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Factors Affecting Bulblet Growth of *Lilium* sp. - Tracking Ontogenic Development and Bulb Production *in vitro*

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Abstract

Poor growth of bulblet and ontogenic development are the major problems of adventitious bulb production of lilies *in vitro* through explant culture. Ontogenic development from the juvenile phase to the adult vegetative phase strongly correlates with bulblet growth of lily, and it is important to understand how lily bulblets grow *in vitro*. This study was at aimed to determining an effective *in vitro* culture process for lily to track bulblet growth, ontogenic development, and bulb production. In the results, bulblet performance was higher with the increase in the amount of medium; however, bulblet size was not satisfactory and was in the juvenile phase under an inducing condition. The threshold weight at which 100% ontogenic development was about 300 mg. Avoiding depletion of sucrose in the media is the key to achieving desired bulblet growth and ontogenic development *in vitro* and indicates growth could be sustained by subculture. The circumference of bulblets increases with its weight after subcultured *in vitro*. Therefore, a threshold circumference of about 3.2 cm in proportion to bulblet fresh weight of 300 - 350 mg may be an indicator of ontogenic development.

Introduction

The genus *Lilium* ranks fourth in the world for the production of cut flowers (Grassotti and Gimelli 2010). The *in vitro* propagation of lily bulb production through scale explant culture is the high-volume vegetative propagation method (Bahr and Compton 2004, George et al. 2008) as an alternative to conventional methods for producing lilies (Skorić et al. 2012). Regardless of its advantages, slow growth and smaller bulb size are the major problems of adventitious bulb production of lilies *in vitro* (Islam et al. 2017). In tissue

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culture of bulbous crops, bulblet size is crucial (De Klerk et al. 1992, Langens-Gerrits and De Klerk 1999). The size of regenerated bulblets depends on two factors: (a) the size of the explant; the larger the explant, the larger the bulblets that are regenerated (Langens-Gerrits et al. 2003a, Islam et al. 2017), and (b) the role of starch converted from sucrose in the medium (Varshney et al. 2000, Kumar et al. 2005, Cheesman et al. 2010, Islam et al. 2017).

On the other hand, there are three ontogenic phases: juvenile, adult vegetative, and flowering phase, during the development of lily bulbs (Rees 2012, Langens-Gerrits et al. 2003b). Ontogenic development from the juvenile to adult vegetative phase strongly correlates with bulblet growth (particularly size > 300 mg) of lily (Langens-Gerrits et al. 2003b, 1996b). Recent studies reported that bulb size is an essential factor for floral transition also (Lazare and Zaccai 2016). However, it is not known as to the supply of nutrients *in vitro* is adequate or not? *In vitro* subculture is a possible alternative to growing *in vivo*? Hence, this study was at aimed determining an effective *in vitro* culture process to track the bulblet growth, ontogenic development, and bulb production of lily.

Materials and Methods

Clean and healthy bulbscales of lily cultivars 'Stargazer' and 'Casablanca' were rinsed in 70% ethanol, sterilized in 1% (w/v) NaOCl for 30 min, and rinsed three times in sterile de-ionized water. Bulblets were regenerated from scale explants (Fig. 1) cultured with the adaxial side on medium T_1 (10 ml), T_2 (15 ml), T_3 (20 ml), T_4 (30 ml), and T_5 (50 ml) per explant including 4.4 g/l MS medium with vitamins, 6%, 60 g/l sucrose, NAA 50 µM/l and 7 g/l micro-agar for 14 weeks at 25°C under a 16/8 hrs dark/light condition in the tissue culture growth chamber, laboratory of floriculture and vegetable sciences at Kochi University, Japan from April, 2017 to October, 2018.

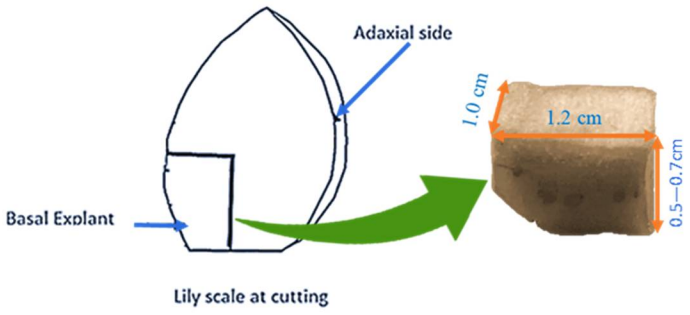

Fig. 1. Schematic drawing of explants cut from lily scales.

