

## SCIENTIFIC ARTICLE

## 6-Benzylamino purine outperforms Kinetin and Thidiazuron in ameliorating flower longevity in *Calendula officinalis* L. by orchestrating physiological and biochemical responses

Mohammad Lateef Lone<sup>1</sup> , Sumira Farooq<sup>1</sup> , Aehsan ul haq<sup>1</sup> ,  
Shazia Parveen<sup>1</sup> , Inayatullah Tahir<sup>1\*</sup> 

<sup>1</sup> Plant Physiology and Biochemistry Research Laboratory, Department of Botany, University of Kashmir, Srinagar, India

### Abstract

In view of extending the relatively brief postharvest life of flowers by a range of technologies, the present study elucidates the implication of 6-benzylamino purine (BAP), kinetin (KN) and thidiazuron (TDZ) on postharvest performance and flower longevity of isolated flowers of *Calendula officinalis*. BAP and KN belong to adenine group cytokinins while as TDZ is a diphenyl urea compound having cytokinin like activity. The harvested flowers were supplemented with BAP, KN and TDZ at various concentrations viz., 25, 50, 75 and 100  $\mu\text{M}$  at one day before anthesis (cup shaped) stage. The control was designated by a distinct set of flowers held in distilled water (DW). Our findings revealed substantial enhancement in flower longevity by application of various growth regulators as compared to the control. Vase solutions containing BAP and KN at 50  $\mu\text{M}$  and TDZ at 75  $\mu\text{M}$  (individually) were most effective in improving the longevity of cut *Calendula* flowers. Improvement in flower longevity was primarily associated with high membrane stability index (MSI), upregulated activities of various antioxidant enzymes viz., catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX), besides an attenuated lipoxygenase (LOX) activity in the petals. As compared to control, the treated flowers exhibited higher values of soluble proteins, total phenols and total sugars, besides lower  $\alpha$ -amino acid content in the petal tissues. However, BAP outplayed TDZ and KN in improving the flower longevity of *Calendula officinalis* by maintaining higher physiological and biochemical stability in petals.

**Keywords:** *Calendula*, antioxidant enzymes, senescence, flower longevity, cytokinins.

### Resumo

#### 6-Benzilamino purina supera Kinetin e Thidiazuron no aumento da longevidade das flores em *Calendula officinalis* L. orquestrando as respostas fisiológicas e bioquímicas

Visando a estender a vida pós-colheita relativamente breve de flores de corte por uma gama de tecnologias, o presente estudo elucida a implicação de 6-benzilamino purina (BAP), cinetina (KN) e tidiazuron (TDZ) no desempenho pós-colheita e longevidade de flores isoladas flores de *Calendula officinalis*. O BAP e o KN pertencem às citocininas do grupo adenina, enquanto o TDZ é um composto de difenilureia com atividade semelhante à da citocinina. As flores foram suplementadas com BAP, KN e TDZ em várias concentrações 25, 50, 75 e 100  $\mu\text{M}$  um dia antes do estágio de antese (em forma de copo). O controle foi designado por um conjunto distinto de flores mantidas em água destilada (DW). Nossos resultados revelaram o aumento substancial na longevidade das flores pela aplicação de vários reguladores de crescimento em comparação com o controle. Soluções de vaso contendo 50  $\mu\text{M}$  de BAP e KN e 75  $\mu\text{M}$  de TDZ (individualmente) foram mais eficazes em melhorar a longevidade das flores de calêndula cortadas. A melhora na longevidade das flores foi principalmente associada com alto índice de estabilidade de membrana (MSI), o aumento das atividades de várias enzimas antioxidantes viz., Catalase (CAT), superóxido dismutase (SOD) e ascorbato peroxidase (APX), além de uma atividade atenuada de lipoxigenase (LOX) em as pétalas. Em relação ao controle, as flores exibiram maiores valores de proteínas solúveis, fenóis e açúcares totais, além de menor teor de  $\alpha$ -aminoácidos. No entanto, o BAP superou o TDZ e o KN na melhoria da longevidade da flor de *Calendula officinalis*, mantendo maior estabilidade fisiológica e bioquímica nos tecidos das pétalas.

**Palavras-chave:** Calêndula, enzimas antioxidantes, senescência, longevidade das flores, citocininas.

\*corresponding author: [tahir.inayatullah@gmail.com](mailto:tahir.inayatullah@gmail.com)

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## Introduction

Flowers offer an exclusive window to unveil the physiological, biochemical and molecular changes that occur in cells and tissues while progressing towards senescence. Senescence is an intricate process encompassing systematic and highly co-ordinated phases of inception, progression and completion implicated in an extensive re-programming of gene expression. The process involves shut down of several biosynthetic pathways and up-regulation of many degradative pathways, eventually leading to the cell death (Saeed et al., 2014; Ahmad and Tahir, 2016; Sarwat and Tuteja 2019). In addition to alterations in molecular crosstalk, the flower senescence involve various physiological and biochemical changes such as loss of water from the senescing tissue, leakage of ions, generation of reactive oxygen species (ROS), increase in membrane fluidity/lipid peroxidation, hydrolysis of proteins, nucleic acids and carbohydrates (Shibuya et al., 2016; Salleh et al., 2016; Chen et al., 2018; Hemati et al., 2019). Visible symptoms of petal senescence typically include colour change, rolling and wilting (Bagniewska-Zadworna et al., 2014), while anatomical changes such as collapse of mesophyll cells occurs well before the apparent morphological changes (Wagstaff et al., 2003; Shibuya et al., 2016).

