BIOCHEMICAL AND PHYSIOLOGICAL RESPONSES OF LYCORIS SPRENGERI BULBLETS (AMARYLLIDACEAE) TO EXOGENOUSLY APPLIED N-(2-CHLORO-4-PYRIDYL)-N1-PHENYLUREA (CPPU)

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Abstract

Bulblets of Lycoris sprengeri (Amaryllidaceae) were obtained by cutting. Six concentrations of N-(2-chloro-4-pyridyl)-N1-phenylurea (CPPU) solutions were sprayed on leaves from one-year-old bulblets during their green period. Fresh weight, diameter, carbohydrate content, activity of starch metabolism-related enzymes and levels of endogenous hormones of bulblets were determined. The effects of CPPU treatment on bulblet development and biochemical and physiological indices of L. sprengeri were analyzed using the determined values. The results showed that CPPU treatment at an appropriate concentration promoted the enlargement of L. sprengeri bulblets; the optimal concentration was 7.5 mg L\(^{-1}\). Bulblet growth showed a significant positive correlation with starch content and the activities of soluble starch synthase (SSS) and starch-bound starch synthase (GBSS). Bulblet growth showed an extremely significant positive correlation with the ratio of endogenous gibberellic acid/abscisic acid (GA/ABA). The GA/ABA ratio showed a significant positive correlation with the activities of (α,β)-amylase and GBSS. The exogenous application of CPPU promoted the synthesis and accumulation of starch in the bulblets of L. sprengeri and the activities of starch metabolism-related enzymes; an increase in the endogenous GA/ABA ratio had a synergistic effect.

Key words: CPPU, Bulblet, Starch metabolism, Endogenous hormones.

Introduction

The genus Lycoris (Amaryllidaceae) consists of more than 20 species that are widely distributed in China, Japan, North Korea, Laos, and Burma (China Flora, 1985 – Chinese Academy of Sciences). Lycoris species have been termed “Magic lilies” in Western countries because they never show flowers and leaves simultaneously during their entire life cycle (Adams, 1976). L. sprengeri is unique to China and originated in the coastal areas of East China and Taiwan. L. sprengeri, which has a beautiful flower shape and an elegant floral color, is usually considered an ornamental plant (Xu, 1989). This plant is grown in flower beds, flower borders, and on the edges of forests. L. sprengeri is planted in combined pots, and its flowers are cut for ornamental purposes. L. sprengeri has been extensively applied in urban landscaping and greening (Ji, 2002). However, L. sprengeri has smaller bulbs than other Lycoris species (Zhang et al., 2002). Under natural conditions, L. sprengeri exhibits slow bulb growth and a low reproduction rate. Zhang et al. (2002) reported cutting propagation for L. sprengeri by cutting bulbs but obtained low propagation coefficients ranging from 2.37 to 3.48. The average diameter and fresh weight of two-year-old bulbs from L. sprengeri were significantly lower than those from L. aurea, L. radiata, and L. chinensis. Approximately 4.4% of two-year-old bulbs reached the qualification standards.

N-(2-chloro-4-pyridyl)-N1-phenylurea (CPPU) is a cytokinin-like substance with 10- to 100-fold higher levels of biological activity than 6-benzylaminopurine (Zhang & Chang, 2010). Significant increases in the number of lily cells and cell layers, as well as bulb growth and development, were observed by applying CPPU (Watanabe, 1989). In our previous studies, Xiao et al. (2013) demonstrated that 5 mg L\(^{-1}\) CPPU treatment on L. radiata bulblets accelerated nutrient metabolism, starch accumulation and bulblet enlargement. Work by She et al. (2014) demonstrated an optimal CPPU concentration of 1.0 mg L\(^{-1}\) CPPU for L. aurea. Accordingly, bulblets of L. sprengeri from artificial propagation were used as materials in the current study. The effects of CPPU treatment on the development and biochemical and physiological indices of L. sprengeri bulblets were quantified. We correlated bulb growth with the activities of starch metabolism-related enzymes and levels of endogenous hormones. Key factors affecting the enlargement of L. sprengeri bulblets were screened. The results of this study serve as an experimental basis for chemically induced methods to accelerate the growth of bulblets from the genus Lycoris.

