

Senescence Associated Genes Modulating Programmed Cell Death in Plant Leaves

Allah Jurio Khaskheli^{1*}, Muharam Ali¹, Muhammad Ibrahim Khaskheli², Niaz Ahmed Wahocho³, Shahla Baloch¹, and Juang Hong Li⁴

¹Department of Biotechnology, Sindh Agriculture University, TandoJam-70060, Pakistan

²Department of Plant Pathology, Sindh Agriculture University Tandojam-70060, Pakistan

³Department of Horticulture, Sindh Agriculture University Tandojam-70060, Pakistan

⁴Collage of Agriculture and Biotechnology, China Agriculture University, Beijing, P.R. China

*Corresponding Author Email: allahjurio@sau.edu.pk

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ABSTRACT

The aim of this study was to evaluate the effects of various promoters and inhibitors of leaf senescence in *Rosa hybrida* by applying different concentrations of plant hormones under controlled tissue culture conditions. The study sought to identify hormonal treatments that can delay or accelerate senescence, with the goal of improving the postharvest quality and longevity of rose foliage. The plants were grown by tissue culture techniques, which were free from disease. Regenerated plants with well-defined leaves, were infiltrated with hormones and the untreated plant leaves used as control. After the infiltration, all plants were kept under growth conditions at 25±2°C and phenotypical characteristics were observed regularly. Further, the round discs of rose leaves were prepared from two opposite of same sized leaves collected from tissue cultured plants and discs of same size were made. Molecular studies were performed on discs of leaves for further clarification involved regulatory mechanism. The results showed various narrative phenotypes with elevated level of transcripts. The phenotype was characterized by yellowing of leaves, observed in both treated and untreated samples. Notably, leaves treated with Ethylene, ABA, and NaCl exhibited more pronounced yellowing, particularly in the lower regions of the plants, accompanied by a reduced number of tillers at the plant base. To assess the senescence progression level, key markers gene expression of *SAG12* (a senescence-associated gene) and *photosynthesis-associated genes (PS)*, as well as content of chlorophyll and leakage of ions were analyzed. The results highlighted that hormonal treatments play a significant role in regulating leaf senescence. Promoter treatments accelerated the onset of senescence and reduced chlorophyll content, while inhibitor treatments preserved chlorophyll levels and delayed the senescence process. Furthermore, photosynthesis, essential for plant growth and development, was significantly disrupted in promoter-treated leaves, underscoring the pivotal influence of hormonal regulation on senescence

KEYWORDS: Ethylene, Gene Expression, Inhibition, Promoter, Senescence.

INTRODUCTION

Leaf senescence is a complex process in which leaf cells undergo aging in response to various internal and external signals. Though, this response enhances the coordinated senescence to overall vigor of plants by integrating environmental and physiological conditions within a specific ecological condition. However, by modifying the timing of onset, progression rate, and characteristics of senescence, the plants optimize their adaptability. Environmental factors influencing leaf senescence encompass both abiotic and biotic elements (Lim *et al.* 2005).

The knocking out of specific genes plays a significant role in the senescence of various plant organs including fruits, flowers and leaves. Further, leaf senescence is an active process characterized by hundreds of differential gene expressions. It is believed that various transcription factors act as key components of the regulatory network controlling this process.

Although, genome-wide studies of gene expression identified numerous *Arabidopsis* genes encoding transcription factors that are upregulated during the process of leaf senescence (Chen *et al.*, 2002; Guo *et al.*, 2004; Lin and Wu, 2004; Buchanan-Wollaston *et al.*, 2005; Balazadeh *et al.*, 2008). Whereas, by analyzing the senescence phenotypes of knockout mutants only a few transcription factors among these have been demonstrated to be involved in regulating the leaf senescence and by identifying downstream target genes.

During senescence, the expression of *photosynthesis-associated genes (PAGs)* are downregulated, while numerous *senescence-associated genes (SAGs)* are upregulated. In-depth studies on the identities and expression patterns of SAGs reveal that leaf senescence is regulated by highly intricate regulatory network (Hensel *et al.*, 1993; Gan and Amasino, 1997; Nam, 1997; Buchanan-Wollaston,

2005). In *Arabidopsis* plant, leaf longevity of individual leaves determined by the age of plant and its prominent role showing in determining the senescence specific leaves area (Hensel *et al.*, 1993; Oh *et al.*, 1997; Nooden and Penney, 2001; Jing and Weiss, 2002), further plant longevity also initiate the influence on floral and whole-plant senescence (Levey and Wingler, 2005). In present study, we tried to understand the mechanism involved in leave senescence associated with different genes and phenotype involvement, we also investigated the specific role of senescence inhibitors and promoter regulating hormonal efficiencies in response to aging of leaves.

