

## Obstruction of Water Uptake in Cut Chrysanthemum Stems after Dry Storage: Role of Wound-Induced Increase in Enzyme Activities and Air Emboli

U. van Meeteren<sup>1, a</sup> and L. Arévalo-Galarza<sup>2</sup>

<sup>1</sup>Horticultural Supply Chains Group Dept of Plant Sciences, Wageningen University, Marijkeweg 22, 6709 PG, Wageningen, The Netherlands

<sup>2</sup>Colegio de Postgraduados, Carretera Mexico Texcoco km 36.5, Montecillo, CP 56320, Mexico

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### Abstract

Hydraulic conductance of cut chrysanthemum stems was lowered by the aspiration of air as well as by a wound-induced plant response. By measuring the hydraulic conductance of stem segments in which air could be introduced into and/or removed from the xylem vessels at various times after harvest, we showed that the two processes, air aspiration and wound-induced reactions, occur independently. The pronounced xylem occlusion after a longer period of dry storage is due to the progress of the enzymatic wound-induced reaction in time superimposed on emboli due to aspired air. The wound-induced blockage was also present when air entrance was precluded from harvest. Measurements of enzyme activities in stems at time intervals from harvest showed that the activity of L-phenylalanine ammonia-lyase (PAL) increased after wounding in contrast to the activities of peroxidase and polyphenol oxidase. This suggests a major role of PAL in the xylem occlusion caused by wounding of the flower stem.

### INTRODUCTION

Water uptake of cut flowers can be obstructed by several reasons. In cut chrysanthemum stems was shown that as well air aspired into the opened xylem vessels at the cut surface as enzymatic reactions induced by wounding of the stem at harvest lower the hydraulic conductance of the cut flower stems (van Meeteren, 1992; van Doorn and Cruz, 2000; van Meeteren et al., 2006). Often this hindering of xylem water transport results in wilting of the leaves already during the first days of vase life (van Meeteren, 1989). A wound-induced occlusion was most pronounced after a dry storage period of several hours at 20±1°C (van Meeteren et al., 2006). Unclear was if the wound-reaction is stimulated by the presence of air. Van Doorn and Cruz (2000) suggested that the presence of air in stems promote wound-induced blockage. Their conclusion was based on observations of the occurrence of leaf wilting. Leaf wilting, however, is the result of a non-steady state balance between water uptake and loss. Water uptake is affected as well by hydraulic conductance as by the water potential difference along the stem. The water potential difference is the result of water uptake and loss, and will change as soon as hydraulic conductance changes. Whether a decrease in hydraulic conductance reaches a critical level (shown by leaf wilting) is therefore depending on transpiration rate and on the overall value of the conductance, which is the result of the number of xylem vessels, vessel diameter distribution, (partly) blockage of vessels by air emboli and/or bacteria and wounding-induced enzymatic blockage. An extra complicating factor is that transpiration rate will decrease when water potential decreases because of stomata closure. By measuring the hydraulic conductance of isolated stem segments, in which air could be introduced into and/or removed from the xylem vessels at various times after harvest, we investigated a possible role of air presence in the occurrence of the wound-induced enzymatic blockage.

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<sup>a</sup> uulke.vanmeeteren@wur.nl

Previous results showed that the wound-induced enzymatic blockage was considerably delayed by inhibitors of peroxidase and polyphenol oxidases (van Doorn and Vaslier, 2002; van Meeteren et al., 2006). To our knowledge, there has been no report where measurements of enzyme activities in stems of chrysanthemum or other cut flowers have been correlated with cutting or xylem blockage. In line with the above mentioned inhibitor studies, we measured the activities of peroxidase and polyphenol oxidases in stem segments before and after cutting and after a dry storage period (when wounding-induced enzymatic blockage will be present). L-phenylalanine ammonia-lyase (PAL) is another enzyme which activity has been reported to increase after wounding of plant tissue (Camm and Towers, 1977; Ke and Saltveit, 1989; Campos-Vargas and Saltveit, 2002). Therefore, we included measurements of PAL activities at various time intervals from harvest in our studies.

