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Physiological Changes During Cold Storage of *Lilium ledebourii* (Baker) Boiss. Bulb, a Rare Indigenous Species to Iran

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Abstract

Lilium ledebourii is a native and endangered rare species in Iran. It requires a cold period for proper growth and flowering. This experiment was performed to investigate the effects of cold storage on changes of endogenous abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellic acid (GA_3), sucrose, fructose, glucose, and total saponin during dormancy of *Lilium ledebourii* bulbs. The bulbs of lily were stored at 1 or 7 ºC, for 0, 30, 60, 90, and 120 days, and after each period removed from storage and analyzed using HPLC system. The results indicated that, cold storage at 1 ºC for 120 days decreased the levels of ABA while, 7 ºC in the same duration increased its content, slightly. The levels of IAA increased at 1 ºC storage, while remained almost constant at 7 ºC. Cold treatment at 1 ºC increased the levels of GA_3, while storage at 7 ºC caused slight decrease in its content. Sucrose and fructose concentrations increased in both temperatures during the storage, while minor reduction in glucose content was observed. Storage at 7 ºC for 120 days induced the highest content of total saponin. The magnitude of changes in hormones and soluble sugars measured at 1 ºC was greater than that at 7 ºC.

Keywords: Cold period, Hormone, Lily, Saponin, Soluble sugars.

INTRODUCTION

Lilium ledebourii (Baker) Boiss. (Liliaceae), with the local name of Susan -e- chelcheragh is an endangered scarce species endemic to Iran which is distributed on the heights of Damash and Amarlou in Guilan province, and Khanqah in Ardabil province. This species is highly resistant to cold, and begins to sprout even when the land is covered with snow (Padasht Dehkaei, 2004).

A literature search showed that, no comprehensive investigation has so far been carried out on the physiology of this species. However, numerous reports were found on the physiology of other species of *Lilium* genus (Farsam *et al*., 2003).

Most lily bulbs overcome adverse environmental conditions by the development of dormancy. In the bulbs that develop dormancy in winter, dormancy is released by low temperatures (Langens-Gerrits *et al*., 2003). However, breaking dormancy occurs by complex procedures that cannot transmitted from one species to another. In many species, determination of the chilling requirements is important factor in their cultivation, especially in commercialization of the newly known geophytes (Dole, 2003). Low temperature treatment imposes changes on metabolic activities in *Lilium* bulbs (Roh, 1996). It is suggested that, levels of growth promoters or growth inhibitors within the bulbs rise or fall during the cold storage (Sharma *et al*., 2016).

Due to the different reactions of the species to low temperatures, there are often contradictions about the increasing or decreasing of the endogenous growth regulators levels. For example, Liu *et al*. (2014) reported decreasing in the levels of endogenous abscisic acid (ABA) during vernalization in *Lilium* Oriental hybrid 'Sorbonne' while, the results reported by An *et al*. (2010) suggested increasing in the ABA content in *Lilium* 'Siberia' at low temperature. Also, Park and Lee (1992) reported an increase in the content of endogenous indole-3-acetic acid (IAA) in garlic while, Okubo *et al.* (1988) stated that, the level of endogenous IAA decreased in Easter lily bulbs at cold period.

In regard to gibberellic acid (GA_3), some researchers stated that, gibberellins had no role in the induction or release of dormancy (Rebers *et al.*, 1996). However, Takayama *et al*. (1993) reported that, the level of gibberellin A_{24} (GA_{24}), a precursor of gibberellin A_4 (GA_4), increased during low temperature and GA_4 is a key factor in dormancy break of *Lilium elegans*. Endogenous plant growth regulators affect different aspects of growth and development. ABA and GA alleviate biotic and abiotic stress (Wang *et al*., 2018), and auxin is the main factor in the temperature-dependent growth of geophytes therefore, it is important to study their changes during the chilling period (Khodorova and Boitel-Conti, 2013).

Another important physiological changes happening during low-temperature conditions in bulbs are alterations in carbohydrates (Nikolic *et al*., 2008). During chilling, carbohydrate reserves are mobilized and used as energy source for the organism metabolism (Langens-Gerrits *et al*., 2003). It is reported that, endogenous levels of sucrose in bulbs are linked to dormancy development (Aguettaz *et al*., 1990). Furthermore, phytochemical experiments of *Lilium ledebourii* by Farsam *et al*. (2003) indicated high quantity of saponins in bulbs and lower concentration in flowers. Ncube *et al*. (2011) reported that, the amount of total saponin in bulbous plant samples was higher in winter compared to the other seasons. Saponins apply for pharmaceutical, nutritional and industrial uses (Szakiel *et al*., 2011).

