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Tipburn Incidence and Ca Acquisition and Distribution in Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn.) Cultivars under Different Ca Concentrations in Nutrient Solution

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Abstract: Tipburn (calcium (Ca) deficiency disorder) is a major problem in the production of lisianthus cultivars. However, few studies have investigated the influence of different Ca concentrations in nutrient solution on tipburn incidence and Ca acquisition and distribution. Thus, it remains unclear why some cultivars exhibit tipburn under high Ca concentrations. To address this, we used three lisianthus cultivars 'Azuma-no-Kaori' (AK), 'Celeb Wine' (CW), and 'Voyage Yellow' (VY) and compared tipburn incidence and Ca acquisition and distribution under different Ca concentrations in a nutrient solution (low (40 ppm), moderate (80 ppm), and high (120 ppm) Ca). Tipburn severity and incidence in AK and VY significantly decreased with increasing nutritional Ca concentrations; the Ca concentrations in each organ and Ca acquisition competence (RGR_{Ca}) increased at higher nutritional Ca concentrations. In contrast, tipburn incidence in CW was 100% for all treatments. In CW, Ca acquisition competence and Ca concentrations in most organs increased with increasing nutritional Ca concentrations, but the Ca concentrations in the tips of the upper leaves did not differ significantly between treatments. Thus, our results suggest that the cause of tipburn under sufficient Ca conditions is an inability of the plant to distribute Ca to the tips of its upper leaves.

Keywords: Ca deficiency; *Eustoma grandiflorum*; growth analysis; path analysis

1. Introduction

Lisianthus (*Eustoma grandiflorum* (Raf.) Shinn.) is a species in the family Gentianaceae that originated in the warm regions of the southern United States and northern Mexico. Its cultivars are mainly supplied in the form of cut flowers. The wholesale value of lisianthus cultivars ranked fifth among cut flowers in Japan in 2017. In addition, most of the lisianthus cultivars produced around the world are bred by Japanese seed companies.

The occurrence of tipburn (calcium (Ca) deficiency disorder) is a major problem for the production of lisianthus cultivars because it causes shipment delay, deterioration in plant quality, and serious economic losses. The cause of tipburn has been widely investigated in commercial crops such as lettuce [1–5], Chinese cabbage [6–9], strawberries [10–13], and lilies [14–16].

Recent studies on lisianthus cultivars have shown that tipburn is mainly caused by the inability of the plant to translocate adequate amounts of Ca to the tips of the upper leaves, which is associated with an increase in the distribution of Ca in the roots [17,18]. It has been suggested that changes in plant and organ growth rates have little effect on the incidence of tipburn in lisianthus [18]. However, few studies have compared tipburn incidence and Ca acquisition and distribution under different

Ca concentrations in nutrient solution for lisianthus cultivars. Thus, it remains unclear why some lisianthus cultivars exhibit tipburn even under conditions of high Ca concentrations.

Acquisition competence of elements (e.g., calcium, carbon, nitrogen) has been quantified by applying growth analysis methods [17–21]. In addition, path analysis has been conducted to quantify the relevance of the relative growth rate (RGR) and its components (e.g., leaf area ratio (LAR), net assimilation rate (NAR), specific leaf area (SLA), and specific nitrogen absorption rate (SAR) in roots) in plant ecology [22–25]. Under different nutritional Ca concentrations, Ca distribution to the upper leaves seems to be affected by changes in Ca acquisition. However, no studies to date have quantified the roles of Ca acquisition and distribution under different nutritional Ca concentrations. Thus, in this study, we attempted to address this knowledge gap by applying growth and path analyses.

The aims of the present study were to elucidate the influence of different Ca concentrations in nutrient solution on tipburn incidence and Ca acquisition and distribution in three lisianthus cultivars and to discuss the causes of tipburn under high Ca concentrations. In addition, path analysis was conducted to quantify the roles of Ca acquisition and distribution.

