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Scientific Article

Boric acid as a potential substitute for conventional ethylene antagonists in mitigating postharvest flower senescence of *Digitalis purpurea*

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Abstract

In the floriculture industry, postharvest senescence is one of the glaring challenges restricting the marketability of cut flowers. Hence, maintaining good quality of cut flowers and extending flower longevity are considered to be the most crucial factors in the cut flower trade. Therefore, to gain better understanding of the specific physiological and biochemical aspects of petal senescence we conducted an experiment to evaluate the efficacy of Boric acid (BA) on flower longevity in excised flowers of *Digitalis purpurea* L. Isolated buds were harvested at stage IV i.e, 1 day before anthesis and divided into 5 sets, with one set of buds held in distilled water (DW) designated as control. The other 4 sets were supplemented with 24h pulse treatment of different concentrations of BA viz., 50, 100, 150 and 200 µM. The application of BA at 150 *µM* concentration was found to be most effective in increasing flower longevity by about 4 days as compared to control. The enhanced longevity coincided with higher values of floral diameter, fresh mass, dry mass and solution uptake. Flowers with delayed senescence also retained higher soluble proteins, sugars and phenols in addition to lower bacterial density compared to control. Moreover, this ameliorated flower longevity has also been shown to be positively associated with increased activities of various antioxidant enzymes viz., superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) and reduced activity of lipoxygenase (LOX).

Keywords: Flower longevity, lipoxygenase, senescence, sugars.

Resumo

Ácido bórico como potencial substituto para os antagonistas convencionais do etileno na mitigação da senescência da flor na pós-colheita de *Digitalis purpurea*

Na indústria da floricultura, a senescência pós-colheita é um dos desafios que restringe a comercialização das flores de corte. Portanto, manter a boa qualidade das flores de corte e prolongar a longevidade das flores são considerados os fatores mais importantes no comércio de flores de corte. Portanto, para obter uma melhor compreensão dos aspectos fisiológicos e bioquímicos específicos da senescência das pétalas, conduzimos experimento para avaliar a eficácia do ácido bórico (BA) na longevidade das flores em flores cortadas de *Digitalis purpurea* L. Botões isolados foram colhidos no estágio IV, ou seja, 1 dia antes da antese e dividido em 5 conjuntos, sendo um conjunto de gemas mantido em água destilada (DW) designado como controle. Os outros 4 conjuntos foram suplementados com tratamento de *pulsing* de 24 horas de diferentes concentrações de BA (50, 100, 150 e 200 µM). A aplicação de BA na concentração de 150 µM foi considerada mais eficaz no aumento da longevidade das flores em cerca de 4 dias em comparação com o controle. O aumento da longevidade coincidiu com os maiores valores de diâmetro floral, massa fresca, massa seca e absorção de solução. Flores com senescência retardada também retiveram proteínas, açúcares e fenóis mais solúveis, além de menor densidade bacteriana em comparação com o controle. Além disso, a longevidade melhorada da flor também mostrou estar positivamente associada ao aumento das atividades de várias enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX) e redução da atividade da lipoxigenase (LOX).

Palavras-chave: longevidade da flor, lipoxigenase, senescência, açúcares.

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Introduction

Flowers are very interesting and complex commodities used in vase displays, flower bouquets and indoor decorations, etc. These are susceptible to major post-harvest losses due to their limited vase life and highly perishable nature (Thakur et al., 2020). Floriculture researchers have developed various technologies for different flowers in order to maximize the attractiveness of flowers for a longer period of time. These include pre-cooling, selection of appropriate storage methods and storage temperature, and standardization of holding solutions for a specific cut flower (Gupta et al., 2018). To improve the vase life of cut flowers, clarifying the factors inducing their senescence will be important. Senescence is used to describe a series of events that culminate in cell death at the end of a development period, including structural deterioration and macromolecule degradation (Lekli et al., 2017). It is a programmed mechanism that does not occur at the same time in all floral organs and petals are the first tissues to display signs of senescence based on their unique biological role (Wang et al., 2020). Reactive oxygen species (ROS) accumulation is closely related to flower senescence. The excessive production of ROS damages the cells and accelerates senescence in flowers. Plant cells are generally protected against ROS produced during oxidative stress by an antioxidant enzyme system such as SOD, CAT and APX activity (Li et al., 2020). A number of experiments concerning antioxidant properties have been conducted in order to increase the longevity of flowers (Fonseca et al., 2017). The flower senescence is governed by the interplay of several growth regulators viz. ethylene, cytokinins, gibberellic acid, auxins, jasmonic acid, salicylic acid and abscisic acid (Iqbal et al., 2017).