In the first subculture, 90 juvenile bulblets of two lily cultivars, 'Stargazer' and 'Casablanca' (45 from each cultivar), were harvested from the previous explant culture *in vitro*. Thirty Stargazer bulblets were subcultured in MS supplemented with 3% sucrose, and 30 Casablanca bulblets were subcultured in MS supplemented with 6% sucrose for

16 weeks at 25°C under a 16/8 hrs dark/light condition. At the same time, 15 juvenile bulblets (mixed of both cultivars) were subcultured at 25°C for 12 weeks and subsequently 4 weeks at 15°C under a 16/8 hrs dark/light condition in MS supplemented with 6% sucrose (16 weeks *in vitro*) to track their growth and ontogenic development. The remaining 15 bulblets (mixed of both cultivars) were placed under an inducing condition *ex vitro* (in soil) for 16 weeks under a 16/8 hrs dark/light condition to track their growth and ontogenic development.

In the second subculture, 30 bulblets harvested from the first subculture (15 from each cultivar and fresh weight ranging from 147.2 to 368.1 mg) were again cultured at 25°C for 12 weeks and subsequently 4 weeks at 15°C in MS supplemented with 6% sucrose for a total of 16 weeks under a 16/8 hrs dark/light condition *in vitro* to assess their growth and ontogenic development. Then *in vitro* regenerated bulblets were kept at 5°C for 10 weeks on moist filter paper in Petri dishes to break dormancy (Langens-Gerrits and De Klerk, 1999) and then planted in pots (9 cm in diameter and 15 cm in depth) containing fertilized soil with 380, 290 and 340 mg/l of N : P : K (Tanekura No. 42, Sumirin Agricultural Industry Co., Ltd., Japan). The pots were then placed in a greenhouse with optimum environmental conditions (20°C, 16 hrs light). After 16 weeks, the bulblets were uprooted and data were collected.

Apical meristems were fixed in 5% glutaraldehyde in phosphate buffer (pH 6.8) and rinsed in the same buffer. They were then dehydrated in an ethanol series and embedded in Spurr's resin. Longitudinal sections, made on a rotary microtome (Yamato Kohki Industry Co., Ltd., Saitama, Japan), were mounted on glass slides, stained with toluidine blue, and examined under a light microscope (Olympus BX51TRF; Olympus Optical Co. Ltd., Japan). Sucrose depletion over time in the medium during lily bulblet regeneration *in vitro* was measured using a pocket refractometer (PAL-J; Atago Co. Ltd., Tokyo, Japan), with the result appearing as Brix%. Degrees Brix (°Bx) is the sugar content of an aqueous solution. The sugar content of 1°Bx is 1 g of sucrose in 100 g of solution and represents the strength of the solution as a percentage by mass.

The initial weight of the bulblets was measured before subculture *in vitro* and planting in soil. The growth rate during the study was calculated as follows:

(Final weight-Initial weight)/Initial weight = Growth rate (x)

And then multiplied by 100 = Percentage growth rate

Bulblets circumference (cm) was measured using the formula:

$\text{Circumference} = \pi \times \text{diameter} = 2 \times \pi \times \text{radius}$.

The results are expressed as a mean ± standard error (SE). For all comparisons, each value represents an average of 30 replicates, and statistical analysis was performed using one-way ANOVA.

Results and Discussion

This study was at aimed tracking bulblet growth, ontogenic development, and bulb production to determine an effective *in vitro* culture process of lily. Ontogenic development from the juvenile phase to the adult vegetative phase of lily strongly correlates with the bulblet size (Langens-Gerrits et al. 2003b), and so, most studies suggested culturing to lily scale explants in a medium containing high sucrose concentration during regeneration of bulblets (Islam et al. 2017, De Klerk 2009, Kumar et al. 2005, Langens-Gerrits et al. 2003a). These studies raised the question 'why not increase the amount of medium and thus the total sucrose content' to increase bulblet size and therefore advance ontogenic development. Present authors hypothesize that applying a higher amount of medium rather than higher sucrose eliminates the risk of increasing medium osmolarity, called phytotoxicity (Staikidou et al. 2005), and would be more beneficial for bulblet growth because carbon supply is more important (Podwyszynska 2012). Accordingly, authors looked at the bulblet performance of lilies using different amounts of medium with 6% sucrose concentration: T_1, 10 ml of the medium; T_2, 15 ml; T_3, 20 ml; T_4, 30 ml; and T_5, 50 ml.