MATERIAL AND METHODS

Planting materials and growth circumstances:

Initially, the growing buds of young rose (*Rosa* hybrid sp.) plant were grown by tissue culture techniques, which were free from disease. Plants were kept for two months under optimum growth conditions; about one month on shooting media (MS Basal supplemented with 2mg/L of BAP, 0.25mg/L of IAA and 1.5 mg/L of GA3) and one month on rooting media (1/2 MS Basal supplemented with IAA), as they become ready for infiltration. When the plant becomes ready, possessed proper root with well-defined leaves, they were infected with hormones and the untreated plant leaves were used as control. After the infection, all plants were kept under growth conditions at 25±2°C and phenotypical characteristics were observed regularly. Further, the round discs of rose leaves were prepared from two opposite of same sized leaves collected from tissue cultured plants and discs of same size were made.

Preparation of different concentrations of ABA: 50mM, 100mM and 150mM of ABA were composited and measured in culture bottle, mixed the solution with quantity of sterilized double distilled water (SDDW). The dissolved solution was relocated to 250ml of volumetric flask and the final volume of the solution was made up to the mark with sterilized distilled water. The solution was stored in the refrigerator for further experimentation.

Preparation of different concentration of Ethylene: In order to make different concentrations of ACC (ethylene), 5mM, 7mM and 10mM were measured and mixed into 200ml of distilled water.

Preparation of different concentrations of NaCl: 50mM, 100mM and 150mM of NaCl were measured and transferred to culture bottle, mixed the solution in sterilized distilled water. 250ml of volumetric flask was used and transferred the mixed solution in it, volume was made up to mark with remain sterilized distilled water. The solution was stored in the refrigerator for further experimentation.

Preparation of different concentrations of BAP: 50mM, 100mM and 150mM of BAP were composited and measured in culture bottle, mixed the solution with quantity of sterilized double distilled water (SDDW). The dissolved solution was relocated to 250ml of

volumetric flask and the final volume of the solution was made up to the mark with sterilized distilled water. The solution was stored in the refrigerator for further experimentation.

Ion leakage measurement: In order to measure the leakage of ion in leaves, avoiding the central midrib, approximate 7-9 mm of discs were made using the middle part of both outer and inner leaves. The prepared discs were transferred and placed in distilled water and washed by shaking for one hour to remove ions from the surface. Subsequently, 30 discs were placed in each culture plate containing 10ml of sterilized double distilled water. Plates were placed in the growth chamber under controlled condition. After 24 hours interval, the aqueous solution was collected and measured the conductivity through conductivity meter (Guo *et al.*, 2004).

Measurement of chlorophyll content: Fresh leaves of tissue cultured rose plants were processed immediately after collection. The collected leaves were chopped into fine pieces and 0.5g was measured using an analytical balance. 10ml of 80% acetone was added in measured material and standardized it using homogenizer. The extract was obtained and diluted using 9 ml of 80% acetone. The spectrophotometer performed to measure the content of chlorophyll in pigment of leaves (Guo *et al.*, 2004).

Extraction of DNA and Semi-quantitative PCR: For seme-quantitative PCR (sqPCR) analyses, about one microgram of total RNA was used to synthesize complimentary DNA (cDNA) by using Powerscript reverse transcriptase (Clontech, CA, USA) kit by following the manufacturer's procedures. PCR reaction was carried out in a 25µl volume containing 1µL cDNA, 2.0µL of 10mM dNTP, 2.5µL PCR Buffer 1µL each primer (Table 1.), 0.25 µL Taq DNA polymerase. The PCR reaction was cycled in a GeneAmp PCR system 2720 machine (Applied Biosystems). The PCR reactions were carried out by following cycle as: initial denaturation at 94 °C for 5 min and 33 cycles of 30s at 94 °C, 30 s at 57 °C, 45s at 72°C followed by incubation at 72°C for 5 min (Hee *et al.*, 2010).

Statistical analysis: The results were analyzed statistically according to designs of analysis of variance. The obtained average means of various treatment were associated by Least Significant Difference (LSD) @ 5% level of probability. Student edition software package 8.1 was used in analyzing the data.

