



Chlorocholine chloride and paclobutrazol treatments promote carbohydrate accumulation in bulbs of *Lilium* Oriental hybrids ‘Sorbonne’

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Received Dec. 8, 2010; Revision accepted Apr. 24, 2011; Crosschecked Oct. 18, 2011

Abstract: The present study was to test the hypothesis that the plant growth retardants chlorocholine chloride (CCC) and paclobutrazol (PBZ) could improve the carbohydrate accumulation in lily bulbs by enhancing photosynthetic capacity and changing endogenous hormones. Plants of *Lilium* Oriental hybrids ‘Sorbonne’ were treated with a foliar spray of CCC or PBZ (both at 300 mg/L) solution, at six weeks after planting (6 WAP). The morphological parameters, endogenous hormone contents (gibberellic acid (GA), abscisic acid (ABA), and indole-3-acetic acid (IAA)), and carbohydrate contents were measured from 6 to 18 WAP, at 2-week intervals. The results showed that CCC increased the biomass of leaves and stems which might produce more photoassimilates available for transportation and utilization. However, PBZ treatment suppressed vegetative growth and favored photoassimilate transportation into bulbs. A slight delay of bud and anthesis formation was observed in both treated plants. CCC and PBZ treatments substantially enhanced the sucrose contents in leaves probably due to the increase of chlorophyll contents. Treatment with CCC or PBZ decreased GA but increased IAA contents in lily bulbs which might stimulate starch accumulation and formation of new scales. Our experiment suggested that CCC or PBZ treatment is an effective method to promote carbohydrate accumulation in lily bulbs.

Key words: Carbohydrate, Chlorocholine chloride, Gibberellic acid, *Lilium* Oriental hybrids ‘Sorbonne’, Paclobutrazol
 doi:10.1631/jzus.B1000425 Document code: A CLC number: S682.2^{†9}

1 Introduction

Lilium Oriental hybrids ‘Sorbonne’ is one of the most popular bulb flower varieties on the Chinese market. Bulb is the sink organ of the lily where starch is the dominant reserve energy source for plant growth. Starch is degraded into sucrose which can be transported and utilized for vegetative growth of source organs and formation of inflorescence. Carbohydrates flow back to bulb and are converted into storage materials during and after blossoming when new scales begin to form.

Chlorocholine chloride [(2-chloroethyl)trimethylammonium chloride] (CCC) and paclobutrazol [(2R, 3R+2S, 3S)-1-(4-chlorophenyl), 4-dimethyl-2-(1,2,4-triazol-1-yl)-pentan-3-ol] (PBZ) are two anti-gibberellin growth retardants that have been extensively used to manipulate growth in plants (Menhenett, 1984; Jiao *et al.*, 1986; Sharma *et al.*, 1998a; 1998b; Berova and Zlatev, 2000). CCC is an anti-gibberellin growth regulator based on its inhibition of the conversion from geranylgeranyl pyrophosphate (GGPP) to ent-kaurene, an early step in gibberellic acid (GA) biosynthesis (Wang *et al.*, 2009). Previous studies demonstrated that CCC could effectively promote tuberization and improve the starch content in tubers of potato (Sharma *et al.*, 1998a; 1998b). This could be

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explained by the following reasons. Firstly, GA was revealed to cause a remarkable reduction in the activity of adenosine diphosphate-glucose pyrophosphorylase (AGPase), a key rate-limiting starch synthesis enzyme (Mares *et al.*, 1981). Treatment with CCC may counteract this reduction in starch synthesis by blocking GA synthesis. Secondly, CCC treatment could have a long-term impact on plant growth by changing endogenous hormone contents. Wang and Xiao (2009) found that foliar spraying of CCC could noticeably decrease GA and abscisic acid (ABA) contents in potato leaves, which in turn increased chlorophyll contents and stimulated photosynthetic rate (Sharma *et al.*, 1998a). PBZ is a triazole compound well known for its anti-GA effects also based on the inhibition of ent-kaurene biosynthesis. Foliar spray of PBZ treatment could control excessive vegetative growth (Tekalign and Hammes, 2005), and improve photosynthetic capacity and water balance in leaves (Berova and Zlatev, 2000; Abdul Jaleel *et al.*, 2007b; Mobli and Baninasab, 2008), thereby redirecting more photoassimilates to sink organs like the bulb (Yim *et al.*, 1997; Tekalign and Hammes, 2005). Thus, we hypothesize that CCC and PBZ may potentially improve the carbohydrate accumulation in lily bulbs due to changes in morphogenesis, photosynthetic capacity, and phytohormonal balance.