Flower longevity and quality are important factors that determine the economic and market value of ornamental flowers (Hegazi, 2016). The key to extend the flower longevity is to understand the biological processes governing postharvest physiology, respiration, transpiration, hormonal imbalance and the activation of enzymes associated with petal discoloration (Wojciechowska et al., 2018). Cytokinins act as the front line anti-senescence hormones that improve the longevity in both the ethylene sensitive and insensitive cut flowers (van Doorn and Woltering, 2008; Ahmad and Tahir, 2016; Iqbal et al., 2017). The anti-senescence role of cytokinin make it the hormone of considerable demand in the postharvest technology to improve the vase performance of cut flowers. Cytokinin delay senescence by increasing sink activity, promote transport and maintain increased amounts of metabolites in flower petals, besides preventing lipid peroxidation of membranes (Radio et al., 2017; Iqbal et al., 2017). Cytokinins may alternatively

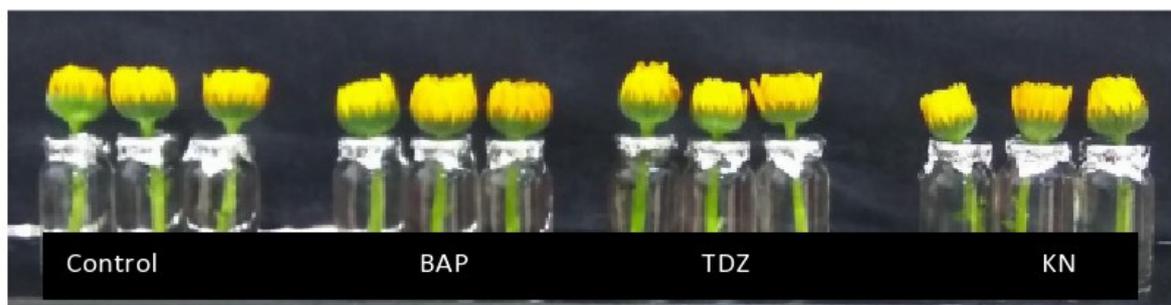
extend the longevity of flowers by enhancing the levels of antioxidant compounds such as phenols and anthocyanins, which function as scavengers of ROS produced during petal senescence (Trivellini et al., 2015). Molecular studies report that cytokinins defer senescence through auto-regulation of cytokinin biosynthesis through isopentenyl transferase gene using a promoter senescence-associated gene 12 (*SAG12*). In this regard, *SAG12*:IPT gene has been extensively evaluated in different species, and all of them evidenced delayed senescence (Xiao et al., 2017).

*Calendula officinalis* (marigold) of asteraceae (compositae) family is grown worldwide as an ornamental plant with great potential in floriculture industry as cut flower because of its vivid and attractive colours (Chopde et al., 2015). However the brief postharvest life of the flower diminishes its potential market value. *Calendula* is least sensitive to ethylene (Kondo et al., 2017). Pertinently, oxidative stress is reported as the main cause of limited vase life in ethylene insensitive flowers (Rahmani et al., 2015) and implication of cytokinins on oxidative stress underlying flower senescence in *Calendula* is yet to be explored. In this regard, an experiment was set-up to ascertain the implication of adenine type cytokinins viz., benzylamino purine (BAP) and kinetin (KN) and a diphenyl urea compound; thidiazuron (TDZ) having cytokinin like activity on oxidative stress associated with flower senescence in *C. officinalis* by evaluating various physiological and biochemical parameters with the ultimate aim to improve its postharvest performance and flower longevity.

## Materials and Methods

### Plant material and treatments

Fresh flower buds of *Calendula officinalis* were harvested at 900 h from Kashmir University Botanical Garden (KUBG) at one day before anthesis stage. After transported to the laboratory, the flowers were re-cut to a uniform stalk length of 4 cm under distilled water and divided into four sets. Three sets were supplied with BAP, KN and TDZ (individually) of different concentrations viz., 25, 50, 75 and 100  $\mu$ M. Another set of flower buds held in distilled water (DW) designated the control (Figure 1).



**Figure 1.** Cup shaped (one day before anthesis) stage of *Calendula officinalis* used for the present study

Each treatment was represented by ten replicates. The experiment was conducted under controlled conditions with relative humidity (RH) of 60%-70%, 12 h light period a day and average temperature of  $21 \pm 2$  °C. The first day of experiment when flowers were subjected to different treatments was designated as day zero. Visual assessment and changes in various parameters occurred in the flowers during the experiment were recorded on day 2 and 4 of transfer of flower buds to the respective vase solutions.

#### Evaluation of flower longevity and floral diameter

The average flower longevity of the cut flowers was calculated from second day of experiment until the last flower lost its market value. The floral diameter was measured as average of two perpendicular measurements across the floral head on day 2 and 4 of the experiment.

#### Evaluation of membrane stability index (MSI)

The MSI of petal tissue evaluated in the form of electrolyte leakage was determined by employing Sairam (1994) method. 0.5 g of petal tissue was incubated in 25 ml of ion free water at 25 °C for 30 minutes and 95 °C for 15 minutes. The conductivities of the petal tissue incubated at 25 °C (C1) and 95 °C (C2) were obtained on Elico CM180 conductivity meter. MSI was calculated by using the formulae:

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

#### Determination of total phenols and sugar fractions

1 gram chopped petal tissue from each treatment was macerated and centrifuged. An appropriate aliquot taken from the supernatant was used to estimate these parameters. Swain and Hillis (1959) method was used for quantification of phenols using gallic acid as standard. Nelson's (1944) protocol was employed for quantification of reducing sugars using glucose as standard. Estimation of total sugars was determined by converting non-reducing sugars into reducing sugars using invertase enzyme. Amount of non-reducing sugars was determined by evaluating the difference between total and reducing sugars.

