

# Effects of pre-harvest applications of different source of calcium on the cell wall fractions and stem bending disorder of Gerbera (*Gerbera jamesonii* L.) cultivar flowers

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**Key words:** catalase, cellulose, lignin, membrane stability index, superoxide dismutase.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

**Abstract:** Gerbera flower belongs to the composite family and is one of the top five cut flowers in the world in terms of production and consumption, which has a great economic value in the international flowering industry. This study was designed to evaluate whether calcium pre-harvest application, provided through 0, 0.5, 1 and 1.5% of calcium chloride (CaCl<sub>2</sub>) and calcium nitrate (CaNO<sub>3</sub>), could extend the day of stem bending of gerbera cut flower. In the present study, we used two gerbera cultivars 'Intense' and 'Rosaline' as resistant and sensitive to stem bending, respectively. For evaluation of associated traits with stem bending, the produced flowers were kept in a vase solution containing 200 mg/L hydroxyquinoline with temperature conditions of 20°C. The results showed that day of stem bending of flowers extended 9.62 and 10.37 days by application of 1% CaCl<sub>2</sub> for 'Rosaline' and 'Intense' respectively. All treatments were effective in the increasing relative water content of flower due to increase water uptake. The results also revealed that the calcium pre-treatment delayed flowers senescence and increased antioxidant enzyme activity. Application of calcium resulted in an increase in membrane stability index in the cut flowers of both cultivars, providing evidence for delay of senescence in calcium-treated cut flowers. Also, results showed that calcium application significantly increased lignin, cellulose and hemicellulose content of both cultivars. The maximum and the minimum lignin and cellulose content were observed in resistant and sensitive cultivars, respectively. In general, pre-harvest application of calcium (especially 1% CaCl<sub>2</sub>) with increasing of antioxidant enzyme activity and stem lignification led to decreasing of stem bending disorder in both cultivars.

## 1. Introduction

Gerbera is one of the most important ornamental plants in the world

used widely as cut flower or potted flower (Çelikel and Reid, 2002; Perik *et al.*, 2014). Gerbera, followed by rose, chrysanthemum, tulip, and lily, occupies fifth position in top ten cut flower in world in terms of its production and consumption (Nair *et al.*, 2006; Perik *et al.*, 2014). The longevity or postharvest life of gerbera flowers depends on the extent of stem bending and the lack of stem strength (Naing *et al.*, 2017; Nazarideljou and Azizi, 2015). In this regard, although flowers of some gerbera cultivars are apparently not wilted or faded, their stems have drooped due to their inappropriate stem strength; and based on flower quality and grading, flowers with stem bending more than 30° are not worth maintaining (Çelikel and Reid, 2002; Perik *et al.*, 2014). The lack of mechanical protection and cell wall lignification (especially secondary cell wall) as well as lowering stem strength are of more likely reasons for emerging stem bending in gerbera (Perik *et al.*, 2012). In addition to its remarkable effect on stem hardening and growing gerbera uprightly, lignin plays an utmost role in promoting water flow inside xylem vessels (Vanholme *et al.*, 2010). Also, there is a negative relationship between the rate of stem lignification and the extent of stem bending (Perik *et al.*, 2012). Accordingly, increasing stem lignification directly influences stem strength and thereby reducing stem bending of gerbera cut flowers (Nazarideljou and Azizi, 2015).

In past years, many attempts have been made to increase postharvest life and quality of cut flowers through different techniques. In cut flowers industry, inappropriate condition and inadequate nutrition often lead to a reduction in the quality and quantity of produced gerbera flowers; and regarding these issues can economically improve gerbera flowers production (Khangoli, 2001). Calcium is an essential element affecting on plant growth and development; and its accumulation in plants facilitates pectin polymers' linkage so as to improve mechanical strength of stem in line with reducing stem bending and extending flowers vase life (Gerasopoulos and Chebli, 1999; Hepler, 2005; Li *et al.*, 2012). The role of intra and intercellular calcium on cell metabolism change is attributed to its effect on the membrane structure and function and cell walls (Ferguson and Drobak, 1988). The results of previous studies showed that calcium, due to its role in the synthesis of pectin located in cell wall of xylem vessels, facilitates water translocation in stem and inhibits stem bending (Van leperen and Van Gelder, 2006). Also, applying calcium as pre or postharvest treatments caused to