Therefore, in order to further understand of chilling requirement of this species, this study was established to determine the endogenous levels of ABA, IAA, GA_3, soluble sugars, and total saponin in *Lilium ledebourii* bulbs stored at cold temperature.

MATERIALS AND METHODS

Plant materials

Lilium ledebourii bulbs were collected from Kojoor, located in Nowshahr in the North of Iran, in late September 2018 and immediately sent to the laboratory of horticultural sciences in

Zanjan University.

Bulbs (20 to 24 cm of circumference) were packed into polyethylene bags containing moist coco peat. Bags were placed either at 1 or 7 for 0, 30, 60, 90, and 120 days. At the end of each step of storage time, a number of bulbs were selected, cleaned and frozen at -80 °C to posterior analysis.

Plant hormones extraction, purification, and quantification

ABA, IAA, and GA_3 extraction of samples was performed as described by Kim and Kim (2005). Briefly, 50 g plant sample were extracted at 4 for 16 h with 100 ml of 80% methanol. The extract was centrifuged at 4 at 15000 rpm for 30 minutes. Methanol was evaporated in vacuum, and then 3 g of polyvinylpolypyrrolidone (PVPP) was added and adjusted to pH 2.7 with 1 N HCl. After addition of 50 ml 100% ethyl acetate, the aqueous phase was discarded and the ethyl acetate phase evaporated and passed through a C18 cartridge. The cartridge was washed with 2 ml 20% methanol. The solute was passed through a 0.45 µm filter. The separation was carried out by an isocratic HPLC (Unicam-Crystal -200 UK) system with a diode array detector. ABA was purified using a C18 column (Diamonsic, 250 mm × 4.6 mm, 5 µm) (Li *et al*., 2010). A C18 column (HiQ SiL, 250 mm × 4.6 mm, 5 µm) was used to separation of IAA (Hou *et al*., 2008). GA_3 purification was carried out using a C18 column (Zorbax SB, 150 mm × 2.1 mm, 3.5 µm) (Ma *et al*., 2008). Quantification was obtained by comparing peak areas with those of the external standards.

Soluble sugars and total saponin extraction, purification, and quantification

Sugars extraction of samples was referred to the method of Shin *et al*. (2002). Sugars were separated on a Eurokat H-10 µm column (300 mm × 8 mm) and a refractive index detector. The mobile phase was distilled water at a flow rate of 0.7 ml/min. Sugars were identified and quantified by their retention time in comparison with external standards.

Extraction of total saponin was carried out according to Wu *et al.* (2010) with minor modification. Briefly, dried bulbs were grounded and defatted in petroleum ether (60-90 °C) by soxhlet extraction method. The samples were extracted with 50% methanol. Total saponin concentration was determined with UV-756 spectrophotometer (Shimadzu-UV-160A-Japan) at 408 nm.

Statistical analysis

Factorial experiment based on completely randomized design with three replicates was used in the experiment. Data were subjected to analysis of variance (ANOVA) using MSTAT-C statistical software. Means were compared by the Duncan's multiple range test at $P<0.05$ probability level.

RESULTS AND DISCUSSION

Endogenous hormones changes

The results of analysis of variance showed that temperature, storage duration and their interaction had a significant impact on concentrations of endogenous growth regulators (Table 1). According to fig. 1A and table 2, the levels of endogenous ABA in bulbs decreased at 1 °C, while at 7 its levels were higher at 90 and 120 days.

Decreasing in the levels of ABA during the low temperature has been reported in a study by Zhang and Jia (2014) in lily bulblets. In lily bulbs, dormancy is probably associated to the high content of ABA and poor respiratory activity. Thus, the low temperatures are required to reduce ABA levels and resume growth (Kilsdonk, 2002). The correlation between the decrease of endogenous ABA and dormancy break has also been reported in tulip bulbs (Podwyszynska, 2012). As shown in results, significant reduction in levels of ABA in the end of cold storage at 1 °C indicated the ending of dormancy in *Lilium ledebourii* bulbs.

Table 1. The variance analysis of the effect of temperature and storage duration on concentrations of ABA, IAA, GA_3, sucrose, fructose, glucose and total saponin in *Lilium ledebourii* bulbs.