In this paper, we examined the regulation of carbohydrate accumulation in bulbs of *Lilium* Oriental hybrids 'Sorbonne' in response to CCC and PBZ treatments, and measured the associated changes in morphogenesis, photosynthetic capacity, and endogenous hormones.

2 Materials and methods

2.1 Materials and cultivation methods

The *Lilium* Oriental hybrids 'Sorbonne' bulbs (16–18 cm in perimeter) were purchased from Zhejiang Hongyue Seed Company, China. The plants were raised in the Botanical Garden on the Huajiachi Campus of Zhejiang University on Sept. 10, 2007. The soil was a mixture of peat moss and perlite (2:1) with 10 g APEX fertilizer (14-14-14) per bed, and no fertilizer was used afterward. Twelve bulbs were planted at a distance of 10 cm to a depth of 15 cm in one standard nursery bed (20 cm×40 cm×60 cm). The

nursery beds were watered and weeded regularly in order to ensure healthy growth of the plants. At six weeks after planting (6 WAP), a solution of CCC or PBZ (300 mg/L) was applied separately by foliar spray until obvious drops appeared on the leaves. The plants were uprooted randomly at 6, 8, 10, 12, 14, 16, and 18 WAP, washed and separated into leaves, stems and bulbs, and then used for analysis of growth and biochemical constituents. All treatment trails consisted of five independent replicates.

2.2 Morphological parameters and chlorophyll contents

The length of the main stem and the length of its branches up to the tips were measured and added as the total stem length. Leaf area of the plant was measured using a LICOR photoelectric area meter (model LI-3100, Lincoln, USA) and the area was expressed in mm² per plant. The weights of stems, leaves and flower buds were summed up as total shoot weight per plant. Fresh weight of bulbs and total shoot weight were determined by using an electronic balance. Chlorophyll contents were estimated by the method of Arnon (1949).

2.3 Assays for sucrose and starch

Samples containing 0.5 g fresh lily bulbs were homogenized in 5 ml 80% ethanol, and centrifuged for 5 min at 4000 r/min. The supernatant was recovered and the pellet was re-extracted twice more using 5 ml 80% ethanol. The upper aqueous phase was removed and collected by using anthrone at 0.2% (2 g/L). The extraction and determination of starch levels were based on the method of McCready *et al.* (1950). The pellets were ground with 5 ml 30% perchloric acid and centrifuged for 15 min at 10000 r/min. The supernatant containing starch was removed and the pellets were re-extracted twice more. The supernatants were combined and the pellets were discarded. The absorbance was determined at 620 nm in a digital ultraviolet spectrophotometer.

2.4 Assays for GA, ABA, and IAA

The endogenous contents of GA, ABA and indole-3-acetic acid (IAA) were each analyzed by a specific enzyme-linked immunosorbent assay (ELISA). The hormone ELISA kits were developed at the College of Crop Sciences, China Agricultural

University. Samples were collected at 0 °C, immediately frozen in liquid nitrogen, and stored at -80 °C. The extraction, purification, and determination of endogenous hormones were performed as described by Hao *et al.* (2001).

2.5 Statistical analysis

Data analysis was undertaken using the SPSS 11.5 software package. The contents of chlorophyll and endogenous hormones were subjected to a one-way analysis of variance (ANOVA; Duncan's test) to compare the differences between treated and untreated plants. A *P* value less than 0.05 was considered significantly different.

3 Results

3.1 Effects of CCC and PBZ on morphological parameters of lily plants

The lily bulbs were planted and the morphological parameters were carefully observed. The whole growth period was mainly divided into three stages, i.e., vegetative growth period (0–6 WAP), initial and full blossoming period (6–16 WAP), and post-blossoming period (16–18 WAP). The biomass of stems and leaves significantly increased during the vegetative growth period. Visible buds appeared at 6 WAP when the plants entered the blossoming period. The carbohydrate contents in bulbs collapsed at the initial blossoming period. However, the carbohydrates started to re-accumulate during the full blossoming period and maintained a constant increase until the post-blossoming.