#### Quantification of soluble proteins and $\alpha$ -amino acids

For quantification of proteins, 1 gram of petal tissue was macerated in 100 mM phosphate buffer (pH 7.2) comprising NaCl (150 mM), ethylene diamine tetraacetic acid (1 mM), Triton X-100 (1%), glycerol (10%), polyvinyl

pyrrolidone (10%) and Dithiothreitol (1 mM). The mixture was subjected to centrifugation at 12,000 g at 4 °C in a refrigerated centrifuge for 15 minutes. Employing Lowry et al. (1951) protocol, a suitable aliquot taken from the supernatant was used for quantifications of proteins. Estimation of  $\alpha$ -amino acids was performed by employing Rosen's (1957) protocol using glycine as standard.

#### Extraction and determination of enzyme activities Superoxide dismutase (SOD)

SOD activity was determined by observing the inhibition of photochemical reduction of nitroblue tetrazolium (NBT), employing Dhindsa et al. (1981) protocol. The reaction mixture contained sodium carbonate (50 mM), NBT (75  $\mu$ M), ethylene diamine tetraacetic acid (0.1 M), methionine (13 mM) in 50 mM phosphate buffer (pH 7.8) and 0.1 ml of the enzyme extract in a final volume of 3 ml. The reaction was initiated with the addition of 2  $\mu$ M riboflavin and keeping the test tubes in water bath at 25 °C and illuminating with a 30 W fluorescent lamp. The test tubes were kept in dark after the reaction was stopped by switching off the light. Other identical unilluminated test tubes served as blanks. Absorbance was obtained at 560 nm by using spectrophotometer. One unit of SOD activity was defined as the amount of the enzyme that prevents 50% photoreduction of NBT to blue formazan in comparison to the reaction mixture kept in dark. The SOD activity was expressed as units  $\text{min}^{-1}\text{g}^{-1}$  protein.

#### Catalase (CAT)

CAT activity was assayed by Aebi (1984) protocol. Petal tissue (1 g) was macerated and homogenized in 100 mM potassium phosphate buffer of neutral pH containing ethylene diamine tetra acetic acid (1 mM). The reaction mixture contained 50 mM potassium phosphate buffer of neutral pH,  $\text{H}_2\text{O}_2$  (12.5 mM), enzyme extract (50  $\mu$ l) and distilled water to make the volume to 3 ml. With the addition of  $\text{H}_2\text{O}_2$ , reaction was initiated and the catalase activity was assayed by determining  $\text{H}_2\text{O}_2$  consumption for 3 minutes at 240 nm and expressed as  $\mu\text{M H}_2\text{O}_2$  red.  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein.

#### Ascorbate peroxidase (APX)

Chen and Asada (1989) protocol was employed for determining APX activity. Petal tissue (1 gram) was macerated in 100 mM sodium phosphate buffer containing

ascorbate (5 mM), glycerol (10%) and ethylene diamine tetraacetic acid (1 mM). The APX activity was determined in 1 ml reaction mixture containing 50 mM potassium phosphate buffer of neutral pH, ascorbate (0.1 mM), H<sub>2</sub>O<sub>2</sub> (0.3 mM) from absorbance decrease due to ascorbate oxidation. The absorbance was recorded at 290 nm for 3 minutes and expressed as  $\mu\text{min}^{-1}\text{mg}^{-1}\text{protein}$ .

#### Lipoxygenase (LOX)

LOX activity was ascertained by employing Axerold et al. (1981) protocol. Petal tissue (1 gram) was homogenized in extraction buffer comprising 50 mM potassium phosphate buffer (pH 6.5), polyvinyl pyrrolidone (10%), Triton X-100 (0.25%) and phenyl methane sulfonyl fluoride (1 mM). Reaction mixture of 1 ml contained Tris-HCl buffer (50 mM) of pH 6.5 and linoleic acid (0.4 mM). By adding 10  $\mu\text{l}$  of crude petal extract to the mixture, reaction was started and absorbance was recorded at 234 nm for 5 minutes by spectrophotometer. The activity was expressed as  $\mu\text{Mmin}^{-1}\text{mg}^{-1}\text{protein}$ .

#### Statistical Analysis

A completely randomized design was performed on the data collected from experiment. The investigation was repeated twice to minimize the variance between the variables. Complete statistical analysis was done and Least significant difference (LSD) was computed at P 0.05 using SPSS ® (SPSS version 16 software; Chicago USA).