As a result, performance of explants was higher with the increase in the amount of medium (Table 1) because of the rise in the amount of sucrose *in vitro*. 'Stargazer' and 'Casablanca' both lily cultivars performed in the same way in case of regeneration percentages (Table 1). Leaf and fresh root weight increased with the increase in the amount of medium, and so, the balance between roots and shoots *in vitro* influences bulblet production (Table 1). Bulblet fresh weight and number also increased with the increase in the amount of medium (Table 1). Besides, all bulblets were juvenile and developed rosette leaves under the inducing condition *ex vitro* (Fig. 6, data not shown).

Table 1. Performance of lilies cultured *in vitro* with differing amounts of medium.

Lily cultivar	Amount of medium (ml)	Regeneration %	Leaf FW (mg)	Fresh root wt. (mg)	Root: Shoot	Mean bulblet FW (mg)	No. of bulblets (avg.)
Stargazer	T_1	86	144.9 ± 7.9	274.2 ± 9.2	1.89	110.6 ± 4.1	2.1
	T_2	88	205.2 ± 11.3	404.2 ± 15.1	1.97	109.8 ± 6.5	2.3
	T_3	81	464.4 ± 17.1	432.9 ± 19.2	0.93	122.4 ± 5.2	3.2
	T_4	86	531.4 ± 24.1	487.1 ± 20.1	0.92	128.7 ± 5.7	3.0
	T_5	80	403.8 ± 16.2	448.2 ± 13.5	1.10	133.2 ± 7.0	3.0
Casablanca	T_1	84	188.6 ± 8.4	312.3 ± 7.5	1.66	104.7 ± 5.0	2.0
	T_2	81	233.1 ± 15.3	367.2 ± 19.1	1.58	103.5 ± 4.7	2.5
	T_3	80	450.9 ± 22.1	440.1 ± 19.4	0.98	127.2 ± 6.6	3.0
	T_4	86	487.4 ± 21.2	530.1 ± 20.7	1.09	130.5 ± 6.7	3.1
	T_5	83	483.5 ± 12.6	510.2 ± 21.4	1.05	137.1 ± 6.4	3.2

(T_1, 10 ml of medium; T_2, 15 ml; T_3, 20 ml; T_4, 30 ml; T_5, 50 ml. FW, fresh weight; avg., average).

According to Rodrigues-Falcon (2006), sucrose influences cell division at the stolon swelling, so the most important factor is storage organ formation. Apart from the positive interaction, bulblet size (fresh weight) was not satisfactory and was below 150 mg (Table 1).

Histological observation of a longitudinal section of the apical meristem revealed that these juvenile bulblets showed only leaf primordia attached to the basal plate, and no tunica-corpus structure in the juvenile apical meristem (Fig. 5a) indicated the absence of ontogenic development. According to Takayama et al. (1983), lily bulblets regenerating *in vitro* on scale segments may undergo the vegetative phase change as they may form stems after planting in soil. Still then, all the bulblets were juvenile under an inducing condition. When bulblets weigh less than 300 mg, they sprout with a few rosette-type leaves (juvenile) instead of leaves with a stem (adult vegetative) (Islam et al. 2017, Langens-Gerrits et al. 2003b), so it was evident that there were no adult vegetative bulbs. Histological observation of a longitudinal section revealed that these juvenile bulblets had only leaf primordia attached to the basal plate, and no tunica-corpus structure in the juvenile apical meristem (Fig. 5a) indicated the absence of ontogenic development. The transition from juvenile to adult vegetative *in vitro* is characterized by the development of the tunica corpus structure consist of a distinct epidermal (L_1) and sub-epidermal (L_2) layers called the 'tunica' and an inner layer (L_3) called the corpus (Fig. 5b) with increased mitotic activity in the apical meristem (Langens-Gerrits et al. 2003b, 1996a,b, Rees 2012).

