µM), ABA (100 µM) + ST (50 µM) and ABA (100 µM) + GA (150 µM)

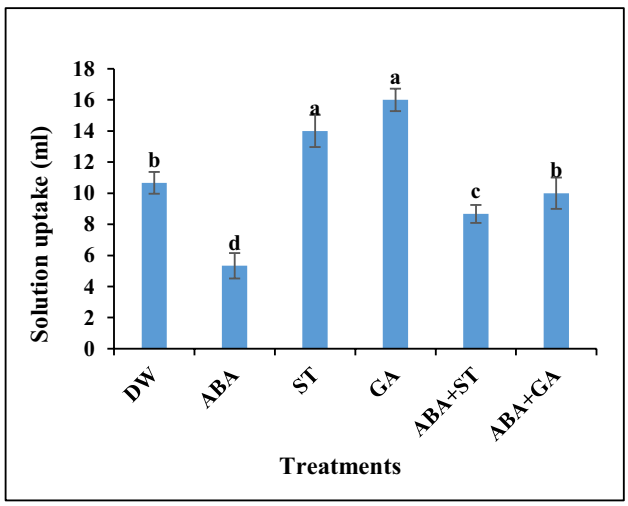


Fig. 8 Solution uptake in the flowers of *C. officinalis* treated with ABA (100 µM), ST (50 µM), GA (150 µM), ABA (100 µM) + ST (50 µM) and ABA (100 µM) + GA (150 µM). The data infer the mean of three replicates (n = 3). Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

treatments. The least uptake was observed in ABA-treated flowers followed by the control. The flowers held in DW (control) and treatment containing ABA + GA in combination exhibited similar solution uptake (Fig. 8).

Lipoxygenase (LOX) Activity and Membrane Integrity Index (MII)

The petal tissues (ray florets) of flowers subjected to 50 µM ST and 150 µM GA manifested a significant reduction in LOX activity as compared to the control. The maximum LOX activity was registered in petal tissues treated with 100 µM ABA. The flowers immersed in DW (control) and the solutions containing the combined concentrations of ABA + ST and ABA + GA registered comparable LOX activity to that of the control. However, the comparisons across the days demonstrated a significant increase in the LOX activity from D2 to D4 of the flower development. A minimal increase in LOX activity was registered in the petal tissues supplied with ST and GA. Notably, the flowers supplemented with ST and GA demonstrated significantly improved MII in floret tissues. The flowers treated with ABA showed the least MII followed by the control. The flowers held in solutions containing the combined concentrations of ABA + ST and ABA + GA demonstrated comparable MII to that of the control. However, when compared across the days, the MII was found to decrease significantly from D2 to D4 of the flower development with the least decrease in GA- and ST-treated flowers (Fig. 9a, b).