increase the vase life of many cut flowers (Gerasopoulos and Chebli, 1999; Hepler, 2005; Li *et al.*, 2012; Geshnizjany *et al.*, 2014). In spite of existing such documents, the effects of calcium on reducing stem bending of gerbera cut flowers are in needs of more investigations. Therefore, in present research, the effect of different calcium concentrations in forms of chloride and calcium nitrate on stem bending and other related parameters in two gerbera cultivars were scrutinized.

## 2. Materials and Methods

This research was conducted using a factorial experiment in completely randomized design (CRD) with four replicates and fourteen plots in each replicate and five pots in each plot (totally 280 pots) in local commercial greenhouse (Persian Gulf, Tehran, Iran).

Based on the sensitivity to stem bending, two cultivars of gerbera, namely 'Intense' as a tolerant cultivar and 'Rosaline' as a sensitive cultivar to stem bending (Nazarideljou and Azizi, 2015), were used in this experiment. Gerbera seedlings were cultivated in pots with 18 cm diameter filled with coco peat and perlite (1:1) maintained in greenhouse with 20-25/15-18°C (day/night), 70±5% relative humidity and 14 h with a light intensity of 40  $\mu\text{mol}^{-2} \text{m}^{-2} \text{s}^{-1}$  (Naing *et al.*, 2017). Before receiving the treatments, the seedlings were feeded based on a nutritional formula used by most commercial greenhouses in Iran (Table 1).

Table 1 - Nutrient elements used in gerbera plant fertilization during excremental period

Elements	Content (mmol/L)
<i>Macro-elements</i>	
Nitrogen	10
Phosphorous	1.8
Potassium	5.5
Magnesium	2
Sulphate	3
<i>Micro-elements</i>	
Iron	40
Magnesium	5
Zinc	5
Copper	1
Molybdenum	1
Boron	30

After planting seedlings in pots and undergoing their perfect establishment, experimental seedlings received calcium chloride and calcium nitrate treatments at three levels (0.5%, 1%, and 1.5%) in form of foliage spray (Mehran *et al.*, 2007) while controls received distilled water. The flowers were harvested when two to three rows of ray floret got color but the stamens still did not open. After preparing of flowers stem in the length of 40 cm, they were placed in the Erlenmeyer containing 500 cc distilled water and hydroxiqinolin sulphate (HQS) in concentration 200 mg/L. Then, they were transferred into the ambient temperature and a light with intensity of  $28 \mu\text{mol}^{-2} \text{m}^{-2} \text{s}^{-1}$  and 60% relative humidity (Gerasopoulos and Chebli, 1999). The treatments were measured on 9<sup>th</sup> of experiment.

Stem bending was determined by assessing the accompanying change in the position of the flower head as described previously (Perik *et al.*, 2012). Ten excised petals per each flower were weighed (fresh weight, FW) and placed in distilled water in the dark for 6 hours to allow them to reach full turgidity and, hence, to determine their turgid weight (TW). These samples were then dried at 70°C for 24 h and their dry weight (DW) was recorded. Finally, relative water content was calculated using the  $\text{FW-DW}/\text{TW-DW}$  (Abdolmaleki *et al.*, 2015).

Membrane stability index (MSI) was recorded using an electrical conductivity meter based on Ezhilmathi *et al.* (2007). Petal samples were rinsed and immersed in 10 ml of distilled water. The samples were incubated at room temperature for 60 min with shaking (150 rpm). The electrical conductivity of the solution (EC1) was read after shaking using a conductivity meter. Samples were incubated in 95°C water bath for 20 min the second reading (EC2) was taken after the solutions had cooled to room temperature. MSI was calculated as  $[1-(\text{EC1}/\text{EC2})] \times 100$ .