2. Materials and Methods

2.1. Plants

The lisianthus cultivars used in this study were 'Azuma-no-Kaori' (AK) (Sakata Seed Corporation, Yokohama, Japan), 'Celeb Wine' (CW) (Sumika Agrotech Co., Ltd., Osaka City, Japan), and 'Voyage Yellow' (VY) (Sakata Seed Corporation), which were selected from a group classified in a previous study [17]. AK has been classified as tipburn–sensitive with high Ca requirements, and CW has been classified as tipburn-sensitive with low ability to acquire Ca and distribute it to the tips of its upper leaves; VY has been classified as tipburn-sensitive with low ability to distribute Ca to the tips of its upper leaves.

Seeds were sown in plug flats (406 cells per tray) filled with seedling propagation medium (Metro Mix 350; Sun Gro Horticulture, Agawam, MA, USA) on 17 March 2018. The seeded trays were maintained in a germination room at 24 °C under a 14 h light/10 h dark photoperiod. After two weeks, the trays were transferred to a controlled environmental system (phytotron), and the plants were grown under the following conditions: 25 °C (light period) and 20 °C (dark period), 60 ± 5% humidity, 400 ppm CO_2, 14 h light (225 ± 25 µmol m^{-2} s^{-1}) and 10 h dark. An irrigation system in the phytotron supplied a nutrient solution by bottom watering for 30 min at once a day. The nutrient solution was made by dissolving nutrient salts in distilled water (KNO_3 (Fujifilm Wako Chemicals U.S.A. Corporation, Richmond, VA, USA): 0.202 g/L, $Ca(NO_3)_2 \bullet 4H_2O$ (Fujifilm Wako Chemicals U.S.A. Corporation): 0.236 g/L, $NH_4H_2PO_4$ (Fujifilm Wako Chemicals U.S.A. Corporation): 0.038 g/L, $MgSO_4 \bullet 7H_2O$ (Fujifilm Wako Chemicals U.S.A. Corporation): 0.123 g/L, Otsuka-house No.5 L (OAT Agrio Co., Ltd., Tokyo, Japan): 0.4 mL/L) and its nutrient concentrations were same as that used in previous studies [17,18,26]. Five weeks after transfer to the phytotron, plugs were transplanted into 0.25 L polyethylene pots filled with Metro Mix 350.

2.2. Experimental Design

Individuals of the three cultivars were assigned randomly to either low (40 ppm Ca), moderate (80 ppm Ca), or high Ca treatments (120 ppm Ca). Plants in the low Ca treatment were supplied with the same nutrient solution as described above. In order to minimize the influence of incremental changes in nutrients other than Ca, $CaCl_2$ (Fujifilm Wako Chemicals U.S.A. Corporation) was added to the nutrient solution in the moderate (added $CaCl_2$: 0.147 g/L) and high Ca treatments (added $CaCl_2$: 0.294 g/L). Although the Cl concentrations in the nutrient solutions for these treatments (moderate: 71 ppm Cl, high: 142 ppm Cl) appear to be high, these values are lower than the reference values for raw water for nutrient solutions in Japan. The supplied nutrient solutions were replaced every two weeks. All plants were maintained in the same phytotron under the environmental conditions described above.

At the start of the experiment, five pots of each cultivar were randomly sampled in triplicate. Plant dry weights and Ca concentrations in all plant organs were measured. No tipburn was observed in any of the cultivars at that stage. Eight weeks later, a total of 15 pots (5 pots × 3 replicates) were sampled from all treatments, and the tipburn severity and incidence and Ca acquisition and distribution were investigated. Harvested plants were washed with distilled water and divided into roots, stems, and leaves to measure Ca concentrations and dry weights. In addition, to evaluate vertical Ca distribution in each cultivar, the 1st (upper), 4th (middle), and 7th (lower) leaves on the main stem (leaf position was counted from the top to the base) were distinguished.

2.3. Tipburn Severity and Incidence

Whole leaves were scored using an arbitrary tipburn severity index with ranking from 0 to 1 (0, asymptomatic; 0.2, deformed leaf margins; 0.5, leaf-tip chlorosis; 1, leaf-tip necrosis) [26]. The severity of tipburn in each plant was defined as

$$\text{Tipburn severity} = \sum \{(\text{severity index} \times \text{leaf number})/\text{whole leaves number per pot}\} \times 100 \quad (1)$$

The incidence of tipburn (tipburn incidence) was expressed as the percentage of plants in a cultivar exhibiting tipburn symptoms.