### Results

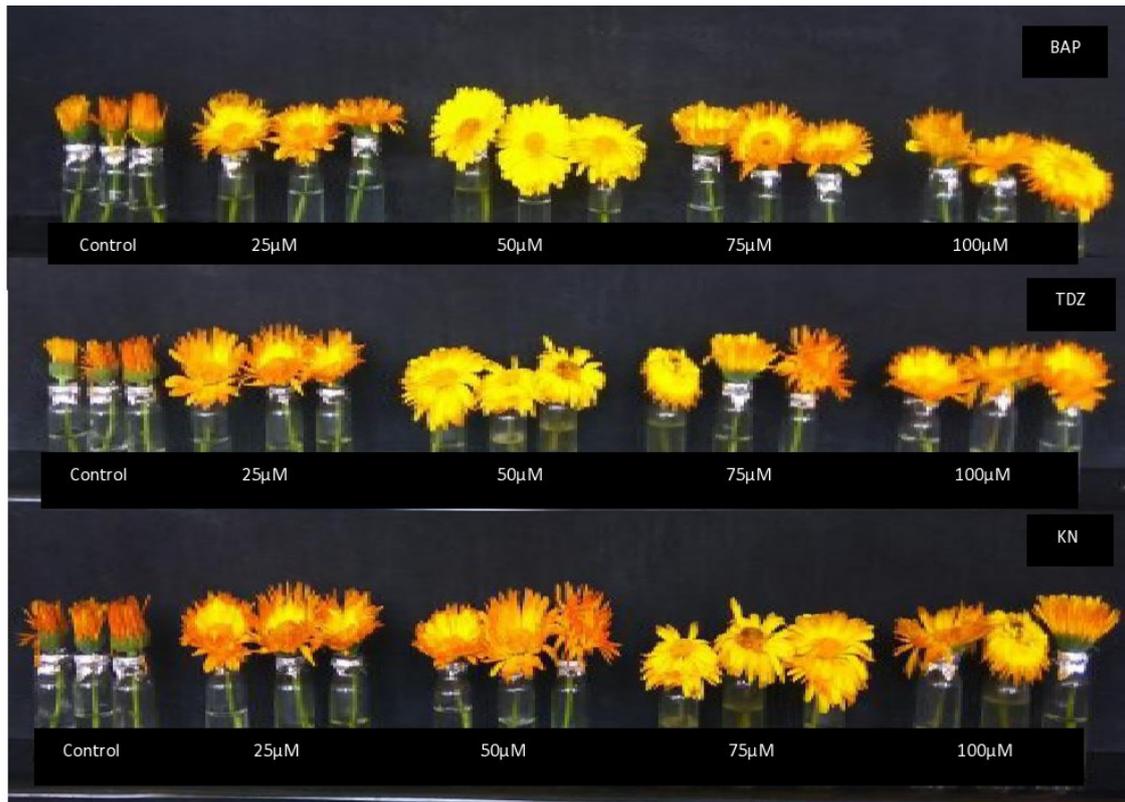
Based on the present investigation, significant differences were revealed among the growth regulators (KN, BAP and TDZ) tested at various concentrations in extending the

longevity of cut *Calendula* flowers. The prolonged flower longevity commensurate with up-regulated activities of antioxidant enzymes, high membrane stability, elevated total phenols, total sugars and soluble proteins, besides a decreased  $\alpha$ -amino acid content and LOX activity in the tissue samples. Application of BAP and KN at 50  $\mu\text{M}$  and TDZ at 75  $\mu\text{M}$  resulted in maximum enhancement in flower longevity and improved postharvest performance of cut *Calendula* flowers considerably. The petal tissue from the samples treated with the concentrations above and below of optimal concentrations (50  $\mu\text{M}$  BAP and KN) and (75  $\mu\text{M}$  TDZ) showed a significant decrease in all the parameters investigated except LOX activity and  $\alpha$ -amino acids which showed a marked increase. However, BAP profoundly increased the flower longevity and was most effective in improving postharvest performance of *Calendula* flowers as compared to other growth regulators and control.

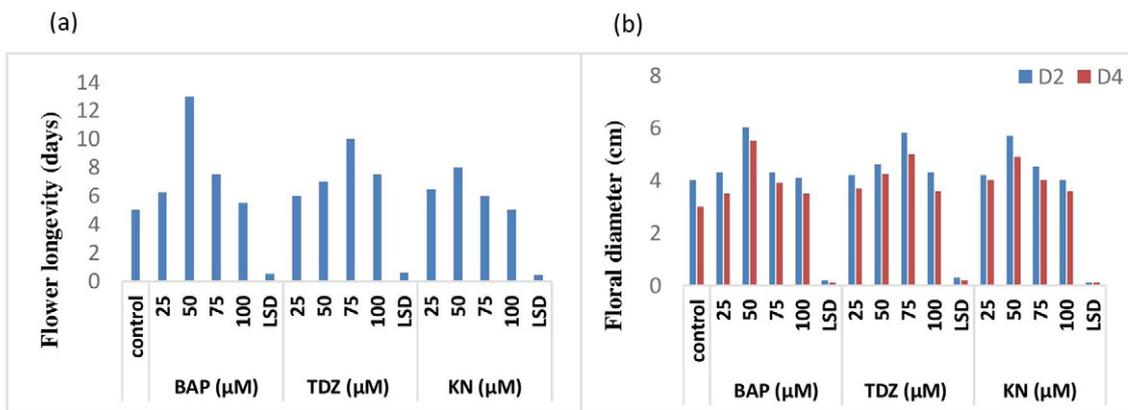
#### Flower longevity and floral diameter

Flower senescence was characterized by loss of petal turgidity leading to shrinkage and upward erection of petals in the form of needles. The petals however didn't abscise, remained on the flower pedicels till complete wilting. The flower buds held in distilled water (control) lasted for 5 days only, whereas application of growth regulators increased the longevity of cut *Calendula* flowers markedly. The longevity of flowers held in 50  $\mu\text{M}$  BAP, 75  $\mu\text{M}$  TDZ and 50  $\mu\text{M}$  KN solutions recorded was 13, 10 and 8 days respectively, revealing BAP as most efficacious cytokinin in improving the longevity of cut *Calendula* flowers (Figure 2).

Head diameter of flowers held in various test solutions showed a significant increase as compared to control which however was observed to decrease gradually from day 2 to 4 (Figures 3a, b).



**Figure 2.** Effect of different concentrations of BAP, TDZ and KN on postharvest performance of isolated flowers of *Calendula officinalis* on day 7 of transfer to the test solutions.

