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Effects of Salicylic Acid Concentration and Exposure Time on the Surface Fungal Load of Tulip Bulbs

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of this study was to determine the impact of various SA concentrations and exposure times on the surface fungal load (SFL) of tulip bulbs.
Place and Duration of Study: Department of Plant Protection, Kocaeli University, in the year of 2022.
Methodology: The tulip bulb (*Tulipa gesneriana* L. cv. Royal Ten) used in the study was obtained from an officially registered flower bulb company in Türkiye. SA solutions ranging from 0 to 1600 ppm were prepared and used to treat tulip bulbs for 1, 6, 12, and 24 hours. Microbiological analysis involved assessing fungal growth inhibition through colony counts on potato dextrose agar plates after incubation. Statistical analysis, employing ANOVA and Scheffe tests, evaluated significant

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differences in fungal growth inhibition across different SA concentrations and exposure times.
Results: According to the results, the SFL of tulip bulbs decreased with increasing SA concentrations. Additionally, SA treatment significantly lowered SFL according to the initial log CFU at all exposure times except for one-hour exposure. The EC50 of SA values changed as a function of the exposure times, which ranged from 194 to 23 ppm against the SFL of tulip bulbs. These results suggest that SA may be a promising candidate for the treatment of fungal diseases in tulips.
Conclusion: SA demonstrated effective antifungal properties compared to synthetic fungicides, suggesting it could mitigate storage diseases in tulip bulbs. Recommendations included using 1600 ppm SA for six hours or 800 ppm SA for 24 hours to manage fungal contamination before storage or planting of tulip bulbs.

Keywords: Tulipa spp.; salicylic acid; antifungal effect; surface fungal load.

1. INTRODUCTION

Natural beauty and distinctiveness are two qualities that distinguish ornamental plants. These plants' beautiful blooms, foliage, and fruits draw a lot of attention due to their attractive colors and shapes. Grown for a variety of decorative uses, ornamental crops include potted plants, woody ornamentals, cut flowers or cut leaves, bulbs, and corms [1].

Plant diseases greatly reduce the quantity and/or quality of a variety of ornamental crops, therefore have severe economic consequences. The appearance of ornamental plants is extremely important, especially for cut flowers and potted plants. Visual disease symptoms, as well as pathogen-induced growth inhibition, have a significant impact on the quality and market value of flowers. As a result, ensuring quality features in these plants is critical as demand for industrial uses grows [2]. In plants, fungi are responsible for around 70% of the diseases, the rest are brought on by bacteria, viruses, and viroids. Fungal diseases frequently result in a substantial decrease in crop quality and production, up to 30–40% of the total yield potential [3]. According to estimates, fungi cause the greatest harm and are mostly responsible for pathogen-driven host losses, deteriorating ornamental flower market prices, and visual quality.

Tulip is one of nature's most attractive flowers and the world's greatest ornamental geophyte crop. Tulip belongs to the family Liliaceae and has 100-150 species. It is the most popular bulbous ornamental due to its vivid color and beautiful blossoms. Tulips are ideal for cut flowers, beds, and pots, and are planted in both open and closed locations [4]. Tulips can be grown from seeds, but the bulb is where most of the flowers are grown for commerce. Vegetative commercial propagation is slow. Therefore, once they are introduced into the production cycle, soil-borne diseases and pests can infect and result in significant losses. Major fungal diseases in tulips are caused by *Botrytis tulipae* [5], *Fusarium oxysporum* f.sp. tulipae [6], *Penicillium* spp. [7], *Pythium* spp. [8], *Rhizoctonia solani* [9], and *R. tuliparum* [10], *Corticium rolfsii, Sclerotinia bulborum, Phytophthora* spp., *Gloeosporium thumenii* Sacc. f. tulipae [11]. Fungal diseases are frequently controlled through the use of chemical fungicides that are only effective for a few diseases and are occasionally non-specific [2]. Furthermore, excessive chemical use leads to microbial resistance to these chemicals, which is extremely undesirable owing to health and environmental safety concerns [12]. Therefore, alternative control methods should be developed. In terms of sustainable methods of plant disease control, natural fungicides are gaining popularity due to their favorable environmental characteristics, availability, and cost.