Digitalis purpurea (foxglove, common foxglove, purple foxglove) is a species of essential medicinal flowering plant in the family *Plantaginaceae* and is well known as the original source of heart medicine digoxin (Rad et al., 2020). A rapid upsurge in the production of ethylene by floral tissue after pollination, accompanied by accelerated corolla senescence and abscission, has been recorded in these flowers (Stead et al., 1983). In ethylene sensitive flowers, ethylene antagonists such as aminooxyacetic acid (AOA), silver thiosulfate (STS), aminotriazole (AT) and amino vinyl glycine (AVG) delay the senescence of the flowers (Nisar et al., 2015). These compounds are not only expensive, but also pose certain toxicological risks (Serrano et al., 2001). Boric acid (BA) may therefore be an effective substitute and a strong competitor to these ethylene antagonists in cut flower industry due to its biodegradable, inexpensive and non-toxic nature (Serrano et al., 2001; Khattab et al., 2017). During climacteric respiration, the activity of 1- aminocyclopropane-1-carboxylic acid synthase (ACC synthase) and ACC oxidase increase, which convert S-adenosylmethionine (SAM) to ACC and ACC to ethylene, respectively (Sadka et al., 2019). Pertinently, BA prevents ethylene production by inhibiting the activity of ACC synthase and ACC oxidase (Moon et al., 2020). Despite being susceptible to ethylene, the application of pulsing and preservative solutions to prolong post-harvest life of *Digitalis* cut flowers has not been adequately studied. The present experiment was therefore, designed to elucidate the role of BA as an effective substitute to ethylene antagonists in mitigating postharvest senescence in *Digitalis purpurea* L. cut flowers.

Material and methods

Fresh flower buds of *Digitalis purpurea* collected from healthy plants growing in the Kashmir University Botanic Garden (KUBG) were utilized in this study. Isolated buds at one day before anthesis stage with their cut ends immersed in distilled water were transported to the laboratory. The flowers were divided into five sets, each set comprising of 15 vials with 10 ml of respective solutions. The first set of buds designated as control was transferred to vials containing DW. The harvested flowers of other 4 sets were held in BA solution of different concentrations (50, 100, 150 and 200 µM, respectively) for 24 hours and then transferred to distilled water. The day on which isolated buds were subjected to different treatments was designated as day zero (D0). The analysis of various physiological and biochemical parameters was performed on day 2 (D2) and day 4 (D4) from the petal tissues after transfer to their respective test solutions. The experiment was performed under stable conditions with a relative humidity (RH) of 60% ± 10%, a light duration of 12-h per day and an average temperature of 23 ± 2 °C.

Assessment of flower longevity and flower Diameter

The average longevity of the flowers was counted from the day of transfer of isolated flowers to their respective testing solutions, and assessed to be terminated when the last flower lost its ornamental value. During the experiment, floral diameter was recorded on day 2 and day 4 as the mean of two perpendicular measurements across the flower.

Fresh Mass, Dry Mass and Water Content

Fresh mass and dry mass were recorded on day 2 and day 4 after transfer of excised buds to holding solutions. For the determination of fresh mass, five flowers were taken at fully open stage, whereas the same flowers were oven-dried at 70 °C for 48 h for calculation of dry mass. The water content was measured as difference between fresh mass and dry mass.

Bacterial density and solution uptake

The bacterial density was calculated by recording the optical density (absorbance) of 1 mL of holding solution collected from each treatment including control at 600 nm using PC-based UV-VIS spectrophotometer (Systronics) by taking *Escherichia coli* as standard (1 OD = $8x10^8$) (Naing et al., 2017). Solution uptake (mL) was evaluated as the difference between the volume of vase solution at the end of the experiment and total volume of vase solution.

Membrane stability index (MSI)

The MSI evaluated in the form of solute leakage of the petal tissues was calculated by incubating 100 mg of petal tissue in 5 mL deionized water at 25 °C for 30 min and 100 °C for 15 min (Sairam 1994). MSI was computed as under

$$MSI = [1 - C1/C2] \times 100$$

C1 represents the conductivity of the samples incubated at 25 °C and C2 represents the conductivity at 100 °C, after recording the values on Elico CM180 Conductivity meter.

Protein estimation

For the estimation of proteins, 1 g of petal tissue was macerated in 100 mM phosphate buffer of pH 7.2 containing 10% polyvinyl pyrrolidone (PVP), 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and 1 mM Dithiothreitol (DTT). The mixture was centrifuged utilizing a refrigerated centrifuge at 12,000 *x*g at 5 °C for 15 min. Proteins were estimated from a suitable volume of aliquot taken from the collected supernatant following the method of Lowry et al. (1951).