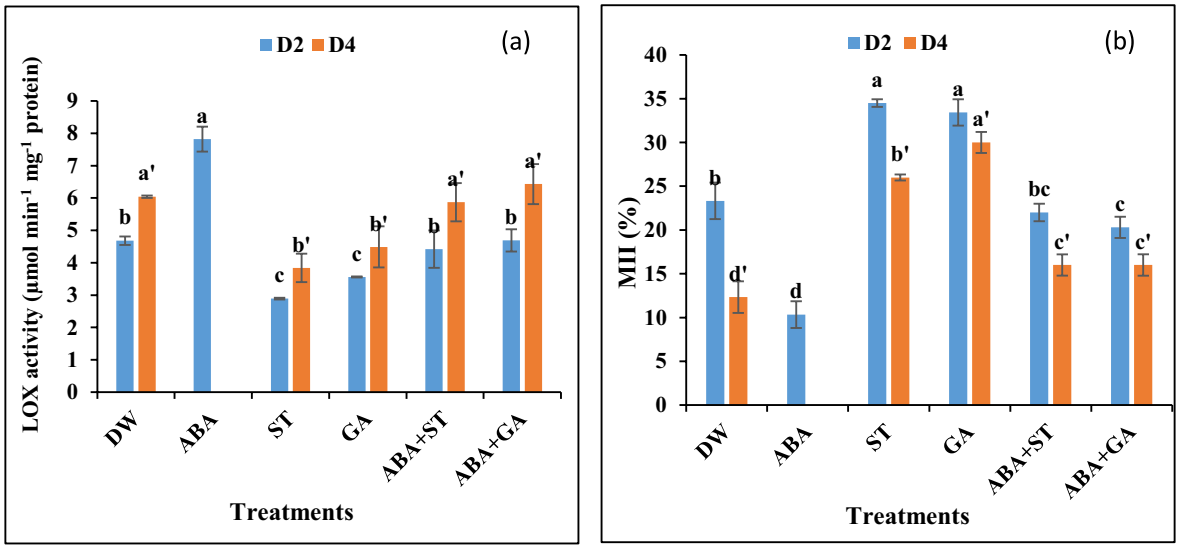


Fig. 9 Variations in LOX activity (**a**) and MII (**b**) in the ray florets of *C. officinalis* treated with ABA (100 µM), ST (50 µM), GA (150 µM), ABA (100 µM) + ST (50 µM), and ABA (100 µM) + GA (150 µM). The flowers treated with ABA (100 µM) survived only for 3 days, therefore, the data pertaining to this treatment were recorded only on D2 of the experiment. The data infer the mean of three replicates ($n = 3$), each repetition contained petals collected from multiple flowers of the same treatment. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

Soluble Proteins and Specific Protease Activity (SPA)

The flowers supplemented with ST and GA recorded significantly higher content of soluble proteins. A minimal protein content was recorded in the ABA-treated flowers. The flowers treated with the solutions that combined ABA + GA showed insignificant differences from the control. However, treatment comparisons across the days revealed a significant decrease in the soluble protein content from D2 to D4 of the flower development. On the other hand, the flowers treated with ST and GA showed a significant reduction in SPA. The highest SPA was documented in the flowers supplied with ABA. Flowers held in solutions with the combined concentrations of ABA + ST and ABA + GA recorded similar protein and SPA patterns to that of the control. In contrast to soluble proteins, the SPA demonstrated a significant increase from D2 to D4 of the flower development (Fig. 10 a, b).

Reducing Sugars and Hydrogen Peroxide (H_2O_2) Content

The flowers treated with GA and ST recorded significantly higher content of reducing sugars. The least sugar content was observed in ray florets treated with ABA. The sugar content, however, decreased significantly from D2 to D4 of the flower development. The flowers treated with combined solutions of ABA + GA and ABA + ST recorded comparable reducing sugar content patterns to that of the control. On the contrary, the flowers treated with GA and ST recorded lesser H_2O_2 content in the ray florets. GA was relatively more effective in preventing H_2O_2 accumulation followed by ST. The maximum H_2O_2 content was recorded in ABA-treated flowers followed by the control. The ray florets treated with the combined application of ABA + GA showed relatively reduced H_2O_2 content than those held in ABA + ST which recorded an insignificant difference from the control. However, the ray florets demonstrated a significant increase in H_2O_2 content from D2 to D4 of the flower development, with flowers treated with GA demonstrating a lesser increase followed by ST as compared to other treatments (Fig. 11 a, b).

Antioxidant Enzyme Activities

The SOD, CAT, and APX activities were significantly amplified in the ray florets of flowers treated with the GA and ST. GA was shown to be the most effective treatment for enhancing the CAT and APX activities, while SOD activity was equally increased by both GA and ST. On the other hand, the flowers treated with ABA registered reduced activities of these enzymes as compared to the control and other treatments. However, comparisons across the days revealed a significant decline in the activities of these enzymes from D2 to D4 of the flower development with a minimal decrease in GA- and ST-supplied flowers (Fig. 12a–c).