2.4. Measurement of Dry Weight and Ca Concentration

Samples of plant organs were dried at 70 °C for 72 h and weighed. To evaluate morphological responses to different Ca concentrations, leaf mass ratio (LMR; leaf mass/whole-plant mass), stem mass ratio (SMR; stem mass/whole-plant mass), and root mass ratio (RMR; root mass/whole-plant mass) were calculated. Ca concentrations were determined using a Z-5300 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Ltd., Tokyo, Japan). To evaluate horizontal Ca distribution for each leaf position, Ca concentrations of the top one-fifth (leaf tip) and the remainder (leaf base) of each leaf (the 1st (upper), 4th (middle), and 7th (lower) leaves) were analyzed separately. To calculate total Ca concentrations, we quantified the whole-plant Ca content by adding the Ca content (Ca concentration × dry weight) of each organ. Total Ca concentrations were calculated by dividing the whole-plant Ca content by the mass of the whole plant. Ca concentrations of whole leaves were similarly determined.

2.5. Ca acquisition Competence

The Ca acquisition competence (RGR_{Ca}) for each plant was defined as

$$RGR_{Ca} = (\ln W_{Ca2} - \ln W_{Ca1})/(T_2 - T_1) \quad (2)$$

where W_{Ca1} and W_{Ca2} are the whole-plant Ca content at times T_1 and T_2, respectively. In addition, the specific Ca absorption rate per unit root mass (SAR_{Ca}) and the root mass ratio per whole-plant Ca content (RMR_{Ca}) were calculated using the following equations [17–19,27].

$$SAR_{Ca} = (W_{Ca2} - W_{Ca1})/(T_2 - T_1) \times (\ln W_{R2} - \ln W_{R1})/(W_{R2} - W_{R1}) \quad (3)$$

$$RMR_{Ca} = (W_{R2}/W_{Ca2} + W_{R1}/W_{Ca1})/2 \quad (4)$$

where W_{R1} and W_{R2} are the root dry weights at times T_1 and T_2, respectively.

2.6. Statistical Analysis

Data were analyzed using SPSS v.22.0 (IBM Corp. Japan, Tokyo, Japan). A one-way ANOVA (analysis of variance) was conducted to assess the effects of the treatments. Differences in mean values were evaluated using Tukey's b (homoscedasticity assumed) or Dunnet T3 (homoscedasticity not assumed) tests. The mean values are presented. For the path analysis, SPSS Amos 25 (IBM Corp.) was used. Pathway analysis was performed on Ca concentrations in the tips of the upper (1st) and lower (7th) leaves, Ca acquisition competence (RGR_{Ca}), and its components (SAR_{Ca} and RMR_{Ca}).

3. Results and Discussion

3.1. Tipburn Severity and Incidence

The tipburn severity and incidence for each cultivar and treatment are shown in Figure 1. For AK and VY, tipburn severity and incidence decreased at higher Ca concentrations. In contrast, CW exhibited tipburn in all plants (tipburn incidence = 100%) and tipburn severity was higher than 10% even in the high Ca treatment. These results indicate that Ca application is not an effective approach to eliminate tipburn in some lisianthus cultivars.