Estimation of sugar fractions and phenols

One g of chopped petal tissue from each treatment was fixed in hot 70% ethanol, macerated and centrifuged thrice. From the suitable aliquot taken from the supernatant total phenols, reducing, non-reducing and total sugars were estimated. Total phenolics were quantified by Swain and Hillis method (1959) using gallic acid as standard. Nelson's method (1944) was employed for estimating reducing sugars with glucose acting as standard. For the estimation of total sugars, non-reducing sugars were converted to reducing sugars by invertase. Amount of non-reducing sugars were calculated from difference between total and reducing sugars. Total phenols and sugar fractions (reducing, non-reducing and total sugars) were determined at day 2 and 4 of the transfer of the flowers to the respective test solutions.

Enzyme extraction and assays

Superoxide dismutase activity (SOD)

One g of petal tissue was homogenized and thoroughly mixed with 0.1 mM potassium phosphate buffer (pH = 7.8) containing 0.5% (v v^{-1}) Triton X-100, 0.1 mM EDTA and 1% PVP. The mixture was centrifuged at 15,000*x*g for 10 min. The collected supernatant was first filtered through Mira cloth and then used for the enzyme assay. The activity of SOD was calculated following the method of Dhindsa et al. (1981), by observing the inhibition of photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM sodium carbonate, 0.1 mM EDTA, 75 *µM* nitroblue tetrazolium (NBT), 0.1 mL of the enzyme extract, and 13 mM methionine in 50 mM phosphate buffer (pH = 7.8) in a total volume of 3 mL. To the reaction mixture adding of 2 µM riboflavin and putting the test tubes in water bath at 25 °C and illuminating with a 30 W fluorescent lamp initiated the reaction. The test tubes were kept in dark after the reaction was stopped by switching off the light. Other identical, unilluminated test tubes acted as blanks. Absorbance was measured at 560 nm. The amount of enzyme that prevents photoreduction of NBT to blue formazan by 50% compared to the reaction mixture kept in darkness without the enzyme extract has been defined as one unit of SOD activity. The SOD activity was expressed as units min^{-1} mg^{-1} protein.

Catalase activity (CAT)

Catalase activity was calculated following the method of Aebi (1984). 1 g of petal tissue was macerated and homogenized in 100 mM potassium phosphate buffer of pH 7.0 containing 1 mM EDTA. The reaction mixture contained 50 mM potassium phosphate buffer (pH = 7.0), 50 µL enzyme extract, 12.5 mM H_2O_2, was added with distilled water to make the final volume to 3 mL. Addition of H_2O_2 initiated the reaction and the catalase activity was assayed by determining H_2O_2 consumption for 3 minutes at 240 nm and was expressed as µmol H_2O_2 red $min^{-1}mg^{-1}$ protein.

Ascorbate peroxidase activity (APX)

Petal tissue of 1 g was macerated and homogenized in 100 mM sodium phosphate buffer containing 10% glycerol, 1 mM EDTA and 5 mM ascorbate. The APX activity was determined in 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH = 7.0), 0.3 mM H_2O_2 and 0.1 mM ascorbate. The reduction in the absorbance was noted for 3 min at 290 nm (Chen and Asada 1989).

Lipoxygenase activity (LOX)

LOX activity was determined by employing the method of Axerold et al. (1981). 1 g of petal tissue was thoroughly mixed in 1 mL extraction buffer containing 50 mM potassium phosphate buffer (pH = 6.5), 10% polyvinyl pyrrolidone (PVP), 0.25% Triton X-100, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). The 1 ml reaction mixture contained 50 mM Tris–HCl buffer (pH = 6.5) and 0.4 mM linoleic acid. Addition of 10 µL crude petal extract to the reaction mixture initiated the reaction and absorbance was recorded at 234 nm for 5 min. The activity was expressed as µmol min^{-1} mg^{-1} protein.

Statistical Analysis

During the experiment, a completely randomized experimental design was adopted. Treatment means were compared via analysis of variance using SPSS (SPSS version 16; Chicago, USA). Standard error between the replicates was also calculated. The Duncan's multiple range test (DMRT) has been applied to the data to separate the means.

Results

Flower longevity, flower diameter

The application of 24-h pulse duration of 150 µM BA resulted in substantial increase in the flower longevity against control. The average lifespan of the flowers of *D. purpurea* was about 6 days when transferred to DW (control). The longevity of flowers held in 50, 100, 150 and 200 µM BA recorded was 8, 7, 10 and 9 days respectively, revealing 150 µM BA as most efficacious concentration in accentuating the longevity of *Digitalis* cut flowers (Figure 1).