Materials and Methods

The experiment was conducted in a bulb and perennial root germplasm resource garden at Zhejiang University from March 2013 to July 2013. The experimental materials included L. sprengeri bulblets from bulb cuttings in June 2012 (average fresh weight: 0.63 g; average diameter: 8.81 mm). Two weeks before the experiment, bulblets with two to three leaves, consistent growth status, and no pests or diseases were planted in pots, with five bulblets per pot. Beginning on March 27, 2013, five concentrations of CPPU were applied three times to the leaves of the entire plants (Table 1) once every week. Three replicates were established. The pots were sheltered from rain, and conventional management procedures were adopted. When all leaves had fallen (after 12 weeks of treatment), the underground bulblets were dug out of the ground to determine morphological, biochemical, and physiological indices. Sampling was performed once before treatment (March 27, 2013). The bulblets were sampled 12 weeks after leaf spray of different concentrations of CPPU, labeled C0 to C5. The bulblet samples were treated as follows: mud was washed off; aged brown scales were...
removed; pictures were taken; roots were cut; bulbs were wiped clean with water; fresh weight and diameter were measured; bulbs were cut into pieces with scissors and mixed evenly; 0.5 g of each sample was wrapped in tinfoil, frozen in liquid nitrogen for 30 min, and stored in an ultra-low temperature freezer at -75°C.

From the washed bulblets, a 1 cm² piece of the bulb was cut from the second layer of fresh scales, counting from outside to inside. The samples were fixed in formalin/acetic acid/alcohol, stained in periodic acid-Schiff (PAS) reagent and observed under a microscope for starch granule distribution (Hu & Xu, 1990). The sucrase, total soluble sugar, and starch content in bulblets were measured using the anthone colorimetric method (Zhang, 2001). The activities of amylases were determined using 3,5-dinitrosalicylic acid colorimetry (Li, 2000). The activity of starch synthase was measured using Fangmin’s method with some modifications (Zheng et al., 2012). The levels of endogenous hormones were determined using the ELISA method. The ELISA test kit was provided by the Center of Crop Chemical Control in China Agricultural University.

The data were statistically analyzed using Excel 2007 and SPSS 18.0. Duncan’s test was performed using one-way ANOVA. Bivariate correlation analysis was performed based on data from C0 to C5 before and after treatment.

**Results**

**Bulblet growth:** Twelve weeks after CPPU treatment at six different concentrations, the fresh weight of the bulblets in C4 treatment was significantly higher than that in C0; the bulblet diameter after C1 treatment was significantly larger than in C0. No other obvious changes were observed in other treatments in comparison with C0 (Table 2).

**Carbohydrate content in L. sprengeri bulblets:** The starch concentration in L. sprengeri bulblets was significantly higher (1.55 times) in C4 than in C0 12 weeks after CPPU treatment; other treatments showed no such difference from C0. C5 significantly decreased the total soluble sugar content in the bulblets, with no other significant changes in other CPPU treatments. Changes in sucrose content were similar to those in total soluble sugar content for all treatments compared to C0 (Table 3).

The microstructure of the bulb scales was observed after PAS staining, and uneven starch granule size distributions were observed in the scales. Most starch granules were small and round. In each cell of the bulb scale in the C0 treatment, the average number of starch granules was 5.6. Under 400x magnification, the average number was approximately 96.3. The average number of starch granules in each cell of the scales in C4 was 7.7. Under 400x magnification, the average number was 124.7(Fig. 1).

**Activities of starch metabolism related enzymes in L. sprengeri bulblets:** The β-amylase activities in bulblets in the CPPU treatments significantly increased compared to the control. The highest activity for β-amylase was found in C4, which was 7.25-fold higher than C0. The tendency of activity changes in (α+β)-amylase was consistent with that of β-amylase (Fig. 2).