RESULTS

Hormonal efficiency regulates the leaves senescence activity: In preliminary stage, the ethylene competency with different concentrations on the senescence of rose leaves was tested. At every two days interval, phenotypic analysis of discs was measured throughout the entire experiment. In the beginning stage after infiltrations, a very slight yellowing of leaves was observed. In contrast leaves treated with normal water, we further found and noticed that using

ethylene hormonal interpretation; the senescence approach was accelerated outstandingly and increased significantly the yellowing of leaves and fading the green. After one week (10-15days) of infiltration, the discs of leaves becoming yellow and high faded were observed in ethylene treated leaves, instead of using distilled water, where impartial slightly reduced and declined the senescence activity (Figure 1). Further, to confirm it deep controlled mechanism regulating senescence, deviations happened through gene

expression, chlorophyll content, ion leakage measurement, the *RhSAG12* gene for expression as markers for senescence associated was measured and assessed. Interestingly, exceptional changes we found significantly in ethylene promote leaves senescence as compared to that of all concentrations. Likewise, ion leakage and expression of *RhSAG12* gene increased promptly (Figure 6) and decreased chlorophyll concentration was observed during senescence of leaves.

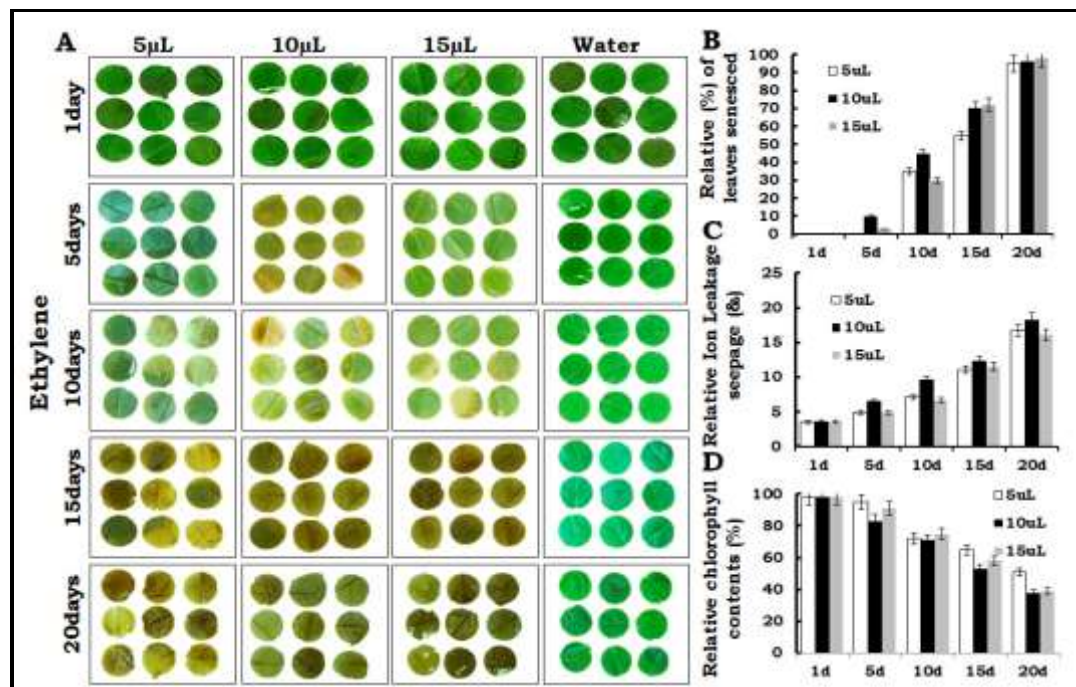


Figure 1. Effect of different ethylene concentrations on discs of rose leaves. (A) Phenotypic analysis of affected leaves. (B) Encountering the number of leaves senesced. (C) Ion leakage measurement. (D) Chlorophyll content measurements from leaf discs. Error bar on column denotes the standard deviation (SD) of the mean average from for the date of three different replications.

ABA signaling suppresses the leaves senescence:

The efficiency of ABA on leaves senescence approach for rose plant was also tested. The senescence in ABA treated leaves started early, usually the yellow shading was initiated after 5-10 days of infiltration, compared to that of leaves infiltrated with water. Furthermore, in the beginning days of infiltrations a slight yellowing of leaves was observed. However, after 20 days we had found that during ABA phenomenal senescence most of the leaves became yellow.