## MATERIAL AND METHODS

### Plant Material

Chrysanthemum (*Chrysanthemum* × *morifolium* Ramat. cvs. Cassa and Vyking) plants were grown in a greenhouse at Wageningen University in 14-cm diameter plastic pots containing a commercial potting soil. An average 18 h photoperiod was maintained until the plants had formed 15-17 leaves longer than 0.5 cm. Thereafter, a 9 h photoperiod was maintained until harvest. Pots were transported from the greenhouse to the laboratory when the flowers had reached commercial maturity. In all experiments, stems were cut under water, at the root-shoot junction. This prevented uptake of air at the cut surface.

### Vase Solution and Dry Storage

When cut flower stems or stem segments were placed in water, an aqueous solution of 0.7 mM CaCl<sub>2</sub>, 1.5 mM NaHCO<sub>3</sub> and 50 μM CuSO<sub>4</sub> (van Meeteren et al., 2000) was used. This solution will be indicated as 'vase solution'. For dry storage treatments (Table 1, Fig. 1), flowers were placed in a vase life evaluation room on tables for 1h to cause air aspiration. Thereafter the stems were stored into a plastic bag to prevent further drying out, at 5 or 20°C for 24 h.

### Hydraulic Capacity of Stem Segments with Air Aspiration and Air Removal

The capacity of a stem segment to conduct water, or hydraulic capacity  $Q_h$  (Reid et al., 2005), and the effect of air aspiration and air removal on  $Q_h$  was measured as described before (van Meeteren et al., 2006). For measuring  $Q_h$  22 cm long stem segments were used. The 22 cm long stem segments were prepared under water according to van Ieperen et al. (2001), to prevent air-entrance into the xylem conduits at the cut stem surface.

**1. General Measurement Procedure.** A typical measurement procedure consisted of several phases including an initial measurement of the capacity of a stem segment to conduct water ( $Q_h$ ) and the effect of air uptake into the xylem conduits on  $Q_h$  as described by van Meeteren et al. (2006). To investigate the role of the length of the period that air was present in the xylem conduits, the period without water supply varied from 3 min. to 10 h. To measure if the loss of  $Q_h$  after reapplying water following a dry period was due to the actual presence of air or to another process induced by cutting and/or by air uptake, all air that might still be present in the stem segment was removed by water infiltration under low pressure.

**2. Hydraulic Capacity Measurements.** The capacity of a stem segment to conduct water, or hydraulic capacity  $Q_h$  (Reid et al., 2005), was determined from measured flow rates ( $Q$ ) through the leafless segment under a 40 kPa partial vacuum using the apparatus described by van Ieperen et al. (2000). The water sucked through the stem segment was the above-mentioned 'vase solution'.

**3. Air Embolization and Vacuum Infiltration of Stem Segments.** Air aspiration was induced by removing the water supply from the basal cut end while maintaining the

tension at the top end for the length of the dry period, with a maximum of 5 min. In long dry storage treatments the tension device at the top of the stem segment was removed after 5 min., after which the top end was sealed with a closed piece of tubing. This was done because the treatment was only meant to result in air aspiration at the cut surface, and maximal air aspiration in flowering stems is already almost saturated after a few minutes of exposure of the cut surface to air (van Doorn, 1990; van Ieperen et al., 2001). In such prolonged dry treatments, the stem segments were left hanging on a laboratory stand after the initial 5 min. of air aspiration. After a dry storage, the segment was reconnected to the measuring device and the 'vase solution' was reapplied to the base of the stem segment.  $Q_h$  was then measured for approximately 1.5 h. Thereafter, to remove air from the stem segments, the segments were disconnected from the measuring device and placed under 'vase solution' at low pressure (25-30 mbar) for 30 min., after which  $Q_h$  was measured again. Reconnection of the segment end to the subtending tube was also done under water in order to prevent the entrance of air into the open xylem conduits.