Petal samples (0.2 g) was homogenized with 2 ml of 50mM phosphate buffer (pH=7) containing 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF) and 2% polyvinylpyrrolidone (PVP) (w/v) in ice water-bath and then centrifuged at 12000 g for 20 min at 4°C. The supernatant was collected and used for enzyme assay with a UV-Visible spectrophotometer (Cary 100, Varian, USA).

The activities of enzymes were determined according to standard methods previously reported for CAT (EC1.11.1.6) (Aebi, 1984) and SOD (EC 1.15.1.1) (Giannopolitis and Ries, 1977). Enzyme activities were expressed as enzyme units.mg<sup>-1</sup> protein. Protein content was determined according to

the method of Bradford (1976).

The cell wall materials of the proximal end of flower stem were fractioned following the method of Li *et al.* (2012). Briefly, the stems were ground into fine powder in liquid nitrogen and extracted with 95% alcohol, then washed twice with boiling alcohol and methyl alcohol: chloroform (1:1, v/v), respectively. Finally, the cell wall residues were dried over night at 50°C and used for analysis. Hemi-cellulose content was determined by the phenol colorimetric assay (Dubois *et al.*, 1956) and the cellulose content was measured by the anthrone colorimetric assay (Updegraff, 1969). Lignin content was determined following the method of Müse *et al.* (1997). All measurements were reported as  $\mu\text{g}/\text{mg DW}$ . Stem calcium concentration was determined by atomic absorption spectrophotometer (Abdolmaleki *et al.*, 2015).

The data were analyzed using SAS software and the comparing means was carried out using Duncan multiple range test at 5% probability and the drawing figures were carried out using excel 2013 software.

### 3. Results and Discussion

#### Stem bending

The results of variance analysis showed that the main effects of treatment and cultivar ( $p < 0.01$ ) and their interaction effects ( $p < 0.05$ ) were significant on stem bending (Table 2). All calcium treatments, except 0.5% calcium nitrate in 'Intense', significantly increased the days until onset of stem bending. Also, calcium chloride had better effect on stem bending in 'Rosaline'.

Stem bending is the most important factor affect-

Table 2 - Analysis of variance (ANOVA) for cultivar, calcium treatment and their interactions effects on evaluated dependent variables in gerbera cut flower

Dependent variable	Cultivar (C)	Treatment (T)	C × T
Stem bending	**	**	*
Relative water content	NS	**	NS
Membrane stability index	**	**	*
Catalase activity	**	**	**
Superoxid dusmutase activity	*	**	*
Lignin content	**	**	*
Cellulose content	**	**	**
Hemicellulose content	NS	**	*
Calcium content	**	**	NS

\*\* and \* represent significance at the 1 and 5% probability levels, respectively, and ns represents non-significance at  $p < 0.05$ .

ing the quality of cut flowers, flower vase life, and flower loss during harvesting in gerbera. Genetic, nutrition, storage temperature, and the lack of water balance in xylem vessels are of the most important factors facilitate stem bending (Van Meeteren, 1978; Perik *et al.*, 2012; 2014). Reduction in rigidity of the stems carrying flower is associated with the reduction in the formation of lignin compounds. In this regard, it has been stated that the higher-vase life cultivars of *chrysanthemum* contain more lignin compared to lower-vase life ones (Lv *et al.*, 2011). It has been described that tolerance to stem bending is contributed to the presence of a sufficient amount of sclerenchyma and lignin in the stems of flowers (Perik *et al.*, 2012). So, each factor being able to increase the stem rigidity also reduce stem bending. Calcium ions maintain cell wall rigidity as they bind to pectin molecules, thereby increasing cell wall stiffness. The positive effects of calcium chloride on the reduction of stem bending in gerbera were proved by Gerasopoulos and Chebli (1999). Also, Perik *et al.* (2014) reported that calcium treatments decreased stem bending in gerbera cultivars likely due to enhancing stem rigidity. It seems that calcium inhibits vascular blockage and escalates stem strength in line with reduction in stem bending and consequently increasing flower vase life (Fig. 1).

