The Feeding of Lily Bulblets Regenerating From Scale Explants *In vitro*: The Roles of Carbohydrates in the Scale Explant and in the Medium

MSc Thesis (Major)

Md. Saiful Islam

Chair group:
ORNAMENTAL, TISSUE CULTURE AND GENE TRANSFER
(PBR - 80436)

THESIS NO.: .....

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October, 2014

WAGENINGEN UR
For quality of life

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Summary

This research deals with the growth of lily bulblets *in vitro* and then role of starch. The main factors determining growth of bulblets *in vitro* prove to be sugar concentration in the medium and explant's position on the scale. The latter is related to internal transport of sucrose from the apical to the basal side which plays a vital role for bulblets growth irrespective of cultivars used in this study, namely, Star Gazer and Santander. Bulblets growth correlates with uses of starch granules reserves inside explants tissue and sucrose in the medium. Mobilization of explants starch reserves that accumulated from the medium plays an important role in growth and development of the photosynthetic apparatus and influence bulblets growth. Cold treatment exert slowdown or stoppage effect on sprouting and bulblets growth is due to immobilization of starch to be used for regeneration and growth of bulblets during culture *in vitro;* similar to the natural process of induce dormancy. Vascular tissues have a major role in the transport of solutes *in vitro* and yet, application of a sugar together with the auxin promotes vascular differentiation. The increase of size and number of vascular bundles inside explants tissue *in vitro* point to the correlation between starch accumulation from the medium and both explants and bulblets growth. Therefore, the explants growth reveals an interesting subject for further research as they find out the positive influence on bulblets growth in this research.

Keywords: Feeding of Lily Bulblets, Regeneration, Scale explant, Carbohydrate, *In vitro*

1 Introduction

Lily bulb scales readily regenerate bulblets basally when isolated from the parent bulb (De Klerk, 2014). This feature is obviously important for vegetative propagation of commercial cultivars. Vegetative propagation, by tissue culture technology, termed "*in vitro* propagation" or "micro-propagation" seems to run efficiently in lily and has reached 50-100 million bulblets per annum (De Klerk, 2014). *In vitro* propagation technology for lily bulblets regeneration appears as the gateway to the commercial growers for producing disease-free plants. Beside this, it has also significant advantages for breeders because of very rapid propagation. Regardless of these advantages, *in vitro* propagation of lily has some major problems. Slow growth of lily *in vitro,* even though only incidentally recognized is most important among them. Growth of lily bulblets *in vitro* is not fast in comparison to growth in soil though all the environmental conditions are favourable (De Klerk, 2014).

According to Langens-Gerrits *et al.,* 1998; **"**In tissue culture of bulbous crops, large bulblets should be produced**"**. Hence, the sizes of bulblets that are being produced are crucial. The size of the regenerated bulblets depends upon the size of the explant: the larger the explants, the larger the bulblets that are regenerated (Langens-Gerrits *et al.,* 2003). In other words, lily scale explant size influenced bulb growth during the complete culture period. However, besides the sucrose concentration the factors that determine bulblets growth are largely unknown. As a matter of fact, we even do not know in general how plantlets cultured *in vitro* achieve growth (De Klerk, 2014). Clear understanding of the growth mechanism of lily bulblet regenerated *in vitro* would be helpful to improve the optimal growth conditions of lily bulblets.

Vascular tissues have a major role in the transport of solutes in *in vitro* cultured plants just as they have in plants growing *ex vitro* (De Klerk, 2014). Uptake of sucrose mainly occurs through the cut surfaces since the epidermis is relatively impermeable because of a wax layer. An increase in sucrose concentration obviously stimulates uptake (µg sucrose) from the medium hence, bulblets growth (Langens-Gerrits *et al.,* 2003). Moreover, according to Langens-Gerrits *et al.,* 2003; "Sucrose taken up in the explant was mainly recovered at the basal side of the explant, where regeneration occurs". This indicates that, internal transport of sucrose from the apical to the basal side may play a role. One can also say, when complete scales of lilies are propagated cultured in a moist

environment ('scaling'), starch mobilization proceeds from the apical to the basal region (Miller & Langhans, 1990).