Treatment with foliar spray containing CCC or PBZ had significant effects on morphological parameters over the entire growth process. Leaf areas of all plants increased during the vegetative growth stage and initial blossoming period. A marked enhancement in leaf area was observed in CCC-treated plants from the blossoming period onward (Fig. 1a). While CCC treatment significantly increased leaf number and total stem length since initial blossoming period, PBZ treatment decreased the stem length by 21.12% compared to control plants at 18 WAP (Figs. 1b and 1c). Based on our visual observation, CCC treatment delayed the bottom leaf senescence during the blossoming period and the leaf number of

CCC-treated plants was 19.64% higher than that of controls at 14 WAP (Fig. 1b). Similar to the increase in leaf number and total stem length, the total shoot weight and the bulb weight of CCC-treated plants were generally higher than those of controls especially during the blossoming period (Figs. 1d and 1e).

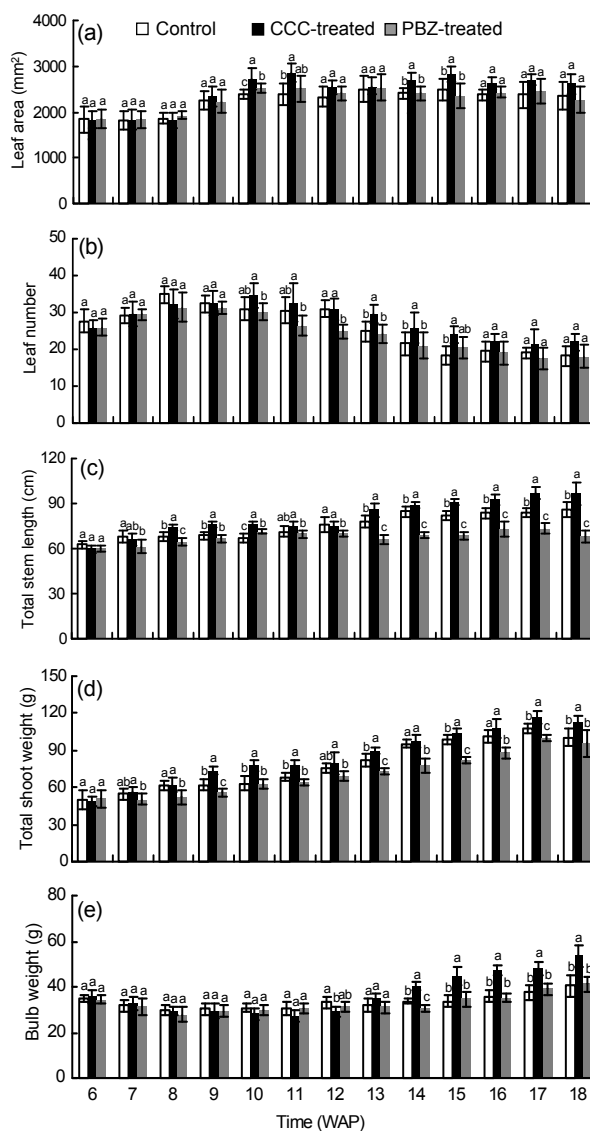


Fig. 1 Effects of CCC and PBZ treatments on the morphological parameters at different growth stages

(a) Leaf area; (b) Leaf number; (c) Total stem length; (d) Total shoot weight; (e) Bulb weight. Total stem length was recorded as the length of the main stem and its branches up to the tips. The weights of stems, leaves, and flower buds were summed to yield the total shoot weight. Values are presented as mean±standard deviation (SD) of five samples in each group. Values not sharing a common superscript letter differ significantly at $P \leq 0.05$

In addition to the delay of leaf senescence induced by CCC treatment, the timings of bud and anthesis formation were also changed by CCC and PBZ treatments. The number of visible bud in untreated plants was higher than that in treated plants at the initial blossoming period (Fig. 2a). In addition, visible anthesis was first observed in untreated plants at 12 WAP (Fig. 2b), indicating that CCC and PBZ treatments both postponed and suppressed bud and anthesis formation in lily plants.