### Enzyme Activities

Lyophilized plant material was used for the measurement of the activities of peroxidases and polyphenol oxidases. The lower 4 cm of the cut stems were frozen in liquid nitrogen, followed by freeze-drying. Ground samples were stored at  $-20^{\circ}\text{C}$ . For the measurements of PAL, 2 cm stem segments were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**1. Peroxidases.** Peroxidases (POD; EC 1.11.1.7) are measured according to the procedure of Castillo et al. (1994). Forty mg of stem powder was dissolved in 1 ml of 0.1 M succinic-lactic buffer (pH 4.5) and centrifuged at 18200 g for 5 min. The supernatant was taken as enzyme source; 150  $\mu\text{l}$  of enzyme source was mixed with 10  $\mu\text{l}$  of EDTA and then added to 730  $\mu\text{l}$  of succinic-lactic buffer mixed with 100  $\mu\text{l}$  of MBTH-DMAB substrate solution (0.07 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 1 mM 3-(dimethylamino) benzoic acid (DMAB)) and 100  $\mu\text{l}$   $\text{H}_2\text{O}_2$ . Absorption was measured at 590 nm.

**2. Polyphenol Oxidases.** Polyphenol oxidase (PPO; EC 1.14.18.1) activity was measured using L-3,4-dihydroxyphenylalanine (L-Dopa) in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH). Forty mg of stem powder was dissolved in 1 ml of 50 mM phosphate buffer (pH 6.5) and centrifuged at 18200 g for 5 min. Thereafter 50  $\mu\text{l}$  of supernatant was mixed with 53  $\mu\text{l}$  3.5 mM sodium dodecyl sulphate (SDS) and 15  $\mu\text{l}$  milliQ-water. This mix was added to a glass cuvette containing a mix of 20  $\mu\text{l}$  DMF (2% v/v), 100  $\mu\text{l}$  MBTH (5 mM) and 780  $\mu\text{l}$  L-Dopa (14 mM). Total polyphenol oxidase activity was measured at 484 nm.

**3. L-Phenylalanine Ammonia-Lyase.** For measuring L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity the stem segments were taken from the  $-80^{\circ}\text{C}$  storage, weighed, cooled with liquid nitrogen and grinded in a cooled ball mill during 25 s. The ground sample was put as fast as possible in a plastic centrifuge tube, containing 400 mg of insoluble polyvinylpyrrolidone (PVPP). The tube was kept in liquid nitrogen until the extraction procedure was carried out. Extraction was done by adding 8 ml, 50 mM borate buffer (pH 8.5) containing 5 mM 2-mercaptoethanol. The mixture was vortexed, placed in ice for 30 min. and centrifuged at 18200 g for 10 min. at  $4^{\circ}\text{C}$ . A mixture of 800  $\mu\text{l}$  extract and 400  $\mu\text{l}$  L-phenylalanine was incubated at  $40^{\circ}\text{C}$  for 2 h. After incubation the reaction was stopped by adding 10  $\mu\text{l}$  5 N trichloroacetic acid (TCA); the samples were centrifuged 5 min. The formed *trans*-cinnamic acid was measured by HPLC.

## RESULTS AND DISCUSSION

After 24 h, the hydraulic capacity ( $Q_h$ ) of cut chrysanthemum stem segments stored at  $20^{\circ}\text{C}$  was decreased to about 20% of the initial value, whether they were placed with their cut stem ends continuously in water or stored dry (Fig. 1). There was no decrease in  $Q_h$  when stem segments were stored at  $5^{\circ}\text{C}$  placed in water, while dry storage at  $5^{\circ}\text{C}$  resulted in a  $Q_h$  of 70% of the initial value. Rehydration of the dry stored stem

segments (by removing the air from the xylem vessels under low pressure) resulted in a recovery of  $Q_h$  in stem segments stored dry at 5°C, while it resulted only in a small increase in  $Q_h$  in segments stored dry at 20°C. This demonstrates that the decrease in hydraulic conductance after dry storage at 5°C is totally caused by the presence of air emboli, while at 20°C an occlusion is formed 24 h after cutting that is not caused by the presence of air in the xylem vessels. From these results it is likely that there were two processes involved in the occlusion of xylem vessels; one of these processes is diminished by low temperature, while the other is related to the presence of air. This agrees with earlier findings (van Meeteren et al., 2006).

When air emboli were removed before measurement of  $Q_h$ ,  $Q_h$  decreased over time after cutting to the same extent whether the xylem vessels were continuously filled with water, filled with air for 3 min. or filled with air for 6 or 10 h (Fig. 2). Also the period between cutting and air aspiration (immediately, 6 or 10 h after harvest) did not result in a difference in the loss of conductance (Fig. 2).