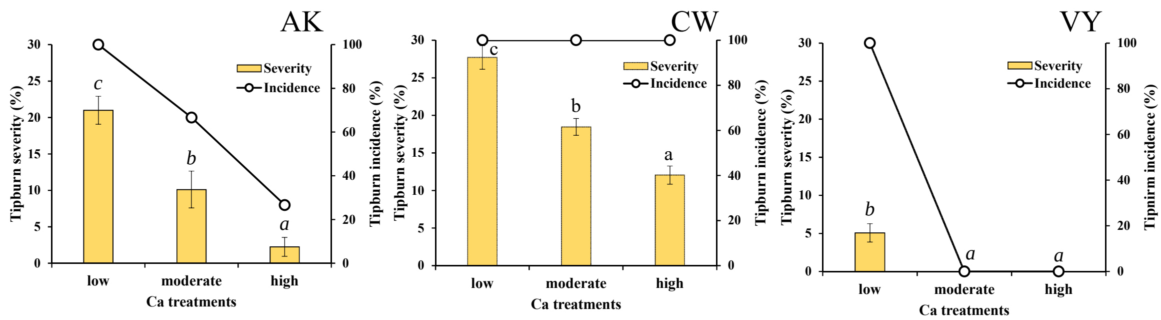

Figure 1. Tipburn severity and incidence for three lisianthus cultivars and Ca concentration treatments at the end of the experiment. Mean values ($n = 15$) are shown, and significant differences among them are indicated by different letters (normal type: Tukey's b test, $p < 0.05$; *italic type*: Dunnet T3, $p < 0.05$). Cultivars: 'Azuma-no-Kaori' (AK), 'Celeb Wine' (CW), 'Voyage Yellow' (VY).

3.2. Plant Growth

Every cultivar exhibited the lowest plant dry weight and RMR in the low Ca treatment (Table 1). There were no significant differences in the LMR of any cultivar between treatments. The SMR of each cultivar tended to decrease with increasing nutritional Ca concentration.

Table 1. Plant dry weight, leaf mass ratio (LMR), stem mass ratio (SMR), and root mass ratio (RMR) for each cultivar and treatment at the end of the experiment.

Cultivars	Treatments	Dry Weight (g)		LMR (g/g)		SMR (g/g)		RMR (g/g)	
AK	low	3.7	a	0.56	n.s.	0.20	c	0.24	a
	moderate	5.4	b	0.55		0.18	b	0.27	ab
	high	5.0	b	0.55		0.15	a	0.30	b
CW	low	4.2	a	0.63	n.s.	0.18	b	0.19	a
	moderate	5.2	ab	0.61		0.16	a	0.23	ab
	high	5.9	b	0.59		0.16	a	0.24	b
VY	low	4.6	a	0.57	n.s.	0.14	*b*	0.29	n.s.
	moderate	5.5	b	0.57		0.11	*a*	0.31	
	high	5.4	b	0.57		0.12	*ab*	0.31	

Mean values ($n = 15$) are shown, and significant differences among them are indicated by different letters (normal type: Tukey's b test, $p < 0.05$; *italic type*: Dunnet T3, $p < 0.05$). n.s. represent no significant differences among the treatments (ANOVA, $p > 0.05$). Cultivars: 'Azuma-no-Kaori' (AK), 'Celeb Wine' (CW), 'Voyage Yellow' (VY).

The results for dry weight (Table 1) and tipburn severity and incidence (Figure 1) demonstrate that plant growth inhibition by tipburn occurrence was reduced at high Ca concentrations (moderate and high). Increases in RMR under high Ca concentrations can be attributed to increases in plant dry weight and water consumption of the plant. In addition, Ca and Cl excess disorder were not observed in this experiment, indicating that differences in Cl concentrations in the nutrient solution had little effect on plant growth.

3.3. Ca Acquisition Competence

Total Ca concentration, RGR_{Ca}, SAR_{Ca}, and RMR_{Ca} of each cultivar and treatment are shown in Table 2. Total Ca concentrations and RGR_{Ca} of each cultivar increased with increasing nutritional Ca concentration and there were significant differences in those values between low and high Ca treatments. The SAR_{Ca} of each cultivar increased similarly to total Ca concentrations and RGR_{Ca}. In contrast, no increases in RMR_{Ca} with increasing Ca concentration were observed in any of the cultivars.

Table 2. Total Ca concentrations, Ca acquisition competence (RGR_{Ca}), specific Ca absorption rate per unit root mass (SAR_{Ca}), and root mass ratio per whole-plant Ca content (RMR_{Ca}) for each cultivar and treatment at the end of the experiment.