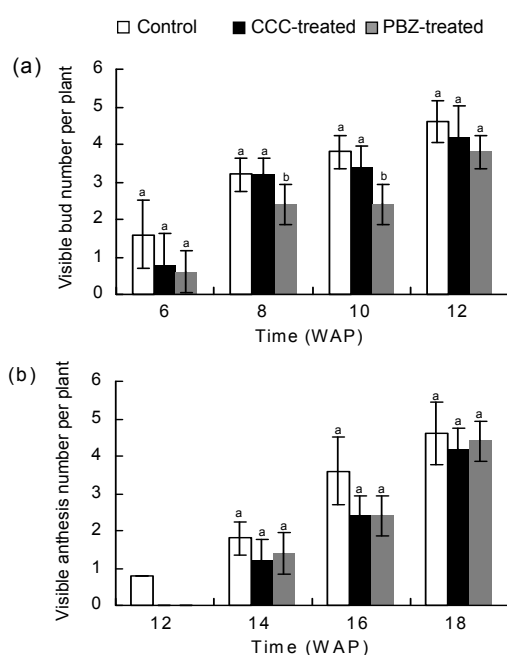


Fig. 2 Effects of CCC and PBZ treatments on the timings of bud and anthesis formation

Visible bud and anthesis numbers per plant were measured to demonstrate the timing of bud/anthesis formation. No visible anthesis was observed in treated plants at 12 WAP. Values are presented as mean±SD of five samples in each group. Values not sharing a common superscript letter differ significantly at $P \leq 0.05$

3.2 Effects of CCC and PBZ on chlorophyll contents of lily leaves

CCC and PBZ treatments both enhanced chlorophyll a and b contents and inhibited the reduction that normally occurred during vegetative growth period (6–10 WAP). A sharp decrease in chlorophyll contents still appeared in all plants after blossoming, but CCC and PBZ treatments proved to be efficient in promoting the chlorophyll b content at 14 WAP. The chlorophyll b contents of CCC-treated and PBZ-treated plants were 793.82% and 536.98% higher than that of control plants at 18 WAP, respectively (Table 1). Consequently, the total chlorophyll contents of treated plants were significantly higher than those of controls after blossoming (Table 1).

3.3 Effects of CCC and PBZ on endogenous hormones in lily bulbs

The GA content of the control bulbs decreased dramatically with age, reaching a minimum value at the start of blossoming period and remaining low after blossoming (Fig. 3a). Both CCC and PBZ evoked an even more prolonged and extreme decrease in GA content. GA content was 89.53% lower in CCC-treated bulbs and 90.6% lower in PBZ-treated bulbs compared to controls at 18 WAP (Fig. 3a). The ABA content of control plants was stable during vegetative growth period and initial blossoming period, but increased considerably after blossoming. CCC treatment increased the ABA content largely in bulbs, and it was 55.26% higher than that of control at 14 WAP. However, an appreciable reduction of ABA content was observed in PBZ-treated bulbs at all growth periods (Fig. 3b). With regard to the IAA content in lily bulbs, it was maintained at a low level in control plants through all growth periods. Both CCC and PBZ treatments increased the IAA content dramatically

Table 1 Effects of CCC and PBZ treatments on chlorophyll contents of lily leaves at different growth stages

Time (WAP)	Chlorophyll a content (mg/g FW)			Chlorophyll b content (mg/g FW)		
	Control group	CCC group	PBZ group	Control group	CCC group	PBZ group
6	0.71±0.05a	0.76±0.05a	0.72±0.02a	0.42±0.06a	0.40±0.08a	0.40±0.06a
10	0.48±0.03c	0.81±0.01a	0.72±0.02b	0.21±0.03c	0.47±0.05a	0.36±0.06b
14	0.75±0.04b	0.86±0.06a	0.88±0.01a	0.30±0.05c	0.53±0.08a	0.42±0.05b
18	0.23±0.06c	0.41±0.01a	0.35±0.01b	0.04±0.02c	0.32±0.05a	0.22±0.03b

FW: fresh weight. Values (mean±SD) followed by different letters differ significantly at $P \leq 0.05$

following the vegetative growth period. The IAA contents of CCC-treated and PBZ-treated plants reached their maximum values at 201.92 and 154.96 ng/g fresh weight (FW), respectively, at 10 WAP and then declined moderately (Fig. 3c). CCC treatment was superior to PBZ treatment for enhancing IAA content in lily bulbs.