After CPPU treatment for 12 weeks, the activities of AGPase and starch-bound starch synthase (GBSS) in L. sprengeri bulblets in C3 was significantly higher than in the control. The activities of SSS in L. sprengeri bulblets in C1 were significantly higher than in the control.However, AGPase activity slightly decreased in C5. No significant changes were found for other CPPU treatments (Fig. 3).

### Table 1. Concentrations of CPPU for treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg L⁻¹)</td>
<td>0</td>
<td>1.0</td>
<td>2.0</td>
<td>5.0</td>
<td>7.5</td>
<td>10.0</td>
</tr>
<tr>
<td>C represents CPPU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Bulblet diameter and fresh weight of L. sprengeri bulblets 12 weeks after CPPU treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diameter (mm)</th>
<th>Fresh weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>10.21abc</td>
<td>1.35abc</td>
</tr>
<tr>
<td>C1</td>
<td>11.45a</td>
<td>1.75ab</td>
</tr>
<tr>
<td>C2</td>
<td>9.91c</td>
<td>1.31c</td>
</tr>
<tr>
<td>C3</td>
<td>11.29ab</td>
<td>1.66abc</td>
</tr>
<tr>
<td>C4</td>
<td>11.18ab</td>
<td>1.77a</td>
</tr>
<tr>
<td>C5</td>
<td>10.49abc</td>
<td>1.44abc</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Different letters in each column indicate significant differences (p<0.05) according to Duncan’s Multiple Range Test.

### Table 3. Carbohydrate contents in bulblets of L. sprengeri12 weeks after CPPU treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sucreose content (mg g⁻¹ FW)</th>
<th>Content of total soluble sugar(mg g⁻¹ FW)</th>
<th>Starch content (mg g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>59.00 ± 5.8abc</td>
<td>83.239 ± 6.7abc</td>
<td>84.93 ± 4.6 abc</td>
</tr>
<tr>
<td>C1</td>
<td>68.34 ± 9.0a</td>
<td>82.847 ± 8.8abc</td>
<td>89.29 ± 13.7b</td>
</tr>
<tr>
<td>C2</td>
<td>64.34 ± 0.8abc</td>
<td>84.651 ± 4.6abc</td>
<td>100.27 ± 4.5b</td>
</tr>
<tr>
<td>C3</td>
<td>68.73 ± 2.1a</td>
<td>86.298 ± 3.4abc</td>
<td>104.82 ± 3.8b</td>
</tr>
<tr>
<td>C4</td>
<td>62.46 ± 2.9abc</td>
<td>91.082 ± 5.1a abc</td>
<td>131.33 ± 2.6b</td>
</tr>
<tr>
<td>C5</td>
<td>47.08 ± 8.0b</td>
<td>63.396 ± 3.7a abc</td>
<td>84.14 ± 7.9b</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Different letters in each column indicating significant differences (p<0.05) according to Duncan’s Multiple Range Test.
Endogenous hormone levels in *L. sprengeri* bulblets:

12 weeks after CPPU treatment, the zeatin riboside (ZR) levels in the bulblets of C1 to C5 were significantly higher than in the control, with the ZR level of C4 reaching 1.50-fold that of the control. The indole acetic acid (IAA) levels in the bulblets of C2, C3, and C5 significantly increased in comparison with the control; the IAA level of C5 was 1.39-fold higher than the control. Twelve weeks after CPPU treatment, the gibberellic acid (GA), abscisic acid (ABA), and jasmonic acid (JA) levels in the bulblets of each treatment significantly decreased in comparison with those of C0. The GA level of C2 was 0.62-fold that of C0. The ABA and JA levels of C3 were 0.48 and 0.64 times that of C0, respectively (Table 4).
Fig. 3. Starch-synthesizing enzyme activity in *L. sprengeri* bulblets 12 weeks after CPPU treatment. A, B, C: Starch-synthesizing enzyme activity in *L. sprengeri* bulblets. Each value represents mean ± SD. Lines with different letters indicate significant differences (p<0.05) according to Duncan’s Multiple Range Test.