Besides to that, leaves placed in normal distilled water, the hormonal regulations and concurrence for senescence was accelerated exceptionally and increased significantly yellowing process of leaves. Subsequently the treated disc of leaves flattered to yellow after 8 to 10 days of infiltration, higher faded was observed in ABA treated leaf senescence. Whereas, leaves flattered rapidly to yellow till 20 days, rather than in distilled water, where leaves consistently stayed green till 15 days (Figure 2). Moreover, these

changes were confirmed by measuring the variation occurred in gene expression *RhSAG12* (a senescence associated gene), decline of ion leakage and increase/decrease of chlorophyll content for senescence progression (Figure 5-6). Surprisingly, diverse results we found as compared to that sampled without treated (control) using normal sterilized distilled water. Likewise, sudden decline in content of chlorophyll and strengthen of gene expression (*RhSAG12*) and persistence increase of ion leakage were observed throughout the ABA treated senescence of leaves. The final stage of aging, specialty in flower development and leaves growth is the process of senescence that ultimately proceeds with the execution of any floral organ of plant. Reason that senescence is natural process controlled and regulated by endogenous signaling at development stages, meaning this process may lead us to gain in deep understanding involved controlled mechanism specifically for promoting the approach of leaves senescence

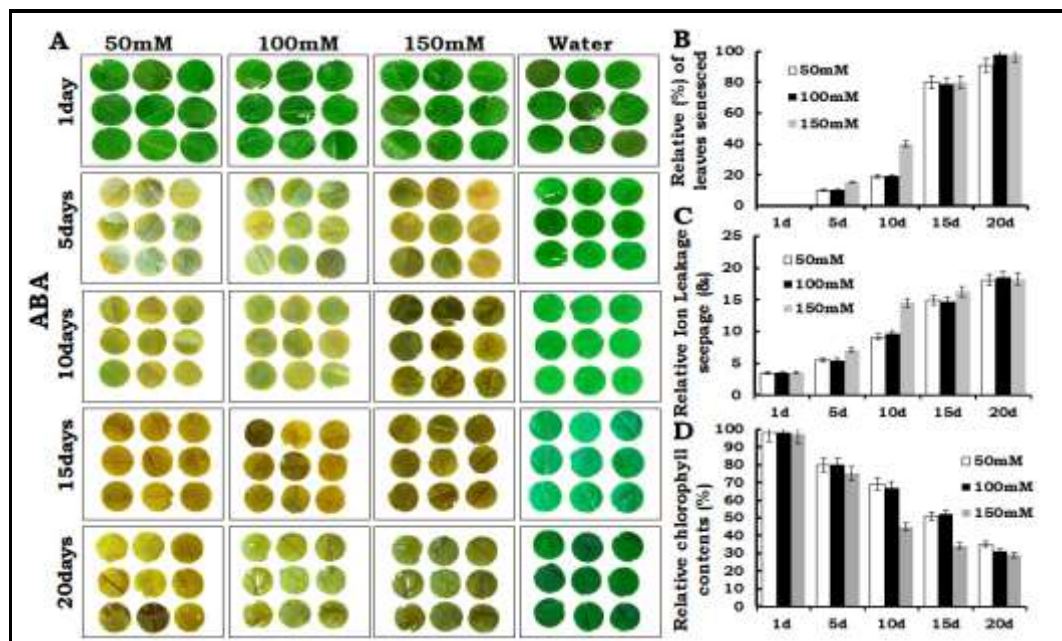


Figure 2. Effect of different ABA concentrations on discs of rose leaves. (A) Phenotypic analysis of affected leaves. (B) Encountering the number of leaves senesced. (C) Ion leakage measurement. (D) Chlorophyll content measurements from leaf discs. Error bar on column denotes the standard deviation (SD) of the mean average from for the date of three different replications.

Senescence prompter slightly inhibits the leaves senescence: Further, in order to understand the mechanism of inhibitor in delaying the senescence we assessed some hormones which are the inhibitor of senescence such as 6-BA. It was noticeably observed that the content of chlorophyll of treated leaves under 6BA as well as normal distilled water treated leaves were elaborated significantly (Figure 4). 6-BA treated leaves showed constant content of chlorophyll almost throughout the experimental time interval. Whereas, as

compared to natural process of innate senescence, the inhibitor of senescence also showed same concentration of chlorophyll. In this regard in order to confirm it further, the *PAGs* photosynthetic genes expression was performed and noticed the higher expression was notably shown in senescence occur under the treatment of inhibitor. While slightly lower expression of *PAGs* in innate and higher expression levels of *SAG12* observed significantly in senesced leaves (Figure 6 and 7).

