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Research Article

Evaluation of some changes in biochemical parameters of Iranian lily (*Lilium ledebourii* var. Kelardasht Salehi1) bulbs during a growth and development cycle

Mehdi Salehi[1], Vahab Jafarian[2*], Kamal Gholamipourfard[3], Maryam Hosseinnia[2].

[1]Department of Horticultural Science, Faculty of Agricultural Science, University of Guilan, Rasht, Iran
[2]Department of Biology, Faculty of Sciences, University of Zanjan, Zanjan, Iran
[3]Department of Plant Production, College of Agriculture and Natural Resources of Darab, Shiraz University, Darab, Fars, Iran
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Abstract

Understanding the relationships between biochemical contents and morpho-phenological aspects of a plant during life cycle would be helpful for doing precise agricultural practices through its cultivation. In the present study, various biochemical parameters were evaluated in relation to bulb storage, shoot growth and florogenesis in Iranian lily (*Lilium ledebourii* var. Kelardasht Salehi1). Ten plants from each sampling were examined at intervals of 10 days throughout an annual life cycle. Results showed that starch hydrolysis commenced in the late stage of storage and reached the maximum level at flower initiation time. It was concomitant with the increase of amylase activity, indicating that the change of starch content in the bulbs was regulated by amylase. The accumulation of soluble carbohydrates in the bulbs during shoot growth and stem elongation period (from 50 days after planting to the next 50 days) did not result from starch hydrolysis. A great deal of starch accumulation occurred mainly just during ten days before shoot withering. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that the protein bands during bulb storage were different from those after planting. Moreover, it was revealed that a certain protein was expressed in bulb at anthesis stage. Our results recommend that the protection of aerial organs until complete withering seems to be necessary for higher energy reservation in the bulbs of *L. ledebourii*.

Keywords: Amylase, Bulb dormancy, Carbohydrate, Florogenesis, Iranian lily, SDS-PAGE

Introduction

Iranian lily (*Lilium ledebourii* var. Kelardasht Salehi1) is a popular, but an endangered ornamental plant growing at altitudes between 1750-2300 m in scanty parts of Hyrcanian forests, north of Iran. Most recently, the plant was molecularly studied, and its 5.8S rDNA sequence was registered in GenBank with accession number KX495217.1 (Salehi *et al*., 2019). The florogenesis process of the plant was anatomically depicted through SEM (scanning electron microscope) by our research group. We found that flower initiation taken place in the apical meristem of the plant about 10 to 20 days after bulb planting when the shoot reached approximately 5-10 cm in height (Salehi *et al*., 2018). Various ornamental geophytes differ not only in the environmental requirements for flowering, but also in the time of florogenesis process, therefore, among them, the changes of biochemicals content occur either at different times or in a different manner. The annual cycle of Iranian lily is as follows: bulbs sprout in spring, mid of April, flowers oppear usually two to three months later, from June to July, and shoot withering takes place at August. The natural habitat of the plant has severe winter and moderate temperatures during spring and summer. Iranian lily has not yet been domesticated despite its valuable and beauty appearance (Mirmasoumi *et al*., 2013; Salehi *et al*., 2018).

In the bulbous plants, no visible external growth occurs during quiescence period. In fact, it is well known that the processes of organogenesis in many geophytes occur in underground buds during the quiescence period (Kamenetsky and Rabinowitch, 2002). Previous studies have shown the changes in many biochemicals and hormones inside the quiescent organ. In lilies, the majority of reserved carbohydrates in bulbs belongs to starch (Shin *et al*., 2002; Legnani *et al*., 2010). It has been proved that, in both deciduous and perennial species, starch as an energy source contributes during budding, flowering, pollination, and fruit setting (Klein *et al*., 2016; Tixier *et al*., 2017). In addition, starch hydrolysis leads to the production of

*Corresponding Author, Email: v.jafarian@znu.ac.ir

soluble carbohydrates that cause the survival of perennial structures (Fernandez *et al*., 2018). As the principle source of carbon storage and cellular energy, the amount of starch is significantly related to the growth and development of the whole plant in lily (Marinangeli and Curvetto, 1997; Zhang *et al*., 2007), narcissus (Chen, 1986), and potato (Wang *et al*., 2008). It has been widely proved that amylase hydrolyzes starch (Sun and Henson, 1991). Specifically, in the plants that flowering occurs before leaf emergence, starch hydrolysis is considered as a main strategy for providing energy source (Kuhn, 2006). Different proteins including enzymes have been revealed to express in different amounts during various stages of a plant life cycle (Mousavi *et al*., 1999; Chung *et al*., 2006).