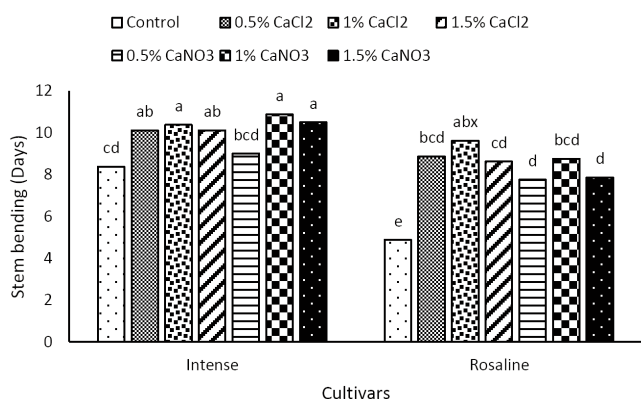


Fig. 1 - The effects of calcium treatments on day of stem bending of two gerbera cultivars. Bars represent standard error of four replicates. Values with the different letters are significantly different according to Duncan's Multiple Range Test at P<0.05.

### Relative water content

The results showed that just treatment had a significant effect on relative water content (RWC). The effects of cultivar and its interaction with treatment were not statistically significant (Table 2). Compared to controls, all applied calcium treatments effectively

increased RWC and among all applied treatments, 1% calcium chloride had the better effect on RWC while 1.5% calcium nitrate gained the lowest effects on RWC (Fig. 2).

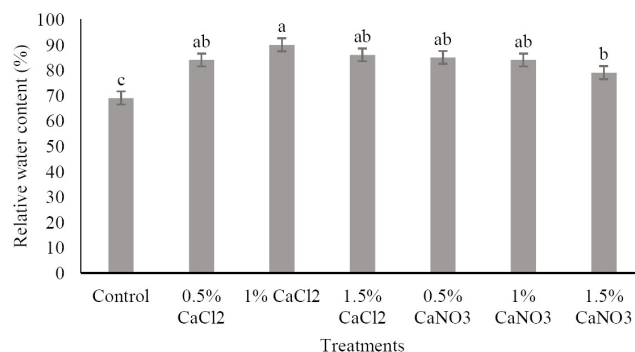


Fig. 2 - The main effects of calcium treatments on petals relative water content of two gerbera cultivars. Bars represent standard error of four replicates. Values with the different letters are significantly different according to Duncan's Multiple Range Test at P<0.05.

The results of this research are in agreements with those obtained by Cortes *et al.* (2011) who reported that calcium treatments increased the relative fresh weight of cut flowers. In gerbera, applying calcium treatments extended flowers vase life by enhancing water uptake by stem and maintaining flower water content (Geshnizjany *et al.*, 2014). Also, it has been stated that the flower fresh weight and solution absorption decreased whereas flower wilting increased, these mainly occur due to water loss by different organs of flowers (Borochoy and Woodson, 1989; Urban *et al.*, 2002). In other words, when the stem vessels are blocked, flower spent the absorbed water through transpiration and subsequently reduced RWC (Urban *et al.*, 2002; Van Meeteren, 1978). In results, upholding a proper water uptake by cut flowers is of crucial issues in order for extending the vase life of flowers (Urban *et al.*, 2002). Whenever the rate of transpiration exceeds more than that of water uptake, water deficiency occurs and leads to flower wilting and reduction in flower weight (Van Meeteren, 1978). In current research, the significant effect of calcium treatments on increasing the petal RWC of cut flowers was observed, it can be resulted from a reduction in stem blockage owing to increasing lignin biosynthesis.

### Membrane stability index

The results of variance analysis revealed that the main effects of cultivar and treatment as well as their interaction cultivar × treatment on MSI was significant (Table 2). Also the mean comparison of the

interaction effect revealed that calcium treatments used in both cultivars significantly increased MSI. It was found a significant different among calcium treatments of both forms on MSI. The highest rate of MSI was observed in 'Intense' cultivar by 1% calcium chloride which increased as much as 13% to controls, but the highest rate of MSI was found in 'Rosaline' cultivar by applying 1% calcium nitrate which increased as much as 15% to controls (Fig. 3).