S.o.V	df	MS						
		ABA	IAA	GA_3	Sucrose	Fructose	Glucose	Total saponin
Temperature (T)	1	1749.56**	5677.376**	547.841**	4141.875**	254.625**	19.683*	640.322**
Storage duration (S)	4	3848.941**	1932.399**	238.495**	1343.182**	86.199**	33.255**	120.234**
T × S	4	3851.057**	1816.583**	189.678**	588.48**	17.951*	8.186*	205.714**
Error	20	77.036	10.006	6.024	16.328	5.583	4.33	19.236
CV (%)		8.92	9.92	8.49	7.64	9.22	13.93	10.6

*and **: Significant at P<0.05 and P<0.01, respectively.

Table 2. Effects of temperature and storage duration on concentrations of endogenous abscisic acid (ABA), indole - 3- acetic acid (IAA), gibberellic acid (GA_3), sucrose, fructose, glucose, and total saponin in *Lilium ledebourii* bulbs.

Treatment		ABA (µM g^{-1} FW)	IAA(µM g^{-1} FW)	GA_3(µM g^{-1} FW)	Sucrose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)	Glucose (mg g^{-1} FW)	Total saponin (mg $10g^{-1}$ FW)
Temperature (°C)	1	119.12 b	45.65 a	33.2 a	64.63 a	28.55 a	15.75 a	36.75 b
	7	134.39 a	18.14 b	24.65 b	41.1 b	22.72 b	14.13b	46 a
Storage duration (day)	0	121.33 b	25.33 c	33.73 a	33.13 c	20.6 c	13.4b	33.4 b
	30	157.63 a	14.1 d	27.92 b	46.02 b	25.2 bc	18.2 a	44 a
	60	142.15 a	21.7 c	22.45 c	66.6 a	31.1 a	13.4 b	43.58 a
	90	122.34 b	38.58 b	23.6 bc	69a	24.7 bc	16.75ab	43.13 a
	120	90.35 c	59.8 a	36.95 a	49.67 b	26.65 ab	13 b	42.83 a

*In each column, means with the similar letter(s) are not significantly different (P < 0.05) using the Duncan's multiple range test.

According to fig. 1A, an ascending trend in ABA levels was observed in the 30th day of 1 °C storage and 90th day of 7 °C, and afterward the levels of ABA decreased. These results are consistent with those in Wang *et al*. (2013) for *Lilium* OT hybrid 'Manissa'. They reported that ABA level increased during early storage period, and then decreased in the fifth week. They reported that, increase in the levels of ABA could be a form of adaptation to stress conditions. ABA can induce the production of some proteins involved in adaptation to cold stress (Taiz and Zeiger, 2012).

Considering the significant effects of temperature and storage duration on IAA concentration, the results demonstrated that, the levels of endogenous IAA in bulbs increased sharply at 1 °C storage in 90 and 120th day, while at 7 °C increase was slight (Fig. 1B and Table 2).

Increase in the levels of endogenous IAA during the cold storage has been reported by Tsukamoto (1971) in Easter lily. Such changes indicate the preparation of scales for production of pedicle and flowering (Hartmann *et al.*, 1990), and could be related to the end of the dormancy period (Mirzakhani, 2010).

In the present study, the maximum level of IAA was verified in the treatment of 1 for 120 days, while bulbs maintained at 7 presented an almost constant level of IAA during storage (Fig. 1B). In contrast, Sun *et al*. (2005) reported that in *Lilium davidii* bulbs, the higher temperatures (6

and 10) resulted in more increase in IAA levels compared to the lower temperature (2). These contrasting reports could be due to that various genotypes have different cold responses (Dole, 2003).

As shown in fig. 1B, at 1, IAA content had a trend of decrease in the 30th day of storage, and then increased until the end of the experiment. At 7, the reduction trend was evaluated until the 90th day of cold period, and afterward increased slightly. This result is in accordance with Gu *et al*. (2020) in *Lilium* Oriental hybrid 'Sorbonne', that reported IAA content decreased in the middle of cold period, then increased afterward. This reduction in IAA level could be due to the cold stress that decreases the activity of auxin transporters in a mechanism that is unknown (Shibasaki *et al*., 2009). After adaptation to the stress conditions, activity and movement of water and transporters increase in order to supply the required compounds to growing meristems (Khodorova and Boitel-Conti, 2013).