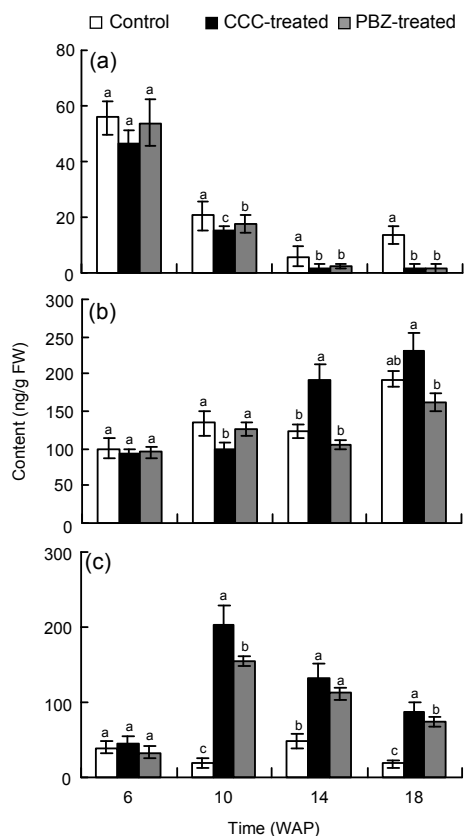


Fig. 3 Effects of CCC and PBZ treatments on the GA (a), ABA (b), and IAA (c) contents in lily bulbs at different growth stages

Values are presented as mean \pm SD of five samples in each group. Values not sharing a common superscript letter differ significantly at $P \leq 0.05$

3.4 Effects of CCC and PBZ on carbohydrate contents in leaves and bulbs

In control plants, the sucrose content of leaves declined during the initial blossoming period and then increased steadily during the full blossoming period. Both CCC and PBZ treatments promoted the sucrose content in leaves by a considerable coefficient during the full blossoming period (Fig. 4a). The starch content of leaves in all plants maintained a stable level throughout the whole growth period (Fig. 4b).

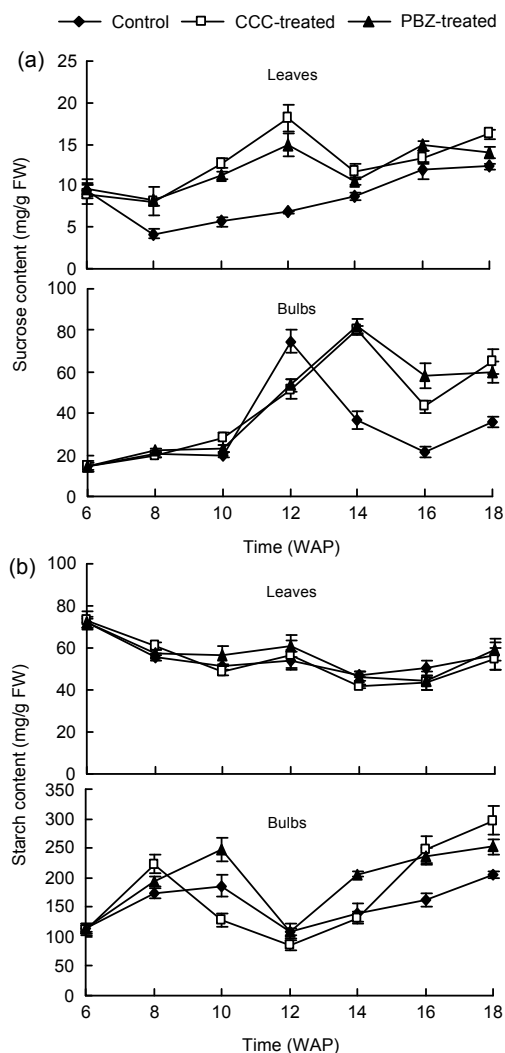


Fig. 4 Effects of CCC and PBZ treatments on the sucrose (a) and starch (b) contents in leaves and bulbs at different sampling stages

Values are presented as mean \pm SD of five samples in each group

The sucrose content in bulbs of control plants was stable during the vegetative growth period and then increased abruptly to reach its maximum value at 12 WAP. The value decreased to a low level after that and increased stably after blossoming (Fig. 4a). The starch contents in all plants increased with age during the vegetative growth period. However, a remarkable reduction in starch content was observed in all plants during 10–12 WAP, which coincided with the increase in sucrose content indicating that starch was hydrolyzed into sucrose during 10–12 WAP. However, the balance switched to sucrose-to-starch conversion as the starch content sustained a constant

increase beyond 12 WAP. The bulb starch contents reached their maximum values at 18 WAP and the starch contents of CCC-treated and PBZ-treated plants were 44.83% and 22.96% higher than that of control, respectively (Fig. 4b).