The actual presence of air in the xylem vessels resulted in a large reduction of  $Q_h$ , as well immediately after cutting ( $t_0$ ) or after storage of the stems in water for 8 h ( $t_8$ ) (Fig. 3). When the aspirated air was removed by rehydration under low pressure,  $Q_h$  was restored to  $95.0 \pm 0.7\%$  of the initial value when this procedure was applied at  $t_0$ . When the aspiration of air and subsequent rehydration were applied 8 h after harvest ( $t_8$ ),  $Q_h$  was respectively  $31.7 \pm 1.1\%$  (after 5 min. of air presence) and  $79.4 \pm 0.8\%$  (after rehydration); this last value was not significantly different from the value of these stem segments measured just before air entrance ( $72.6 \pm 3.0\%$ ). The  $Q_h$  of control flowers (no air uptake) measured after 8 h in water did not significantly differ from the  $Q_h$  8 h after air entrance for 5 min. followed by rehydration at  $t_0$ . These results showed that the actual presence of air emboli caused a decrease in  $Q_h$  independent of the time after cutting (harvest). When air emboli are present shortly after cutting, the emboli are the only cause of hindering the water transport in the stem; when air emboli are introduced some hours after cutting, the resistance for water transport due to emboli is superimposed on a time-dependent increase in resistance. After a period of 8h dry storage, the  $Q_h$  was decreased to  $9.5 \pm 0.2\%$ , which restored to  $65.0 \pm 2.0\%$  by rehydration (Fig. 3).

The results of Figures 1, 2 and 3 endorse the conclusion that a xylem occlusion starts to develop after cutting; the development rate of this occlusion is temperature dependent and is also initiated when air entrance in the xylem vessels was continuously precluded from harvest. In an earlier paper we reported that a plant-induced xylem blockage became more serious upon increasing the length of the dry period (van Meeteren et al., 2006). The present results demonstrated that this was not due to the length of the dry period but to the time since cutting (irrespective of dry storage or placed in water) and the temperature during this period. Van Doorn and Cruz (2000) found that there was a difference in time of the appearance of leaf wilting between stems cut in air and stems cut under water. This difference could be attributed to the resistance for water transport by air aspirated at the moment of cutting.

Based on studies with inhibitors, it was argued that the time-dependent xylem occlusion is an enzyme-mediated wounding response (van Doorn and Cruz, 2000; van Meeteren et al., 2006), probably including peroxidase and phenoloxidase activities (van Doorn and Vaslier, 2002). However, as can be seen in Table 1, the activities of peroxidase and phenoloxidases measured in 4 cm-stem segments including the cut surface, were at a high level before cutting; the activities did not increase after cutting with or without 24 h storage. In contrast, the activity of L-phenylalanine ammonia-lyase (PAL) increased about five- to fifteen-fold (chrysanthemum 'Cassa' and 'Vyking', respectively) within the first day after cutting (Fig. 4). The PAL activities that we measured in chrysanthemum stems and its increase were similar to the values reported for wounding of potato tubers (Smith and Rubery, 1979) and lettuce leaves (López-Gálvez et al., 1996). In 'Cassa' the activity was already at its maximum 6 h after cutting of the stem (shorter time intervals were not analyzed); according to Figures 2 and 3 the decrease in hydraulic capacity starts 7-8 h after cutting (at 20°C). These results suggest that increase in PAL activity is involved in

the plugging of cut chrysanthemum xylem vessels. The cultivar Vyking is more vulnerable for wounding-induced blockage compared to 'Cassa' (unpublished own results); the higher PAL activity in 'Vyking' after wounding in Figure 4 suggests that the vulnerability is related to the magnitude of the wounding-induced increase in PAL. However, the variability between different experiments (batches of flowers) is rather large (unpublished), which is likely due to the many factors like light, N-depletion, age and development (Camm and Towers, 1977; Olsen et al., 2008) that affect PAL activity. More data are needed to be sure about differences between cultivars in the magnitude of the wounding-induced increase in PAL.

In what way an increased PAL activity results in impairment of the hydraulic capacity is unknown. The strong inhibitory effect of tropolone (an inhibitor of tyrosinase, a phenoloxidase) on the wounding-induced xylem blockage (van Meeteren et al., 2006) suggests that phenoloxidases are involved in the downstream processes leading to the blockage. This is agreement with the findings of inhibitory studies by van Doorn and Vaslier (2002). Our results (Table 1) suggest that the activities of phenoloxidases or peroxidase are not the rate limiting wounding-induced steps in the occurrence of the xylem blockage.