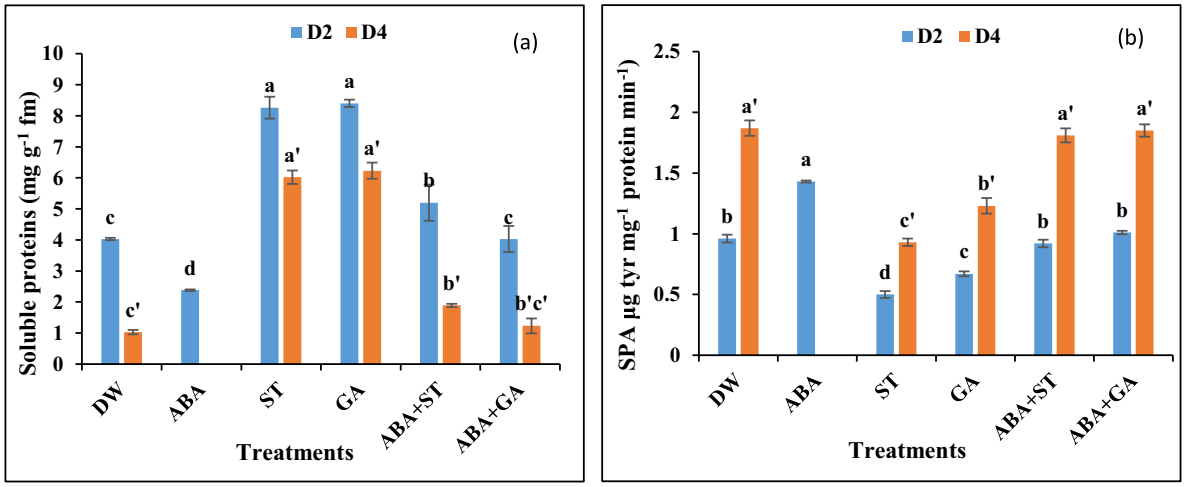


Fig. 10 Variations in soluble proteins (**a**) and SPA (**b**) in the ray florets of *C. officinalis* treated with ABA (100 µM), ST (50 µM), GA (150 µM), ABA (100 µM) + ST (50 µM), and ABA (100 µM) + GA (150 µM). The flowers treated with ABA (100 µM) survived only for 3 days, therefore, the data pertaining to this treatment were recorded only on D2 of the experiment. The data infer the mean of three replicates ($n = 3$), each repetition contained petals collected from multiple flowers of the same treatment. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