To the best of our knowledge, no biochemical study has been accomplished on the bulbs of *L. ledebourii* during annual growth and development cycle. Thus, the present study was performed to reveal the relationships between the biochemical contents and the morpho-phenological aspects of Iranian lily during annual life cycle. This knowledge would be helpful for doing accurately agricultural practices during cultivation of the plant.

Materials and methods

Plant material: The bulbs of *L. ledebourii* grown naturally in the district of Kelardasht, in Mazandaran province (N: 36° 32’14.3’’; E: 51° 3’27.53’’, altitude 2270 m), were collected in late Aguest 2017, after shoot withering. They were transferred to Biology lab of the University of Zanjan, and placed in a well-drained medium containing coco peat: perlite in a ratio 1:1. Subsequently, for vernalization and breaking dormancy, the bulbs were subjected at 3^{o}C for 2 months in storage. During cold storage, the bulbs media were always kept humid. Afterwards, they were separately planted in pots (35 cm in height and 25 cm in diameter) in a greenhouse under 20/17^{o}C day/night temperatures where they received weekly full Hoagland nutritive solution. Bulb samples were taken every 10 days started at the beginning of cold storage and ended after complete shoot withering. The bulbs were washed with water and frozen at -70 °C for biochemical analyses.

Biochemical analyses: For SDS-PAGE protein electrophoresis, bulb samples were ground with a pestle and mortar in the presence of liquid nitrogen. Then, 0.5 g of well powdered fresh bulbs and 500 µL extraction buffer (10 mM phosphate buffer (pH 7.0) containing glycerol 10% and 3 mM β-mercaptoethanol) were thoroughly mixed by vortexing, and then centrifuged at 9000 ×g for 5 min at room temperature. After centrifugation, the supernatants in each tube were transferred to another tube, and stored at -20 °C for gel electrophoresis. Then, the extracted soluble proteins were fractionated through one-dimensional SDS-PAGE by 5% and 10% stacking and running gels based on the method of Laemmli (1970). After reaching the tracking dye to the bottom, the gels were overnight stained in 0.25% Coomassie brilliant blue-G250, followed by destaining for 45 min in acetic acid and methanol.

The extraction of enzyme was accomplished according to Koussa *et al*. (2005). Generally, 0.5 g of tissue was crushed into 1 mL of ice-cold phosphate buffer (50 mM, pH 7.0) containing 10% glycerol and β-mercaptoethanol (3 mM). The extracts were centrifuged for 15 min, at 4 °C and 9000 ×g. Then, the supernatants were used for determining both total protein and enzyme activity. Total protein was determined according to Bradford (1976). For enzyme assay, the reaction mixture contained 20 µL of enzyme extract, 50 µL of a 0.1% starch solution and 30 µL of 50mM phosphate buffer (pH 7.0). The enzymatic reaction was conducted for 15 min at 37 °C. The degradation of starch was stopped after 30 min by adding 100 µL of DNS (Dinitrosalicylic acid) reagent. The absorbance was done at 540 nm by a spectrophotometer (Shimadzu, Japan). The activity of amylase was calculated based on a standard calibration curve (0 to 200 mgmL^{-1}) of maltose.

Total soluble carbohydrate (TSC) was determined by the colorimetric test according to Dubois *et al*. (1956) with some modifications. Briefly, TSC were extracted from 0.5 g of bulb sample through extracting once with 8 mL of 80% methanol at 90 °C, and then it was extracted again with 8 mL double distilled water at 65 °C. The extractions were applied for determining the TSC quantified at 620 nm absorption. The measurement of starch concentration was done using the perchloric acid method described by Robin *et al*. (1991). The pellet remaining after carbohydrate extraction was again extracted three times with 5 mL of 35% (v/v) perchloric acid accompanied with shaking slowly for 15 min. The extracts were centrifuged at 9000 ×g for 5 min. Then, the supernatants were collected in tubes and added to 20 mL distilled water. Afterwards, 5 mL of anthrone reagent (0.175%, w/v, in 75% cold sulfuric acid) was added to 1 mL of the extract. The mixture was boiled for 12 min and then placed on ice. The absorbance was read at 620 nm. We used standard calibration curve (0 to 200 mgmL^{-1}) of maltose.