Fig. 1 (A and B) showed that the changes of IAA content were contrary to ABA content. This result has been reported by Okubo *et al*. (1988) for *Lilium longiflorum* and Chen *et al*. (2007) for onion. Increase in the levels of one hormone and decrease in another establish a hormonal balance which, in turn, controls the transition from physiological state of dormant to active in dormant buds (Taiz and Zeiger, 2012).

Storing bulbs at 1 increased the levels of GA_3, whereas 7 decreased it (Fig. 1C). Increase in the levels of endogenous gibberellins under the low temperature is reported by Aung and Hertogh

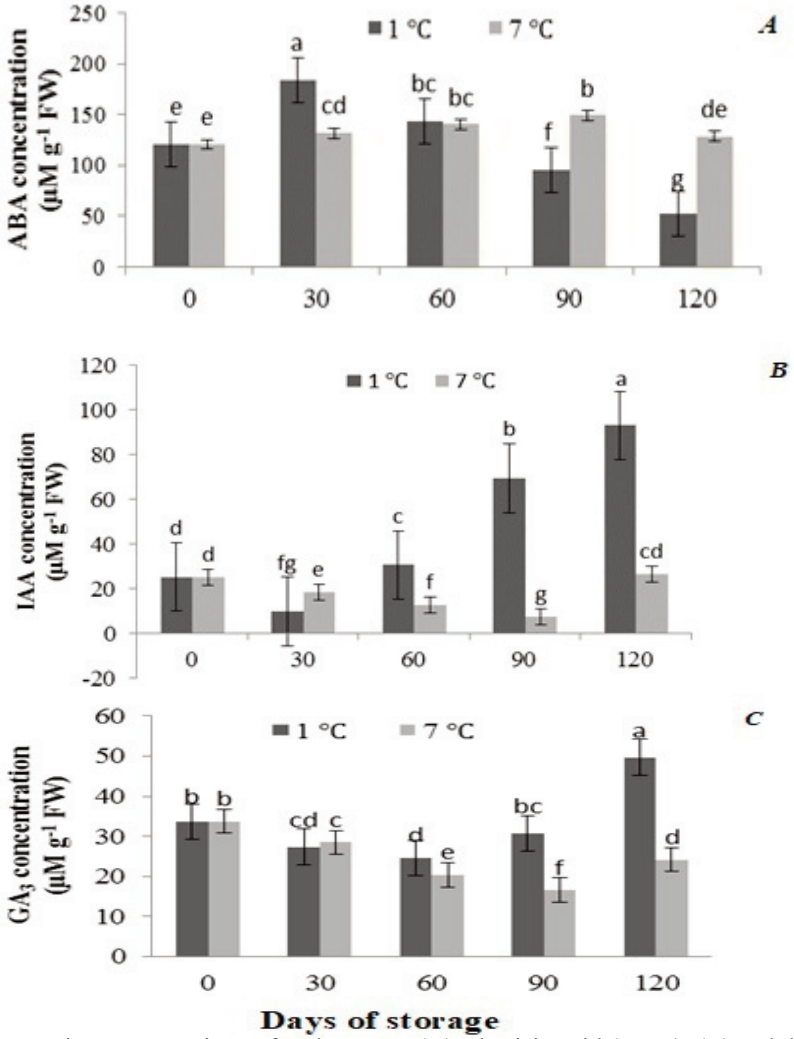

Fig. 1. Changes in concentrations of endogenous (A) Abscisic acid (ABA), (B) Indole -3- acetic acid (IAA) and, (C) Gibberellic acid (GA_3) in *Lilium ledebourii* bulbs at 1 or 7 during 0, 30, 60, 90, and 120 days.

(1967) in tulip bulbs.

According to fig. 1 (A and C), the obvious inverse relationship between GA_3 and ABA is observed. This relation has been described by previous researchers.

At low temperature, when ABA content declines and GA_3 content rises, the ratio of GA_3 to ABA increases that induces the production of essential RNA for sink-source transport of amino acids and sugars (Mornya and Cheng, 2013). This relationship could explain the increase in the sugars contents at 90 and 120 days of cold storage at 1 °C (Fig. 2).

Compared to IAA and ABA, the levels of GA_3 showed fewer changes during cold storage, especially at 7 °C (Fig. 1). It is due to the different nature of endogenous GA_3 in geophytes during the dormancy and cold storage (Kamenetsky and Okubo, 2013). Nie *et al*. (2016) also suggested that, during chilling treatments or winter time in *Asparagus officinalis*, levels of GA_3 remained constant.