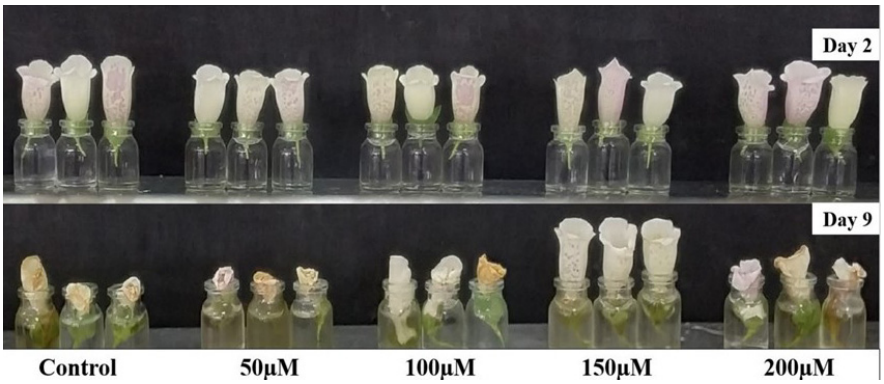

Figure 1. Vials in triplicates arranged from left to right represent the effect of various concentrations of BA on the flower longevity of isolated flowers of *Digitalis purpurea* at second day (day 2) and 9th day of experiment (day 9). Control includes the buds directly transferred to distilled water.

The diameter of flowers supplemented with 150 µM BA was higher than the control which then decreased gradually with the progression of time from D2 to D4 (Figure 2).

Fresh mass, dry mass and water content

Increased fresh mass, dry mass and water content was observed in the flowers treated with BA with maximum in the flowers held in 150 µM BA. All these parameters displayed a decreasing pattern from D2 to D4 (Figure 3).

Bacterial density and solution uptake

Treatment of BA substantially enhanced solution uptake by reducing bacterial density in vase solutions as shown in (Figure 4). Minimum bacterial density in 150 µM BA resulted in maximum solution uptake thereby improving flower longevity.

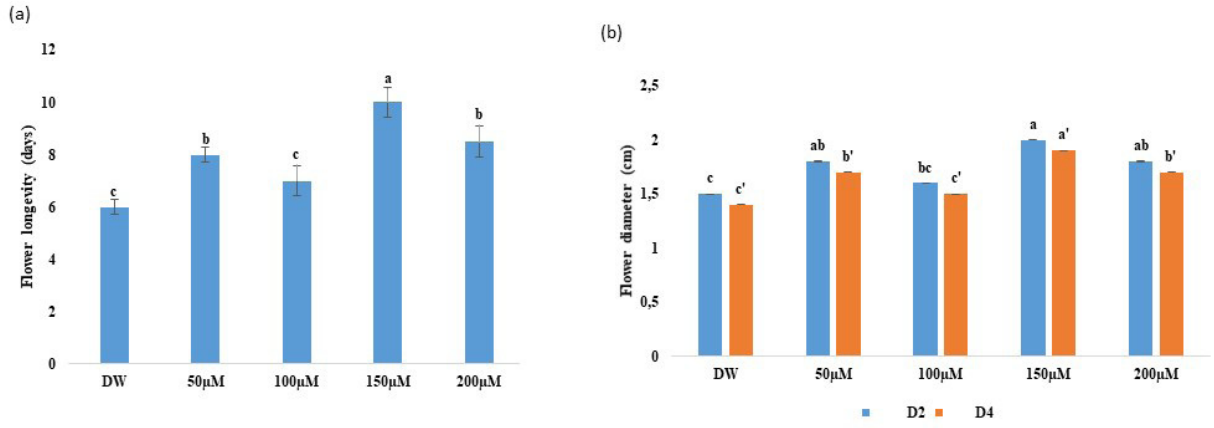

Figure 2. Flower longevity (a) Flower diameter (b) of isolated flowers of *Digitalis purpurea* held in distilled water (control) and treated with different concentrations of BA. Each value is the mean of 3 replicates and error bars represent ± SE (standard error). Bars with different letters differ significantly at $p < 0.05$ by DMRT.