Statistical analysis: All biochemical results were reported as the average of three replicates ± SE. Data were exposed to analysis of variance (ANOVA) by statistical software (SAS, version 6.12). Duncan’s Multiple Range Test was used for means separation with a level of significance $P < 0.05$.

Results and discussion

The anatomical stages occurred in the apical meristem were depicted through SEM and its results were published (Salehi *et al*., 2018). Different biochemicals measured consecutively from the beginning of bulb storage to the shoot withering had different changes in various growth and development stages. Table 1 shows the results of analysis of variance regarding to the changes in the measured traits at different growth and

Table 1. Analysis of Variance (ANOVA) of the measured biochemical traits of Iranian lily (*Lilium ledebourii* var. Kelardasht Salehi1) bulbs at different growth and development stages.

Source of variation	Df	Total protein (mg/mL)	Starch (mg/g F.W.)	Amylase activity (Unit/mg protein F.W.)	Total soluble carbohydrates (mg/g F.W.)
Treatment (growth stages)	21	890.987**	9258.501**	0.0087**	820.2**
Error	44	1.861	3.149	0.0063	1.148
CV (%)		1.544	1.705	14.3853	1.606

NS: Non-significant, *Significant at 0.5%, **Significant at 0.1%

development stages. This Table indicates that there are significant differences at 0.1% level between treatments (growth stages) for the measured biochemical parameters.

As Fig. 1 shows, the amount of total protein changed significantly ($P \leq 0.01$) at the final 10-days of cold storage period. So that, it significantly increased (up to 140 mg/mL) during rooting and flower initiation stages. Afterwards, it decreased until flower senescence. The amount of total protein again increased (up to 100 mg/mL) in the bulbs of Iranian lily from flower senescence to shoot withering stage.

The banding patterns of polypeptide in SDS-PAGE of total protein extracted from bulbs of the plant showed differences in the protein expression during various stages (Fig. 2). According to the SDS-PAGE, certain polypeptide bands were highly observed at the final of storing period. In addition, an intensive polypeptide band occurred during rooting and flower initiation period which was different from the bands of storing period. Moreover, the polypeptide band was a few thicker at flowering stage than the other stages.

As Fig. 3 shows, at storing period, the bulbs contained considerable amounts of starch (about 180 mg/g F.W.). However, it gradually decreased at final 10-days of storing period and suddenly reduced by about 70 and 50 mg/g F.W. at rooting and flower initiation stages, respectively. The highest starch accumulation (about 200 mg/g F.W.) occurred in the bulbs of *L. ledebourii* at shoot withering period.

At the same time, the activity of amylase increased gradually at final stages of storing period and reached the maximum of 7.7 (Unit/mg protein F.W.) at rooting stage (Fig. 4). The minimum activity of amylase in the bulbs was observed from flower senescence to the end of shoot withering.

Contrary to starch, the content of TSC was negligible at storing period. While, it dramatically increased (up to 100 mg/g F.W.) at rooting stage. The content of TSC in the bulbs of *L. ledebourii* decreased significantly ($P \leq 0.01$) from flower senescence to the end of shoot withering and reached about 50 mg/g F.W. (Fig. 5).

Among various stages, total proteins were highest at rooting and flower initiation stages. A characteristic polypeptide has been characterized by SDS-PAGE in *Hyacinthus orientalis* L. cv. Anna Marie which assumed to be a main storage protein in the bulblets (Chung *et al*., 2006). The effects of temperature treatment in "bulbous plants" during storage on defining the subsequent growth and flowering were due to the change of carbohydrate metabolism, bulb hormones content, enzyme activities, etc. (Haaland, 1974). Charles-Edwards and Rees (1975), have reported that in tulip, the amount of soluble carbohydrates in the mother bulbs was in parallel to the length of low temperature to which the bulbs have been exposed and this amount determines the shoot growth. In *Nerine fothergillii*, the amounts of carbohydrates have been evaluated; however, there is an indication of various carbohydrate concentrations resulted from different storage temperatures. In *L. longiflorum*, the carbohydrate analysis showed that the translocation of soluble sugars from scales to the basal plate occurred at rooting stage. It has been found that, in cereal grain, the secretion of hydrolysis enzymes was stimulated by gibberellin after exposure to low temperatures (Salisbury and Ross, 1992). It seems that one of the main factors needed for starch hydrolysis in the bulbs of *L. ledebourii* is cold storage. Moreover, the carbohydrate metabolising enzyme in tulips has been supposed to be activated after low temperature via hormonal control (Moe and Wickstrom, 1973). In plants, carbohydrate reserves refer to seasonal fluctuations; during the growing season, they accumulate late and when the plant sprouts in spring, they are utilized (Yang and Hori, 1980). In some trees in winter, some part of the stored starch is hydrolyzed into soluble carbohydrates via starch degrading enzymes (Sperling *et al*., 2017). While, in *L. ledebourii* no significant starch hydrolyzing occurred during quiescent period. From the results and discussion, it could be inferred that in *L. ledebourii*, the storage treatments influence the emergence and flower initiation by conversing the starch content into soluble carbohydrates. During the early stages of bulb storage, no considerable changes happened in amylase activity, starch and soluble carbohydrates. However, as storage period went on, the biochemicals entered into some changes. It seems that cold storage is necessary for breaking down the starch content. In another words, cold treatment might induce stimulators and reduce the inhibitors. It has been reported that cold treatment leads to starch hydrolysis concomitant with sucrose accumulation (Mohamed *et al*., 2010). Based on our results, changes in starch content in the bulbs seem to be regulated by amylase. So that, as amylase activity