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## **Tables**

Table 1. Activity of peroxidases (POD) and polyphenol oxidases (PPO) in the lower 4 cm stem segments measured after harvest (Fresh), after flower stems have been placed with their cut ends in vase solution for 5 h (5 h water) and after dry storage of 24 h following the placement in vase solution (5 h water + 24 h dry).

Flower treatment	POD activity (nktal/g DW)	PPO activity (nktal/g DW)
Fresh	531 a	619 a
5 h water	80 b	269 b
5 h water + 24 h dry	58 b	146 c

## Figures

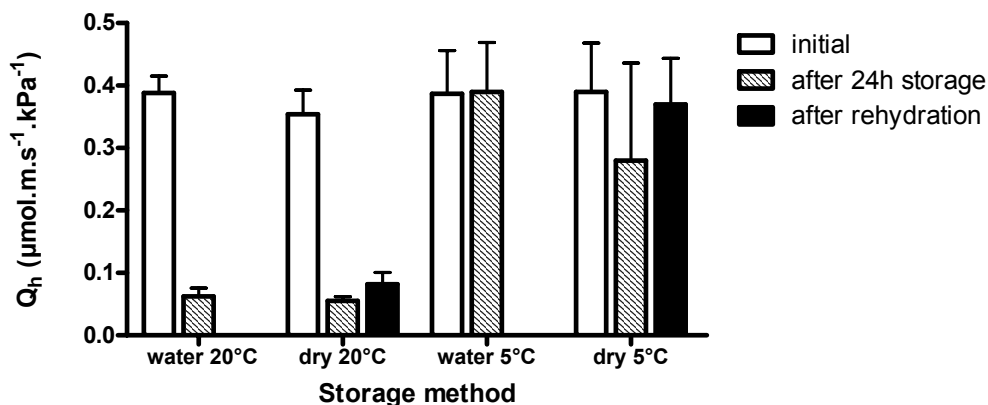


Fig. 1. Hydraulic capacity ( $Q_h$ ) of stem segments. Segments were placed with their cut end in vase solution (water) or submitted to a 24 h dry period, at 20 or 5°C. After 24 h, segments exposed to a dry storage treatment were rehydrated under low pressure;  $Q_h$  was measured before and after rehydration. Vertical bars S.E.M. ( $n=4$ ).

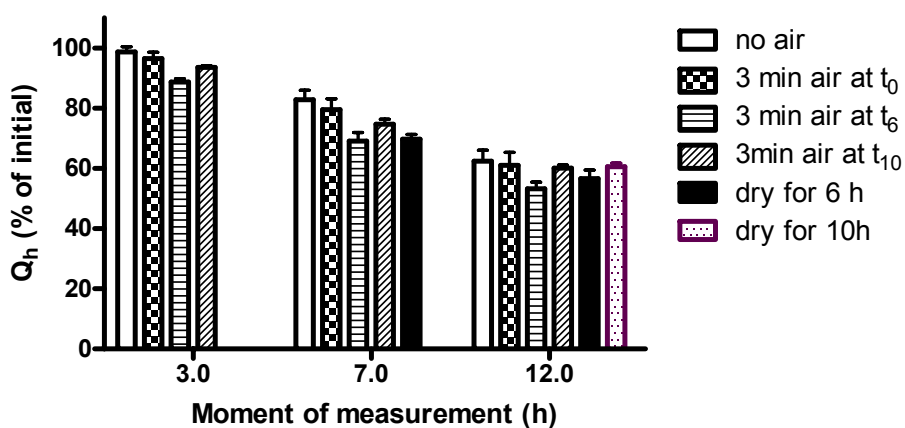


Fig. 2. Hydraulic capacity ( $Q_h$ ) of stem segments measured 3, 7 and 12 h after harvest at 20°C. Segments were continuously placed with their cut end in vase solution (no air), or 0 h ( $t_0$ ), 6 h ( $t_6$ ) or 10 h ( $t_{10}$ ) after harvest air was sucked into the stem for 3 min. followed by air removal under low pressure, or submitted to a 6 or 10 h dry period followed by air removal. Vertical bars represent S.E.M. ( $n=4$ ).