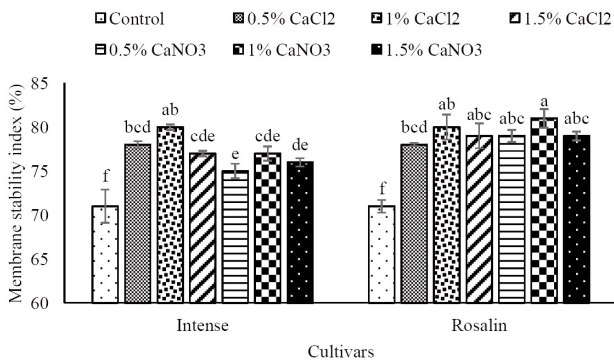


Fig. 3 - The effects of calcium treatments on petals membrane stability index of two gerbera cultivars. Bars represent standard error of four replicates. Values with the different letters are significantly different according to Duncan's Multiple Range Test at  $P < 0.05$ .

In general, the senescence in plants is an oxidative and controlled process including biological, physiological, hormonal, and structural changes destroying macro molecules such as protein, nucleic acids, and lipids (Iqbal *et al.*, 2017). The lack of balance between generating reactive oxygen species (ROS) and cleaning ROS by plant defensive systems paves the way for inducing oxidative stresses which inflict damage on different parts of cells and destroy them. The destruction of cell membrane is one of the processes which intensifies in the presence of different ROS, resulted in increasing cell electrolyte leakage (Abdolmaleki *et al.*, 2015). In our research, pretreatment of different sources of calcium increased MSI in petals of gerbera cut flowers. calcium is supposed to alleviate the adversary effect of ethylene on initiating cell senescence through inhibiting cell membrane destruction and consequently to extend the vase life of cut flowers (De Capdeville *et al.*, 2005). Also, the structural and functional roles of calcium on maintaining cell membrane and cell walls have been proved (Ferguson and Drobak, 1988). In other words, according to the results of our experiment, calcium treatments were found to enhance antioxidant enzyme activities resulted in decreasing oxidative stress and preserving cell membrane structure. Our

results are in agreement with those of Abdolmaleki *et al.* (2015) who reported that applying calcium pre-harvest treatment retarded damage to cell membrane and hence increased vase life of rose cut flowers. It appears that calcium treatments directly increase cell wall stability and indirectly influence enzyme processes in order to increase the content of MSI in gerbera cut flowers.

#### CAT and SOD activity

The results showed that the interaction effects of cultivar  $\times$  treatment was significant in terms of catalase (CAT) and superoxide dismutase (SOD) (Table 2). Compared to controls, all applied treatments significantly increased CAT in both cultivars and the effects of the treatments in both cultivars were the same (Fig. 4A). Also, calcium nitrate and calcium chloride significantly improved SOD activities on both cultivars. The highest SOD activity was observed in 'Intense' cultivar by using 1.5% calcium nitrate, whereas in 'Rosaline' cultivar it was found by applying 1.5% calcium chloride (Fig. 4B).

The process of senescence in cut flowers is usually accompanied with a modulation in the antioxidant enzymes' activities (Borochoy and Woodson, 1989).