Cultivars	Treatments	Total Ca Concentrations (mg-Ca/kg-DW)		RGR_{Ca} (mg-Ca mg-Ca^{-1} week^{-1})		SAR_{Ca} (mg-Ca g-DW^{-1} week^{-1})		RMR_{Ca} (mg-Ca/g-DW)	
AK	low	2.7	a	0.59	*a*	6.1	a	0.11	
	moderate	3.9	b	0.68	*b*	8.8	b	0.10	n.s.
	high	4.4	b	0.69	*b*	9.1	b	0.10	
CW	low	2.7	a	0.60	a	7.4	a	0.10	b
	moderate	3.8	b	0.67	b	9.7	b	0.09	a
	high	4.5	c′	0.71	b	11.2	c	0.09	a
VY	low	2.7	*a*	0.66	a	6.4	a	0.10	b
	moderate	3.8	*b*	0.73	b	8.7	b	0.09	ab
	high	4.6	*c*	0.75	b	10.8	c	0.08	a

Mean values ($n = 15$) are shown, and significant differences among them are indicated by different letters (normal type: Tukey's b test, $p < 0.05$; *italic type*: Dunnet T3, $p < 0.05$). n.s. represent no significant differences among the treatments (ANOVA, $p > 0.05$). Cultivars: 'Azuma-no-Kaori' (AK), 'Celeb Wine' (CW), 'Voyage Yellow' (VY).

Although we hypothesized that Ca acquisition of tipburn-sensitive cultivars would not increase under high Ca concentrations, our results demonstrated that, in fact, Ca acquisition in all of the cultivars increased with increasing nutritional Ca concentration. Thus, the changes in Ca acquisition under different Ca concentrations appeared to have little effect on tipburn incidence for the lisianthus cultivars studied. In addition, Ca acquisition competence was strongly affected by a change in SAR_{Ca}. This finding suggests that variation in Ca acquisition under different Ca concentrations is mainly a result of physiological responses (SAR_{Ca}) rather than morphological responses (RMR_{Ca}).

3.4. Ca Distribution

3.4.1. Ca Concentrations of Whole Leaves, Stems, and Roots

The Ca concentrations of each organ for the three cultivars are shown in Table 3. Ca concentrations of all the organs in each cultivar showed a tendency to increase with increasing nutritional Ca concentration. These results were consistent with the results for RGR_{Ca} and total Ca concentrations.

3.4.2. Ca Concentrations of Upper (1st), Middle (4th), and Lower (7th) Leaves

For AK and VY—which showed lower tipburn incidence and severity with increasing nutritional Ca concentration (Figure 1)—Ca concentrations in the tips and bases of every leaf position increased at higher Ca concentrations (Table 4); similar results were seen in the other organs in AK and VY (Tables 2 and 3). In contrast, in CW—which had a tipburn incidence of 100% under all treatments (Figure 1)—Ca concentrations in the tips of the upper (1st) and middle (4th) leaves did not differ significantly between treatments, although concentrations in the leaf bases significantly increased with increasing nutritional Ca concentration (Table 4). These results indicate that lisianthus cultivars, which exhibit high tipburn incidence and severity under high nutritional Ca concentrations, have limited ability to distribute adequate Ca to the tips of the upper leaves; this was clearly the cause of tipburn under sufficient Ca concentrations. Lee et al. [28] reported that the expression of genes encoding Ca^{2+} vacuole transporters in cabbage cultivars is associated with accumulation of Ca^{2+} in the vacuoles of leaf cells. To elucidate why tipburn-sensitive lisianthus cultivars cannot distribute adequate Ca to the tips of upper leaves, the roles of Ca distribution and gene expression should be investigated in the future. In addition, further researches on the effect of different Ca supply on tipburn incidence in the other cultivars also need.

Table 3. Ca concentrations of whole leaves, upper stems, lower stems, and roots for each cultivar and treatment at the end of the experiment.