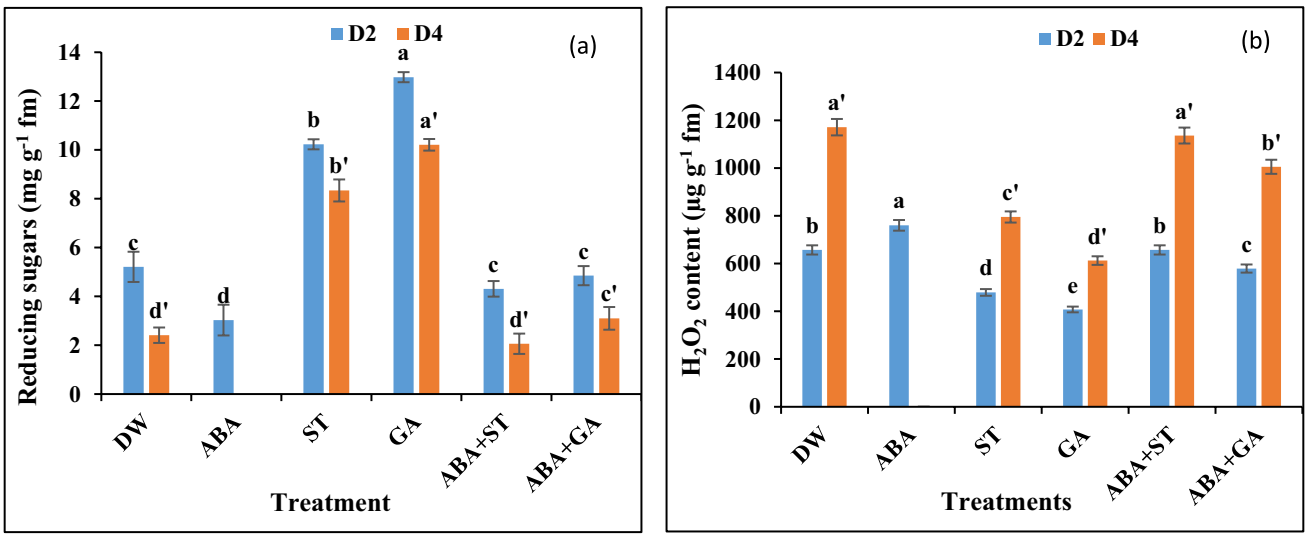


Fig. 11 Variations in reducing sugars (**a**) and H_2O_2 content (**b**) in the ray florets of *C. officinalis* treated with ABA (100 µM), ST (50 µM), GA (150 µM), ABA (100 µM) + ST (50 µM), and ABA (100 µM) + GA (150 µM). The flowers treated with ABA (100 µM) survived only for 3 days, therefore, the data pertaining to this treatment were recorded only on D2 of the experiment. The data infer the mean of three replicates ($n = 3$), each repetition contained petals collected from multiple flowers of the same treatment. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

Discussion

During the current investigation, no climacteric rise of ethylene was recorded in *C. officinalis* with the progression of flower development towards senescence. Additionally, the ethylene inhibitors (STS and AOA) were ineffective in delaying its senescence, suggesting that ethylene does not seem to play a major role in stimulating the senescence of this flower. In contrast, typically, ethylene-sensitive flowers manifest ethylene production throughout various developmental stages, including early, blooming, and senescence phases. However, such flowers exhibit an abrupt increase in ethylene production and a subsequent rise in respiration at the onset of petal senescence, hence

