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Factors affecting bulblet growth of *Lilium sp.* – A timeline for bulblet to bulb production via *in vitro* vs conventional pathway

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Factors affecting bulblet growth of *Lilium sp.* – A timeline for bulblet to bulb production via *in vitro* vs conventional pathway

Md. Saiful Islam[1*] and Kazuhiko Shimasaki[2]

Abstract

Lily–belong to the genus *Lilium* is one of the top cut flowers worldwide. Production and propagation of bulblets *in vitro* is an important approach for high-volume production, but not proved satisfactory. Hence, the aim of this study was to describe and compare the performances of morphological characteristics of the lily bulb *in vivo* produced by *in vitro* and conventional culture method and compare the production timelines *in vitro* vs conventional culture method. In results, it seems clear that *in vitro* re-culture of lily bulblet was able to be sustained and maintained its growth and so, ontogenic development after their performance in soil. Our results demonstrate that in the conventional pathway, the course of bulblet growth to bulb and ontogenic development took 3–4 growing seasons to reach the adult flowering phase. On the other hand, along with the re-culture *in vitro*, the course of bulb growth and ontogenic development took 1–2 growing seasons to reach the adult flowering phase because of the increasing initial bulb size and advancement of ontogenic development. Other than bulblet production through bulb scale explant, this study represents the first report on the method of *in vitro* bulb production of lily through re-culture by *in vitro* pathway with a comparison of the timelines and ontogenic development obtained from *in vitro* versus conventional pathway.

Keywords**:** *Lilium* sp.; *In vitro* protocol; Conventional pathway; Bulb production; Timeline

Introduction

The genus *Lilium* are among the top cut flowers in the world. Lily bulb production is a highly complex process, which cannot be fully understood by analysis at any one specific method (i.e. conventional, *in vitro*). Various studies were done on techniques, to analyze the complex multivariate data sets to break down the complexity in a simpler means to a better understanding of the complex effects of the variables involved during the *in vitro* bulb production on the *in vitro* culture and the *in vivo* performance to an improvement of the process. In general bulb production under conventional process is done by 'scaling' (scaling—excised scales are placed in moistened vermiculite to produce small bulblets generally, 1–4 per scale) (Langens-Gerrits and De Klerk, 1999). On the other hand, the scaling process is very slow and requires a longer period of time to bulblet growth (size) and ontogenic development. While we're on the subject, It should be noted that the ontogenic development strongly correlates with the bulb size (Langens-Gerrits et al., 2003b) and there is three ontogenic phases, juvenile, vegetative adult and flowering phase during the development of lily bulb (Rees, 2012; Langens-Gerrits et al., 2003b). In juvenile phase lily plants characterizes by sprouting with rosette leaves with tiny bulblets and the vegetative adult phase by leaves on elongated stem (Islam et al., 2017; Langens-Gerrits et al., 2003b). The flowering phase during the development of lily bulb comes after the bulb has reached the maturity and size with unfolding of several leaves and shoot elongation (Miller, 1992; Lazare and Zaccai, 2016). In contrast, similar to scaling *in vitro* propagation technique of lily bulb production through scale explant on artificial culture medium supplemented with all essential nutrients and carbon source is the prolific vegetative propagation method (Bahr and Compton, 2004) in a short period of time (George et al., 2008). Considering both the *in vitro* and conventional method, the key features may account to understand and identify pros and cons is associated with bulblet size and time, which is the most important factors. Irrespective of production method, the bulb size at planting affects

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growth and development of lily (Lazare and Zaccai, 2016) and a critical size is positively correlated with ontogenic development and flower quality (Kim et al., 1997; Lazare and Zaccai, 2016). In conventional method, this shift of ontogenic development take seasons or years (Ziv and Naor, 2006; Lazare and Zaccai, 2016). Again, when *in vitro* produced bulblets were planted in soil, the ontogenic development occurs after one or two growing seasons (Islam et al., 2017; Langens-Gerrits et al., 2003a). Though, production and propagation of bulblets *in vitro* is an important approach for high-volume production, but this technique has not proved satisfactory for commercial producers and growers, due in part to the lengthy production time similar to the conventional method. At this point, 'is there any alternative to the growing seasons *in vivo*?' became the utmost vital question. An answer to the question possibly will open up a new era for rapid and high-volume production techniques of lily bulb *in vitro*. On the other hand, there are relatively few studies on lily bulblets growth and ontogenic development *in vitro*(Islam et al., 2017) and have not addressed the time-frame for bulb production or resulting plant quality, aspects that are important for commercial applications. Hence, the aim of this study was to (a) describe and compare the performances of morphological characteristics of the lily bulb *in vivo* produced by *in vitro* and conventional culture method, (b) compare the production timelines *in vitro* vs conventional culture method, and (c) describe the procedures to open up a new era for rapid and high-volume production techniques of lily bulb through *in vitro* pathway.