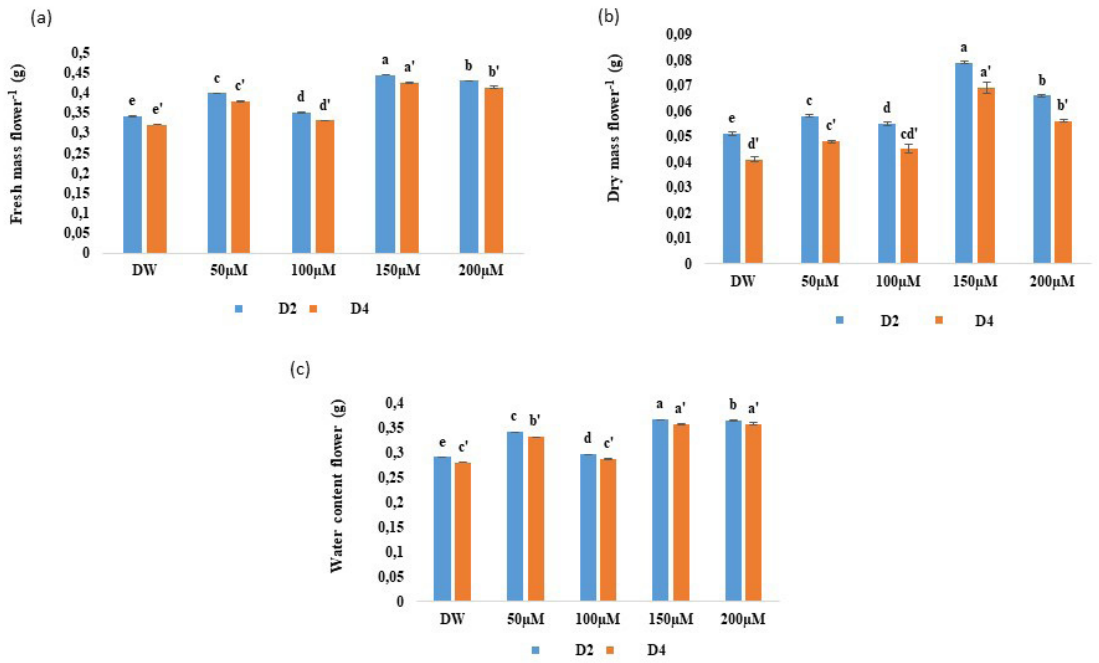

Figure 3. Effect of different concentrations of BA on Fresh mass (a) Dry mass (b) Water content (c) in petal tissues of isolated flowers of *Digitalis purpurea*. Each value is the mean of 3 replicates and error bars represent ± SE (standard error). Bars with different letters differ significantly at $p < 0.05$ by DMRT.

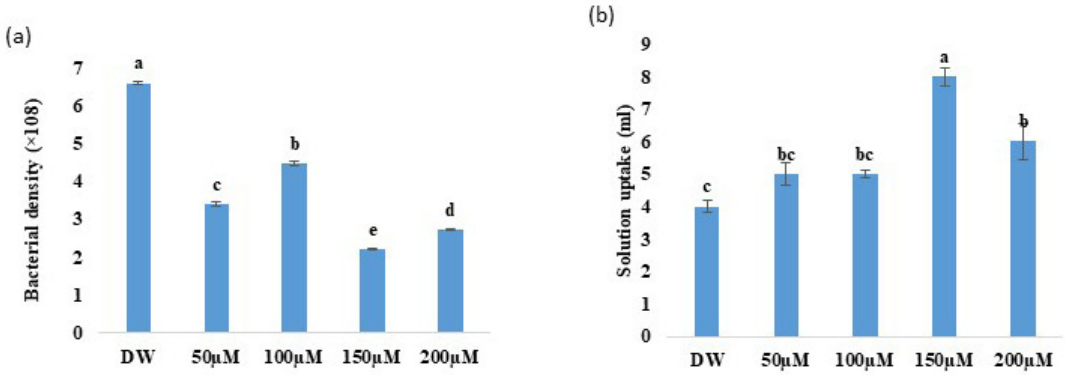

Figure 4. Effect of different concentrations of BA on Bacterial density (a) Solution uptake (b) in petal tissues of isolated flowers of *Digitalis purpurea*. Each value is the mean of 3 replicates and error bars represent ± SE (standard error). Bars with different letters differ significantly at $p < 0.05$ by DMRT.

Membrane stability index (MSI) and **Soluble proteins**

Treatment of isolated flowers of *D. purpurea* with BA pulse revealed that membrane stability was significantly influenced by all treatments with the highest value from samples held in 150 µM BA. In addition to MSI, the soluble protein content also increased with increase in BA concentration, having maximum level in the flowers treated with 150 µM BA. However, both MSI and proteins decreased gradually with the passage of time from D2 to D4 (Figure 5).