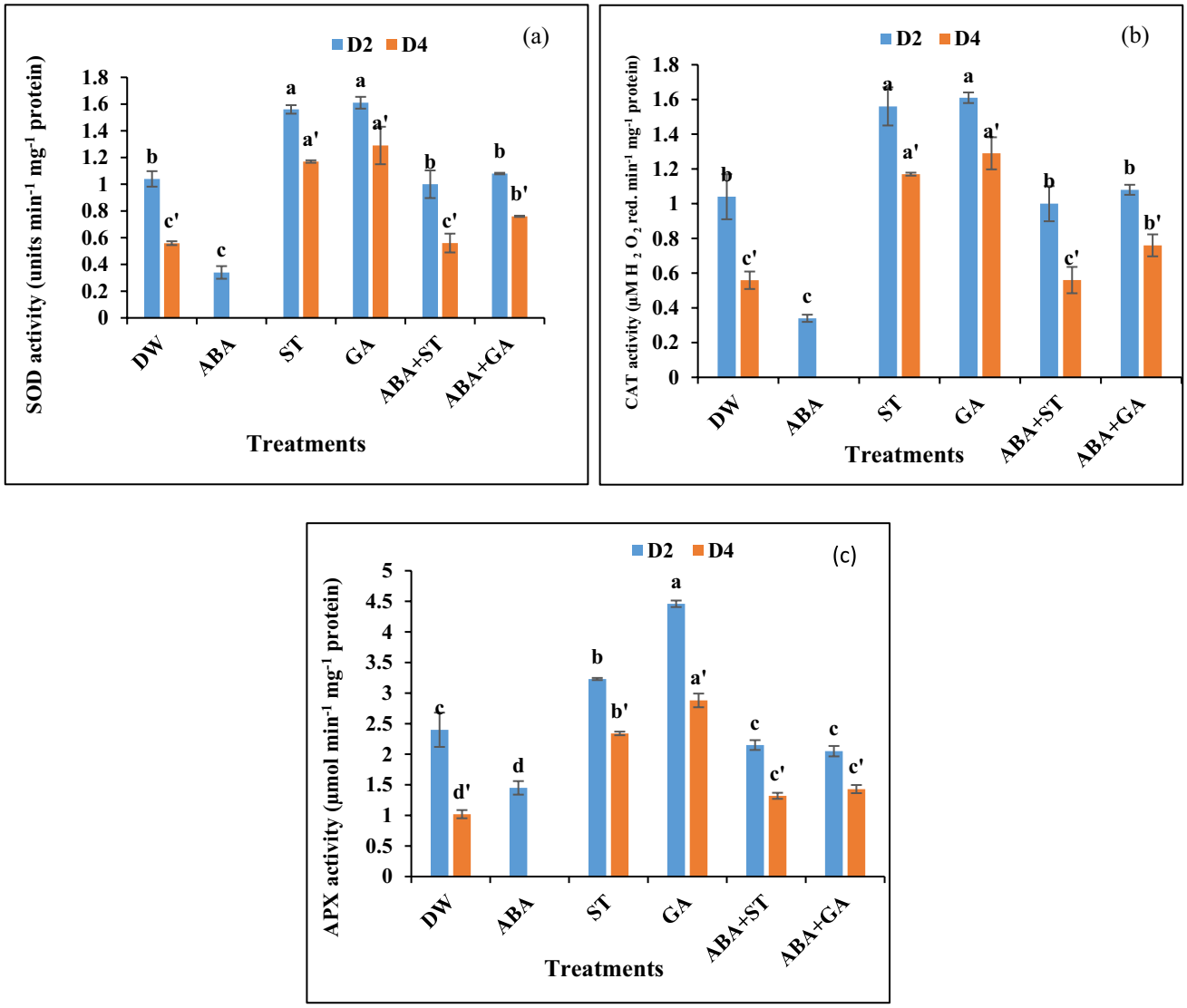


Fig. 12 Variations in SOD (**a**), CAT (**b**), and APX (**c**) activities in the ray florets of *C. officinalis* treated with ABA (100 µM), ST (50 µM), GA (150 µM), ABA (100 µM) + ST (50 µM), and ABA (100 µM) + GA (150 µM). The flowers treated with ABA (100 µM) survived only for 3 days, therefore, the data pertaining to this treatment were recorded only on D2 of the experiment. The data infer the mean of three replicates ($n = 3$), each repetition contained petals collected from multiple flowers of the same treatment. Letters on the bars specify the ± SE (standard error). Bars with distinct letters show significant differences ($P < 0.05$)

categorized as ethylene-sensitive flowers (van Doorn and Woltering 2008; Naing et al. 2021). A sharp increase in ABA content was registered in the ray florets from the fully open (III) stage to the senescent (V) stage, initiating flower senescence in *C. officinalis*. ST effectively reduced the ABA content in the ray florets thereby delaying its senescence significantly. ST has been supplemented in certain fruits such as *Citrus* which significantly prevented ABA accumulation (Cowan and Richardson 1993). This chemical inhibits the molybdenum-requiring enzyme, ABA aldehyde oxidase that catalyzes the last step of ABA biogenesis.

Previous studies, aligning with the results of the current research, demonstrated that ABA accelerates the mechanisms resulting in increased rates of lipid peroxidation and activation of proteases that lead to the decline in membrane stability of the flower tissues (Panavas et al. 1999; Saeed et al. 2014). ABA, as a developmental factor, activates an ABA signaling regulator; *PP2C* (protein phosphatase 2C), which possibly turns on the pathway for "death protein" synthesis and activates hydrolytic enzymes like proteases and proteinases that cause PCD and floral senescence (Zhong and Ciafré 2011). The role of ABA in initiating senescence has been reported in several species, such as