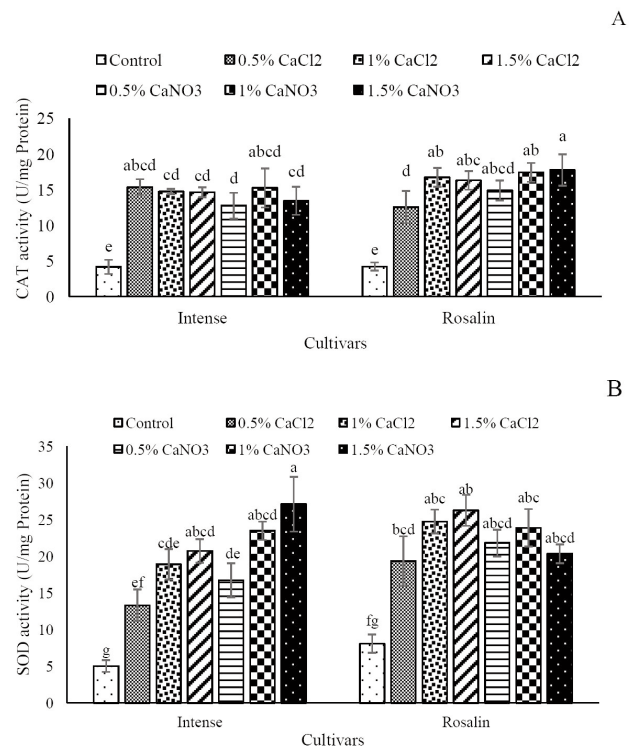


Fig. 4 - The effects of calcium treatments on catalase (CAT) (A) and superoxide dismutase (SOD) (B) activities of gerbera petals. Bars represent standard error of four replicates. Values with the different letters are significantly different according to Duncan's Multiple Range Test at  $P < 0.05$ .

The results of different studies showed that increasing antioxidant enzyme activity extended flower longevity because these antioxidants retarded the process of senescence (Ezhilmathi *et al.*, 2007). It has been demonstrated that calcium treatments, through changing in antioxidants activities, delayed flower senescence and maintained chlorophyll and protein contents in gladiola flower during storage (Sairam *et al.*, 2011). In this respect, Zhao *et al.* (2006) showed that calcium chloride enhanced the activities of CAT and SOD in the cut flowers of *Rosa hybrida* and *Dendrobium phalaenopsis*. In current research, both forms of calcium increased CAT and SOD activities in gerbera flowers (Fig. 4). It is obvious that improving antioxidant activities prevents cell membrane from breakdown and maintains membrane integrity. Calcium may serve as a secondary messenger in a pathway to stimulate the synthesis of antioxidant enzymes (Jiang and Zhang, 2003). With respect to what mentioned above, calcium may indirectly stimulate the antioxidant activities and consequently reduce the oxidative stresses through lessening the peroxidation activity and maintaining the membrane integrity, or it may directly keep the membrane integrity and cell wall and delay the process of senescence in cut flowers.

#### Lignin, cellulose, and hemicellulose

The results showed that the interaction effect of cultivar × treatment was significant on lignin ( $p < 0.05$ ), cellulose ( $p < 0.01$ ), and hemicellulose ( $p < 0.05$ ) (Table 2). Comparing to ‘Rosaline’, more lignin and cellulose were observed in ‘Intense’, but the rate of hemicellulose in both cultivars was not significantly different (Fig. 5). Application of both forms of calcium at all their concentrations significantly improved the rates of lignin and hemicellulose in both cultivars (Fig. 5A and 5B). The highest rate of lignin was found in ‘Intense’ by 0.5% calcium nitrate while it was obtained in ‘Rosaline’ by 1.5% calcium chloride (Fig. 5A). On the other way, all calcium treatments increased cellulose in ‘Intense’ in comparison to control, but just 1% and 1.5% calcium chloride and 0.5% of calcium nitrate increased cellulose in ‘Rosaline’ as comparing to controls (Fig. 5B).

One of probable factors on providing stem bending in cut flowers is contributed to the lack of sufficient lignin formation in cell wall, especially in the secondary wall besides its effect on stem rigidity, causes to take up water continuously by flowers (Vanholme *et al.*, 2010). Peroxidase (POD) and phenylalanine ammonia lyase (PAL) are of key