Materials and methods

Plant materials and bulblet production

Freshly harvested bulbs of three lily cultivars Stargazer, Casablanca, and Cesare were collected from Nakamura-Noen Co., Ltd., Kochi, Japan in November, 2015. Then again, fresh and disease free bulb scale were used during bulblet regeneration via conventional and *in vitro* pathways.

a) Conventional pathway

Conventional process of lily bulblet production is generally known as 'scaling' (Fig. 1). Collected fresh harvested lily bulbs were scaled in December, 2015 and with the new bulblets planted in soil in April 2016. To begin, all of the outer rings of scales were removed and discarded and then, carefully break off the remaining layers of bulbscales. Therefore, set out the "scales" to dry overnight and so, they will air-dry and the broken surface will callous, naturally protecting the scales from fungus. Then lily scales were placed in slightly damp vermiculite in a plastic bag that is loosely folded at the top and avoid to come in direct contact with the plastic bag. To avoid too much moisture the plastic bag was opened several times to allow in more air. After regeneration when bulblets were about the size of green peas they are moved into cold storage for six to eight weeks of temperatures just above freezing as their first winter and later the bulblets were planted in pots and placed in greenhouse.

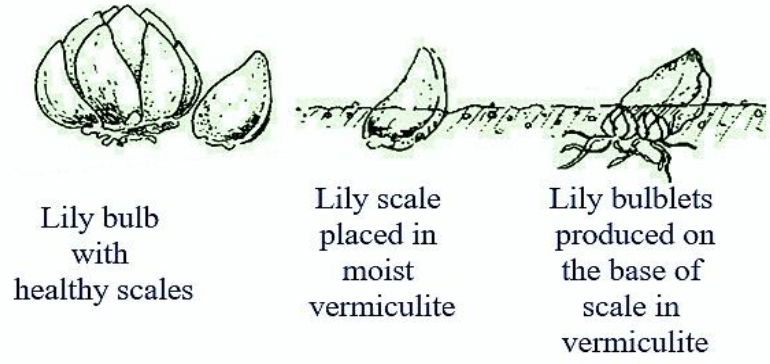

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Figure 1. Schematic drawing of scaling from lily bulb scales

b) *In vitro* pathway

Scale explants of three lily cultivars Stargazer, Casablanca, and Cesare were used during bulblet regeneration *in vitro* in December 2015 and a schematic drawing of the process is shown in Fig. 2. Clean and healthy scales 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 cultured with the adaxial side on 15 mL per explant of MS medium for 12 weeks at 25°C under a 16/8 h dark/light condition. Since, sucrose is the most common carbon source used in plant cells, tissues, and organ cultures, so media with a 6% sucrose concentration has been used as a staple source since Murashige and Skoog (1962) described their MS medium.

Media properties	
MS salt	4.4 g/L MS medium with vitamins (Murashige and Skoog,
PGR	1962)
Sucrose	NAA 50 µM/L
Agar	6%, 60 g/L sucrose
pH	7 g/L micro-agar
	Adjusted to pH 5.8 prior to autoclaving for 15 min

(Adapted from *Islam et al.*, 2017)

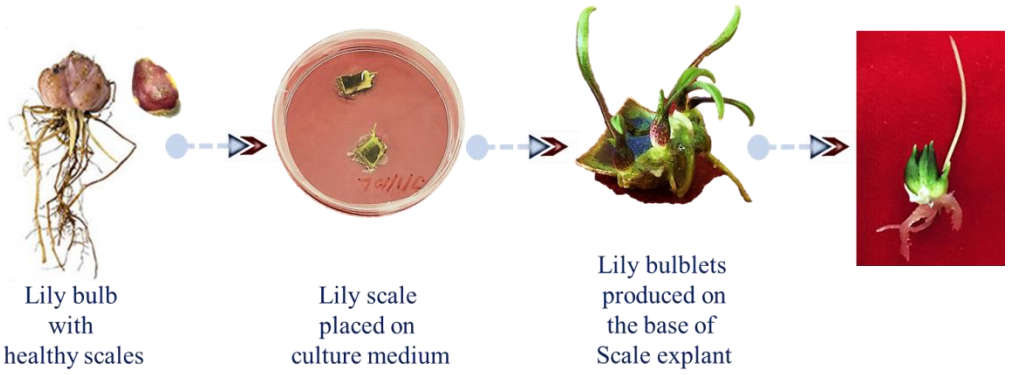

Figure 2. Schematic *in vitro* process of bulblet regeneration from lily bulb scale

Bulblet re-culture *in vitro*

In the first re-culture, tiny bulblets of lily cultivars were harvested from the basic culture *in vitro* were re-cultured in MS medium supplemented with 6% sucrose for 12 weeks at 25°C and subsequently 02 weeks at 15°Cunder a 16/8 h dark/light condition to track their growth and ontogenic development.

In the second re-culture, bulblets were again cultured at 25°C for 12 weeks and subsequently two weeks at 15°C in MS medium supplemented with 6% sucrose for a total of 14 weeks, under a 16/8 h dark/light condition *in vitro* to assess their growth and ontogenic development.

Bulblet grown in soil

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In March 2016; both conventional and *in vitro* grown bulblets were kept at 5°C for 08 weeks on moist filter paper in petri dishes to break dormancy (Langens-Gerrits and De Klerk, 1999) and then later in April 2016 planted in pots (9 cm in diameter and 15 cm in depth) containing fertilized soil with 380, 290, and 340 mg L^{-1} 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 and 16 h light period). After 16 weeks, the bulblets were uprooted and data were collected. Therefore, harvested bulblets were planted again in April 2017 and 2018 following same procedure.

Statistics

The investigation was done in the laboratory of floriculture and vegetable sciences at Kochi University, Japan. The results are expressed as a mean ± standard error (SE). For all comparisons, statistical analysis was performed using one-way ANOVA followed by Tukey's test and $p<0.05$ was considered statistically significant.