4 Discussion

Numerous reports demonstrated that the addition of plant growth retardants including CCC (Sladky and Bartosova, 1990), PBZ (Kozak, 2006), and uniconazole (Ziv, 1990) to an *in vitro* medium could enhance tuberization in various plants. In naturally grown lily plants, growth retardants were extensively used to improve floriculture characteristics, especially to control plant height (Bailey and Miller, 1989). However, little attention has been drawn to the effect of growth retardants on the partition of carbohydrate reserves in different parts of the lily plant, not to mention the internal factors of carbohydrate accumulation in bulbs. Our experiment firstly confirmed that plant growth retardants CCC and PBZ could stimulate the formation of new scales of lily bulbs and promote starch and sucrose contents not only in an *in vitro* environment but also in bulbs during and after the blossoming period. In addition, systematic investigation has been conducted to determine the changes of developmental growth, morphological parameters, photosynthetic capacity, and endogenous hormones, and their association with carbohydrate accumulation in bulbs.

When the leaves were sufficiently mature, the photoassimilates began to accumulate in leaves (Fig. 4a) and returned to bulbs to convert into starch (Miller, 1993). Thus, the delay of inflorescence and leaf senescence caused by CCC and PBZ treatments extended the photosynthetic period, thereby facilitating starch synthesis in bulbs. PBZ treatment was found to suppress plant height in many bulb flowers such as *Caladium*, *Freesia*, *Tulipa*, and *Zantedeschia* (de Hertogh and le Nard, 1993). Similar reduction in stem length, leaf number, and total shoot weight was also observed in our experiment and such reduction has been shown to favor partition of assimilates to sink tissues (Yeshitela *et al.*, 2004; Tekalign and Hammes, 2005). In contrast, CCC treatment increased the biomass of stems and leaves, possibly as a

response to the increased IAA contents (Saniewski *et al.*, 2005). This result is contradictory to previous reports showing the negative effect of CCC on the growth of stems and leaves of various species (Tezuka *et al.*, 1989; Hussain *et al.*, 2006). However, Kirillova *et al.* (2003) suggested that the effects of CCC could largely depend on the physiological state of the plants and the concentration of CCC applied. We are currently conducting tests to determine the specific effects of different CCC and PBZ concentrations on lily plants.

CCC and PBZ treatments increased the chlorophyll contents to a large extent which directly led to enhanced sucrose contents in leaves. For CCC-treated plants, the rise in ABA and cytokinin (CTK) in leaves might account for the increase of chlorophyll contents (Wang and Xiao, 2009). Furthermore, CCC was also shown to increase leaf thickness and ribulose biphosphate carboxylase (RuBisCO) activity, which might also enhance photosynthetic capacity (Tezuka *et al.*, 1989). As for PBZ-treated plants, a thicker epicuticular wax layer, elongated and thicker epidermal cells, thicker palisade, and spongy mesophyll tissue probably led to higher chlorophyll contents (Tsegaw *et al.*, 2005; Abdul Jaleel *et al.*, 2007b). The increase of chlorophyll contents and sucrose content in leaves together with delay of leaf senescence and reduction of stem length could improve transportation of photoassimilates into bulbs.

Plant growth retardants exerted a long-term influence on the bulb scale formation and carbohydrate accumulation probably due to its manipulation on exogenous hormones. GA contents in all lily plants decreased drastically at the start of blossoming period when a massive amount of starch was formed (Figs. 3a and 4b). This is consistent with a possible role of GA as an anti-bulbing hormone (Nojiri *et al.*, 1993; Vreugdenhil and Struik, 1989). In *Lilium* Oriental hybrid 'Casa Blanca', GA content in mother scales was much higher than that in daughter scales, which indirectly proves that low GA content is necessary for bulb formation and filling (Kim and Kim, 2005). Mares *et al.* (1981) observed that GA₃ could substantially reduce the activity of AGPase in the growing tubers of potato, so PBZ and CCC treatments might eliminate the obstacle by blocking GA synthesis thereby enhancing starch accumulation. Suppression of GA might also result in inhibited α -amylase