All bulblets survived and grew well during their first subculture, in particular, their fresh weight. The growth rate of the Casablanca bulblets were 2x (200%), while that of the Stargazer bulblets was 1.5x (150%) (Fig. 2a, b) due to the difference in sucrose concentration in the medium. At the same time, bulblets grown under low temperature *in vitro* performed well with a growth rate of 2.15x (215% of their initial weight; Fig. 2c). Bulblets grown under an inducing condition *ex vitro* (in soil) had a growth rate of 1.9x (190% of their initial weight; Fig. 2d).

After the first subculture *in vitro*, the bulblets were planted in pots and placed in a greenhouse with optimum environmental conditions for 16 weeks. The threshold weight for the bulblet to undergo phase change was investigated, and the lowest bulblet weight to form stems (adult vegetative phase) was 250 - 300 mg bulblets (Fig. 3a). Because of the individual factors in all cases, phase change was prominent in bulblets >300 mg (Fig. 3c-e). There was an exception to the lack of Stargazer bulblets >300 mg due to low sucrose concentration (3%) in the medium (Fig. 3b), resulting in poor ontogenic development. The threshold weight for phase change (juvenile to adult vegetative) was about 300 mg, at which 100% ontogenic development occurred (Fig. 3c-e), which is in line with overall bulblet performances (Fig. 3a).

Subculture of the *in vitro*-produced lily bulblets was done to sustain and track their growth, and ontogenic development as the bulblet growth and development are greatly affected by the amount of sucrose in the medium (Islam et al. 2017, Niimi et al. 2000 and 1997). Bulblet growth rate was satisfactory after the subculture but varied because of

differences in sucrose concentration, temperature, and growth condition (*in vitro* as well *ex vitro*) (Fig. 2a-d). It seems clear that there was nutrient depletion in the lily bulblet regeneration *in vitro* and that bulblet growth was able to be sustained and maintained by subculture in the same way Niimi et al. (1997) found growth reduction of *Lilium rubellum* bulblets by depletion of sugars in the basal medium. Growth may be sustained (stimulated and maintained) by renewing the liquid medium after 4 weeks, mainly when the culture period is more than 8 weeks.

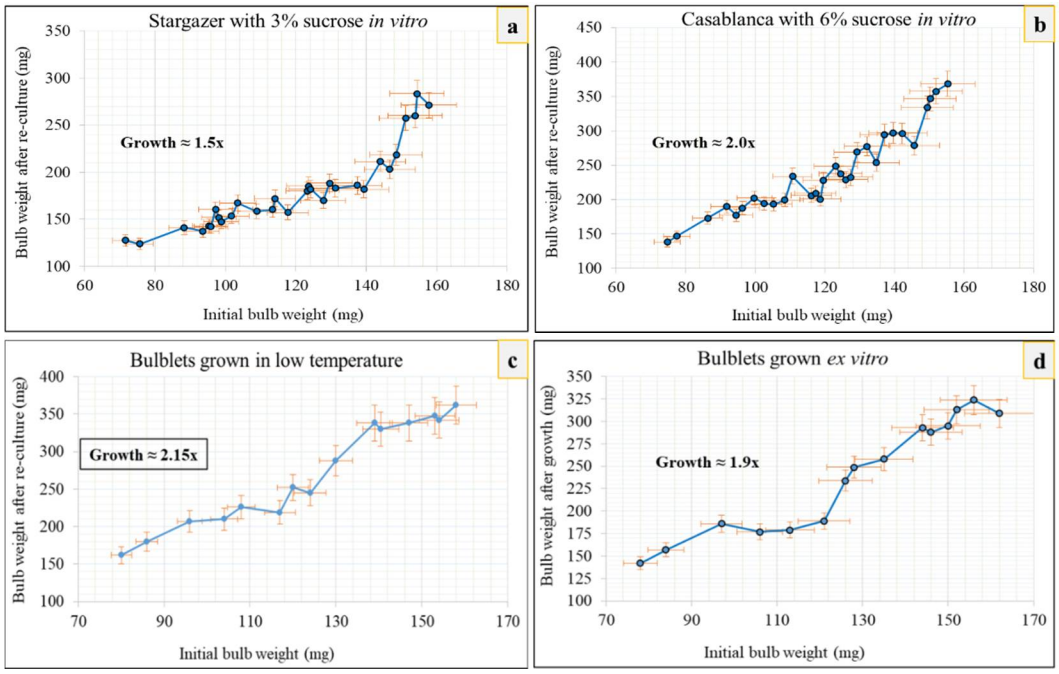

Fig. 2. Performance of lily bulblet with subculture *in vitro* (a, b, c) and *ex vitro* (d) (n = 30 for a, b and n = 15 for c; x = 100% for growth rate).