Results

Assessment of the *in vivo* performance of conventional and *in vitro* produced lily bulblets in consecutive growing seasons

Days to sprout

After 90 days of culture *in vitro*, the bulblets from all three lily cultivars Stargazer, Casablanca, and Cesare were planted in pots and placed in a greenhouse and the data collected for various parameters in three consecutive growing seasons is presented in Fig. 3a—c. Average days to sprout was significantly influenced by both *in vitro* and conventional method, bulblets size in consecutive growing seasons and lily cultivars. In the first growing season, conventional bulblets were sprouted quicker than *in vitro* produced lily bulblets. Among the lily cultivars quickest sprouting occurred in case of Cesare bulblets irrespective of production methods and the slowest with Stargazer bulblets (Fig. 3a).

In the second growing season, both conventional and *in vitro* produced lily bulblets were sprouted quicker than their sprouting in first season because of the rise of initial bulb weight. Again, among the lily cultivars quickest sprouting occurred in case of Cesare bulblets irrespective of production methods in compare to Stargazer and Casablanca bulblets (Fig. 3b).

In the third growing season, both conventional and *in vitro* produced lily bulblets were sprouted quicker than their sprouting in first and second seasons because of the rise of initial bulb weight. Again, among the lily cultivars quickest sprouting occurred in case of Cesare bulblets irrespective of production methods in compare to Stargazer and Casablanca bulblets (Fig. 3c).

Number and types of leaves

In the first growing season, the maximum number of leaves were regenerated in case of *in vitro* produced lily bulblets than conventional bulblets. Among the lily cultivars maximum number of leaves occurred in case of Cesare bulblets irrespective of production methods and the minimum with Stargazer bulblets (Fig. 3a). All sprouted bulblets had rosette-type leaves (Fig. 3a), not the desired sprouts with a stem, due to the initial minute bulblet weight.

In the second growing season, again the maximum number of leaves were regenerated in case of *in vitro* produced lily bulblets than conventional bulblets. In compare to growing seasons number of leaves was much higher than their sprouting in first season because of the rise of initial bulb weight. Among the lily cultivars maximum number of leaves occurred in case of Cesare bulblets

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irrespective of production methods and the minimum with Stargazer bulblets (Fig. 3b). Though, there is a rise of initial bulb weight after first growing season, although all sprouted bulblets had rosette-type leaves (Fig. 3b), not the desired sprouts with a stem, due to the ontogenic development.

In the third growing season, again the maximum number of leaves were regenerated in case of *in vitro* produced lily bulblets than conventional bulblets. In compare to seasons number of leaves was much higher than their sprouting in first and second season because of the rise of initial bulb weight. Among the lily cultivars maximum number of leaves occurred in case of Cesare bulblets irrespective of production methods and the minimum with Stargazer bulblets (Fig. 3c). All sprouted bulblets had desired stem type adult vegetative leaves (Fig. 3c) because of the higher initial bulblet weight and ontogenic development.

Bulb weight after growing seasons and ontogenetic development

Typically, all bulblets increase in weight after each growth seasons (Fig. 3a—c). In the first growing season, the average initial weight were 73.5 mg for conventional and 82.5 mg for *in vitro* produced bulblets. Whereas, at the end of first season final bulblets weight was 180.5 mg (growth rate—2.5x) for conventional and 212.4 mg (growth rate—2.6x) for *in vitro* produced bulblets (Fig. 3a). However, the bulblets weight was little higher in case of *in vitro* produced lily bulblets than conventional bulblets at the end of the first season because of their initial weight which were little higher also at the planting. Among the lily cultivars higher weight of bulblet occurred in case of Casablanca irrespective of production methods (Fig. 3a).

In the second growing season, the average initial weight were 180.5 mg for conventional and 212.5 mg for *in vitro* produced bulblets. Whereas, at the end of second season final bulblets weight was 356.5 mg (growth rate—2.0x) for conventional and 465.0 mg (growth rate—2.2x) for *in vitro* produced bulblets (Fig. 3b). Again, the bulblets weight was higher in case of *in vitro* produced lily bulblets than conventional bulblets at the end of the second season because of their initial weight which were higher also at the planting. Among the lily cultivars higher weight of bulblet occurred in case of Casablanca irrespective of production methods (Fig. 3b).

In the third growing season, the average initial weight were 356.5 mg for conventional and 465.0 mg for *in vitro* produced bulblets. Whereas, at the end of third season final bulblets weight was 1065.0 mg (growth rate—3.0x) for conventional and 1657.0 mg (growth rate—3.6x) for *in vitro* produced bulblets (Fig. 3c). Again, the bulblets weight was higher in case of *in vitro* produced lily bulblets than conventional bulblets at the end of the third season because of their initial weight which were higher also at the planting. Among the lily cultivars higher weight of bulblet occurred in case of Casablanca irrespective of production methods (Fig. 3c).

Along with bulblet size the ontogenic development of lily bulblets is equally important for their economic value as a propagule. In first and second growing seasons all bulblets irrespective of cultivars and production methods sprouted with rosette-type leaves (Fig. 4c—d), not the desired sprouts with a stem, due to the ontogenic development was dependent on the initial bulblet weight at planting (Fig. 4a—b). While in the third season, all sprouted bulblets had desired stem type adult vegetative leaves (Fig. 4f—g) because of the higher initial bulblet weight (Fig. 4e) and so, ontogenic development. However, another season required for the flowering but these lily bulbs are usually used as commercial propagule.