Fig. 4. Ratios of endogenous hormones in *L. sprengeri* bulblets 12 weeks after CPPU treatments. Each value represents mean ± SD. Lines with different letters indicate significant differences (p ≤ 0.05) according to Duncan’s Multiple Range Test.

<p>| Table 4. Levels of endogenous hormones in the bulblet of <em>L. sprengeri</em> 12 weeks after CPPU treatment. |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>GA (mg g⁻¹ FW)</th>
<th>ABA (mg g⁻¹ FW)</th>
<th>IAA (mg g⁻¹ FW)</th>
<th>JA (mg g⁻¹ FW)</th>
<th>ZR (mg g⁻¹ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>4.53 ± 0.04ᵃ</td>
<td>75.49 ± 0.35ᵃ</td>
<td>40.53 ± 0.72ᵃ</td>
<td>47.75 ± 0.72ᵃ</td>
<td>4.58 ± 0.04ᵃ</td>
</tr>
<tr>
<td>C1</td>
<td>3.91 ± 0.01ᵇ</td>
<td>45.37 ± 0.47ᶜ</td>
<td>39.20 ± 0.14ᶜ</td>
<td>44.84 ± 0.55ᵇ</td>
<td>6.52 ± 0.03ᵇ</td>
</tr>
<tr>
<td>C2</td>
<td>2.83 ± 0.01ᶜ</td>
<td>44.17 ± 1.34ᵉ</td>
<td>53.04 ± 0.68ᵇ</td>
<td>34.59 ± 0.23ᶜ</td>
<td>6.69 ± 0.02ᵃᵇ</td>
</tr>
<tr>
<td>C3</td>
<td>3.76 ± 0.03ᵈ</td>
<td>36.41 ± 0.47ᵃ</td>
<td>44.67 ± 0.50ᶜ</td>
<td>30.33 ± 0.43ᵉ</td>
<td>6.58 ± 0.06ᵇ</td>
</tr>
<tr>
<td>C4</td>
<td>4.39 ± 0.03ᵇ</td>
<td>50.91 ± 0.68ᵇ</td>
<td>41.97 ± 0.55ᵈ</td>
<td>30.77 ± 0.29ᵃᵉ</td>
<td>6.87 ± 0.07ᵃ</td>
</tr>
<tr>
<td>C5</td>
<td>4.23 ± 0.02ᶜ</td>
<td>46.74 ± 0.25ᶜ</td>
<td>56.17 ± 0.56ᵃ</td>
<td>32.24 ± 0.70ᵈ</td>
<td>6.55 ± 0.09ᵇ</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Different letters in each column indicate significant differences (p ≤ 0.05) according to Duncan’s Multiple Range Test.
The ratios of ZR/ABA, IAA/ABA, and GA/ABA were calculated to reveal the variation trends for endogenous hormones. These ratios in C1 to C5 significantly increased in comparison with the control 12 weeks after CPPU treatment. All differences reached significance levels, except for the GA/ABA ratio in C2 (Fig. 4).

**Correlation analysis between bulblet growth of L. sprengeri and the physiological and biochemical indices:**

The results indicated that the bulb growth of *L. sprengeri* showed an extremely significant positive correlation with the endogenous GA/ABA ratio. A significantly positive correlation was also found between the contents of starch and the activities of soluble starch synthase (SSS) and GBSS. A significantly positive correlation was observed between the sucrose content in the bulblets and the content of total soluble sugar together with the activities of starch synthase (AGPase, SSS). The activities of α-amylase and (α+β)-amylase in the bulblets showed a significantly positive correlation with the ratios of ZR/ABA, IAA/ABA, and GA/ABA. The activity of GBSS exhibited a significantly positive correlation with the activity of (α+β)-amylase. The activity of GBSS showed an extremely significantly positive correlation with the activity of AGPase and SSS; the activity of SSS and AGPase also showed a significantly positive correlation (Table 5).