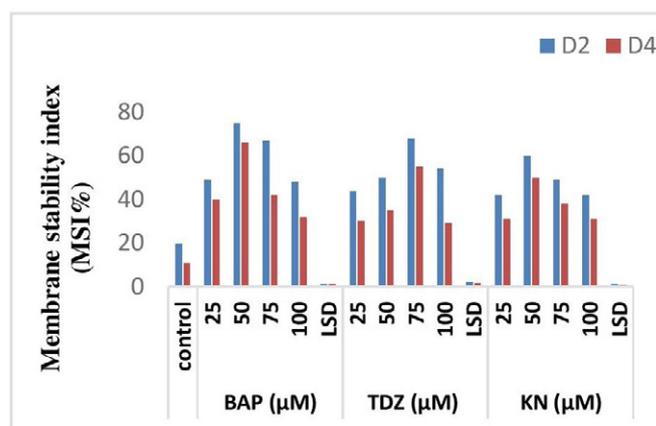


**Figure 3.** Effect of different concentrations of BAP, TDZ and KN on flower longevity (a) and floral diameter (b) of isolated flowers of *Calendula officinalis*. Each value denotes the mean of five replicates for each treatment.

### Membrane stability index (MSI)

Changes in MSI values suggest that with the development of flowers, membranes lose their integrity resulting in outpouring and loss of intracellular compartmentalization. Selected growth regulators in this study revealed their effectiveness in protecting cell membranes. Highest MSI

value was found in the flowers treated with BAP, while those treated with TDZ and KN registered marginally lower values but significantly higher than the control. However, membrane stability in petal tissues was found to decrease gradually from day 2 to 4 as evident from MSI values in Figure 4.



**Figure 4.** Effect of different concentrations of BAP, TDZ and KN on MSI of isolated flowers of *Calendula officinalis*. Each value denotes the mean of three replicates for each treatment.

### Total phenols and sugar fractions

As compared to control, higher phenolic and sugar (total, reducing and non-reducing sugar) content was observed in the flowers held in BAP followed by TDZ and KN (Figures 5a, b). Furthermore, reducing sugar content was found marginally higher than non-reducing sugar content in all tissue samples treated with various growth regulators (Figures 5c, d). However, a significant reduction in both of these parameters was recorded in the flower petals with progression in time from first to second analysis.

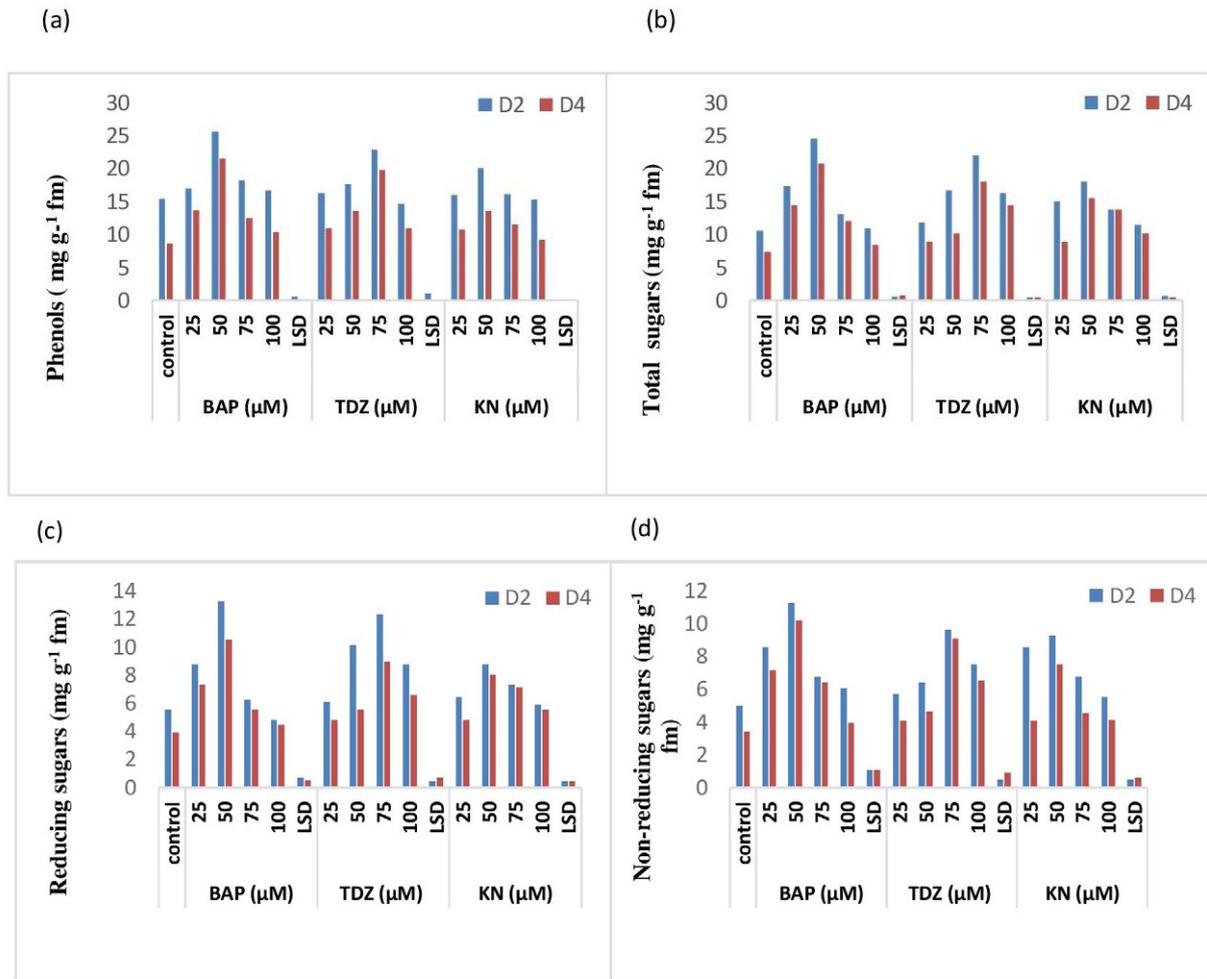
### Soluble proteins and $\alpha$ -amino acid content