The use of salicylic acid (SA) in crop protection has received a lot of attention in recent years. Many laboratories have investigated SA's antifungal properties [13-15]. SA has been found to be effective at controlling fungi such as *Fusarium* and *Rhizoctonia* when applied as a foliar spray [16-19]. It appears to be a reasonable approach because SA is a natural phenolic hormone generated in plants as a signaling molecule that plays an important role in plant defense against many fungi and other diseases via the phenylpropanoid pathway [20]. Its mild application to vulnerable plants may boost their resistance to fungus. SA previously exhibited antifungal activity against *Penicillium expansum* by causing protein and lipid leakage [14]. Other investigations have shown that SA has antifungal efficacy against a variety of postharvest diseases, including *B. cinerea* [21], *F. oxysporum* [18], *R. stolonifer* [22], *R. solani, S. rolfsii* [23],

Macrophomina phaseolinae [24], *Pythium* sp. [25], and *Phytophthora* sp. [26]. In addition to its antifungal properties, SA has been shown to have several other effects on plants. It can stimulate the production of hormones that play a role in plant growth and development, and it has been shown to have a positive effect on the root system of plants. It is important to note that SA should be used with caution, as it can cause phytotoxicity in some plants when used in high concentrations [27].

Moreover, SA applications to tulip bulbs can be effective against possible diseases encountered during their storage. According to several studies, SA treatments in various fruits diminish the prevalence of fungal diseases [21-22, 28-30]. This decrease can be attributed to two factors: first, the antifungal impact of SA [31], and second, the defensive mechanism activated by SA in the plant [32-34]. SA also inhibits the formation of ethylene [35] and lowers disease susceptibility [36]. The main effects of ethylene on tulip bulbs are gummosis (extrusion of amber-colored polysaccharides from bulb scales), flower bud abortion, bulb splitting, increased respiration, poor roots, and accelerated blooming. Axillary bud growth, excessive root hair, a reduction in the deposition of leaf wax, a reduction in height, and a reduction in vigor are further impacts [37]. In addition to the ethylene production of the plant, *Fusarium* spp. in the plant also produces ethylene [38], causing the infection to develop faster. SA increases the resistance of tulip bulbs by preventing the germination and growth of *Fusarium* spores.

The effects of SA treatment on the growth of tulips were investigated in our previous research [27]. Herein, the purpose was to determine the impact of various SA concentrations and exposure times on the surface fungal load (SFL) of tulip bulbs. Once the influence of SA on the SFL has been demonstrated, the effective concentration and exposure time may be advised to the manufacturer prior to tulip bulb planting or storage. Disease occurrences under storage conditions after SA treatment of tulip bulbs can be investigated in a future study.

2. MATERIALS AND METHODS

2.1 Plant Material and Chemicals

The tulip bulb (*Tulipa gesneriana* L. cv. Royal Ten) used in the study was obtained from an officially registered flower bulb company in Türkiye. This cultivar was chosen since it is known to be an ethylene sensitive variety [38]. All chemicals used to prepare solutions and media were obtained from Merck (Darmstadt, Germany).

2.2 Experimental Design and Microbiological Analysis

The bulbs were compared for similarity at the beginning of the study, and several variables, such as circumference, diameter, and weight, were recorded based on the mean of all used bulbs. In average, a bulb was 9.67 cm in circumference, 3.08 cm in diameter, and 17.62 g in weight, according to the measurements.

The molecular weight of the SA ($C_7H_6O_3$) was 138.12 g mol-1 with in ≥99.5 purity. SA solutions were prepared at concentrations of 50, 100, 200, 400, 800, and 1600 ppm, transferred to 380 mL glass jars (Fig. 1a) and sterilized in an autoclave at 121°C for 20 minutes. The jars containing only distilled and sterilized water were used as controls. pH measurements were performed prior to autoclaving and recorded as 4.46, 3.40, 3.20, 3.08, 2.93, 2.75, and 2.57 for distilled water and SA solutions of 50, 100, 200, 400, 800, and 1600 ppm, respectively.

The tulip bulbs were kept in the jars for 1, 6, 12, and 24- hour to see the effect of exposure time (Fig. 1b). At the end of these specified times, each of the tulip bulbs was taken into sterile bags (Fig. 1c) containing 80 mL of sterile saline-peptone solution (8.5 g/L NaCl and 1 g/L peptone) and shaken in a shaker-incubator at 100 rpm for 10 minutes. The tulip bulbs were immersed in and out of SA solutions or sterile-distilled water as the exposure time control group before being transferred to the saline-peptone solution. All treatments were done at room temperature.