**Discussion**

**Effects on growth and carbohydrate content in bulblets:**

Applying CPPU at an appropriate concentration can facilitate the enlargement of *L. sprengeri* bulblets. The fresh weight and starch content in the bulblets of C4 were 1.31-fold and 1.55-fold higher than the control, respectively. The optimal concentration of CPPU was 7.5 mg L\(^{-1}\) in the current study. The microscopic observation of the bulb scales of C4 showed that most starch granules in the cell lumen were small and round 12 weeks after CPPU treatments, indicating high starch metabolism at this stage. In general, smaller starch granules in *Lycoris* species correspond to higher metabolic activities because newly synthesized starch granules are immediately degraded into soluble sugar to be used in plant growth (Wang, 2011).

Similar research has shown that the exogenous application of plant growth regulators affects the starch metabolism of underground bulblets. CPPU is currently used in various crops (Zhao, 2012), vegetables (Wang, 2001; Jin, 2010), and fruit trees (Fang & Wei, 2009; Hou et al., 2012) because it promotes cell division and elongation, bulb enlargement and yield. At the early stage of potato tuber formation, the exogenous application of cytokinins could increase the starch synthesis ability in plants by 25% to 50% (Borzenkova et al., 1998). The addition of IAA into MS increases starch content and starch granule size in potato in vitro (Gukasyan et al., 2005). The CCC treatment of potato promoted the transfer of photosynthetic products to the tubers of potato, thereby promoting tuber growth and increasing yield (Wang et al., 2009). In the current study, the starch content in *L.
CPPU treatment was similar to changes for 12 weeks after CPPU (Xiao et al., 2002). The CPPU treatment significantly increased compared with that of the control treatment, similar to that observed for hybrid rice treated with CPPU (Tang et al., 2002). The CPPU treatment significantly increased the activity of amylase (approximately 2.3- to 3.1-fold) compared with the control. Thus, starch metabolism in the bulblets was active. As an amylase enzyme, β-amylase exhibited the highest activity, indicating that β-amylase is the most important starch decomposition enzyme in L. sprengeri bulblets. This result is consistent with the results of CPPU application in L. radiata (Chang et al., 2013). This finding can also be confirmed from an enzymological perspective; β-amylase is the key enzyme in the bulb growth of Lycoris, as indicated by transcriptomics (Chang et al., 2013).

While studying the effects of CPPU on endogenous hormone levels in L. sprengeri bulblets, the most prominent results were an increase in ZR level and decrease in ABA level. ZR, an important component of cytokinin, promotes cell division. Wang et al. (2005) demonstrated for potato that the ZR level has a significantly positive correlation with tuber yield. At the early stage of carrot fleshy root enlargement (seedling age: 45 d to 75 d) (Yang et al., 2011) and the rhizome formation period of lotus (Xu, 2002), ZR promotes rapid cell division. The exogenous application of CPPU promoted bulblet growth by increasing the level of ZR, as reported for lily treated with salicylic acid (SA) (Fang & Wei, 2009).

Of all plant hormones, endogenous GA has the most definite inhibitory effect on tuber formation (Guo et al., 1991). GA delays the bulb formation of onions (Kato, 1965), inhibits the tuber formation of potatoes (Quan et al., 2001), and hinders the bulblet enlargement of Gradiolus grandavenus (Qian & Yi, 2006). Our results also demonstrated that the endogenous GA levels in the L. sprengeri bulbs in each treatment were lower than in the control after exogenous CPPU treatment at five concentrations (C1 to C5). This finding indicated that CPPU promoted bulblet development by decreasing GA levels, as had been found for other bulbs.