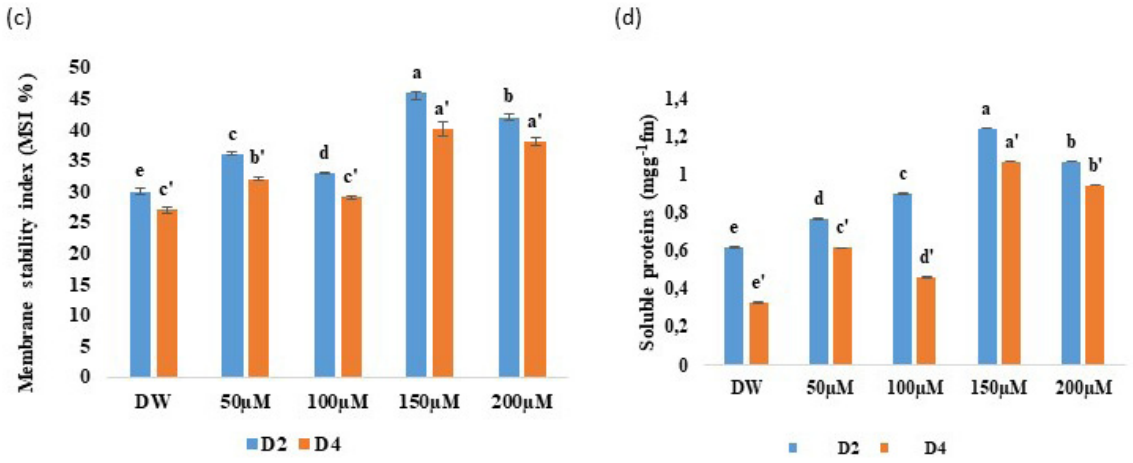

Figure 5. Effect of different concentrations of BA on Membrane stability index (a) Soluble proteins (b) in petal tissues of isolated flowers of *Digitalis purpurea*. Each value is the mean of 3 replicates and error bars represent ± SE (standard error). Bars with different letters differ significantly at $p < 0.05$ by DMRT.

Phenols and sugar fractions

As compared to control, an elevated amount of phenolic and total sugar (reducing and non-reducing sugars) content was observed in the BA treated flowers with maximum in 150 µM BA. Moreover, in all BA treated samples reducing sugar content was found to be considerably higher than non-reducing sugar content. Both phenols and sugars showed a sharp decrease from D2 to D4 (Figure 6).