Flowers treated with various growth regulators registered higher content of soluble proteins in petal

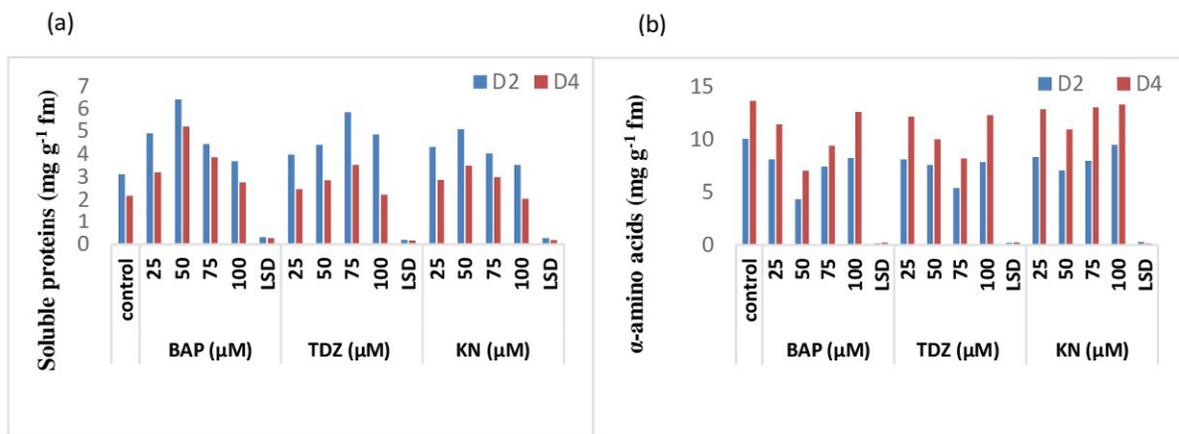
tissues. The petal tissue from the samples treated with BAP showed maximum enrichment of soluble proteins, besides higher reduction in the  $\alpha$ -amino acids followed by TDZ and KN. Maximum  $\alpha$ -amino acid content was recorded in the samples of the control. However, soluble protein content was found to decrease with progression in time from day 2 to 4 with the concomitant increase in  $\alpha$ -amino acids (Figures 6a, b).

### Activities of antioxidant and lipoxygenase enzymes

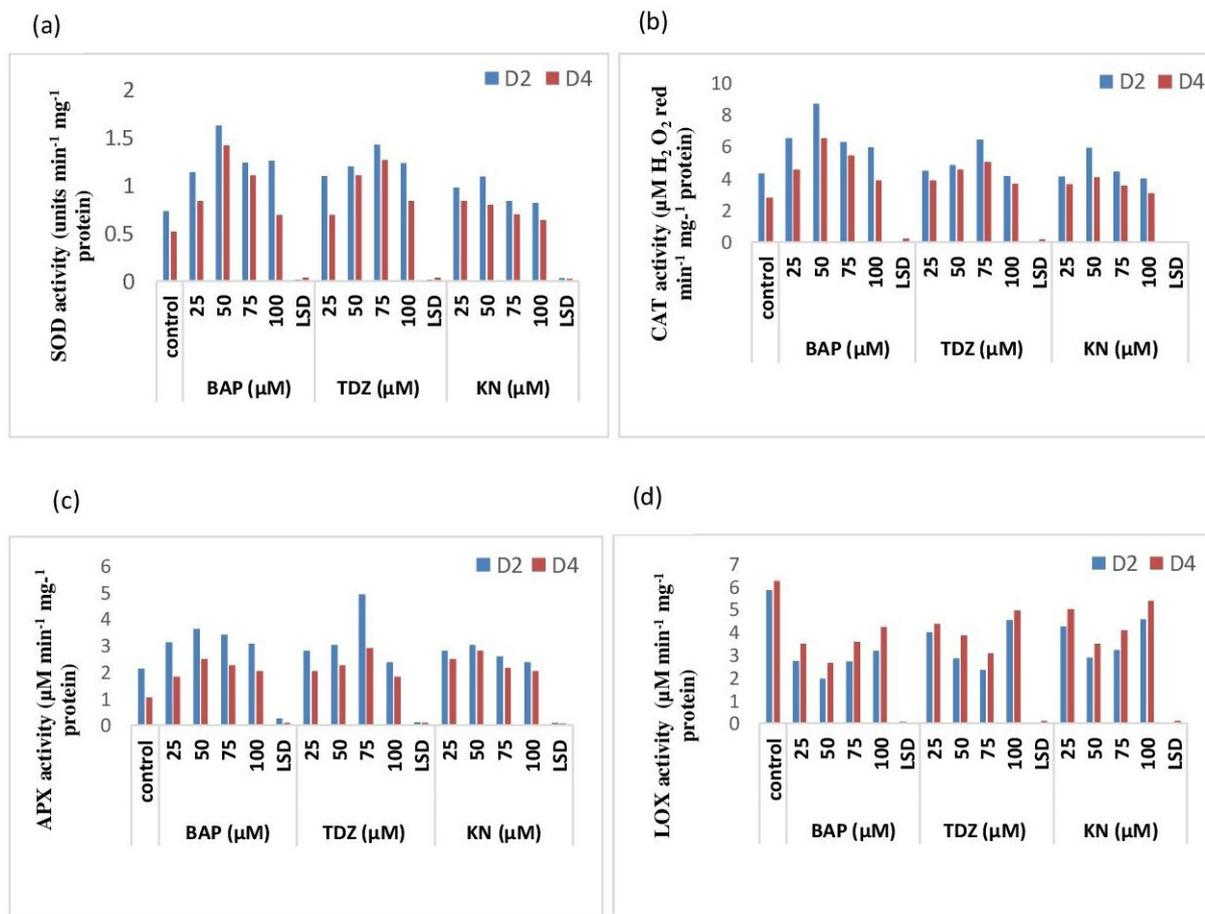
SOD and CAT activities were profoundly augmented in the flowers treated with BAP followed by TDZ and KN, whereas TDZ was more effective in enhancing the APX activity in the tissue samples (Figures 7a, b, c).