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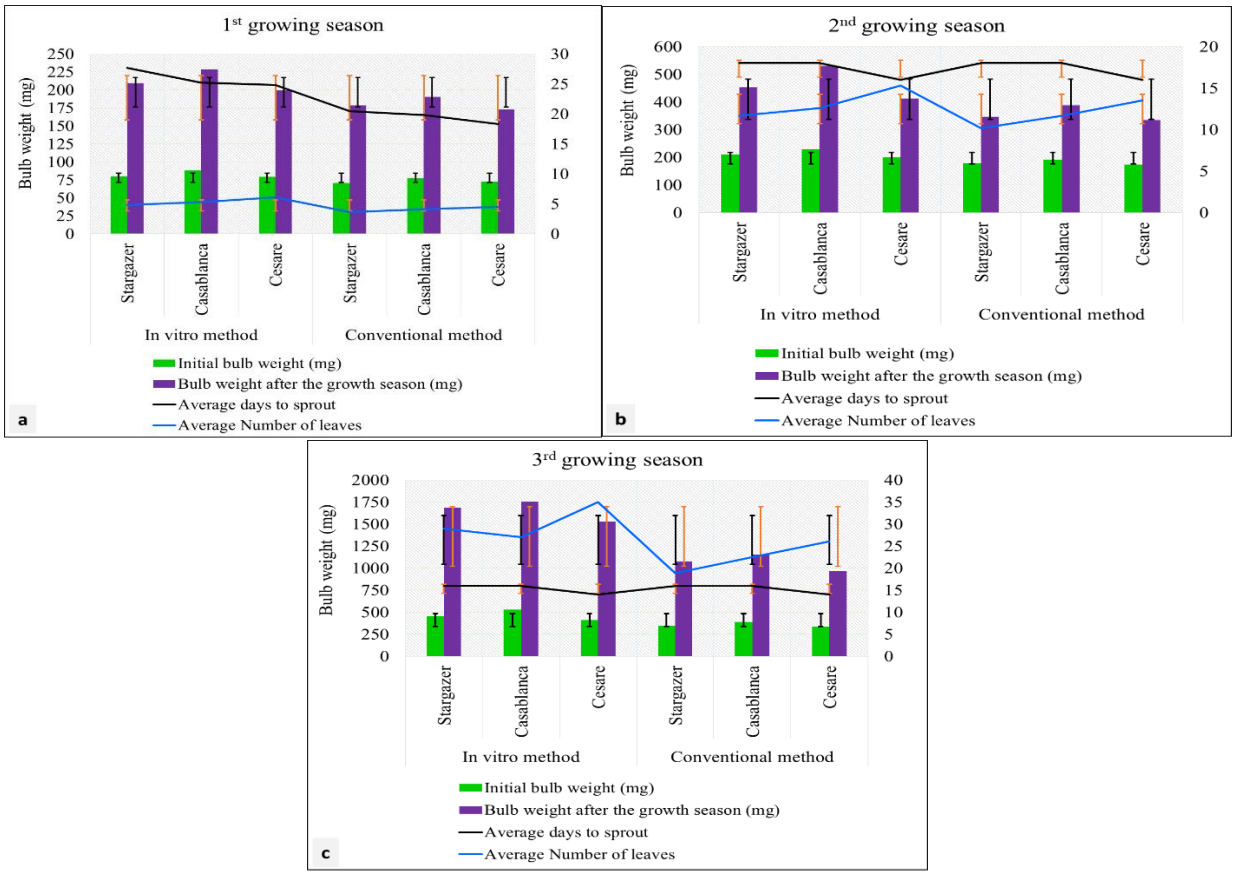

Fig. 3. *In vivo* performance of conventional and *in vitro* produced lily bulblets in consecutive growing seasons (a) after 1st growing season, (b) after 2st growing season, and (c) after 3st growing season.

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Fig. 4. Performance of lily bulblet in consecutive growing seasons *in vivo* (a) conventional bulblet, (b) *in vitro* produced bulblet, (c, d) sprouting with rosette-type leaves because of lower size and so, juvenile bulb at first and second growing season, (e) harvested lily bulb after two growing seasons, and (f, g) lily bulb sprouted with desired stem type adult vegetative leaves because of the advancement in bulb size and ontogenic development.

Assessment of the *in vivo* performance of *in vitro* produced lily bulblets

Days to sprout

All three of *in vitro* produced bulblets (basic cultured, first, and second re-cultured) from all three lily cultivars Stargazer, Casablanca, and Cesare were planted in pots and placed in a greenhouse and the data collected for various parameters throughout the entire growing seasons is presented in Fig. 5a—b. Average days to sprout was significantly influenced by both *in vitro* culture and lily cultivars (Fig. 5a). Among *in vitro* cultures second re-cultured bulblets were quickest because of larger bulb size (Fig. 5a).While in the lily cultivars quickest sprouting occurred in case of Stargazer and the slowest with Casablanca bulblets except basic cultured bulblets (Fig. 5a).

Number and types of leaves

Average number of leaves were significantly influenced by *in vitro* culture and second re-cultured bulblets had maximum leaves because of larger bulb size (Fig. 5a). While in the lily cultivars maximum leaves were formed in case of Cesare and the minimum in Stargazer (Fig. 5a).

