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Postharvest Assessment of Lignifying Enzymes Activity, Flower Stem Lignification and Bending Disorder of Gerbera Cut Flower

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Abstract

Scape bending disorder is the most important factor affecting postharvest loss of gerbera cut flowers. One of the ultimate reasons for gerbera stem bending is lignin, with deformation structural functions and defensive mechanisms. This postharvest experiment was conducted to evaluate the role of phenylalanine ammonia lyase (PAL) and peroxidase (POD) enzymes activity in stem bending of two gerbera cultivars; ('Beaudine' (sensitive)) and ('Aqua'(resistant)). This experiment was based on a completely randomized design with three replications over eight days. Results showed the significant effects of cultivar on stem bending percentage, total phenol content, PAL and POD enzyme activities and lignin content (P<0.05). The 'Aqua' cultivar had the highest phenol and lignin content and the lowest stem bending percentage. The maximum and the minimum PAL and POD enzyme activities were observed in resistant and sensitive cultivars, respectively. Based on the results, induction of PAL and POD enzymes activity, and consequently lignin formation could have direct effects on stem strength and as a result reduce gerbera stem bending disorder.

Keywords: Gerbera jamesonii, peroxidase, phenylalanine ammonia lyase, phenylpropanoid pathway, stem curvature, total phenol.

Introduction

Gerbera (*Gerbera jamesonii* Bolus ex. Hook f.) is the fifth most used cut flower, after roses, chrysanthemums, tulips and lilies based on the world trade data for flowers and decorative plants (Lim *et al*., 2012). Vase life and stem bending are the most important factors to evaluate the postharvest quality of cut gerbera flowers (Celikel and Reid, 2002). Flower stem bending or scape curvature disorder is the most important factor in the postharvest loss of cut Gerberas as has been shown by a number of researches. This postharvest disorder occurs on the flower stem 10 cm below the inflorescence (Willberg, 1973) and is influenced by genetics (Ferrante *et al*., 2007;Nazari Deljou *et al*., 2011), water relations (Van Meeteren, 1978), storage temperature and postharvest handling (Celikel and Ried, 2002), plant nutrition (Gerasopoulos and Chebli, 1999) and some phytohormones (Emongor, 2004).

Recently, one theory for the occurrence of this disorder has implicated lignin formation in the flowering stem during the phenyl propanoid pathway. Perik *et al*. (2014) reported that bending in cut gerbera flowers

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could be related to adverse water relations and lack of stem sclerenchyma development and not due to expansion of the stem central cavity or stem elongation. The phenyl propanoid pathway is one of the most important pathways of plant secondary metabolites, resulting in the formation of phenolic compounds such as lignin (Taiz and Zeiger, 2006). After cell development, lignin as the most frequent natural polymer with a complex structure defends and protects plant cells (Pulse and Schuseil, 1993; Ikeda *et al*., 2002). Lignin is a complex polymer formed by the oxidative polymerization of hydroxycinnamyl alcohol derivatives called monolignols and is composed of phenylpropanoid units (Li *et al*. 2008; Rubin, 2008). Lignin in plants is biosynthesized by catalytic enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (POD) and cinnamyl alcohol dehydrogenase (CAD) whichcatalyse the conversion of phenylalanine to hydroxy- and methoxy-cinnamyl alcohols (Boerjan, 2003; Boudet*et al*., 2003; Vanholme et al., 2010). The low activity of these enzymes in lignin reduction in sclerenchyma cell walls results in the reduced mechanical strength of the stem (Li *et al*. 2009). PAL is the cycle primer in the lignin biosynthesis pathway, the most important enzyme in phenylpropanoid metabolism and trans-cinnamic acid catalysis through deamination of L-phenylalanine and monolignol biosynthesis, their transfer to the cell wall, polymerization and finally the combinationof phenylpropanoid macromolecules such as lignin (Schuster and Retey, 1995). The POD enzymealso completes the polymerization of monolignols and causes lignin formation through oxidation with phenol substances (Imberty*et al*., 1985; Mencarelli*et al*., 1995).