Microbiological analysis was performed on the SA-treated and control group of tulip bulbs to evaluate the antifungal effect of the SA on the tulip bulbs. One ml of each sample was taken and serially diluted with saline peptone solution from 10^0 to 10^5 using a sterile injector. Then one mL of each diluted sample was inoculated on potato dextrose agar (PDA) plates by the spreading method. The plates were incubated at 25 °C for six days. After incubation, the colony count was done (Fig. 1d). The fungal load was expressed as a log CFU/ml. CFU/ml was calculated using the equation:

CFU/ml = (number of colonies x dilution factor) / volume of the culture plate.

Additionally, the percentage inhibition of fungal growth (IFG) was estimated using the equation:

$$IFG = [(N_0 – N_{SA}) \times 100] / N_0$$

in which N_0 and N_{SA} represent the number of the colonies of the fungi in the no SA treatment and in the SA treatment, respectively.

EC50 values (50% maximal effective concentration) were determined as the lowest concentration of SA that inhibited fungal growth and germination in 50% of SFL of tulip bulbs, which was expressed in ppm. EC50 values computed by using Quest Graph™ EC50 Calculator (AAT Bioquest, Inc., Sunnyvale, CA, United States, 2023).

2.3 Statistical Analysis

The experiments were conducted using a Completely Randomized Design (CRD). For each SA concentration and exposure time, three bulbs of tulips with similar sizes and shapes were chosen at random. Therefore the study included seven SA concentrations and five SA exposure times with three repetitions. Furthermore, each repeat was diluted serially six times, resulting in a total of 630 Petri plates to count. Statistical software was used to assess the numbers that were counted (SPSS Statistics 25.0). When significant differences between samples were discovered after performing a variance analysis (ANOVA), the means were compared using the Scheffe test at a *P* =.05.

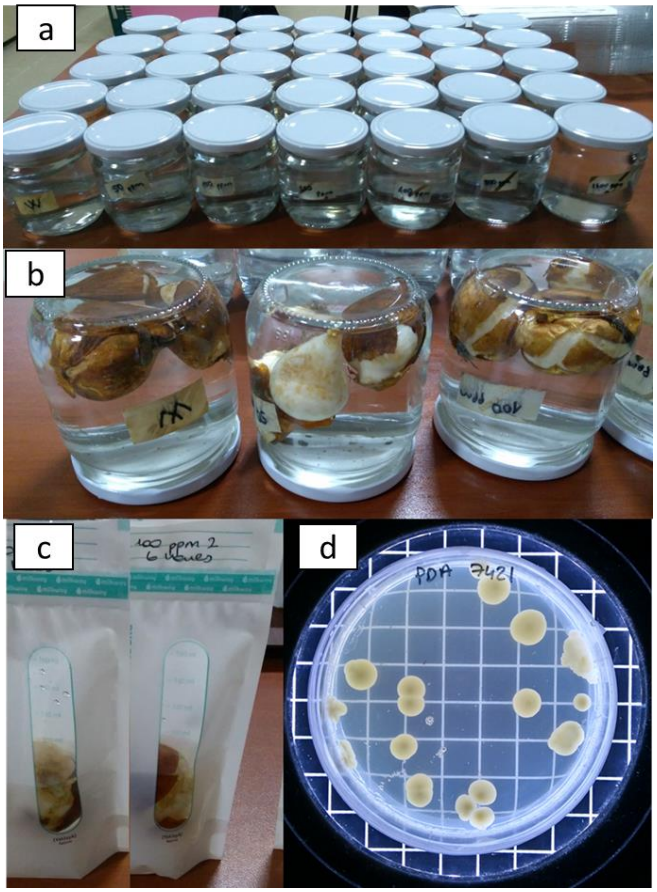

Fig. 1. Experimental design and microbiological analysis a) SA solutions at different concentration (left to right; 0, 50, 100, 200, 400, 800 and 1600 ppm) in 380 mL jars. b) Incubation of the tulip bulbs in SA solutions. c) The sterile bags containing sterile saline-peptone solution and a tulip bulb d) The colonies after 6-days incubation on PDA plates of the samples treated with 800 ppm SA for 24 hours

3. RESULTS AND DISCUSSION

The tulip bulb flora is important for the overall health and development of the bulbs. Some of the microorganisms present on the surface of the bulbs are beneficial, providing a range of functions such as improving the soil quality and helping to suppress the growth of harmful pathogens. Other microorganisms, however, can be harmful to the bulbs and cause diseases such as rot. The reduction in the SFL on the tulip bulbs was investigated in this study using various SA concentrations and exposure times (Table 1).