**Figure 5.** Effect of different concentrations of BAP, TDZ and KN on total phenols (a), total sugars (b), reducing sugars (c) and non-reducing sugars (d) in petal tissues of isolated flowers of *Calendula officinalis*. Each value denotes the mean of three replicates for each treatment.



**Figure 6.** Effect of different concentrations of BAP, TDZ and KN on soluble proteins (a) and  $\alpha$ -amino acids (b) in petal tissues of isolated flowers of *Calendula officinalis*. Each value denotes the mean of three replicates for each treatment.



**Figure 7.** Effect of different concentrations of BAP, TDZ and KN on activities of SOD (a), CAT (b), APX (c) and LOX (d) in petal tissues of isolated flowers of *Calendula officinalis*. Each value denotes the mean of three replicates for each treatment.

Furthermore BAP application resulted in considerable decrease in LOX activity followed by TDZ and KN (Figure 7d). Least antioxidant enzyme activities (SOD, CAT and APX) were assayed in untreated petal tissues (control), besides an increased LOX activity. Activities of these enzymes were found to decline as the flower development progressed with concomitant increase in LOX activity. However, 50 μM BAP was found highly effective in preventing the down-regulation of activities of these enzymes in the petal tissues as pictured in Figures 7a, b, c, d.

## Discussion

Flower longevity is one of the most important characteristics of cut flowers, which is shortened by a number of factors varying in wide range of ornamentals (Vehniwal and Abbey 2019). The current study ascertains the role of BAP, TDZ and KN (as continuous treatments) in regulating senescence and mitigating postharvest losses in cut flowers of *Calendula officinalis*, keeping in view its immense demand in floriculture industry as cut flower. Although, TDZ doesn't belong to BAP and KN type of cytokinins (adenine type), but it possesses cytokinin

like activities, so the three tested growth regulators are discussed together as cytokinins.

Exogenous application of cytokinins and TDZ increased the longevity of cut *Calendula* flowers significantly. Cytokinins could improve flower longevity by affecting many physiological processes such as free radical formation, membrane stability, water balance and protein metabolism (Lama et al., 2013; Salleh et al., 2016; Ahmad et al., 2018; Tahir et al., 2019). Benzyl adenine (BA) application delayed flower senescence by reducing the abscisic acid (ABA) production, which is known to get raised during flower senescence in both ethylene sensitive and insensitive flowers (Trivellini et al., 2011; Ibrahim et al., 2019). Application of Adenine and phenyl urea derivatives have been reported to delay senescence in various cut flowers such as *Iris*, *Dahlia*, *Gerbera*, *chrysanthemum*, *Hemerocallis*, *Eustoma*, *Anthurium* and *Nicotiana* (van Doorn and Woltering, 2008; Danaee et al., 2011; Hatamzadeh et al., 2012; Ahmad and Tahir, 2016; Reid and Wu, 2018; Ahmad et al., 2018; Bergmann et al., 2018; Tahir et al., 2019). Cytokinins application might increase flower dimensions and improve flower coloration by supplying more photosynthates to the flower head. Accumulation of excessive solutes (photosynthates)

establishes a water potential gradient, leading to entry of water into petal tissues making them turgid, consequently increasing the floral head diameter (Reid and Wu, 2018).

*Calendula* is least sensitive to ethylene, and oxidative stress is known to be the main reason of early senescence in ethylene insensitive flowers. Against to this, BAP was highly effective in maintaining a decreased LOX activity in flowers followed by TDZ and KN, which prevented the excessive lipid peroxidation of the membranes as evident from increased MSI values in the tissue samples. The reduced LOX activity resulted in maintenance of adequate phospholipids, proteins and thiols by preventing the seepage of proteases from vacuoles into the cytoplasm (Macnish et al., 2010; Liu et al., 2016a; Dek et al., 2017). Cytokinin mediated enhanced membrane integrity can also be attributed to its involvement in maintenance of high water content, antioxidant system and reduced peroxidation rates in tissues, which are greatly affected by the stresses like senescence (Ahmad et al., 2018; Honig et al., 2018). Studies on senescence regulation in *C. officinalis*, showed that BAP could minimize the reduction in MSI values (Kaur et al., 2015). Moreover, application of BAP to cauliflower and carnations resulted in a marked decrease in lipid peroxidation with concomitant increase in membrane stability of tissue samples (Siddiqui et al., 2015; Ramtin et al., 2019).

High protein levels with concomitant lower  $\alpha$ -amino acid content was maintained in flowers supplied with various growth regulators particularly in BAP treated flowers. Application of postharvest preservatives that downregulate the protease activity can be suggested as a strategy for extending the vase life and minimizing postharvest losses of flowers (Dwivedi et al., 2016). External application of cytokinins was found very effective in delaying senescence of various cut flowers such as *Iris*, *Phalaenopsis* and *Nicotiana* by arresting degradation of proteins and chlorophyll (Ahmad et al., 2018; Chen et al., 2018; Tahir et al., 2019). Indeed, cytokinins extended the longevity of *Andropogon* and *Nicotiana* flowers not only by preventing protein degradation but also by inhibiting the activity of proteases. This suggests that cytokinins delay senescence by downregulating the programmed cell death due to prevention of upsurge of proteasomes (Macnish et al., 2010; Nisar et al., 2015). Exogenous application of BA strongly upregulated some heat shock proteins (HSPs) in petunia flowers which might protect cellular functions or slow down the action of proteases (Veerasingam et al., 2007).