All sprouted bulblets had rosette-type leaves in case of basic culture (Fig. 6a) not the desired sprouts with a stem, due to the initial minute bulblet weight. On the other hand, in case of first, and

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second re-cultured bulblets all of them sprouted with desired stem type adult vegetative leaves (Fig. 6 b, c) because of the higher initial bulblet weight and ontogenic development.

Bulb weight at harvest and ontogenetic development

Typically, all bulblets increase in weight after the season (Fig. 5 a, b). At the start of growing season, the average initial weight were 82.5,318.9, and 587.8 mg for *in vitro* basic cultured, first re-culture, and second re-cultured bulblets (Fig. 5a, b). Whereas, at the end of season final bulblets weight was 212.4, 969.3, and 1753.7 mg for *in vitro* basic cultured, first re-culture, and second re-cultured bulblets (Fig. 5a, b). Therefore, the growth rates were 257, 304, and 298 percent for *in vitro* basic cultured, first re-culture, and second re-cultured bulblets (Fig. 5b). This result of growth rate shows that irrespective of lily cultivars first re-cultured bulblets were higher at the end of the season however, the initial bulblet weight was higher in case of *in vitro* second re-cultured bulblets (Fig. 5a, b).

Then again, along with bulblet size the ontogenic development of lily bulblets is equally important for their economic value as a propagule. After the growing season irrespective of cultivars all bulblets from *in vitro* basic culture sprouted with rosette-type leaves (Fig. 6d), not the desired sprouts with a stem, due to the ontogenic development was dependent on the initial bulblet weight at planting. While all bulblets from *in vitro* first, and second re-culture were sprouted with desired stem type adult vegetative leaves because of the higher initial bulblet weight (Fig. 6e, f) and so, ontogenic development.

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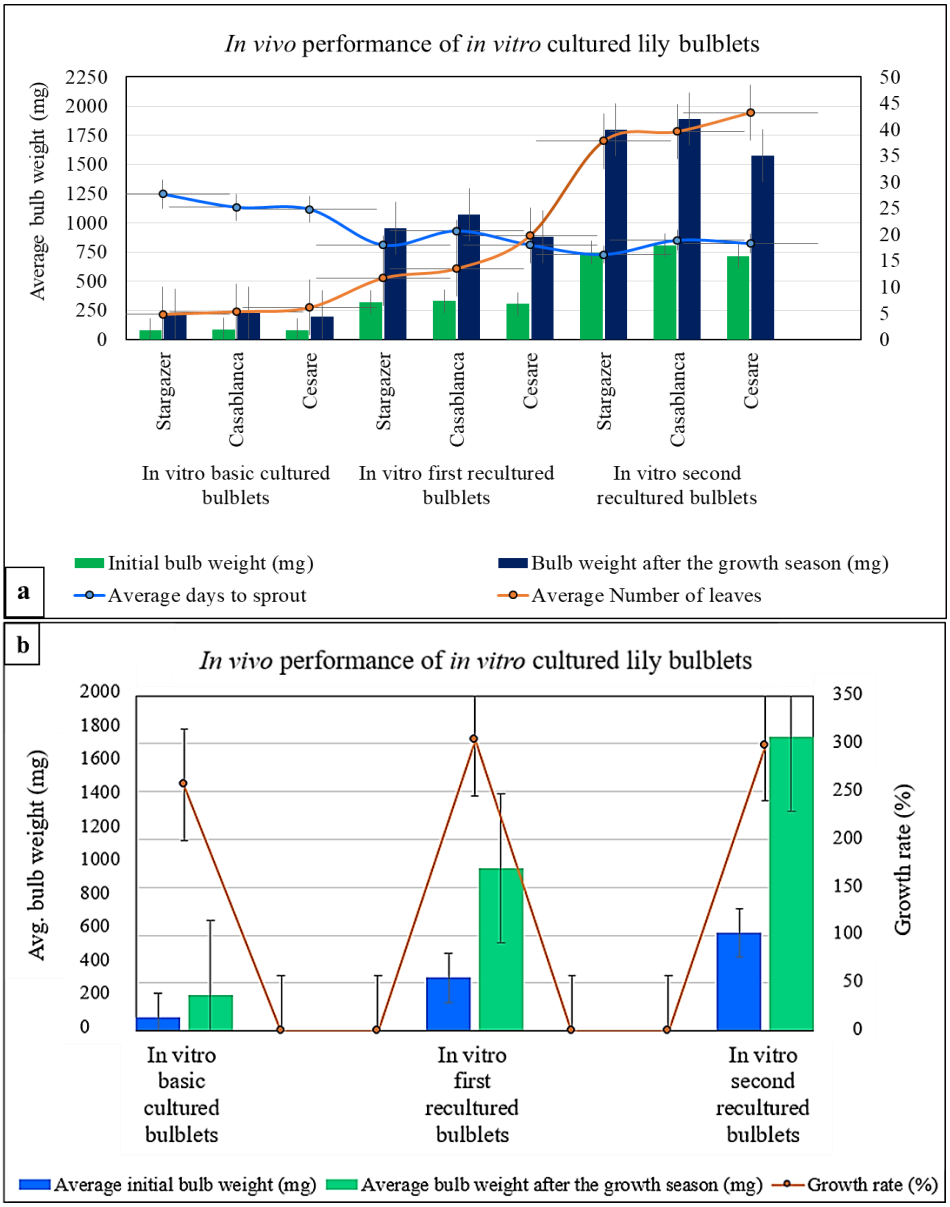

Fig. 5. *In vivo* performance of *in vitro* produced lily bulblets at the end of growing season.