As shown in fig. 1 (B and C), an almost parallel relation in the trends of GA_3 and IAA is observed. It is suggested that, auxin is the only phytohormone which is triggered by cold treatments in bulbs and the levels of auxin regulate genes which encode gibberellin biosynthesis enzymes. In fact, auxin progresses gibberellin biosynthesis (Khodorova and Boitel-Conti, 2013).

Soluble sugars and total saponin changes

The results of the present study manifested that temperature, storage duration and their interaction were significant on sucrose, fructose, and glucose contents (Table 1). The increase in sucrose content of bulbs stored in both temperatures was observed but this increase was abrupt to bulbs maintained at 1 °C (Table 2 and Fig. 2A). Our result is in agreement with Xu *et al*. (2006). They reported that, treatment of *Lilium rubellum* bulbs with low temperature resulted in an increase in soluble sugars contents. When bulbs are exposed to low temperatures, starch is broken down and sucrose accumulated by starch hydrolyzing enzymes (Shin *et al*., 2002). It is suggested that, increase in the endogenous IAA during cold temperature induces growth of meristem. Induction of growth, in turn, triggers increase in respiration, water distribution and starch hydrolysis (Khodorova and Boitel-Conti, 2013).

Storing bulbs at 1 for 120 days induced a 1.7 fold increase in sucrose concentration, whereas storage at 7 for the same duration resulted in a slight increase (Fig. 2A). Similarly, Miller and Langhans (1990) noted that, the change extent of carbohydrate in Easter lily bulbs stored at -1 was bigger than that of the bulbs stored at 4.5. They suggested that, hydrolytic activity is higher at lower temperatures.

It is reported that, soluble sugars start to rise at the beginning of cold temperatures, reach the highest level in full cold conditions, and decrease when are consumed in respiratory process (Mornya and Cheng, 2013). This changing pattern was clearly observed in the changes of sucrose levels at both temperatures in our study and sucrose reached its maximum concentration in the 90^{th} day of storage at 1 °C. According to results (Fig. 2), sucrose is the predominant carbohydrate at the end of dormant period in *Lilium ledebourii* bulbs. It is also likely a transportable sugar within the bulbs and triggers the metabolic activity in the lily bulbs under the cold conditions (Shin *et al*., 2002).

Fig. 2B showed that storing bulbs at 1 for 120 days caused a 1.4 fold increase in fructose concentration and at 7 for the same duration increased slightly. Increase in the level of fructose during cold storage is reported by Zhang *et al*. (2011). It is reported that, the sucrose invertase activity increases during cold treatments, resulting in starch degradation and subsequently reducing sugars (such as fructose and glucose) accumulate (Zhang *et al*., 2011). The accumulation of sucrose and reducing sugars in bulb scales is related to dormancy release and could influence the sprouting and growth of the plant (Xu *et al*., 2006).

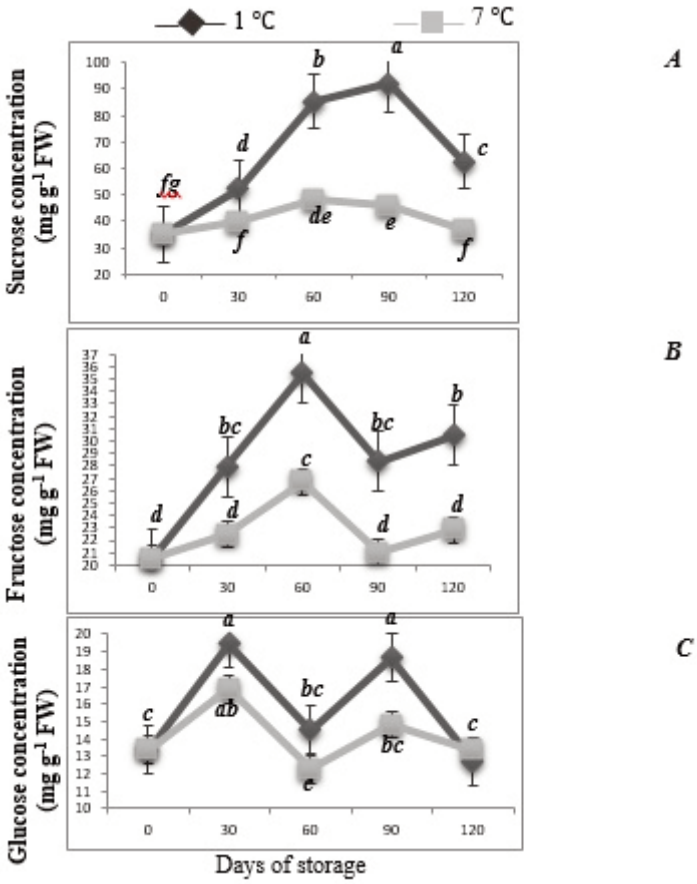

Fig. 2. Changes in concentrations of (A) sucrose, (B) fructose and, (C) glucose in *Lilium ledebourii* bulbs at 1 or 7 during 0, 30, 60, 90, and 120 days.