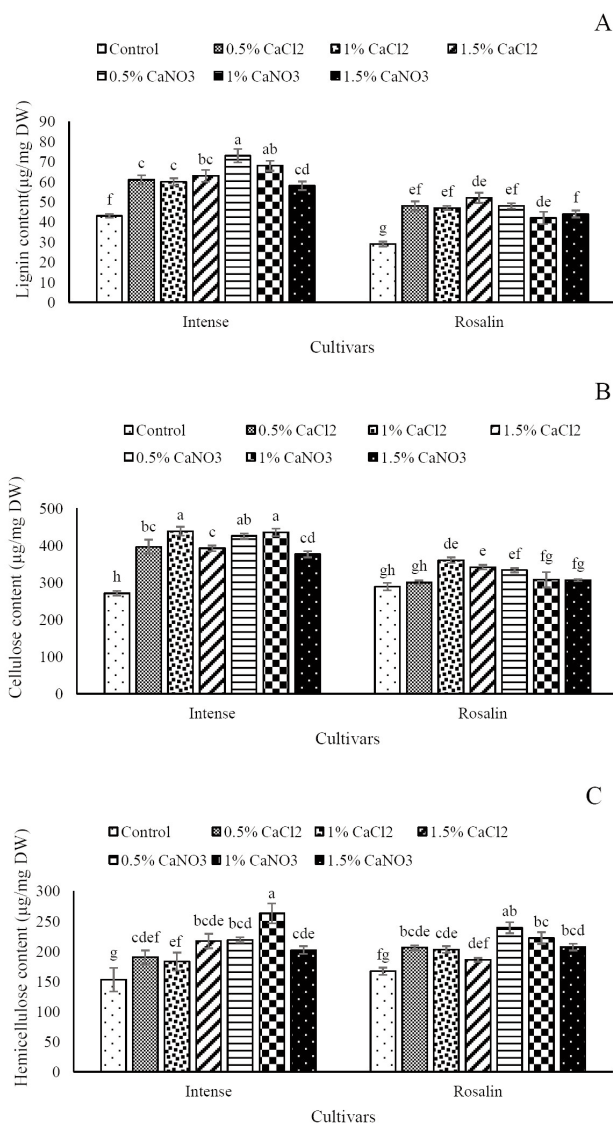


Fig. 5 - The effects of calcium treatments on lignin (A), cellulose (B), and hemicellulose (C) of gerbera petals. Bars represent standard error of four replicates. Values with the different letters are significantly different according to Duncan’s Multiple Range Test at  $P < 0.05$ .

enzymes involving in the pathway of lignin biosynthesis; their roles in stem strength and delaying stem bending in gerbera flowers have been proved (Nazarideljou and Azizi, 2015). It has been reported that the cut flowers prepared from the long-lasting cultivars contain more POD and PAL compared to short -lasting cultivars (Lv *et al.*, 2011; Nazarideljou and Azizi, 2015). In the present research, ‘Intense’ showed a higher tolerance to stem bending than ‘Rosaline’ because of containing more lignin and cellulose. The results of this research also revealed that calcium treatments significantly increased lignin, cellulose, and hemicellulose contents in gerbera. Perik

et al. (2014), by investigating the effects of different treatments on stem rigidity, figured out that using calcium chloride lessened stem bending in gerbera flowers through increasing stem strength resulted from lignin formation. In this regard, Li et al. (2012) reported that applying calcium chloride at pre-harvest stage significantly enhanced the rate of pectin, lignin, cellulose, and hemicellulose in the cut flowers of herbaceous peony. Also, it has been stated that using some materials such as ethylene and salicylic acid, through affecting on the some enzymes like PAL involving in lignin biosynthesis, had a direct influence on stem bending of flower (Ferrante et al., 2007). Also, Naing et al. (2017) reported that applying sodium nitroprusside augmented the gene expression of lignin biosynthesis; and likewise PAL extended flower vase life and diminished stem bending of gerbera flowers.

#### Calcium content

The interaction effect of cultivar × treatment was not significant in terms of calcium, but the main effects of treatment and cultivar were significant (Table 2). The rate of calcium in 'Intense' was observed higher than of that in 'Rosaline' (Fig. 6A). In other words, all calcium treatments significantly increased the amount of calcium in comparison to controls. There was a significant difference among all treatments and 0.5% calcium nitrate and calcium chloride had the lowest effect on calcium content of the stem cut flower (Fig. 6B).