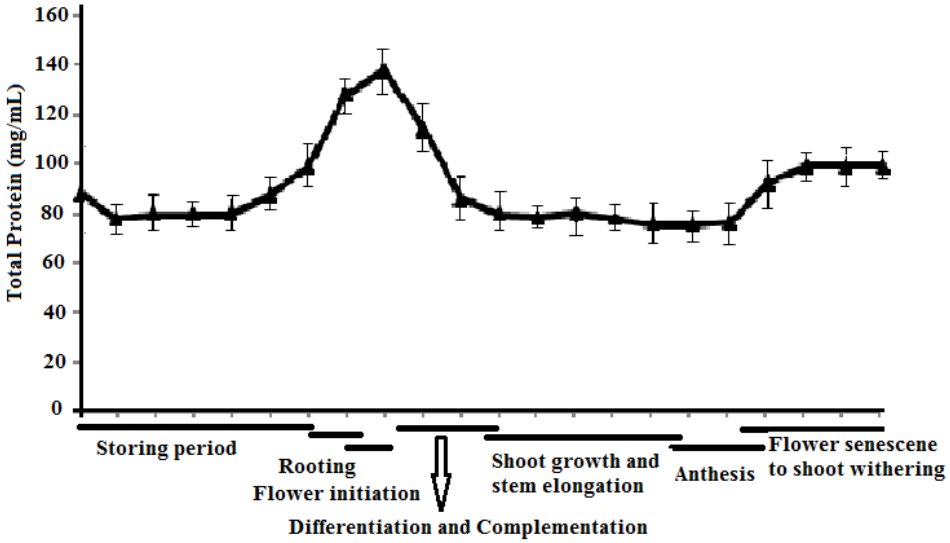

Fig. 1. The alterations of total protein in the bulbs of of Iranian lily (*L. ledebourii* var. Kelardasht Salehi1) during annual life cycle. The distance between bars on X axis represents 10 days. Values are means of 3 replicates ± SD.

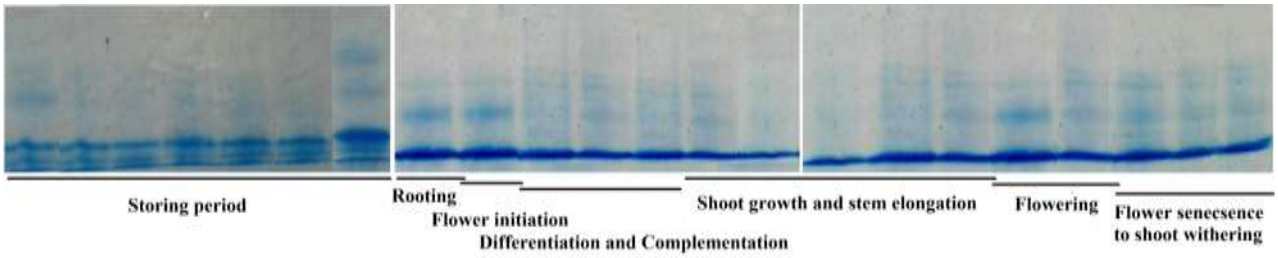

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the protein content in the bulbs of Iranian lily (*L. ledebourii* var. Kelardasht Salehi1) during deifferent life cycles.