Our results demonstrated that at 1, glucose concentration decreased at the end of the experiment. The magnitude of changes in glucose and fructose content was much less than for sucrose (Fig. 2). This finding is consistent with those in Shin *et al*. (2002) for lily bulblets regenerated *in vitro*. Similar results by Miller and Langhans (1990) showed that, hexoses are used for synthesis of sucrose (dominant soluble carbohydrate in bulbs), and, therefore a large content of hexoses could not be expected.

Compared between two storage temperatures, the extent of changes in soluble sugars was more at 1than that at 7 (Fig. 2). In this regards, Shin *et al*. (2002) reported that, lower temperatures (4 °C) induced more activity of α- and β- amylase than higher temperatures (10 and 25 °C).

Total saponin level was significantly ($P<0.01$) influenced by the interaction of temperature and storage duration (Table 1). According to fig. 3, storing the bulbs at 7 for 120 days induced an increase in total saponin concentration, while 1 decreased its content at the same duration. The accumulation of total saponin can be correlated with antimicrobial activities (Wu *et al*., 2010; Ncube *et al*., 2011). It has been reported that the best antimicrobial effects were evaluated in winter and autumn seasons in bulbs of *Tulbaghia violacea* (Ncube *et al*., 2011). However, increased or decreased temperatures have been shown to affect the accumulation of saponin in other plants. These contradictions could be due to the nature of saponin biosynthesis, which is complex and linked to many abiotic and biotic factors (Szakiel *et al*., 2011).

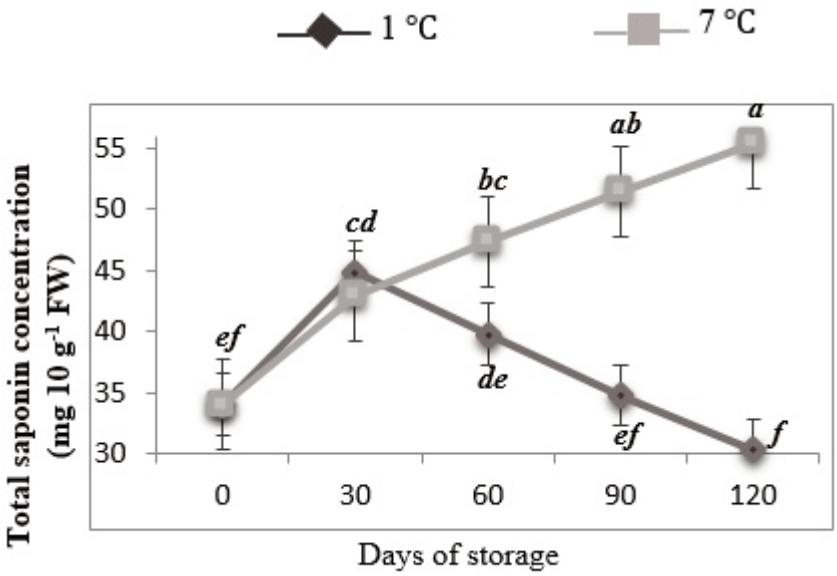

Fig. 3. Changes in total saponin concentrations in *Lilium ledebourii* bulbs at 1 or 7 during 0, 30, 60, 90, and, 120 days.

CONCLUSION

Our findings showed that, decrease in the ABA content and increase in the levels of IAA, GA_3, sucrose, and fructose at the end of chilling period indicate the ending of dormancy and preparation of lily bulbs for germination. The lowest level of ABA and the highest content of IAA and GA_3 were identified at 1 for 120 days. Furthermore, the maximum sucrose content, which is the substantial factor for releasing dormancy, was observed at 1 for 90 days. Therefore, considering the results obtained, storage at 1 for 90-120 days could be critical for dormancy release in *Lilium ledebourii* bulbs. However, there are several known and unknown factors affecting dormancy break in bulbs.

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