One-way ANOVA test is intended to determine the differences between the treatment groups. According to one-way ANOVA, the effect of salicylic acid concentrations on the SFL of tulip bulbs was found to be statistically significant (P<.05) at concentrations of 200 ppm and above. The SFL decreased with increasing SA concentrations. There was no significant decrease in the SFL of tulip bulbs between no SA, 50, and 100 ppm SA concentrations. The next test was post-hoc Scheffe's multiple comparisons to look at a couple of different groups. The results of the Scheffe test showed that 1600 ppm SA treatment significantly lowered SFL according to the initial log CFU/ml at all exposure times except for one- hour exposure. There was no significant difference between the different SA concentration treatments at one-hour exposure.

One-way ANOVA test results also showed that there was a significant difference between the groups treated with different exposure times (P<.05). However, the Scheffe test showed that there was no significant difference between the interaction of treatment no SA, 50, and 100 ppm SA concentrations and all exposure times. The exposure times of 12 hours and 24 hours were significantly different from other exposure times at 200 ppm SA and 400 ppm concentrations, respectively. It can be concluded that the factor of SA concentration and exposure time x SA concentration interaction types affect the decrease in the number of colonies of fungi. The maximum decreases in log CFU/ml were seen at 1600 ppm SA concentrations for all exposure times: 1.26, 1.28, 2.34, 2.42, and 2.29 for no SA exposure, 1, 6, 12, and 24-hour exposures; respectively.

The types of fungal species found on tulip bulbs can be quite variable [14, 18, 21-25]. The values in Table 1 show the decrease in the total SFL for tulip bulbs. The minimum concentration at which no visible growth was observed was defined as the Minimum Inhibition Concentration (MIC). MIC could not be calculated for SA by looking at the values in Table 1 for the SFL of tulip bulbs. However, in the literature, there are some studies that determine the MIC value for possible pathogenic fungi in tulip bulbs, for example, Kong et al. (2016) determined the MIC value of SA against *Fusarium solani* as 1300 ppm [39]. In another study, SA completely inhibited the germination of *Penicillium expansum* conidia at 345 ppm [14]. Other pathogens, such as *R. stolonifer, F. oxysporum, Rhizoctonia solani*, *Sclerotium rolfsii, Macrophomina phaseolinae,*

Table 1. Effect of SA concentrations on fungal surface load of tulip bulbs (log CFU/ml) exposed for 1, 6 , 12, 24-hour, and no exposure (0 hours). Values show the means ± SD of the replicates

SA concentration	Exposure time (hours)*,**				
(ppm)	0	1	6	12	24
0	3,59±0,53aA	3,37±0,94aA	3,87±0,74aA	3,65±0,59aA	3,69±0,84aA
50	2,81±0,53aABC	3,17±0,80aA	3,64±0,54aAB	3,30±0,49aA	3,62±0,88aA
100	3,33±0,53aABC	3,31±0,63aA	3,62±0,46aAB	3,03±0,64aAB	3,37±0,60aAB
200	3,50±0,37aAB	3,04±0,57abA	3,25±0,48abAB	2,63±0,62bAB	2,88±0,48abABC
400	2,56±0,81aBC	3,07±0,51aA	3,24±0,61aAB	2,48±0,55aAB	2,02±0,98aBCD
800	2,89±0,45aABC	2,66±0,38aA	2,76±0,43aB	2,11±0,67abBC	1,40±0,85bCD
1600	2,33±0,60aC	2,09±0,73aA	1,53±0,29aC	1,23±0,48aC	1,42±0,61aD

**Means followed by a common lowercase letter in each row are not significantly different by the Scheffe's test at the P = .05 level of significance*

*** Means followed by a common uppercase letter in each column are not significantly different by the Scheffe's test at the P = .05 level of significance*

Pythium sp., and *Phytophthora* sp., showed similar results, with SA reducing nearly 70% of conidial germination and growth at a minimum concentration of 345 ppm [22,24]. It was shown that all concentrations of SA ranging from 0 to 690 ppm decreased the fungal growth of *R. stolonifer*. At a concentration of 690 ppm SA, there was a 100% of growth inhibition [22].