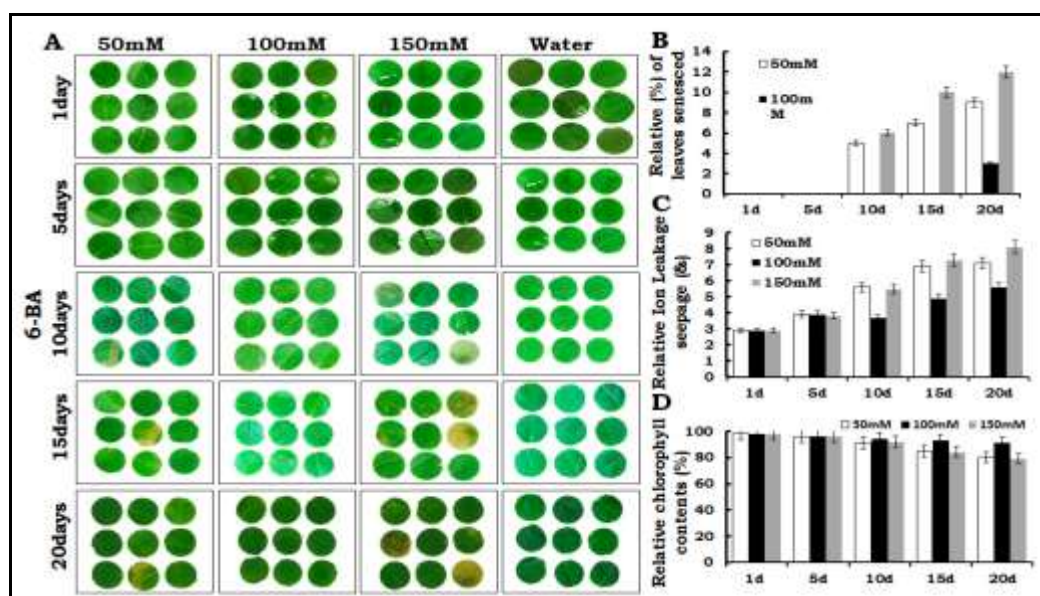


Figure 4. Effect of different 6-BA concentrations on discs of rose leaves. (A) Phenotypic analysis of affected leaves. (B) Encountering the number of leaves senesced. (C) Ion leakage measurement. (D) Chlorophyll content measurements from leaf discs. Error bar on column denotes the standard deviation (SD) of the mean average from for the date of three different replications.

Effectiveness of NaCl and sucrose efficiency on regulation of leaves senescence: It has already been observed and discussed that leaf senescence is a highly complex process involving multiple functions that are regulated by both endogenous and exogenous signals throughout the life cycle of the leaf. However, it is fascinating how senescence is controlled by various inhibitors of regulation. Additionally, leaf senescence is not a single event but consists of continuous, time-dependent transitions in cellular physiology and metabolism. As a result, it is essential to understand senescence from a dynamic perspective. Therefore, in the present study, we observed unexpected findings such NaCl-treated leaves exhibited significantly earlier senescence than those of treated with normal distilled water (Figure 3). After 15 days of infiltration, the majority of the leaves had deceased. In contrast, leaves treated with water remained green, containing higher chlorophyll levels compared to naturally senesced leaves. To validate these observations, we analyzed the expression of photosynthesis-associated genes (PAGs)

in comparison with SAG12. The results showed a significantly higher expression of SAG12 and slightly lower expression of PAGs in naturally senesced leaves. In contrast, the NaCl-treated leaves displayed lower SAG12 expression and higher PAG expression, indicating inhibition of senescence. Additionally, we investigated the effects of different sucrose concentrations as inhibitors of leaf senescence in rose plants. Phenotypic observations of leaf senescence were recorded at two-day intervals. Unlike other hormones, we found that senescence progressed slightly during the entire experiment. Slight yellowing of leaves began from the early days and continued through the end of the experiment, mostly between days 7 and 20. Furthermore, leaves treated with sucrose showed more noticeable yellowing and fading compared to those treated with normal water, which exhibited a reduced senescence rate. The results were further confirmed through the analysis of gene expression, ion leakage, chlorophyll content, and RhSAG12 expression