Ferrante *et al*. (2007) treated Gerbera flowering stems with aminooxyacetic acid (AOA), α-aminooxi-β-phenylpropionic acid (AOPP) and PAL-inhibitors to increase stem bending; however, the amount of lignin in these stems was not analysed. Guoshen*et al*. (2011) reported chrysanthemum cultivars with a long vase-life to contain more vascular elements, lignin and relative amount of water in the stem receptacle. This experiment was conducted to determine the lignifying enzymes and their relationship with lignin formation and postharvest stem bending disorder.

Materials and Methods

Plant materials, treatments, and experiment conditions

This research was performed over eight days after harvest (when more than 70% of flowers started to bend and wilt) on two cultivars (‘Aqua’ and ‘Beaudine’) obtained from a local commercial greenhouse (Pakdasht, Tehran). The cultivars were evaluated for flower stem bending in a factorial experiment (cultivar and time) under a completely randomized design with three replications (9 flowers in each treatment). In order to select cultivars with different stem bending scores, pre-screening postharvest experiment with fourteenGerbera cultivars was performed to select cultivars with different stem bending scores; finally two cultivars with minimum (‘Aqua’) and maximum bending (‘Beaudine’) were selected. All experiments were performed in a postharvest room maintained at 20±°C, 60±5% relative humidity, and 12 h photoperiod provided by fluorescent lamps (25 µmol m^{-2} s^{-1} fluorescent light). In addition, all flowers were placed in 400 ml distilled water in glass tubes and every other day, the distilled water was renewed.

Determination of scape bending

Scape bending was determined and classified based on Celikel and Reid (2002) method. Scape bending was measured daily and expressed with respect to the angle on day 0 of vase-life. The Gerbera flowers were rated as follows: 0 for bending up to 15°, 1 for bending between 15° and 25°, 2 for bending between 25° and 65°, 3 for bending between 65° and 90° and 4 for flowers that bent more than 90°.

Total phenol determination

Total phenols of the proximal and distal end of flower stems were determined by Folin Ciocalteau reagent (McDonald *et al*. 2001). Briefly a dilute methanolic extract (0.5 ml of 1:10 g ml^{-1}) of each sample at fully opened stage (eighth day after harvest) or gallic acid (standard phenolic compound) was mixed with FolinCiocalteureagent (Sigma-Aldrich, Germany) (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (Sigma–Aldrich, Germany) (4 ml, 1 M). The mixtures were allowed to stand for 15 min and total phenols were determined with a colorimeter at 765 nm (Perkin Elmer, UV/VIS, Lambda 25). The standard curve was prepared using different concentrations of gallicacid (Sigma–Aldrich, Germany) in methanol: water (50:50, by volume) ratio. Total phenol values are expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass), which is a common reference compound.

Lignin determination of gerbera cut flower stem

The lignin content of gerbera flower stems was determined by the thioglycolic acid (TGA) method (Bruce and West, 1989). The proximal and distal end (2 g) of the flower stem at fully opened stage (eighth day after harvest) was homogenized in 6 mL of 99.5% ethanol (Sigma–Aldrich, Germany) and the crude extract was centrifuged at 10,000 X g for 15 min. The pellet was transferred to a glass Petri dish, and air-dried overnight. 30 mg of the dried residue was place in a screw-cap tube to which 5 mL of 2N HCl and 0.5 mL of thioglycolic acid (Sigma–Aldrich, Germany) were added and the mixture heated at 100°C for 12 h. After cooling, the mixture was centrifuged at 12,000 X g for 45 min at 4°C. The pellet was washed with 2.5 mL of water and then resuspended in 5 mL of 0.5 N NaOH. The solution was agitated gently at 25°C for 24 h. After centrifugation at 12,000 X g for 30 min, the supernatant was transferred to a new tube. One microliter of concentrated HCl was added to the test tube and the lignin thioglycolate was allowed to precipitate at 48°C for 6 h. After centrifugation at 10,000 X g for 30 min, the pellet was dissolved in 1 mL of 0.5 N NaOH. The absorbance was measured against a NaOH blank at 280 nm using spectrophotometer (Perkin Elmer, UV/VIS, Lambda 25). The amount of lignin was calculated from a linear calibration curve with commercial alkali lignin (Sigma-Aldrich, Germany).