Cultivars	Treatments	Ca Concentrations (mg-Ca/kg-DW)							
		Whole Leaves		Upper Stems		Lower Stems		Roots	
AK	low	1.6	a	0.52	*a*	0.60	*a*	6.79	a
	moderate	2.0	b	0.87	*b*	0.93	*b*	9.44	b
	high	2.2	c	0.96	*b*	1.11	*b*	9.81	b
CW	low	1.6	*a*	0.80	*a*	0.95	a	7.69	a
	moderate	2.1	*b*	1.58	*b*	1.10	b	9.89	b
	high	2.6	*c*	2.24	*c*	1.45	b	11.09	c
VY	low	1.0	a	0.75	a	0.65	a	7.02	a
	moderate	2.2	b	1.19	b	1.00	b	7.51	a
	high	2.7	c	1.70	c	1.13	b	9.35	b

Mean values (n = 15) are shown, and significant differences among them are indicated by different letters (normal type: Tukey's b test, $p < 0.05$; *italic type*: Dunnet T3, $p < 0.05$). Cultivars: 'Azuma-no-Kaori' (AK), 'Celeb Wine' (CW), 'Voyage Yellow' (VY).

Table 4. Ca concentrations in the tips and bases of upper (1st), middle (4th), and lower (7th) leaves for each cultivar and treatment at the end of the experiment.

Cultivars	Treatments	Ca Concentrations (mg-Ca/kg-DW)											
		Upper (1st) Leaves				Middle (4th) Leaves				Lower (7th) Leaves			
		Tips		Bases		Tips		Bases		Tips		Bases	
AK	low	1.77	*a*	1.29	a	1.33	*a*	1.09	a	1.92	a	1.08	*a*
	moderate	2.50	*b*	1.73	b	2.01	*b*	1.88	b	2.28	b	1.57	*b*
	high	3.28	*b*	1.88	b	2.39	*c*	2.13	c	2.42	b	1.74	*b*
CW	low	2.15		1.55	a	1.79		1.77	a	1.63	a	1.43	a
	moderate	1.85	n.s.	2.31	b	1.78	n.s.	2.68	b	1.77	ab	2.01	b
	high	1.85		3.09	c	1.86		3.44	c	2.12	b	2.71	c
VY	low	1.52	*a*	1.36	a	1.75	*a*	1.51	*a*	1.81	a	1.37	a
	moderate	3.52	*b*	1.90	ab	2.27	*b*	2.23	*b*	2.23	b	2.16	b
	high	3.36	*b*	2.72	b	2.45	*b*	2.69	*c*	2.06	b	2.39	b

Mean values (n = 15) are shown, and significant differences among them are indicated by different letters (normal type: Tukey's b test, $p < 0.05$; *italic type*: Dunnet T3, $p < 0.05$). n.s. represent no significant differences among the treatments (ANOVA, $p > 0.05$). Cultivars: 'Azuma-no-Kaori' (AK), 'Celeb Wine' (CW), 'Voyage Yellow' (VY).

3.5. Contributions of Ca Acquisition and Ca Distribution under Different Ca Concentrations

Path models for CW and VY were constructed for all treatments (Figure 2) and were not rejected (CW: χ^2 = 2.362, p = 0.797; VY: χ^2 = 6.329, p = 0.275). These path models provided a good explanation of the contributions of Ca acquisition and distribution for leaf tips (CW: GFI (goodness of fit index) = 0.979, AGFI (adjusted GFI) = 0.937, CFI (comparative fix index) = 1.000, RMSEA (root means square error of approximation) = 0.000; VY: GFI = 0.948, AGFI = 0.843, CFI = 0.986, RMSEA = 0.078). In contrast, the same path model for AK was rejected (χ^2 = 29.066, p = 0.000).