Thirty 'Stargazer' and 'Casablanca' bulblets harvested from first subculture (fresh weight ranging from 147.2 to 368.1 mg) were again subcultured *in vitro* to assess further growth and ontogenic development. Bulblet growth rate (fresh weight) was 3.3x (330%) in the second subculture *in vitro* (Fig. 4), and fresh weight was above the threshold weight (300 mg) at which 100% phase change (juvenile to adult vegetative) occurred. The results showed that bulblet growth (fresh weight) and ontogenic development has a linear relationship with subculture *in vitro* because of renewal and increasing carbohydrate content in the medium and inside bulblet tissues. Histological observation of a longitudinal section revealed that adult vegetative bulblets had epidermal (L_1) and sub-epidermal (L_2) layers called the 'tunica' and an inner layer (L_3) called the corpus (Fig. 5b), indicating ontogenic development. The course of bulblet growth and ontogenic

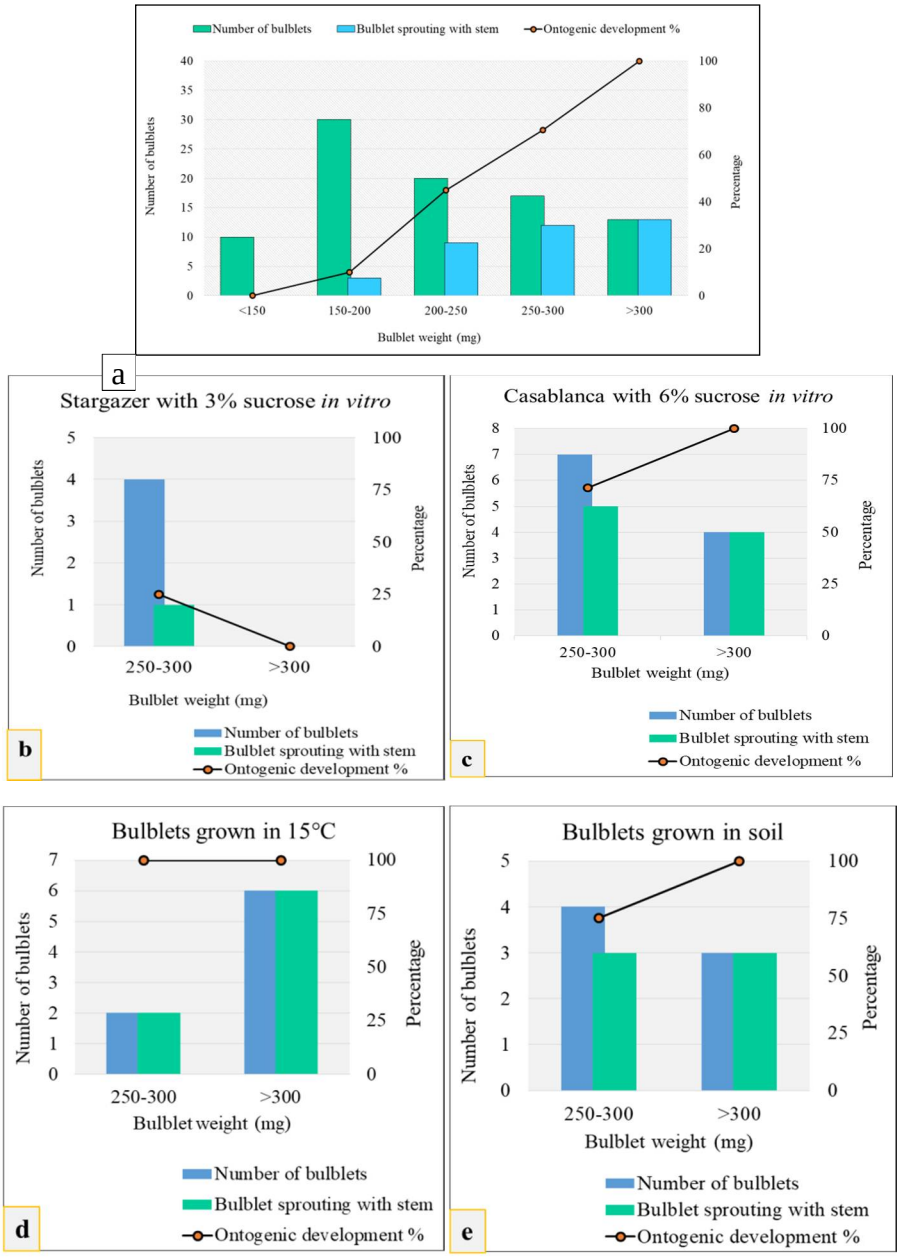

Fig. 3. Threshold weight of lily bulblets for phase change *ex vitro* (a), bulblets subcultured *in vitro* (b, c, d), and bulblets grew in soil (e).

development took 46 - 48 weeks to reach the threshold *in vitro* at which 100% phase change from juvenile to adult vegetative bulblets occurred. In contrast, it took two growing seasons (actually two years because of dormancy) to reach the threshold *in vivo* (Fig. 6).

The initial size strongly affects growth and morphogenesis (transition between various phases, i.e., juvenile, adult, and reproductive) after planting (Kumar et al. 2001; Langens-Gerrits et al. 1996b) and occurs after two or more growing seasons (Islam et al. 2017; Langens-Gerrits et al. 2003a). Therefore, subculture *in vitro* resulted in a high bulblet growth (Fig. 4) in the present study, and it seems clear that a continuous supply of sucrose (or avoiding a decrease in the amount of sucrose in the media) is the key to achieving the desired bulblet growth (size) and ontogenic development (adult vegetative bulblet) *in vitro* because the carbon supply is more important (Podwyszynska 2012). With the upper hand, reduces the period of bulb production from two growing seasons (two years) *in vivo* to two times *in vitro* bulblets culture periods only (Fig. 6). Therefore, according to Boontjes et al. (1981) and Langens-Gerrits et al. (1996a), in lily, stem formation is stimulated in the bulblets when complete scales or scale segments are cultured at 17°C after initial culture at 23°C" indicates that the lower temperature acts as a trigger or signal, inducing the transition from the juvenile to adult vegetative phase (Ishimori et al. 2007, Langens-Gerrits et al. 2003b).

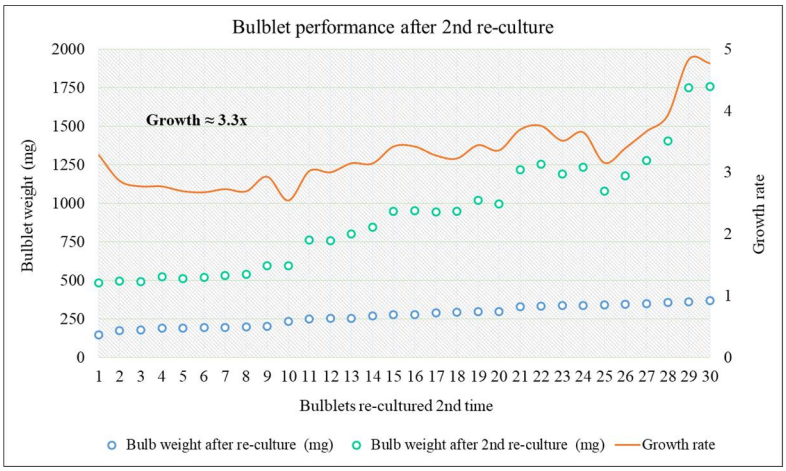

Fig. 4. Performance of lily bulblet with second subculture *in vitro* (n = 30, x = 100% for growth rate) (All bulblet fresh weight above the threshold weight (300 mg) at which 100% phase change).

The amount of medium for bulblet production *in vitro* often proves inadequate because of nutrient depletion, mainly sucrose, which is the source of carbon. In this study, the sucrose depletion was linear over time (culture period) and varied with the amount of medium in the culture vessel (Fig. 7a). Furthermore, the medium with 3% sucrose concentration was depleted much earlier than with 6% sucrose, so sucrose depletion was concentration-dependent as well (Fig. 7b). Therefore, desired bulblet growth and ontogenic development were affected by sucrose depletion in the medium during lily bulblet regeneration *in vitro*. On the other hand, the circumference of 25 adult vegetative bulblets that had reached threshold weight was measured to assess the inter-

relationship with ontogenic development (Fig. 8). Circumference increases with increasing bulblet weight. Therefore, the threshold circumference was about 3.2 cm in proportion to threshold weight (about 300 mg) at which 100% ontogenic development occurred (Fig. 8).