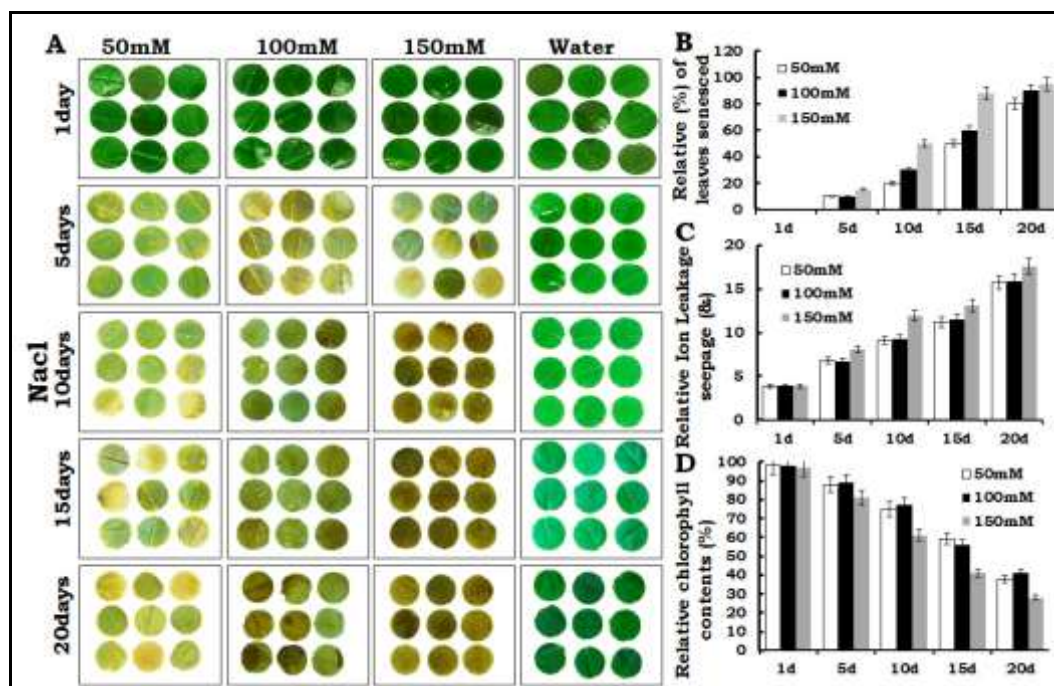


Figure 3. Effect of different NaCl concentrations on discs of rose leaves. (A) Phenotypic analysis of affected leaves. (B) Encountering the number of leaves senesced. (C) Ion leakage measurement. (D) Chlorophyll content measurements from leave discs. Error bar on column denotes the standard deviation (SD) of the mean average from for the date of three different replications.

Quantitative loss of chlorophyll content in senescence leaves: In plants, growth and development is extremely regulated through multistep process, photosynthesis in this regard play and vital role (Tanaka and Makino, 2009). In the present study, the chlorophyll concentration remarkably decreases in

promoter activating treated leaves of rose, compared to that of inhibitor of senescence, which remained normal. Results further indicated that overall gene (*SAG12*) was remarkably higher and slightly lower in *PA* Gene.

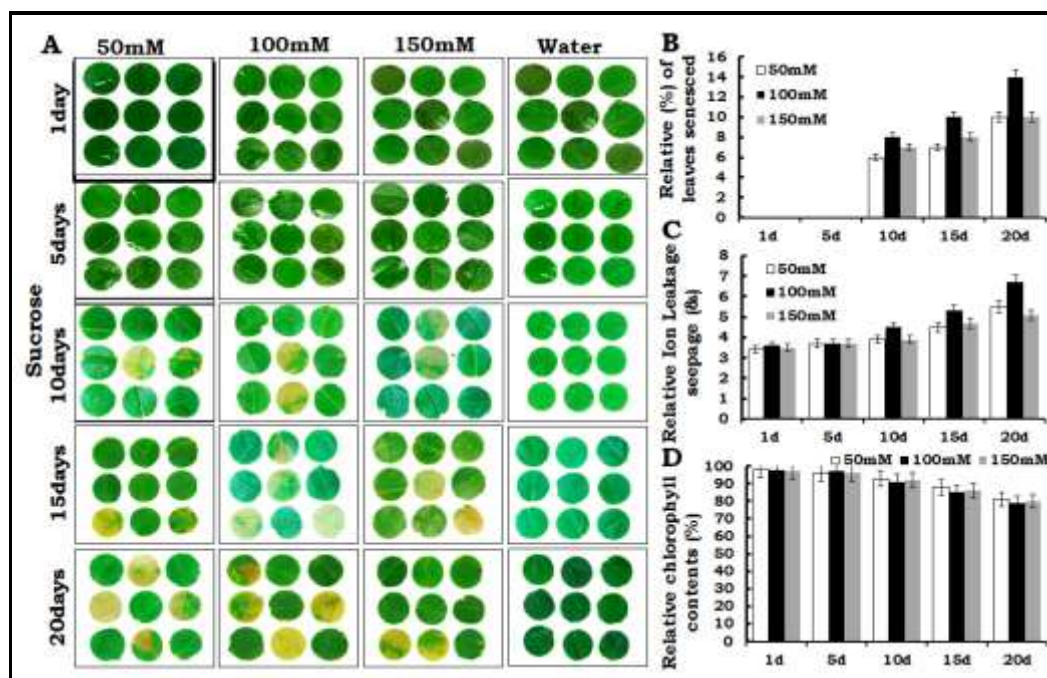


Figure 5. Effect of different sucrose concentrations on discs of rose leaves. (A) Phenotypic analysis of affected leaves. (B) Encountering the number of leaves senesced. (C) Ion leakage measurement. (D) Chlorophyll content measurements from leave discs. Error bar on column denotes the standard deviation (SD) of the mean average from for the date of three different replications.