Determination of protein and enzyme activity

For the measurement of postharvest enzyme activity and protein over eight days from initiation of the experiment (first day to seventh day after harvest), different samples of 10 cm proximal and distal end parts of the Gerbera flower stem were provided and after immediate fixation in liquid nitrogen, were transferred to the freezer (Operon, DFC-84CE, South Korea) at -80°C for the measurement of enzyme activity and protein levels.

Determination of phenylalanine ammonia lyase (PAL) activity

The PAL (EC 4.3.1.5) activity of the two gerbera cultivars was measured following the methods as described by Redman (1999). For this purpose, samples kept at -80°C were used. Phosphate sodium buffer (0.01 M) was used as an extraction buffer and Tris-HCL buffer (0.05 M, pH 8) containing phenylalanine amino acid (6µM) was used as a reactive solution to determine PAL activity. Finally, PAL activity was measured at 290 nm and expressed as mg of cinnamic acid per mg of protein per hour.

Determination of peroxidase (POD) activity

POD enzyme (EC. 1.11.1.7) activity of the two Gerbera cultivars was studied using the Hemeda and Kellin (1990) method. In this method, after emitting the samples from -80 °C, 0.5 g stem texture was pulverized in liquid nitrogen. After transferring the

samples to the falcon, 50 mg polyvinyl pyrrolidone (Merck, Germany) and 3 ml potassium phosphate (100 mM, pH 7) were added to each sample; these were then centrifuged at 4°C and 10,000 rpm for 30 min. 70 µL of the supernatant was then mixed with 750 µL guaiacol, 750 µL phosphate buffer (0.01 M), 1400 µL distilled water and the samples' absorption at 470 nm were recorded. The Bradford (1976) method was used for the measurement of protein in the samples.

Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA) with SAS statistical software (SAS 9.1; SAS Institute); mean comparisons were carried out by Duncan's multiple range test.

Results

Stem bending percentage

Results showed the significant effect of cultivar on stem bending percentage. As shown in Figure 1 'Aqua' showed more stem bending percentage than 'Beaudine.

Stem lignifications and total phenol content

Stem lignification was significantly affected by cultivar (Table 1). Accordingly, 'Aqua', with a lower stem bending score than the'Beaudine' contained a higher lignin content. Similar trends were also observed in total phenol content in both cultivars as the maximum and minimum total phenol content were observed in 'Aqua' and 'Beaudine', respectively (Table 1). Despite non-significant differences between the total phenol and lignin content in the proximal and distal end of the stem in different cultivars, lignin and total phenol contentsin the distal end werehigher than in the proximal end.

PAL enzyme activity of gerbera flower stem

The results are indicative of the significant effects of cultivar on PAL activity. Based on the mean comparisons (Table 1), PAL enzyme activity had a direct and diverse relationship with stem bending disorder as the 'Aqua' with less bending percentage/ score had 1.5 times higher enzyme activity than 'Beaudine' with a higher stem bending percentage/ score. Based on the results from both sensitive and resistant cultivars to stem bending, PAL activity in the distal end was significantly higher than in the proximal end (Table 1).

Time course of postharvest pal enzyme activity

Postharvest PAL enzyme activity in the studied cultivars showed significant and different patterns depending on cultivar (Fig. 2). As a result, PAL activity at seven days after harvest in 'Aqua' and 'Beaudine' was shown to rise and fall, respectively. On the other hand, PAL activity in the 'Aqua' increased to 70% while in the 'Beaudine' it decreased to 35%.

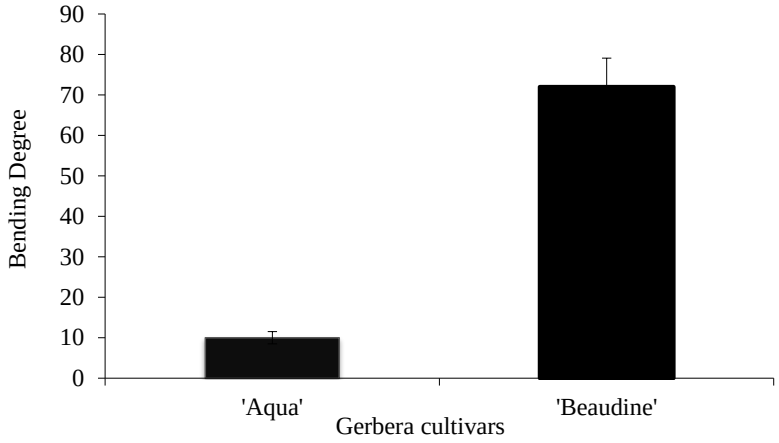

Fig. 1. Effects of cultivars on stem bending percentage

Table 1. PAL, POD enzyme activities, lignin and total phenol content of different flower stem parts of two gerbera cultivars