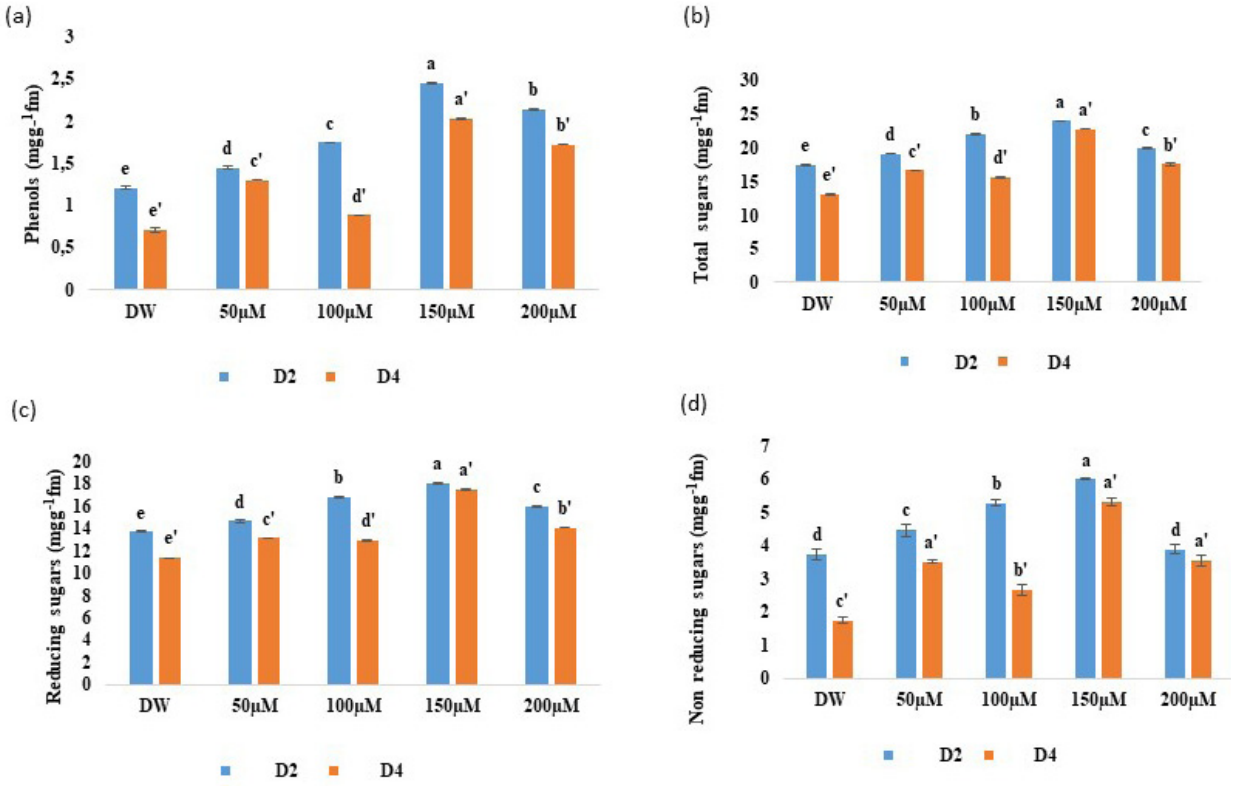

Figure 6. Effect of different concentrations of BA on Phenols (a) Total sugars (b) Reducing sugars (c) Non reducing sugars (d) in petal tissues of isolated flowers of *Digitalis purpurea*. Each value is the mean of 3 replicates and error bars represent ± SE (standard error). Bars with different letters differ significantly at $p < 0.05$ by DMRT.

Activities of antioxidant and lipoxygenase enzyme

The antioxidant activities of the enzymes SOD, CAT and APX were increased in the petal tissues pulsed with 24-h BA. The expression of these enzymes was found to be significantly higher in the 150 µM BA treated floral buds as compared to other holding solutions which however showed a marked decrease with the progression of time from D2 to D4. Treatment with BA resulted in reduced activity of LOX with the least in 150 µM BA treated floral buds. The activity of LOX increased significantly from D2 to D4 (Figure 7).

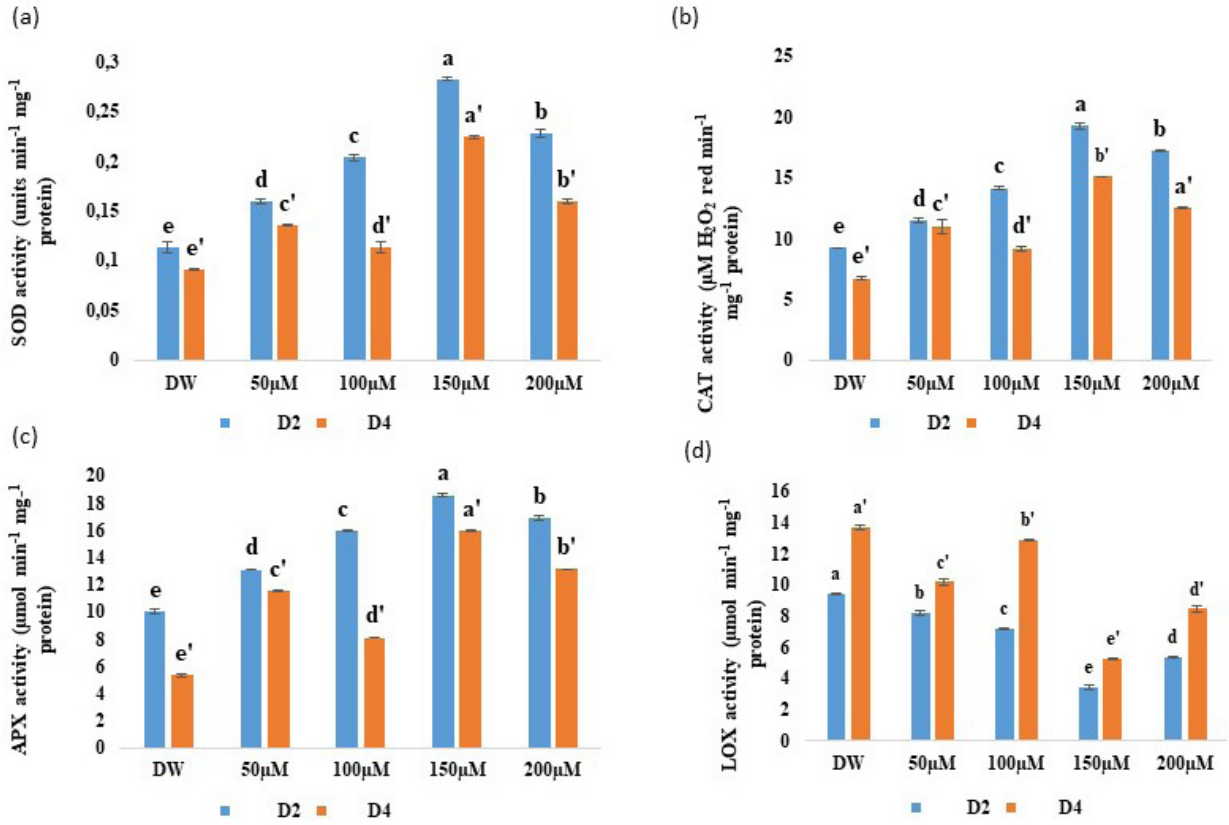

Figure 7. Effect of different concentrations of BA on the activities of Superoxide dismutase (SOD) (a) Catalase (CAT) (b) Ascorbate peroxidase (APX) (c) Lipoxygenase (LOX) (d) in petal tissues of isolated flowers of *Digitalis purpurea*. Each value is the mean of 3 replicates and error bars represent ± SE (standard error). Bars with different letters differ significantly at $p < 0.05$ by DMRT.