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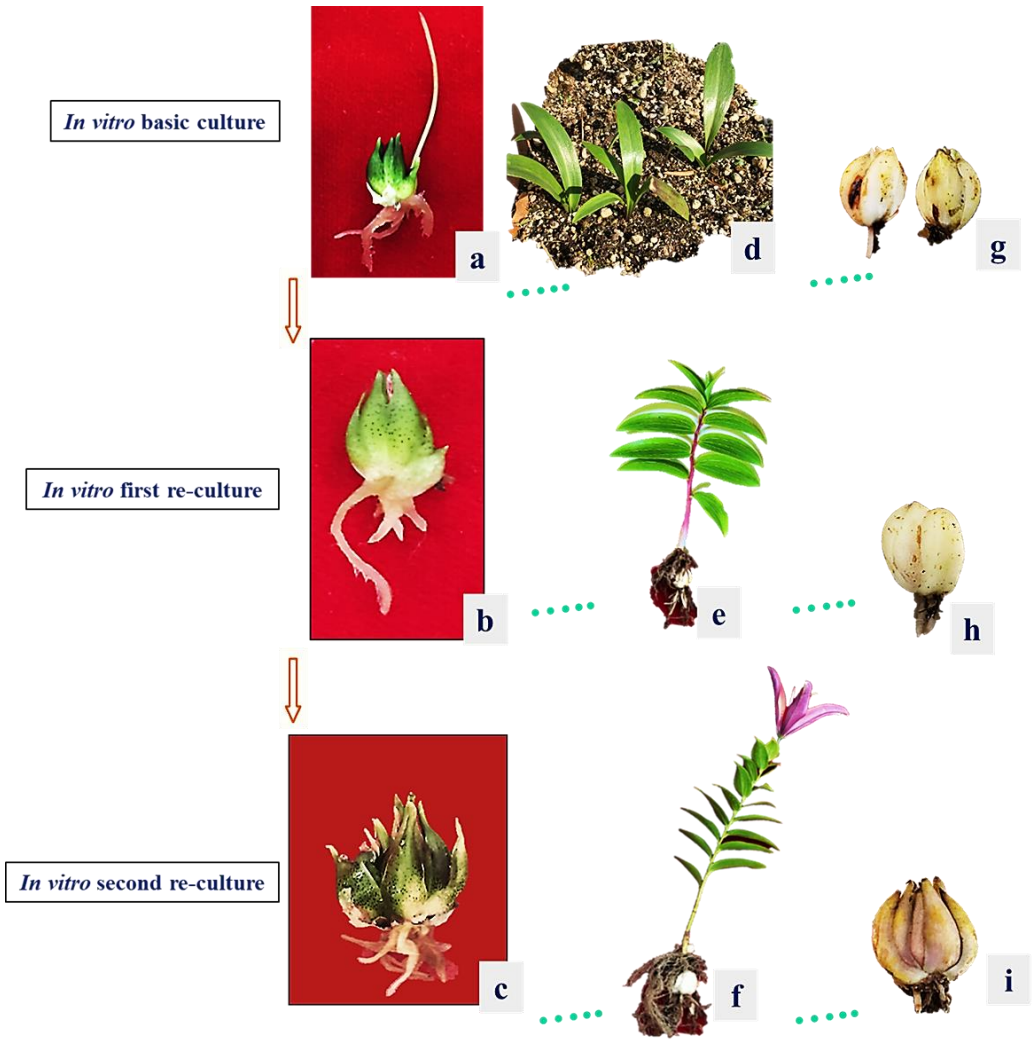

Fig. 6. *In vivo* performance of *in vitro* produced lily bulb at the end of the growing season (a) *in vitro* basic cultured bulb, (b) *in vitro* first re-cultured bulb, (c) *in vitro second* re-cultured bulb (d) *in vitro* basic cultured bulb sprouting with rosette-type leaves because of lower size and so, juvenile bulb (g), (e, f) *in vitro* first and second re-cultured bulb sprouting with desired stem type adult vegetative leaves because of the advancement in bulb size and ontogenic development (h, i)

Assessment of timeline for lily bulb production via *in vitro* vs conventional pathway through various stages of ontogenic development

Conventionally, the course of bulb growth and ontogenic development took 3–4 growing seasons to reach the adult flowering phase (Fig. 7). On the other hand, it took the similar for *in vitro* pathway depending upon the initial bulb size because of their non-uniformity (Fig. 7). However, in compare to conventional, the *in vitro* pathway has more number of bulblets (Fig. 8). Along with the re-culture *in vitro*, the course of bulb growth and ontogenic development took 1–2 growing seasons to reach the adult flowering phase because of the increasing initial bulb size (Fig. 7).