Calcium is an essential element effecting on the plants' growth and development processes in a way that its accumulation in them facilitates creating the connections among pectin polymers; and accordingly it escalates the mechanical strength of stem and lignin production. Eventually, they lead to reducing stem bending and increasing flower longevity (Gerasopoulos and Chebli, 1999; Li et al., 2012). The internal and inter cellular roles of calcium are contributed to its part in plants leading to the changes on cell metabolism as well as its effect on the structure and function of cell wall and membrane (Ferguson and Drobak, 1988). The results showed that application of calcium, due to its role in synthesis of the pectin located in cell wall of xylem vessels, improved water translocation in stem and prevented cut flowers from stem bending (van Ieperen and van Gelder, 2006). In reality, a reduction in the resistance towards water conductivity in plants is one of calcium duties for extending vase life of cut flowers (Cortes et al., 2011). Calcium improves the flower

longevity and this may delay many issues such as physiological phenomena related to senescence, reduction in water uptake, and losing more water from flower through transpiration, and thereby reducing flower fresh weight and stem bending (Gerasopoulos and Chebli, 1999; De Capdeville et al., 2005; Sosanan, 2007). In current research, applying calcium increased calcium concentration in stem of cut flowers. In a similar way, using different forms of calcium at growth stage of herbaceous peony enhanced the internal calcium, lignin formation, and consequently the rigidity of the stems carrying flowers (Li et al., 2012). Nikbakht et al. (2008) reported that calcium accumulation in the stem of gerbera delayed stem bending. Therefore, it seems that an increase in the content of calcium inside stems is associated with the reduction of stem bending and extending flower longevity.

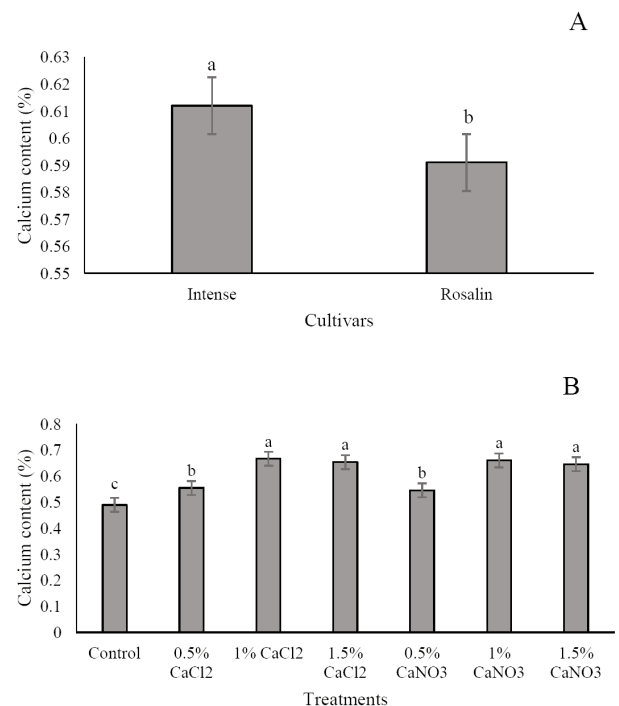


Fig. 6 - The main effects of cultivar (A) and calcium (B) treatments on calcium content of two gerbera flower stem. Bars represent standard error of four replicates. Values with the different letters are significantly different according to Duncan's Multiple Range Test at  $P < 0.05$ .

#### 4. Conclusions

In general, the results showed that applying calcium in different forms of nitrate and chloride reduced the stem bending in gerbera cultivars. Both forms of calcium had a positive effect on gerbera cultivars, but mostly calcium chloride had better effect on stem

bending in comparison to calcium nitrate; and its 1% concentration compared to other its concentrations gained the best results. The positive effect of calcium on stem bending is ascribed to its role on increasing the synthesis of lignin, cellulose, and hemicellulose. The results of this research also showed that the antioxidant enzymes activities were increased while applying calcium treatments led to delaying the senescence processes in flowers.

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