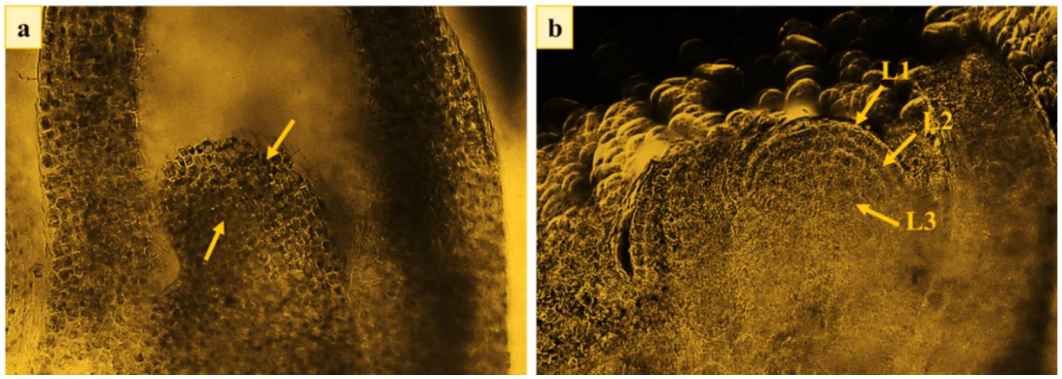

Fig. 5. Histological observation of a longitudinal section of apical meristem of lily bulblets. (Magnification: 100×) (**a**) Juvenile bulblet (only leaf primordia attached to the basal plate, and no tunica-corpus structure in the juvenile apical meristem indicated the absence of ontogenic development), (**b**) Adult bulblet (with a tunica-corpus structure, the first two outer layers L_1 {epidermal layer}, and L_2 {sub-epidermal layer} together are called tunica and the inner layer L_3 [corpus]) under a light microscope.

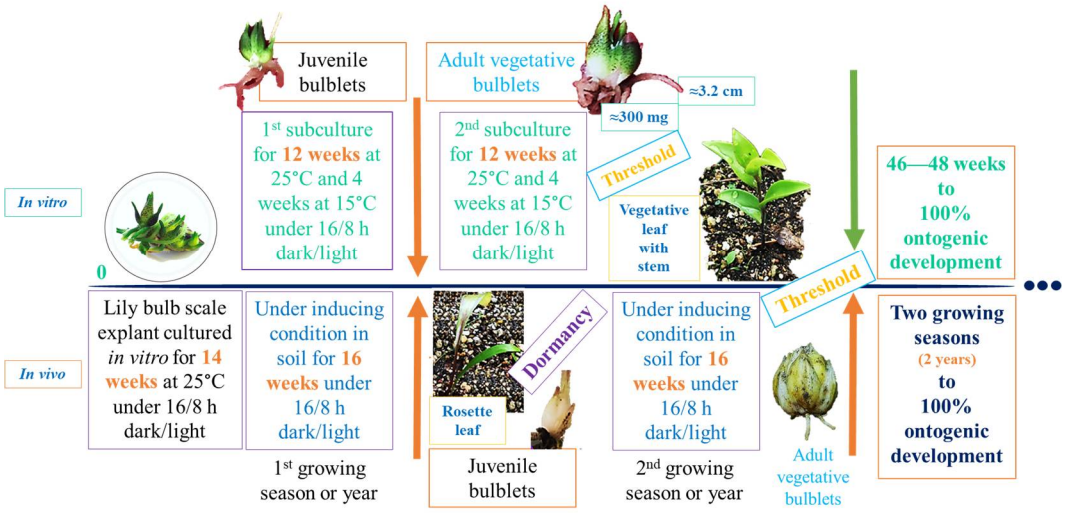

Fig. 6. The course of bulblet growth and ontogenic development *in vitro* and *in vivo*.