Cytokinins activate defense mechanism against postharvest senescence additionally through enrichment of phenols (Schmitzer et al., 2010; Ahmad and Tahir, 2017) as ascertained during the present study. Phenol enrichment has been reported to counter endogenic perturbations, biotic and abiotic oxidative stress in various flowers (Lattanzio et al., 2006; Ahmad and Tahir, 2017). Cytokinin mediated accumulation of phenolic compounds may contribute in strengthening cell wall structures and prevent oxidative damage to membrane lipids and proteins

by modulating the peroxidation kinetics through free radical scavenging (Soleimani Aghdam et al. 2015). A higher phenolic content was assayed in the petal tissue from the samples treated with various growth regulators. Petunia flowers treated with cytokinins also showed a considerable enhancement in phenol accumulation (Trivellini et al., 2015). In Arabidopsis, exogenous application of cytokinins resulted in increased phenol accumulation by stimulating the activation of phenylalanine ammonia lyase (PAL1), chalcone synthase and chalcone isomerase genes, involved in the synthesis of various phenolic compounds (Diekmann and Hammer, 1995).

Higher sugar content was maintained in the tissue samples treated with various cytokinins consistent with the earlier findings which reported a correlation between carbohydrate accumulation and flower longevity by cytokinin application in various flowers such as *Gladiolus*, *Iris*, *Nicotiana*, *Gerbera*, and *Rosa* (van Doorn and Woltering, 2008; Ahmad et al., 2018; Tahir et al., 2019; Hemati et al., 2019; Ramtin et al., 2019). Cytokinins maintain high sugar content in petal tissues by enhancing the sink strength, thus inhibiting the mobilization of sugars from the floral parts to ovary during nutrient remobilization (van Doorn and Woltering, 2008). Furthermore, flower petals like other terminally differentiated sink tissues often contain highly active invertase enzyme, that catalyzes the hydrolysis of sucrose (non reducing sugar) into glucose and fructose (reducing sugars) (Woodson and Wang, 1987).

An increase in ratio of non-reducing to reducing sugars occurs concomitant with petal senescence (Nichols, 1973). Against to this, cytokinins maintained marginally high ratio of reducing to non-reducing sugars in the petal tissues of *Calendula* flowers, contributing to its prolonged vase life.

As mentioned above, oxidative stress caused by production and accumulation of ROS has been recognised as main factor for early postharvest senescence in ethylene insensitive flowers (Rahmani et al., 2015; Dwivedi et al., 2016; Saeed et al., 2016). In order to combat adverse effects of oxidative stress, high antioxidant enzyme activity is prerequisite for efficient scavenging of excessive ROS, that otherwise damage cell membranes and cause flower deterioration. An upregulated activities of SOD, CAT and APX enzymes were recorded in petal tissues supplemented with BAP, KN and TDZ. The association of high antioxidant enzyme activity and flower longevity has been reported in several cut flowers such as carnations, chrysanthemums, red dragon, gerbera, gladiolus and iris (Dwivedi et al., 2016; Ahmad and Tahir, 2016; Naing et al., 2017; Hemati et al., 2019).

Cytokinins improve flower longevity either directly by scavenging the free radicals or alternatively by up-regulating the production and activities of antioxidant enzymes that scavenge ROS (Siddiqui et al., 2015; Liu et al., 2016b; Danilova et al., 2017). Petals with decreased APX activity has been found to show early senescence symptoms in various flowers like daylily, gladiolus and carnation by generation and accumulation of ROS (Mittler

et al., 2004; Saeed et al., 2014). Exogenous application of cytokinins effectively reduced the free radical formation in tobacco plant during senescence (Dek et al., 2017). The role of cytokinins in maintaining the ambient levels of antioxidant enzymes for longer vase life of the flowers has also been demonstrated by application of cytokinin oxidase inhibitors in wall flowers (Bartrina et al., 2017).

### Conclusion and future scope

From the present investigation, the authors conclude that treatment of isolated flower buds with adenine type cytokinins (BAP and KN) and a diphenyl urea compound (TDZ) having cytokinin like activity minimized the postharvest oxidative damage in *C. officinalis* as evident from improved flower longevity and postharvest attributes. BAP was found most efficacious and outcompeted other growth regulators in improving the postharvest performance and longevity of flowers. Based on the findings, it can be argued that the improvement in longevity and quality of isolated flowers of *C. officinalis* by application of BAP, TDZ and KIN could be attributed to up-regulated activities of various antioxidant enzymes, upsurge in MSI and phenolic content, besides lower LOX activity and  $\alpha$ -amino acid content in the petal tissues. Furthermore, maintenance of elevated content of soluble proteins and sugar fractions in the petals by these growth regulators also qualify their role in improving the flower longevity of *Calendula officinalis*.

The study offers immense future scope to mitigate the flower senescence and postharvest losses at molecular level, which will argue the studies of complex interplay and crosstalk of cytokinins and related compounds in this beautiful and fascinating flower. Moreover, studies at molecular level may prove beneficial in gaining an insights into the strategies of flower senescence across the family. Understanding the mechanism of flower senescence in this flower will unveil strategies to modulate it by various chemical formulations and/or molecular interventions for its efficient and productive postharvest output.

### Author contribution

**MLL:** designed and conducted the experiment, assessed results, compiled data and drafted the manuscript. **SF, AuH:** assisted in experimental and laboratory work. **SP:** assisted in statistical analysis of data. **IT:** helped in designing the experiment, supervised at all stages and performed critical revision of the manuscript. All authors participated in discussion of the manuscript.

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