The percentages of inhibition (%I) were calculated for each concentration according to Table 1, and EC50 values (concentration causing 50% inhibition of growth) were obtained from fitting data to a dose-response curve (Fig. 2). The EC50 values of SA for all exposure times were given in Fig. 3.

The EC50 of SA against surface fungi of tulip bulbs ranged from 194 to 23 ppm. EC50 values were less than 100 ppm which means SA showed strong antifungal activity against the SFL of tulip bulbs at higher exposure times. These findings indicate that SA may get its antifungal activity through giving damage to biological membranes [22, 39], and by inhibiting or deactivation specific enzymes [40]. These biological changes could happen simultaneously or independently. Moreover, the antimicrobial effect of SA may be related to cellular respiration disturbances, such as shifting electron flow from the cytochrome pathway to an alternate cyanide-related pathway, partially blocking the primary cellular respiration pathway, and altering the cell energy-generating process [40]. SA can also attach to proteins that can affect the breakdown of intracellular hydrogen peroxide, causing this chemical to accumulate in the cell and inactivate the conidia [41]. Amborabé et al. (2002) revealed that the fungicidal properties of SA derivatives on the growth of *Eutypa lata* and all concentrations of this agent were poisonous for *E. lata*, low (138 ppm) and high concentrations of SA (>276 ppm) showed fungistatic and fungicidal properties; respectively and they found that SA behaves as a dissociating agent causing a change in the trans-membrane pH gradient in membranes of organelles and in the plasma membrane, possibly causing cellular energy loss [13]. In another study it was demonstrated that SA affects directly *P. expansum* conidia by breaking through the cell wall and starting a number of interactions with the plasma membrane. This action disrupts the lipid bilayer and/or damages the proteins that control cellular permeability, which raises the level of reactive oxygen species [14]. Dieryckx et al. (2015) suggested some mechanisms to understand the mechanisms of action of salicylic acid on fungal pathogens. One of the possible mechanisms is a change in pH

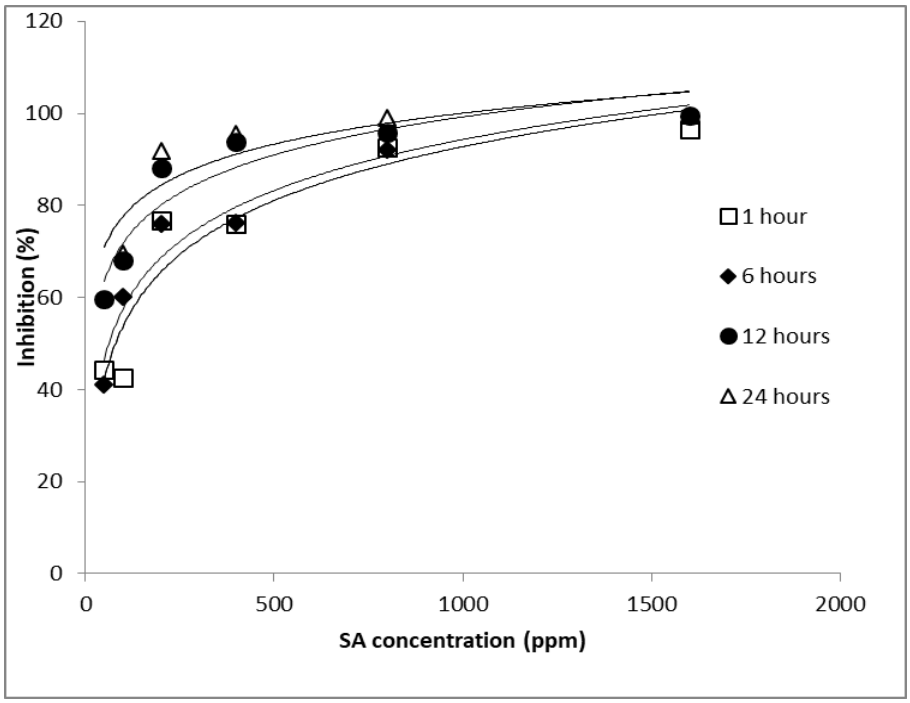

Fig. 2. Dose-response profiles of SA for different exposure times. ϒ: 1 hour, ♦: 6 hours, ●: 12 hours, and Δ: 24 hours