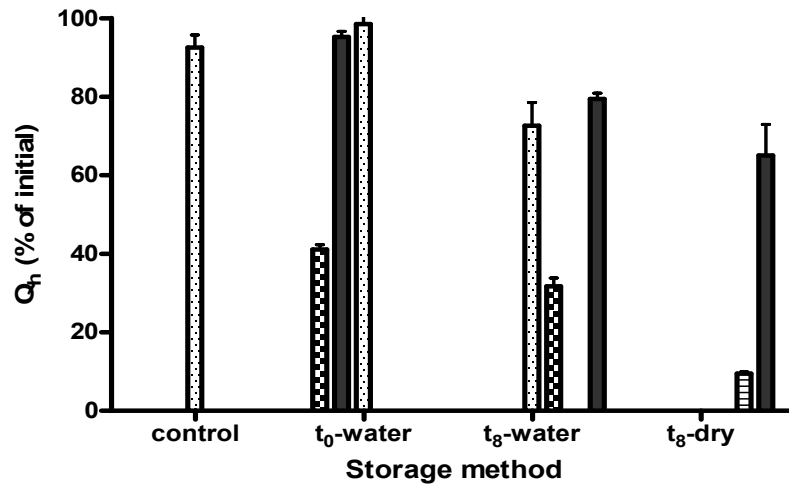


Fig. 3. Hydraulic capacity ( $Q_h$ ) of stem segments of chrysanthemum. Stem segments were placed in vase solution (control,  $t_0$ -water,  $t_8$ -water) or submitted to an 8 h dry period ( $t_8$ -dry), at 20°C. Immediate after cutting ( $t_0$ ) or 8 h after cutting ( $t_8$ -water) air was sucked into the stem for 5 min. Stems exposed to 5 min. of air entrance or to a dry storage treatment were rehydrated under low pressure. For control,  $Q_h$  was measured after 8h storage in vase solution; for  $t_0$ -water,  $Q_h$  was measured after air entrance and after rehydration at  $t=0$  and 8 h later; for  $t_8$ -water,  $Q_h$  was measured before and after air entrance at  $t=8$  and after rehydration; for  $t_8$ -dry,  $Q_h$  was measured after dry storage of 8 h and after rehydration.  $Q_h$  (expressed as percentage of the initial  $Q_h$  (before any storage treatment)) is measured after 8 h in water (▨), after 5 min. of air entrance (▩), after an 8h dry period (▧), or after rehydration (■). Vertical bars represent S.E.M. ( $n=4$ ).

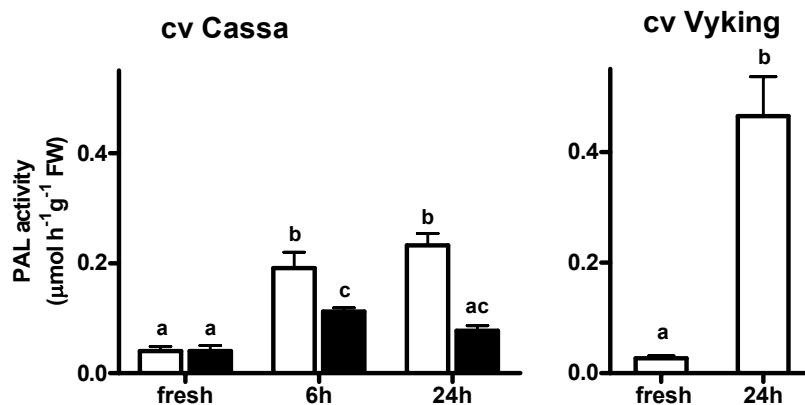


Fig. 4. Activity of PAL in chrysanthemum stems at different times after cutting. Stem samples were taken immediately after harvest (fresh), and after 6 or 24 h of vase life; the samples were taken from 0-2 cm (open bars) or 5-7 cm (solid bars) above the stem cutting surface of the cultivars Cassa (left) and Vyking (right). Vertical bars represent S.E.M. ( $n=12$ ). Columns with different letters were significantly different ( $P < 0.01$ ).