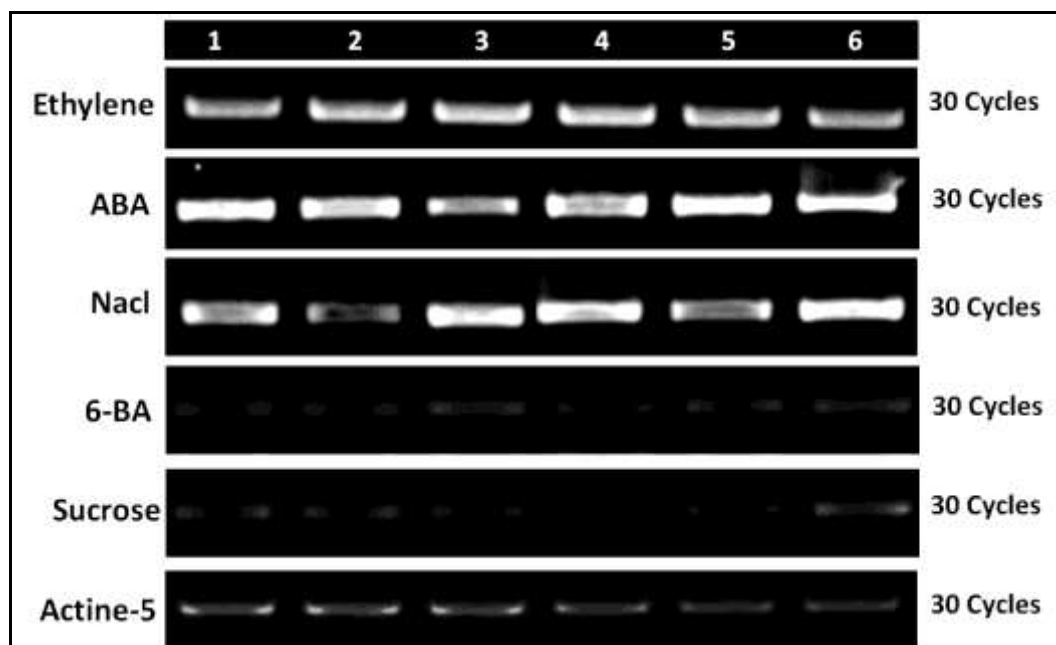


Figure 6. Gene (*SAG12*) expression of hormonal treated leaves. DNA was extracted after 10days of treatment and amplified through Thermo Cycler PCR. The 30 cycles were performed for all treated samples.

Table 1. Forward and reverse primers used in PCR analysis.

Coding	Gene Sequence (5'– 3')
<i>SAG12</i>	F -ACAGAGTCGCAGGAAATGGG
<i>SAG12</i>	R-GGTAAAGCAAGTCCTCGGCC
<i>PSA</i>	F-CATTGTTCCGATGCACCGA
<i>PSA</i>	R-GTGTTGTCGAATCCGATGATC