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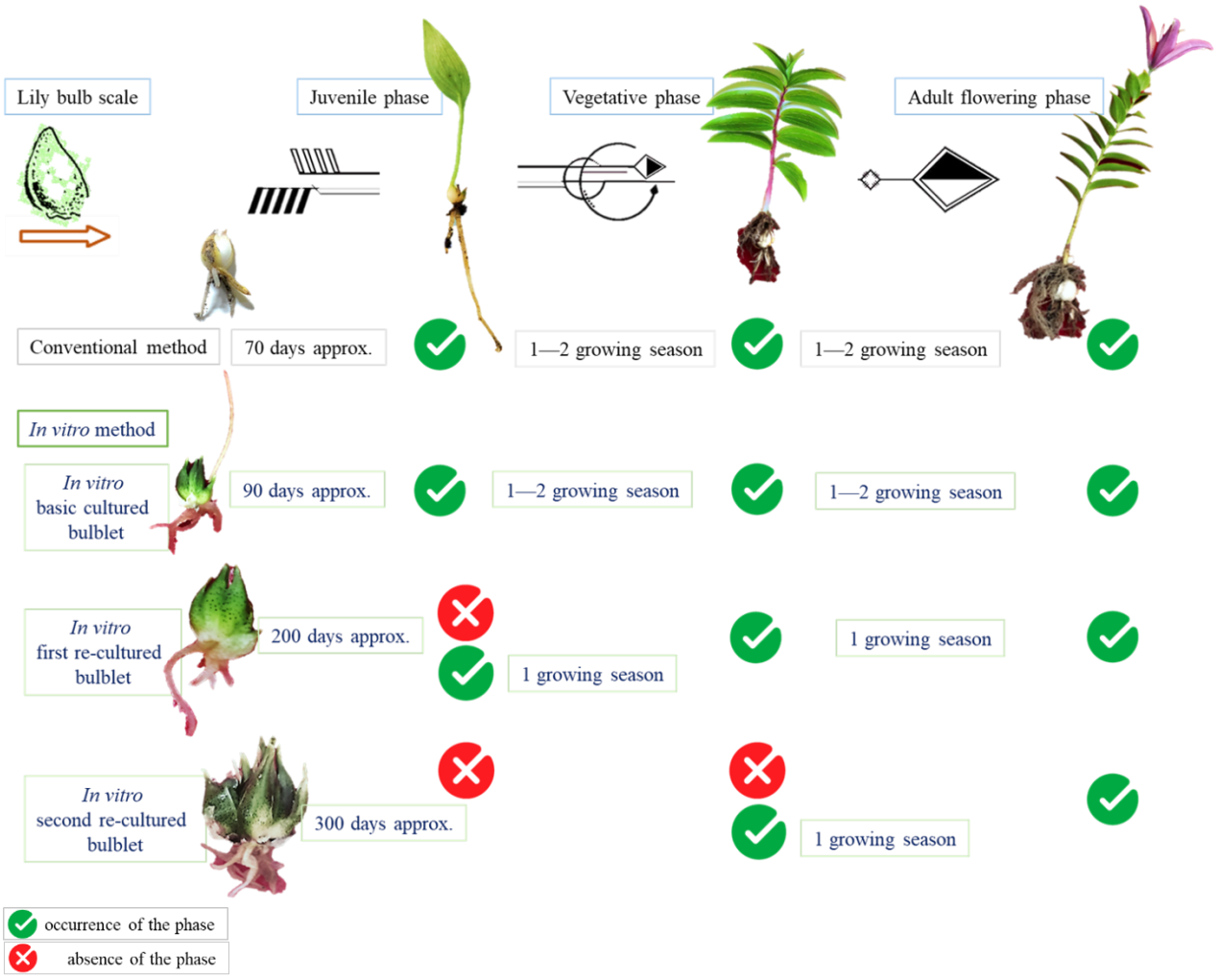

Figure 7. Timeline and course of bulblet growth and ontogenic development through *in vitro* and conventional pathway in soil.

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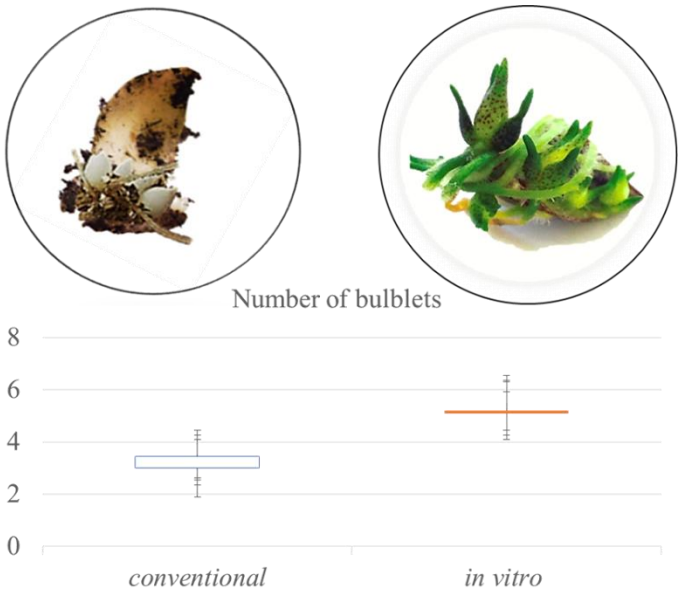

Fig 8. Contrast between the number of lily bulblet production through conventional and *in vitro* pathways