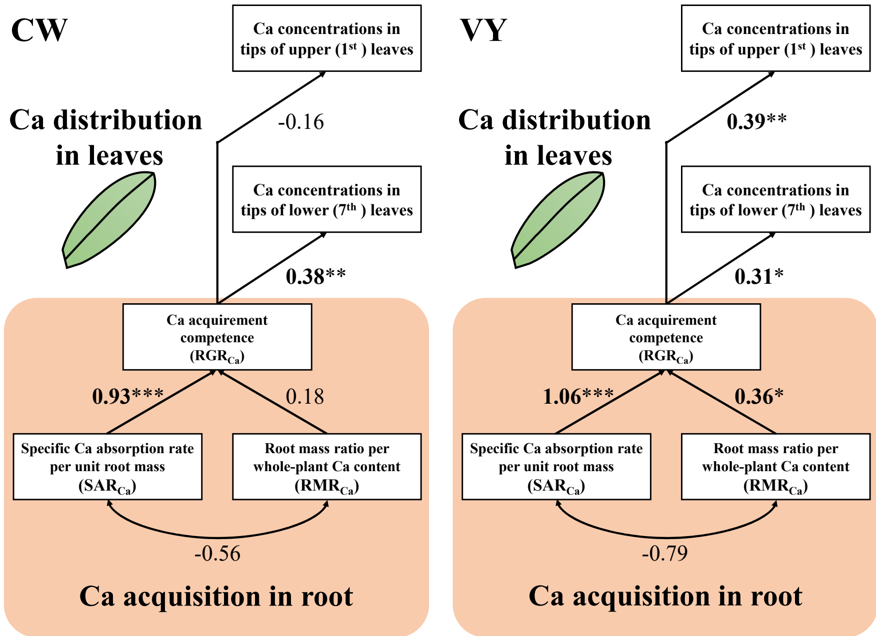

Figure 2. Path model of Ca acquisition competence (RGR_{Ca}), specific absorption rate per unit. root mass (SAR_{Ca}), root mass ratio per whole-plant Ca content (RMR_{Ca}), and Ca concentrations in the tips of upper (1st) and lower (7th) leaves under three different Ca treatments for 'Celeb Wine '(CW) and 'Voyage Yellow' (VY) lisianthus cultivars. Single-arrow lines represent causal relationships and the numbers above the arrows are the standardized path coefficients. Double-arrow lines represent correlations and the numbers above the arrows are standardized correlation coefficients. The significance levels of the path effects are indicated by * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Path standardized coefficients for SAR_{Ca} to RGR_{Ca} of each cultivar were significant and higher than those for RMR_{Ca} to RGR_{Ca} (Figure 2), indicating that Ca acquisition competence was strongly affected by physiological responses (changes in SAR_{Ca}). For VY, the path standardized coefficient for RGR_{Ca} to Ca concentrations in the tips of upper (1st) and lower (7th) leaves were significant and had similar values. In contrast, for CW, the path standardized coefficient for RGR_{Ca} to Ca concentrations in the tips of the upper (1st) leaves was not significant and was distinctively lower than that for the lower (7th) leaves. These results demonstrate that tipburn-sensitive lisianthus cultivars have limited ability to distribute adequate Ca to the tips of upper leaves, although Ca acquisition and Ca distribution to other organs increased with increasing nutritional Ca concentration. In this study, we could not construct an effective path model for all the cultivars although many attempts have been conducted. To address this, in the future, quantification of other physiological and morphological traits also needs to be discussed in more detail.

4. Conclusions

This study investigated the influence of Ca concentrations in nutrient solution on tipburn incidence and Ca acquisition and distribution in three lisianthus cultivars. At higher Ca concentrations, AK and VY exhibited significantly higher Ca acquisition competence (RGR_{Ca}) and Ca concentrations in all organs, and their tipburn severity and incidence declined. In contrast, CW exhibited 100% tipburn incidence under all treatments (Figure 1) and the Ca concentrations in the tips of the upper (1st) and middle (4th) leaves did not differ significantly between treatments although Ca acquisition competence and Ca concentrations in other organs significantly increased with increasing nutritional Ca concentrations. Thus, the cause of tipburn under sufficient Ca conditions is the inability of the plant to distribute Ca to the tips of upper leaves. In addition, path analysis enabled us to describe the roles of Ca acquisition and Ca distribution for the tips of leaves. Use of this analytical method can contribute to our understanding of the interrelationships between multiple physiological and morphological plant traits.

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Conflicts of Interest: The authors declare no conflict of interest.

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