Narcissus, *Hemerocallis*, and *Lilium* (Panavas et al. 1999; Hunter et al. 2004a, b; Arrom and Munné-Bosch 2012a, b; Shibuya 2012). Additionally, the senescent leaves of *A. thaliana* exhibited a substantial upsurge in the expression of genes involved in ABA synthesis (Buchanan-Wollaston et al. 2005). The role of ABA in regulating senescence is not limited to flowers and leaves only, it is also discussed in fruit ripening. For instance, in woodland strawberries, intertwined regulatory loops of ABA synthesis and catabolism were found to regulate fruit growth and ripening (Liao et al. 2018). The fruit ripening was discovered to be accompanied by a decline in both GA and auxin levels.

During the present study, flowers treated with ABA registered a lower content of reducing sugars, accredited to ABA-induced downregulation of invertase and the coordination of source–sink communication in ray florets (Jin et al. 2009; Sane and Khan 2013; Thomas and Ougham 2014). Minimal protein content in ray florets treated with the ABA may be explained by high protein breakdown brought on by high SPA. ABA has been demonstrated to increase the expression of SAGs including *SAG113* (Zhang and Gan 2012), which encodes cysteine proteases. Such mechanisms imply a conflict between initiating the cell death and maintaining cellular functions active enough to allow efficient remobilization in the flower tissues signifying a regulated mechanism of PCD (Rogers 2013).

H_2O_2 can interplay with PGRs such as ABA and ethylene and may alter their role during plant development and senescence (Panavas and Rubinstein 1998; Jajic et al. 2015; Rogers and Munné-Bosch 2016; Zeng et al. 2017; Kong et al. 2018). High H_2O_2 levels at the senescent stage could be attributed to ABA-induced accretion of apoplast ROS (Xia et al. 2015; Qiu et al. 2021). Pertinently, the excessive accumulation of ROS such as H_2O_2 causes oxidative stress, hypothesized as a key elicitor of senescence in ethylene-insensitive flowers such as *Hemerocallis*, *Iris*, and *Chrysanthemum* (Chakrabarty et al. 2007; Rahmani et al. 2015; Ahmad and Tahir 2018). ABA also increased the LOX activity in the ray florets. An increase in LOX activity concomitant with the reduction in SOD, CAT, and APX activities is associated with the senescence of *Iris*, *Gladiolus*, and *Hemerocallis* (Panavas and Rubinstein 1998; Hossain et al. 2006; Ahmad and Tahir 2018). LOX is known to destabilize the membrane integrity by inflicting peroxidative damage on membrane phospholipids (Fukuchi-Mizutani et al. 2000).

In the current investigation, GA significantly prolonged the life of *C. officinalis*. GA, when given separately elevated the antioxidant enzyme activities and improved solution uptake and MII, besides maintaining low H_2O_2 levels in the ray florets. Interestingly, comparable results were noted in *Gerbera*, exhibiting decreased GA levels towards senescence (Emongor 2004; Ge et al. 2019; Li et al. 2022). It is noteworthy that flowers supplemented additively with ABA and GA registered comparable flower life as those held in DW. This demonstrates that GA counteracted the senescence effects of the ABA. GA has been shown to delay senescence in other flowers such as *Hemerocallis, Gladiolus*, and *Iris*, which appears to be associated with a reduction in the activity of endogenous ABA (Hunter et al. 2004b; van Doorn and Woltering 2008). GA and ST enhance the membrane stability of plant tissues, potentially by preserving elevated water levels, fortifying antioxidant defenses, and reducing peroxidation rates, all of which are crucial processes affecting senescence (Kumar et al. 2014; Saeed et al. 2014). An upregulated activity of SOD, CAT, and APX enzymes was registered in the ray florets treated with GA and ST. Consistent with the current finding, GA significantly delayed ABA-induced flower senescence in *Chrysanthemum, Gladiolus*, and *Narcissus* (Hunter et al. 2004a, b; Li et al. 2015; Costa et al. 2016). The association of high antioxidant enzyme activity and flower life in response to GA was reported in other flowers such as gerbera, carnations, gladiolus, red dragon, and iris (Saeed et al. 2014; Dwivedi et al. 2016; Ahmad and Tahir 2018; Naing et al. 2017; Hemati et al. 2019). In *Nicotiana*, GA delayed the senescence by improving the membrane stability and antioxidant system, besides amending the nutritional status of petals (Tahir et al. 2018). The inhibition of ABA and GA biosynthesis, respectively, with fluoridone and paclobutrazol resulted in the reverse effects of these growth regulators in orchestrating senescence in *Gerbera* (Li et al. 2015).