Parameters	'Aqua'A		'Beaudine'	
	Proximal end	Distal end	Proximal end	Distal end
PAL activity (µg cinnamic acid mg^{-1} protein h^{-1})	12.87 ± 0.47aB	7.89 ± 0.23c	8.66 ± 0.31b	5.79 ± 0.19d
POD activity (U mg^{-1} protein)	16.5 ± 0.53a	9.31 ± 0.4b	5.83 ± 0.24c	3.2 ± 0.13d
Lignin content (µg g^{-1}DW)	0.44 ± 0.12a	0.37±0.13b	0.29 ± 0.01c	0.27 ± 0.08c
Total phenol (mg g^{-1} DW)	2.10 ± 0.49a	1.86±0.23b	1.38 ± 0.30c	0.62 ± 0.20d

A) Gerbera cultivars with different stem bending scores. ('Aqua': Resistant cultivar; 'Beaudine': Sensitive cultivar).

B) Different letters in each column indicate significant differences at P≤ 0.05 according to Duncan's multiple range test.

Fig. 2.Postharvest PAL enzyme activity of gerbera cultivars with different stem bending ('Aqua'; resistant- 'Beaudine'; sensitive)

POD enzyme activity of gerbera flowering stem

Based on the results, POD activity was significantly affected by cultivar. The trend and template of POD enzyme activity was the same as PAL enzyme activity as POD activity in the cultivar with less bending ('Aqua') was three times more in the other cultivar ('Beaudine') (Table 1).

Similar trends were also observed in POD activity in the proximal and distal end of the stem in the two studied cultivars (Table 1) as POD activity in lower stem zones or distal end was higher than in the upper zones or proximal end in both cultivars.

Time course of postharvest pod enzyme activity

The trend of peroxidase activity over the eight days after harvest in the studied cultivars varied significantly (Fig. 3). The results from mean comparisons indicated a rising trend in the 'Aqua' while the 'Beaudine' did not show similar trend as its values were uncertain and oscillatory.

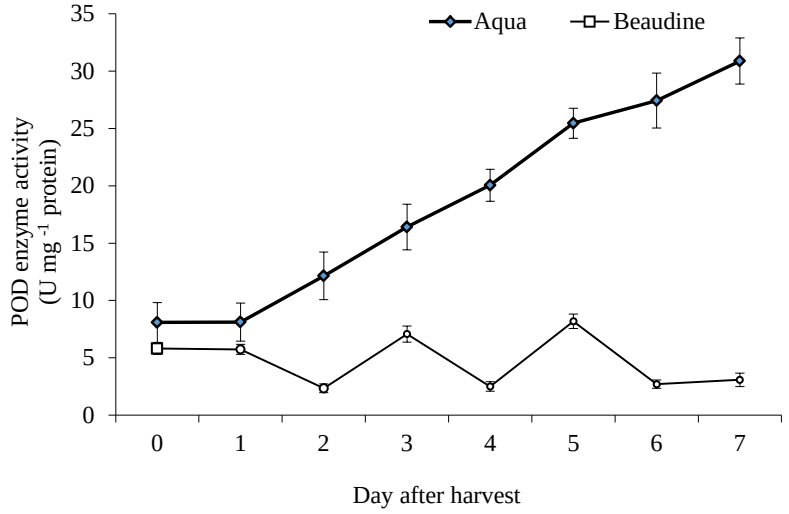

Fig. 3. Postharvest POD enzyme activity of Gerbera cultivars with different stem bending values.('Aqua'; resistant 'Beaudine'; sensitive)