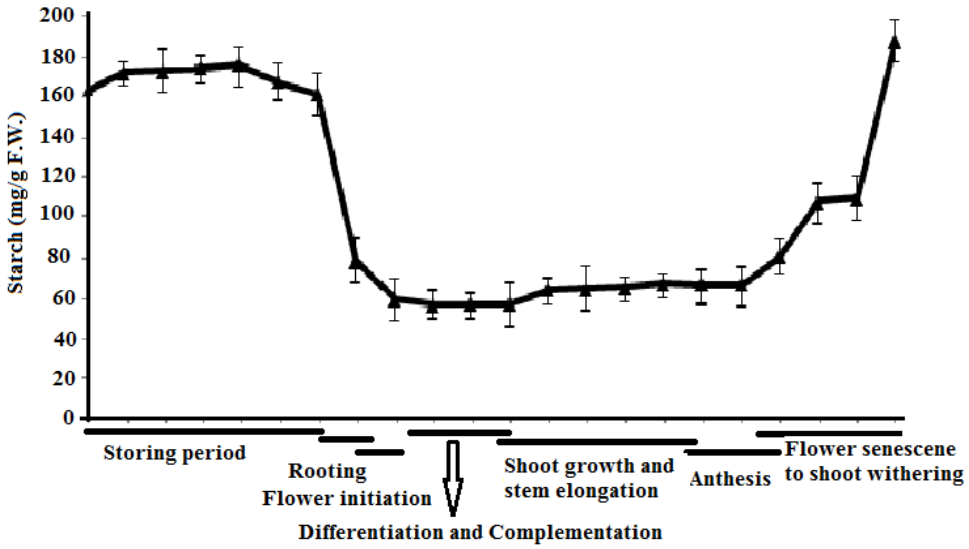

Fig. 3. The alterations of starch content in the bulbs of of Iranian lily (*L. ledebourii* var. Kelardasht Salehi1) during annual life cycle. The distance between bars on X axis represents 10 days. Values are means of 3 replicates ± SD.

increased in the bulbs, the starch content decreased and vice versa. Generally, it has been shown that amylase activity, as the main enzyme in starch hydrolysis, is directly and negatively related to the concentrations of starch in the bulbs of *L. ledebourii*. In *Lilium* Oriental hybrid Sorbenne, it has been reported that the contents of starch in the scales cells were significantly declined from shoot emergence to anthesis. After flowering, the number of starch granules increased dramatically in the bulbs indicating that the bulbs converse into a main sink

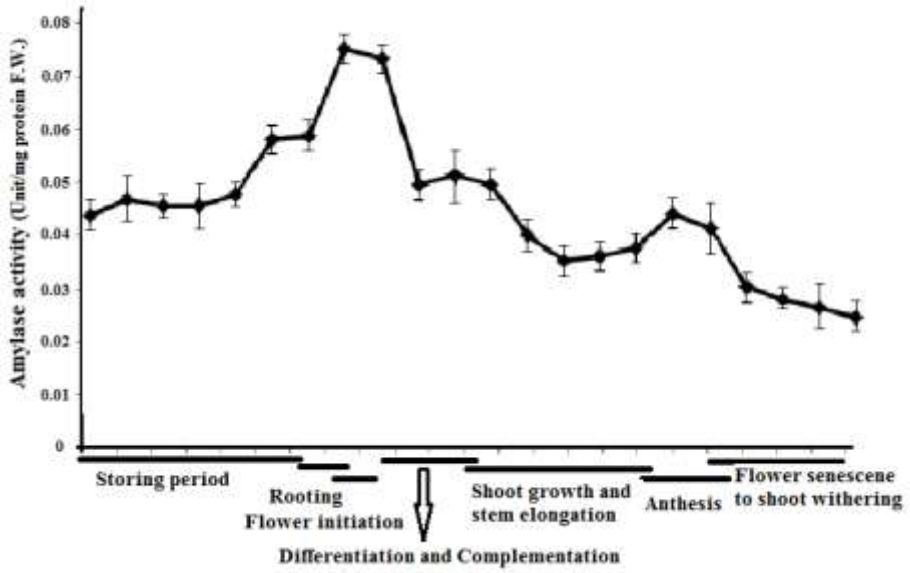

Fig. 4. The alterations of amylase activity in the bulbs of of Iranian lily (*L. ledebourii* var. Kelardasht Salehi1) during annual life cycle. The distance between bars on X axis represents 10 days. Values are means of 3 replicates ± SD.