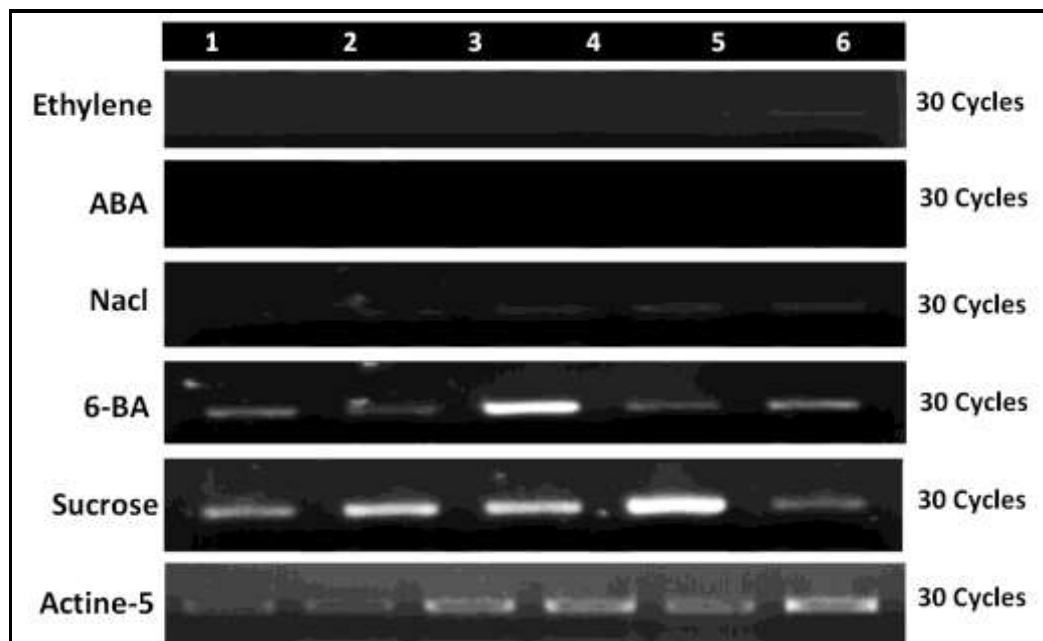


Figure 7. Gene (*PSA*) expression of hormonal treated leaves. DNA was extracted after 10days of treatment and amplified though Thermo Cycler PCR. The 30 cycles were performed for all treated samples.

DISCUSSION

Leaf senescence is a genetically regulated developmental process that ultimately results in cell death (Yoshida, 2003). Under normal growth conditions, senescence does not occur in young leaves. This is likely due to the action of senescence repressors that effectively delay its onset during early leaf development, along with the activation of senescence promoters as leaves mature (Kim *et al.*, 2010). A range of abiotic and biotic factors influences leaf senescence (Chen *et al.*, 2002). To ensure survival and reproduction under adverse conditions, plants exhibit various adaptive traits controlled by intricate regulatory systems.

In this study, the influence of hormonal signals on rose leaf senescence was particularly noteworthy. Observations indicated that treated rose leaves displayed mild yellowing in the early stages of the experiment, particularly in infiltrated leaves. However, as the experiment progressed, leaves treated with senescence-promoting hormones exhibited a pronounced yellow fading compared to those treated with inhibitors. These findings suggest that the effectiveness of both senescence promoters and inhibitors was significant. The observed changes were further validated by measuring ion leakage activity, chlorophyll content, and the expression of *RhSAG12* (a senescence-associated gene) as well as photosynthesis-related genes (*PRGs*), which serve as markers of senescence progression.

The results revealed remarkable differences between hormone-treated and inhibitor-treated leaves, confirming that promoters played a crucial role in accelerating leaf senescence. These findings highlight that leaf senescence is finely regulated to optimize plant fitness by reallocating nutrients from aging leaves. This intricate process involves the differential

expression of numerous genes. Advances in genomics have facilitated the identification of senescence-associated genes (*SAGs*), which show increased expression in senescing leaves and strongly support the conclusions of this study (Buchanan-Wollaston *et al.*, 2003; Andersson *et al.*, 2004; Guo *et al.*, 2004; Zentgraf *et al.*, 2004).

Moreover, diverse interconnected signaling networks downstream of senescence processes are regulated by transcription factors that interact with cis-elements in gene promoters (Lin and Wu, 2004). Gene knockout studies have also demonstrated the significant role of specific genes in the senescence of various plant organs, including leaves, flowers, and fruits (Hee *et al.*, 2010).

Hormonal proficiencies play a critical role in stimulating the instigation of leaf senescence: The role of hormonal pathways in initiating leaf senescence is further supported by findings that link senescence acceleration with decreased photosynthetic activity and reduced chlorophyll content in silenced plants (Quirino *et al.*, 2000; Lim *et al.*, 2007; Hee *et al.*, 2010). Transcription factors from families such as *NAC* and *WRKY* have been identified as critical regulators of senescence (Buchanan-Wollaston *et al.*, 2005). Notably, over 20% of the 109 *NAC* family genes in *Arabidopsis* are specifically induced during developmentally programmed senescence (Buchanan-Wollaston *et al.*, 2005). Collectively, these observations suggest that senescence promoters may play a regulatory role in the initiation of leaf senescence, acting as transcription factors that modulate the activation or repression of genes involved in the progression of this process.

CONCLUSION

Present study concluded that hormonal treatments significantly influence leaf senescence in *Rosa hybrida*. Whereas promoters such as Ethylene, ABA, and NaCl accelerated senescence, as evidenced by increased yellowing, reduced chlorophyll content, and elevated SAG12 expression. In contrast, inhibitors delayed senescence and preserved leaf vitality. These findings highlight the potential of hormonal regulation to enhance postharvest quality and extend the lifespan of rose foliage, offering valuable insights for agriculture practices.

CONFLICTS OF INTEREST

We declare no competing interests affecting the work reported in this study.

AUTHORS' CONTRIBUTION

AJK conceptualized, designed, investigated and wrote original draft. MA and MIK formatted the figures, validated data and formal analysis. NAW and SB reviewed and edited manuscript draft. JHL suggested line of work and provided genes sequences.

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