Discussion

The total phenol and lignin content in the 'Aqua' was 2 and 1.5 times higher than in 'Beaudine', respectively. The observed differences in total phenol and lignin content in the studied cultivars are indicative of genetic control (cultivar) on phenolic compounds such as lignin, which is in agreement with the findings of Ferrante *et al*. (2009) who showed total phenol to be higher in the cultivar with a lower incidence of stem bending. Lignin as a phenolic compound with a complex structure obtained from the biosynthetic pathway of phenylpropanoids plays an effective role in cell strength and resistance (Hatfield and Vermerris, 2001; Cai*et al*., 2006). The function and importance of lignin and sclerenchyma in stem strength is determined when the deficit of expression and synthesis of lignin formation enzymes results in a decrease in lignin and as a result reduces plant strength (Li *et al*., 2009). Guosheng *et al*. (2011) in the anatomical and physiological investigation of the receptacle in the flower stem of chrysanthemum showed that cultivars with a long vase life contained more vascular elements and lignin. Also, despite non-significant differences between phenol and lignin content in the distal and proximal ends in both cultivars, total phenol and lignin contents in the distal end of the flower stem were higher than in the proximal or upper parts of the stem. It is necessary to mention that to achieve best results in relation to the effects of flower stem height on flower vase life and water uptake, after transferring cut flowers to the postharvest room/lab their stems were cut to 40 cm to obtain uniform stems, so that the non-significant differences between distal and proximal ends in case of lignin may be due to removal of the lower zones or basal ends of stems with thicker and higher lignin. This is in agreement with the results of Ferrante *et al*. (2007) who stated that stem strength, and probably lignin content, decreases from the lower part to the receptacle.

As is seen in Table 1, PAL and POD activities in the 'Aqua' were 1.5 and three times more than in the 'Beaudine', respectively. Ferrante *et al*. (2007) during their investigation of the relationship between PAL enzyme and stem bending percentage found the same results as to the relation between enzymatic activity and stem bending percentage. However, they did not assay the lignin quantitatively in order to reach an accurate conclusion.

The evaluation of PAL and POD activities as the most important enzymes in lignin biosynthesis indicates the significant difference between cultivars during postharvest days. Based on this, the results showed that PAL and POD activities in the 'Aqua' showed a rising trend of 72 and 26%, respectively during the seven days postharvest compared to the initial day; while the activity of these enzymes in 'Beaudine' showed an increasing and decreasing pattern. In fact, the 'Aqua' had a higher total phenol and lignin contents than the 'Beaudine'. The activity of enzymes participating in lignin biosynthesis and their relationship with stem bending disorder in different cultivars shows the virtual effect of lignin on postharvest stem bending disorder. The phenylpropanoid pathway is one of the most important pathways in the formation of secondary metabolites, resulting in the formation of different phenols with structural and defensive functions like lignin, phenolic acids and flavonoids in plants (Shen *et al*., 2001). Shen *et al*. (2001) showed that an increase and induction of PAL enzyme results in mRNA enzyme induction, a considerable increase in PAL activity and phenolic compounds like chlorogenic acid, caffeic acid, sinapic acid, ferulic acid and p-coumaric acid. In another experiment, Cai *et al*. (2006) in a study of postharvest lignifying process and activity of three enzymes—PAL, CAD and POD— participating in lignin biosynthesis inloqu at fruits showed fruit strength and lignin content to have increased after harvesting, and that this increase results from an increase in enzyme activity. Of course the activity of the PAL enzyme during the first 72 h after harvest increased and then decreased, while POD and CAD activities constantly increased over eight days postharvest . The results of numerous studies on bamboo buds showed that lignin increase in buds over ten days accompanied increasing PAL and POD activities, but the treatment of 1-MCP results in a reduction in lignin formation with reduced PAL and POD activities (Shen *et al*., 2006; Luo *et al*., 2008a;b).

Conclusion

PAL and POD activities in the 'Aqua' with less stem bending percentage showed an increasing trend after harvest as compared to 'Beaudine'. There is a direct relationship between enzymatic activity and lignin and stem bending percentage enzyme induction activity of enzymes participating in lignin formation using enzymatic inducers or transgenic plants with the capability of lignin formation results in high stem strength which could be an effective strategy towards reducing postharvest bending disorder.

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