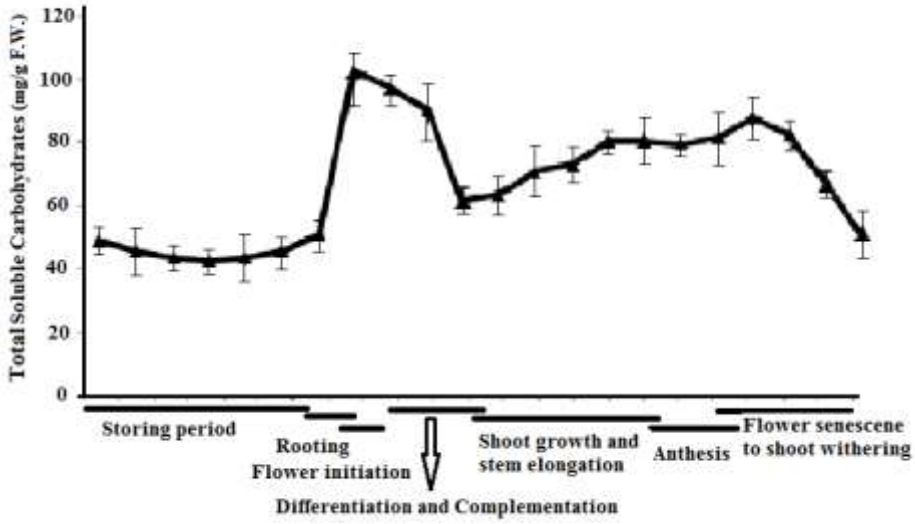

Fig. 5. The alterations of total soluble carbohydrates (TSC) content in the bulbs of of Iranian lily (*L. ledebourii* var. Kelardasht Salehi1) during annual life cycle. The distance between bars on X axis represents 10 days. Values are means of 3 replicates ± SD.

during bulb enlargement. The amylase activity in *Lilium* Oriental hybrid Sorbenne was also reported to be in similar to starch trend. From planting the bulbs to 20 days later, the soluble carbohydrates content significantly increased. According to our anatomical results, the period was simultaneous with rooting of the bulbs and flower initiation (Salehi *et al.*, 2018). Since there is no photosynthetic organ (leaf) in the early stage of planting, the bulbs require the initial carbohydrate energy to induce root and shoot emergence. Apart from the origin of carbohydrates (whether they are provided by leaf photosynthesis of the same season or whether they result from the stored organ from the previous season) they are translocated to the organs of the plant that are not able to provide their nutrition requirement by themselves (Berman and Dejong, 2003). To achieve their development potential, these organs need carbohydrates (Erel *et al*., 2016). At flower initiation time, 10 to 20 days after planting, a little shoot emerges from the plant bulbs. However, it is thought that the new leaves are not able to provide the whole energy needed for phase transition (from vegetative to generative phase). Shin *et al*. (2002) have demonstrated that during early shoot emergence and growth followed by bulb

vernalization, the plant uses stored carbohydrate reserves in bulbs, and the starch content reduces and converses into sucrose, as a major carbohydrate in lily, that is translocated to the aerial organs. However, the accumulation of soluble carbohydrates in the bulbs of *L. ledebourii* during shoot growth and stem elongation period (from 50 days after planting to the next 50 days) do not seem to result from starch hydrolysis, since neither starch decreased at that time, nor amylase had considerable activity during the period. Meanwhile, it has been reported that 98% hydrolysis of lily starch is due to amylase activity (Wu and Liu, 1994).

Conclusion

In conclusion, the bulbs of *L. ledebourii* were assumed to be the main carbohydrate source which provide the primary energy not only for root and shoot emergence, but also for flower initiation and differentiation. SDS-PAGE exhibited that the protein bands during bulb storage were different from those after planting. The accumulation of soluble carbohydrates in the bulbs during shoot growth and stem elongation period was not a consequence of starch hydrolysis. The main part of starch accumulation occurred in bulbs just during some days before shoot withering. The present study recommends that the lily growers should protect aerial organs of the plant until complete withering if they want a higher energy reservation in the bulbs. These information put forth the importance of further biochemical researches on this unique plant during various life cycles. Such studies reveal the importance of the rare geophyte and will persuade scientists to domesticate the plant for ornamental purposes.

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