Discussion

The inclusion of BA in the holding solution enhanced the flower longevity of *D. purpurea* significantly in comparison to control. During this study, 150 µM of BA proved out to be most effective in ameliorating longevity of flowers by 4 days as compared to control. This increased flower longevity could be due to the inhibition of ethylene synthesis by BA, as a result of lower ACC synthase and ACC oxidase activity. BA mediated increase in flower longevity has also been reported in *Tubero*se and *Jasmine* (Baidya et al., 2020; Manimaran et al., 2018). Furthermore, the comparatively increased flower diameter of BA treated flowers can be attributed to the enrichment of carbohydrates in the petal tissues which facilitates increased water influx and thus increases flower diameter, fresh mass, and water content of flowers (Lone et al., 2021). Increased solution uptake of flowers may be justified by the antimicrobial activity of BA which prevents vascular blockage by inhibiting microbial growth and improves hydraulic conductivity. Moreover, decreased microbial growth due to the application of BA has also been reported in *Tuberose, Lisianthus,* cut rose and *Gladiolus* (Khattab et al., 2017).

Increased membrane stability index in BA treated tissue samples may be attributed to reduced LOX activity. This decrease in LOX activity may be due to the preservation of adequate phospholipids, proteins and thiols by preventing the leakage of protease from vacuoles into the cytoplasm (Lone et al., 2021). Increased membrane leakage due to an upsurge in lipoxygenase activity is one of the main events in the senescence of various flowers such as *Iris* (Ahmad and Tahir, 2018).

In this experiment, flowers treated with 150 µM BA showed significantly higher values of soluble proteins compared to control. Elevated protein levels due to the application of BA can be attributed to its role in protein synthesis regulation, thus preserving higher levels of soluble proteins for a longer period of time (Abd et al., 2005). Increased protein content in BA treated flowers has also been documented in various cut flowers such as

Sandersonia, Chrysanthemum (Eason et al., 2002; Balieiro et al., 2018). In addition of retaining an elevated protein content, flowers with delayed senescence also displayed higher phenolic content. Similar findings of increased phenolic content using BA have been recorded in flowers such as *Jasmine* (Lavanya et al., 2016; Manimaran et al., 2018). Higher content of total phenols has been shown to be associated with delayed senescence in *Hemerocallis* (Gulzar et al., 2005).

The onset of senescence is usually marked by a diminution in sugar content as a result of the oxidative process (Cavasini et al., 2018). In our research, the treatment of isolated flowers with BA showed higher levels of various sugars compared to control. These results may be due to the role of BA in delaying leaf senescence by inhibiting climacteric ethylene production, raising the chlorophyll content of the leaves, as well as the vase solution uptake and activating the photosynthesis process (Raffeii and Pakkish, 2014).

Pertinently, application of BA to *Digitalis* flowers augmented the antioxidant potential by increasing the activities of antioxidant enzymes (SOD, CAT and APX). Plant cells are generally protected against ROS generated during oxidative stress by an antioxidant enzyme system such as SOD, CAT and APX activity (Kim et al., 2017). In *Mentha*, and *Cymbopogon* upsurge in activities of antioxidant enzymes due to BA treatment has also been identified (Choudhary et al., 2020). Thus, it can be assumed that increased antioxidant activities in BA treated samples compared to control may be one of the reasons for delaying flower senescence in *Digitalis purpurea*.

Conclusions

The findings revealed that 24-h pulse treatment with a preservative solution containing 150 µM BA was effective in prolonging longevity of *Digitalis* cut flowers by ameliorating various postharvest attributes. This enhanced longevity corroborated with increased activities of antioxidant enzymes, increased protein levels, phenols and total sugars in tissue samples. The current study provides an insight into the role of BA as an effective substitute to expensive and toxic ethylene antagonists in delaying flower senescence and also offers an enormous future scope for studying the comprehensive flower senescence mechanism and corresponding molecular crosstalks involved in this beautiful flower. Understanding these processes will help us to develop more accurate and advanced techniques for increasing the post-harvest output of various cut flowers.

Author contribution

SF: carried out the experiments, obtained results, analyzed and compiled the data; **AUH** and **MLL:** helped in the field and laboratory work; **FA** and **SP:** helped in the statistical analysis of data; **IT:** helped in designing the experiment, supervised the laboratory work and edited the manuscript.

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