Starch is the major storage polysaccharide in *Lilium* bulbs (Matsuo and Mizuno, 1974; Wozniewski *et al.*, 1991). It is a mixture of amylose and amylopectin and is deposited as granules inside plastids (chloroplasts in leaves, amyloplasts in non-photosynthetic tissues). Amylase is an enzyme; it degrades starch through hydrolysis and breaks the alpha-glycosidic linkage between the monomers. According to Mitsui *et al.*, 2010; "α-Amylase probably plays a significant role in starch degradation". This idea is not limited to cereals. α-Amylase, which can act on raw starch, is considered to play the main role in starch degradation including the initial attack on the starch granules (Mitsui *et al.*, 2010). However, recent investigation put forward that the pathway for starch degradation differs with the organs and species. This starch degradation has been observed in cotyledons during germination of starch-storing legume and also in e.g., potato (Mitsui *et al.*, 2010).

During storage at low temperature, starch is hydrolysed in the bulb scales and sugars accumulate (Langens-Gerrits & Miller, 2003). Exposure to low but non-freezing temperatures (4 to 8^0C) induced the net breakdown of starch and the accumulation of sucrose and glucose in lily bulblets regenerated *in vitro* (low temperature sweetening) (Shin *et al.*, 2002). This result the conversion of starch to sugars (Rees *et al.*, 1981). Which indicates that, during this period, preparation for later bulb growth involves mobilization of carbohydrate reserves play an important role in growth and development of the photosynthetic apparatus. Approximately six weeks later, the switch from source to sink took place in the bulblets, which became visible as a deposition of starch inside scales (Langens-Gerrits & Miller, 2003). This indicates that, bulblets growth always exceeded the amount of sucrose taken up.

2 Research Aim, questions and hypotheses

2.1 Research Aim

However, there is no report on starch degradation and growth mechanism during lily bulblets regenerating *in vitro*. The aim of this study is to understand how lily bulblets regenerating from scale explants do grow *in vitro*.

2.2 Research Question

To fulfill the research aim research questions were focused to get better around in progress *in vitro* regeneration and growth of lily bulblets. Those were: a) What is the role of starch in the scale explants? b) Are those starch used for feeding the regenerated bulblets *in vitro*? and c) Do the scale explants grows during culture *in vitro* and has this growth affect bulblets growth?

2.3 Hypotheses

On the basis of research questions and previous findings on related field hypotheses were formulated that, a) Sucrose concentrations in the medium may have influence on lily bulblets regeneration and growth *in vitro*; b) Cold treatment may increase the starch degradation and thus, bulblets regeneration and growth *in vitro*, c) Reserved starch in scale explants may have impact on explants growth and hence, bulblets regeneration and growth *in vitro*.

3 Materials and Methods

3.1 Plant material

Lily cultivars were used for the experiment was *Lilium* oriental hybrids 'Star Gazer' and 'Santander'. Explants were collected from different positions i.e. apical, basal, outer and inner during bulblets regeneration *in vitro* (Figure 1. Schematic drawing of apical and basal explants cut from lily scales; and Figure 2. The Standard micropropagation protocol of lily adapted from De Klerk, 2014). Clean and healthy-looking 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 essentially as described by Langens-Gerrits and De Klerk, 1998. Explants were cultured with their abaxial side on 15 ml per explant of MS-medium (Murashige & Skoog, 1962).