and β -amylase activities as observed in white yam tubers (Abdul Jaleel *et al.*, 2007a). However, an unequivocal role of GA may not be the complete story. Dragičević *et al.* (2008) showed that IAA could reinforce the tuber formation when low GA level was present in potato. In our experiment, the IAA content in control plants remained low throughout all the growth stages, which was consistent with the variation trend in *Gladiolus* (Qian and Yi, 2006). However, the IAA contents in treated plants were pronouncedly higher than that of the control following the blossoming period. Kim and Kim (2005) observed that increased IAA content in daughter scales during bulb filling would probably lead to more vigorous growth of daughter scales compared to mother scales. Thus, CCC and PBZ might improve carbohydrate accumulation by simultaneously decreasing GA and increasing IAA. Increased IAA in response to CCC and PBZ treatments presumably affected the influx of assimilates and sink capacity at the early stages of bulb scale formation by activating cell division and enlargement, and also by stimulating starch synthesis (Borzenkova *et al.*, 1998). ABA is another important endogenous hormone that is involved in bulb growth. Abdul Jaleel *et al.* (2007a) suggested that increased ABA in response to triazoles might stimulate the activity of an important sucrose-synthesizing enzyme sucrose phosphate synthase (SPS), thereby promoting sucrose content. In soybean (Quebedeaux *et al.*, 1976) and wheat (McWha, 1975), a high level of ABA in the immature reproductive organs appeared to be conducive for optimal carbohydrate accumulation. The promotion might be ascribed to the significant correlation between ABA and activities of sucrose synthase (SS), soluble starch synthase (SSS), and starch branching enzyme (SBE) (Yang *et al.*, 2004). Enhanced sucrose-to-starch synthesizing enzyme activities together with increased sucrose content could directly improve starch accumulation in the bulb during scale formation period. Furthermore, the inhibition of tuberization induced by GA was relieved by the addition of ABA in potato (Abdullah and Ahmad, 1980), demonstrating the positive effect of ABA on tuberization and carbohydrate accumulation in sink organs. Xu *et al.* (1998) also proposed that the stimulating effect of ABA might also be due to the antagonism between ABA and GA. In our experiment, CCC treatment evidently increased the ABA content

in lily bulbs especially during the blossoming period which might reinforce the decrease of GA. However, PBZ treatment slightly decreased the ABA content which may account for the lower carbohydrate accumulation in PBZ-treated bulbs relative to CCC-treated bulbs.

CCC treatment exerted an unexpected positive influence on starch accumulation especially after the blossoming period. This enhanced starch accumulation may be mediated by several mechanisms. On the one hand, CCC treatment can improve nutrient uptake from soil, promote water balance, and increase protein synthesis in growing organs (Grossmann, 1990). On the other hand, CCC treatment alters the expression or activity of several starch-related enzymes. For example, a higher SS activity along with higher sucrose content was detected in CCC-treated potato tubers during the active tuber filling stage. This enhanced SS activity could provide abundant uridine diphosphate (UDP)-glucose for its conversion to glucose-L-phosphate, which in turn could enter into the amyloplast, resulting in higher starch content (Sharma *et al.*, 1998b). Furthermore, the increased sink strength in treated bulbs could absorb more photoassimilates for starch conversion. Thus the sucrose and starch contents in CCC-treated bulbs were significantly higher than those of the control during scale formation period.

5 Conclusions

The treatments of growth retardants CCC and PBZ were applied to lily plants only once at the vegetative growth period. This single treatment, however, exerted a profound influence on subsequent development, morphology, chlorophyll contents, and phytohormonal balance, thereby manipulating the carbohydrate accumulation in lily bulbs. While both compounds caused a similar increase in bulb starch, we noted significantly different morphological responses in plants. CCC treatment obviously promoted the biomass of leaves and stems, whereas PBZ treatment depressed vegetative growth by reducing total shoot height and weight. The former facilitated abundant photoassimilate accumulation, whilst the latter probably favored the transport of more photoassimilates to sink organ. Both CCC and PBZ

treatments dramatically elevated the chlorophyll contents and sucrose contents which could be exemplified as promotion of photosynthetic capacity. Although decreased GA content and increased ABA content induced by CCC and PBZ treatments might improve carbohydrate accumulation in bulbs, conclusion regarding the role of endogenous hormone balance in scale formation and carbohydrate accumulation might be premature at this time due to our limited knowledge of biochemical functions of carbohydrate-related enzymes and their relationship with endogenous hormones in bulbs. Both CCC and PBZ treatments were proved to be effective in promoting carbohydrate accumulation in bulbs especially after blossoming.

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