Discussion

The conventional scaling process is slow-moving and so, requires a longer period of time to bulblet growth (size) and ontogenic development (Ziv and Naor, 2006; Lazare and Zaccai, 2016). However, in compare scaling, the *in vitro* technique of lily bulblet production is the prolific method for high-volume production (Bahr and Compton, 2004), but this technique has not proved satisfactory for commercial producers and growers, because of the lack of uniformity is in size (Islam et al., 2017) and lengthy production time similar to the conventional method. Therefore, this study describe and compare the performances of morphological characteristics of the lily bulb grown in soil produced by both *in vitro* and conventional culture method. In the results, conventional bulblets were sprouted quicker than *in vitro* (Fig. 3a) in their first growing season. Whereas, in progress with the growing seasons both conventional and *in vitro* bulblets sprouted quicker than their previous season because of the continuous increasing of size (Fig. 3a—c). Usually, bulblets regenerated on explants *in vitro* vary in size, and the initial size strongly affects growth and development (transition between various phases, i.e., juvenile, adult, and reproductive) after planting (Kumar et al., 2001; Langens-Gerrits et al., 1996b). Therefore, result indicates that the lack of uniformity is in size might had influence on days to sprout in the first growing season in case of *in vitro* bulblets. Similarly, number of leaves were a little higher in case of *in vitro* bulblets, however, in progress with the growing seasons both conventional and *in vitro* bulblets has had more leaves than their previous season because of the continuous increasing of size (Fig. 3a—c). In first and second growing seasons all bulblets irrespective of cultivars and production methods sprouted with rosette-type leaves (Fig. 4c—d), not the desired sprouts with a stem, due to the ontogenic development was dependent on the initial bulblet weight at planting (Fig. 4a—b). While in the third season, all sprouted bulblets had desired stem type adult vegetative leaves (Fig. 4f—g) because of the higher initial bulblet weight (Fig. 4e) and so, ontogenic development. Leaf type is the indicator for the ontogenic development of lily bulblets, however it is hooked on along with bulblet size. According to Islam et al., 2017and Langens-Gerrits et al., 2003b, when bulblets weigh less than 300 mg, they sprout with a few rosette-type leaves (juvenile) instead of leaves with a stem (adult vegetative). At the end of each growing season bulblet weight increases at least a growth rate of—

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2.0x (Fig. 3a—c). In the first and second growing season, all the bulblet was <300 mg while planting in soil and so, few rosette-type leaves (juvenile) (Fig. 4c—d). In the third growing season, all the bulblet was >300 mg while planting in soil and so, profuse leaves with a stem (adult vegetative) (Fig. 4f—g). According to Islam et al., 2017 and Langens-Gerrits et al., 2003a, ontogenic development of lily bulblets (transition from the juvenile to the vegetative adult phase) occur after one or two growing seasons. Moreover, bulblet weight increases at a growth rate of—3.0x (Fig. 3c) which were greatly affected by number of leaves (Islam et al., 2017).

Therefore, in the present study, it seems clear that bulblet size is the key to achieving the desired ontogenic development (adult vegetative bulblet). In the growing season, re-cultured bulblets performance was way better by all means than the basic *in vitro* cultured and conventional bulblets because of the larger bulblet size (Fig. 5a). On the other hand, typically, all bulblets increase in weight after the season and the ontogenic development was satisfactory because of the re-culture. However, *in vitro* basic culture bulblets sprouted with rosette-type leaves (Fig. 6d), not the desired sprouts with a stem, due to the ontogenic development was dependent on the initial bulblet weight after culture and at planting. Since, number of leaves are important for bulb growth profuse leaves were produced by re-cultured bulblets because of larger bulb size (Fig. 5a) sprouted with desired stem type adult vegetative leaves (Fig. 6c). It seems clear that *in vitro* re-culture of lily bulblet was able to be sustained and maintained its growth and so, ontogenic development after their performance in soil. In accordance with ontogenic development, first re-culture is the threshold for adult vegetative phase as the average bulblet weight was about 318.9 mg (Fig. 5a), but for the flowering phase further re-culture required (Fig. 5a and 6f). Hence, we hypothesize that *in vitro* re-culture of lily bulblets significantly promote bulb growth and ontogenic development might be the alternative to the growing seasons *in vivo* and potentially the procedure to open up a new era for rapid and high-volume production techniques of lily bulb through *in vitro* pathway.

Consequently, with the aim to reduce the period of bulblet to bulb production *in vitro* produced bulblets were re-cultured and compared with the production timeline of conventional culture method. In conventional pathway, the course of bulblet growth to bulb and ontogenic development took 3–4 growing seasons to reach the adult flowering phase (Fig. 7). On the other hand, it also took the similar for *in vitro* basic cultured bulblets depending upon the initial bulb size because of their non-uniformity (Fig. 7). However, in compare to conventional, the advantage is that *in vitro* pathway has more number of bulblets (Fig. 8). On the other hand, along with the re-culture *in vitro*, the course of bulb growth and ontogenic development took 1–2 growing seasons to reach the adult flowering phase because of the increasing initial bulb size and advancement of ontogenic development (Fig. 7).Therefore, in the present study, it seems clear that two times re-culture of lily bulblets *in vitro* (approximately 300 days) is likely the substitute to the two growing seasons *in vivo* (conventional) and so, the key to achieving the desired bulblet growth towards bulb production and their ontogenic development (adult vegetative bulblet). With the upper hand, the technique for rapid and high-volume production of clean and healthy lily bulb through *in vitro* pathway.

Conclusion

The results demonstrate that through *in vitro* pathway re-culture of lily bulblets are not only successful for rapid and high-volume production of clean and healthy lily bulb, but also a potential method to achieving the desired bulblet growth towards bulb production and their ontogenic development. Other than bulblet production through bulb scale explant, this study represents the first report on the method of *in vitro* bulb production of lily through re-culture by *in vitro* pathway

with a comparison of the timelines and ontogenic development obtained from *in vitro* versus conventional pathway.

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