The above results make it is clear that continuous bulblet growth and ontogenic development are affected by sucrose depletion in media during lily bulblet regeneration because the carbon supply is more important (Podwyszynska 2012). It should be noted that *in vitro* plants are not fully autotrophic (Kumari et al. 2009; Yaseen et al. 2013). Sucrose in media acts as a fuel source for sustaining photo-mixotrophic metabolism,

ensuring optimal development and restricting photosynthetic efficiency by reducing the levels of chlorophyll pigment, key enzymes for photosynthesis because of the shortage of CO_2 to sustain growth in the absence of sucrose, which is mainly due to limited CO_2 inside the vessel (Gago et al. 2014, Gautheret 1955, Jo et al. 2009, Kozai 1991a, b, Kozai et al. 1992, 1997, Lee et al. 1988, Serret et al. 1996, Yaseen et al. 2013). Hence, bulblet size and ontogenic development are correlated in *Lilium* species *in vitro* and *in vivo* (Matsuo and Arisumi 1978).

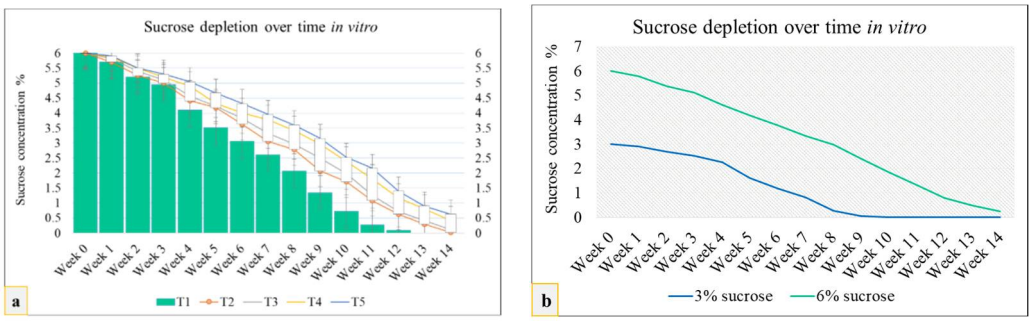

Fig. 7. Sucrose depletion over time and amount of medium (a) and sucrose concentration (b) during lily bulblet regeneration *in vitro* (**T_1**, 10 ml of medium; T_2, 15 ml; T_3, 20 ml; T_4, 30 ml; T_5, 50 ml).

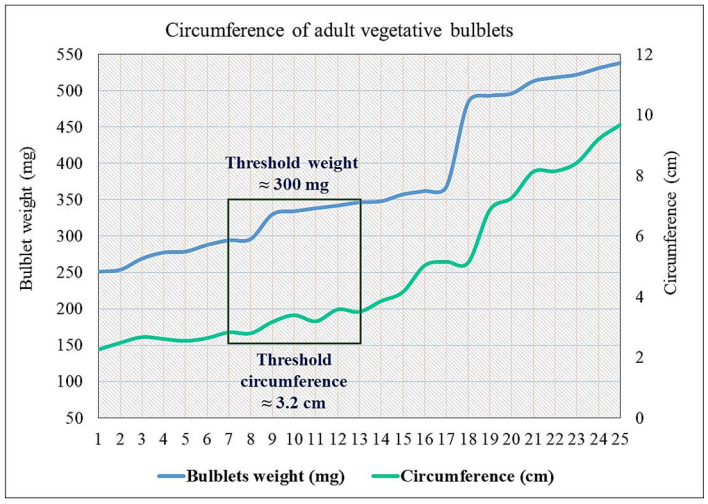

Fig. 8. Lily bulblet threshold circumference for phase change.

The results in the present study reveal that bulblet performance was affected by the amount of medium and also because of the increasing amount of sucrose *in vitro*, and low temperature during the culture period acts as a signal for inducing ontogenic development. However, the amount of medium for bulblet production *in vitro* is often inadequate because of nutrient depletion, particularly sucrose over time, as well as being

concentration-dependent. Avoiding depletion of sucrose in the media is the key to achieving the desired bulblet growth and ontogenic development *in vitro*. Therefore, the *in vitro* subculture course of lily bulblet growth and ontogenic development (46 - 48 weeks to reach the threshold) is a possible alternative than growing *in vivo* performance in soil. As a result, the *in vitro* timeline signifies its efficacy over the conventional method of bulb production.

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