Endogenous ABA inhibits growth and accelerates aging. Many researchers have confirmed the ability of ABA to inhibit garlic bulb enlargement (Li & Zhou, 2002) and the lotus rhizome enlargement (Xu, 2002). In this study, ABA levels in the bulblets of L. sprengeri significantly decreased in C1 to C5, which is similar to our previous study of CPPU application in L. radiata (Xiao et al., 2013). Promoting L. sprengeri bulb growth by decreasing ABA levels is a biochemical pathway for exogenous CPPU application.

Effects on the biochemical indices of bulblets: The activities of starch synthase in L. sprengeri bulblets were increased after CPPU treatment. The activities of AGPase and GBSS after 5mg/L CPPU treatment significantly increased compared with that of the control treatment, similar to that observed for hybrid rice treated with CPPU (Tang et al., 2002). The CPPU treatment significantly increased the activity of amylase (approximately 2.3- to 3.1-fold) compared with the control. Thus, starch metabolism in the bulblets was active. As an amylase enzyme, β-amylase exhibited the highest activity, indicating that β-amylase is the most important starch decomposition enzyme in L. sprengeri bulblets. This result is consistent with the results of CPPU application in L. radiata (Chang et al., 2013). This finding can also be confirmed from an enzymological perspective; β-amylase is the key enzyme in the bulb growth of Lycoris, as indicated by transcriptomics (Chang et al., 2013).

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Correlation analysis of hormone balance and physiological and biochemical indices: The balance of endogenous hormones acts as a regulator for the occurrence and growth of bulblets. At the early stage of garlic bulb formation, the GA/ABA ratio decreases and the IAA/ABA ratio increases (Liu et al., 1997). Xia et al. (2005) suggested that the GA/ABA ratio affects the accumulation and distribution of carbohydrates in tulip bulbs. These researchers suggested that lower GA/ABA ratios contribute to the transport of carbohydrates. In the current study, the ratios of endogenous IAA/ABA, ZR/ABA, and GA/ABA in L. sprengeri bulblets were all higher than those of the control 12 weeks after CPPU treatment. This finding is consistent with the results for SA-treated lily (Fang & Wei, 2009). For L. sprengeri, the GA/ABA ratios in all CPPU treatments were higher than in the control, which was opposite of changes for tulip bulbs but similar to changes for L. radiata (Xiao et al., 2013). Further study is needed to determine whether this finding is caused by the difference in growth stages resulting from the different species and seedlings under investigation.

The indices for bulblet growth, carbohydrate content, activities of starch metabolism-related enzymes and the ratios of endogenous hormone levels were subjected to correlation analysis (Table 5). A significantly positive correlation was found between bulblet growth and starch content and between SSS and GBSS activities. This study is the first to correlate the activities of starch synthase and starch accumulation with the bulblet enlargement of Lycoris. A similar finding was reported for chestnut fruit growth (Xie et al., 2012).

Bulb growth showed an extremely significantly positive correlation with the endogenous GA/ABA ratio in L. sprengeri. This finding indicated that a higher GA/ABA ratio was beneficial to bulb growth. This result differs from that obtained for tulip bulbs (Xia et al., 2005) and lily (Zheng, 2011). In L. sprengeri bulblets, the GA/ABA ratio showed a significantly positive correlation with the activities of (α+β)-amylase and GBSS. Thus, the high GA/ABA ratio was related to the high starch metabolism level of L. sprengeri bulblets, which may be the mechanism for the promotion of L. sprengeri bulb growth.

The activity of β-amylase in the bulb showed an extremely significantly positive correlation with that of (α+β)-amylase. This result is similar to the findings of Chang et al. (2013). GBSS activity showed an extremely significantly positive correlation with the activities of AGPase and SSS, whereas the activities of SSS and AGPase were significantly positively correlated. This finding suggested that the three starch synthases were synergistic. In subsequent experiments, comparison of gene expression levels in L. sprengeri before and after CPPU will provide greater insight into the screening of key genes related to bulblet development.

Acknowledgements

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