GA increases the cell membrane permeability and due to its solute nature reduces the water potential of the sap, which results in enhanced water uptake (Emamverdian et al. 2020). GA diminished the lipid peroxidation in *Gladiolus* and enhanced cell membrane stability, besides inhibiting microbial growth (Singh et al. 2008). The minimal uptake of solution in untreated flowers might induce water stress, triggering the production of ABA, and consequently causing early senescence. GA maintained high protein content in ray florets of *Calendula* by reducing SPA as was found in *Nicotiana* (Tahir et al. 2018). Besides, it has been discovered that GA induces extracellular invertase, which is involved in carbohydrate partitioning, phloem unloading, and growth of sink tissues (Iqbal et al. 2011). GA activates fructose-1,6-bisphosphatase and sucrose phosphate synthase inducing phloem loading. These findings advocate the antagonistic behavior between GA and ABA in regulating protease activity and source–sink relationship during flower development.

During the current study, GA alleviated the oxidative stress in ray florets by ameliorating the antioxidant enzyme activities of SOD, APX, and CAT as opposed to ABA-treated ray florets which registered reduced activities of these enzymes. As stated, the application of GA in *Gladiolus* and *Nicotiana* reduced the peroxidation rates of lipids and increased the activity of antioxidant

enzymes, thereby improving their postharvest life (Singh et al. 2008; Saeed et al. 2014; Tahir et al. 2018). GA can interact with ROS, indirectly by amending the antioxidant system such as SOD, CAT, APX, and polyphenol oxidase activities together called "ROS processing systems." This mechanism maintains the intracellular concentration of ROS at non-toxic levels (Rosenwasser et al. 2010; Aziz et al. 2020; Rosenwasser et al. 2010; Zhu et al 2019; Ahmad et al. 2021; do Nascimento Simões et al. 2018). The higher antioxidant potential obliterates oxidative stress in floral tissues and diminishes ion leakage by restraining the oxidation of fatty acids (Ahmad et al. 2021).

Conclusions and Future Outlook

The key finding of the current investigation established that *Calendula officinalis* is an ethylene-insensitive flower. The increase in ABA content, elevated oxidative stress, loss of cell membrane stability, and attenuation of the antioxidant system were the major events that drive petal senescence in *Calendula officinalis*. Furthermore, our study elucidated the antagonistic administration between GA and ABA in regulating flower senescence. GA and ST combatively counteracted the ABA-induced physiological and biochemical events. Our study suggests that *Calendula officinalis* offers a considerable scope for studying PCD and as such could open new vistas for integrating hormonal signaling with senescence in composite systems of biochemically and molecularly less researched flowers.

Even though great progress has been made in expounding the underlying program of GA signaling and its crosstalk with other phytohormones like ABA, the exact molecular mechanism by which GA orchestrates senescence is still unknown. ROS are emerging as crucial signaling molecules in plant development which function as secondary messengers in collaboration with classical phytohormones. Therefore, it will be interesting to explore how future research on hormonal interaction with ROS may contribute to a better understanding of developmental processes like floral senescence.

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Author Contributions MLL: Literature survey and drafting of the manuscript; SF, AUH, SP, and FA: literature survey and reviewing; IT: Editing and reviewing.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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