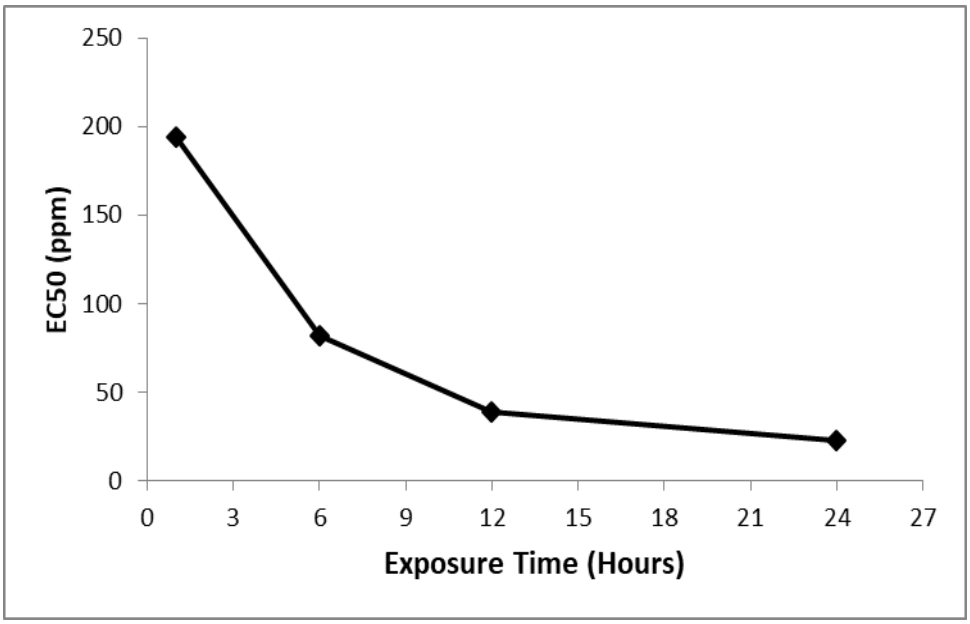

Fig. 3. EC50 values of SA over exposure time based on inhibition percentages calculated using the integral method

regulation in mycelium and a change in environmental pH affects the gene expression profile. In addition, SA may form chelate complexes with metal ions. Thus, an adjustment in metal homeostasis might explain the observed alterations in intracellular proteomes in the presence of SA. The other possibility is that the disruption of mitochondrial respiration may be responsible for the inhibition of fungal growth in the presence of SA. This study also shown that the addition of SA significantly decreased the levels of the enzymes in the internal mycelium proteome that are involved in ROS detoxification: peroxiredoxin, ascorbate peroxidase, manganese superoxide dismutase, and catalase. Therefore, it is possible that the reduction of fungal growth observed in the presence of some harmful compounds arose from an increased ROS accumulation resulting in oxidative stress [42].

4. CONCLUSION

In the current study, the effects of SA concentration and exposure time on the surface fungal load of tulip bulbs were evaluated, and the results showed that the SFL of tulip bulbs decreased as SA concentrations increased. Aside from one-hour exposure, SA treatments considerably decreased the SFL of tulip bulbs according to the initial log CFU/ml at all exposure durations. As a function of the exposure periods, the EC50 of SA values against the SFL of tulip bulbs varied from 194 to 23 ppm. SA revealed promising antifungal activity against the SFL of tulip bulbs instead of synthetic fungicides. The tulip bulb storage disease risks can be greatly reduced when SA is applied prior to entering the storage conditions of the tulip bulbs. We propose to the manufacturer that 1600 ppm SA for a 6-hour exposure or 800 ppm SA for a 24-hour exposure be used to control SFL of tulip bulbs at room temperature prior to planting or storage of the tulip bulbs.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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