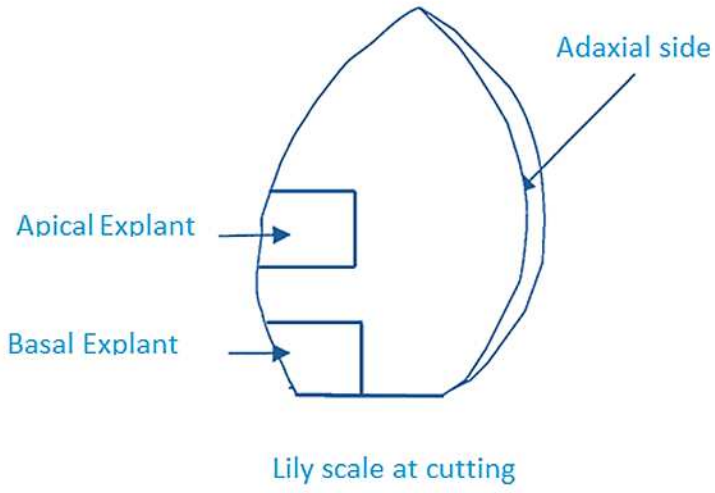

Figure 1. Schematic drawing of apical and basal explants cut from lily scales

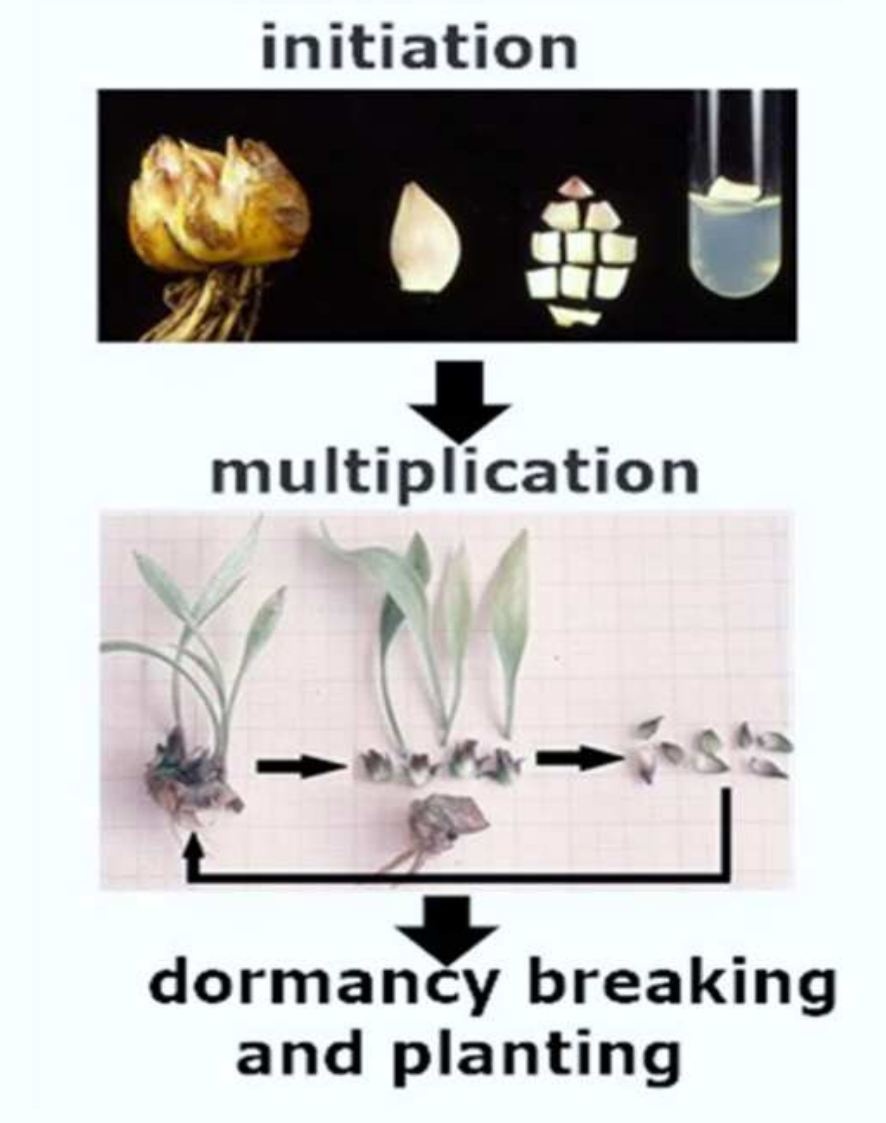

Figure 2. The Standard micropropagation protocol of lily (adapted from De Klerk, 2014)

3.2 Medium preparation

Medium was prepared by 4.4g/L MS medium with vitamins, NAA 50µM/L and 7g/L micro-agar with sucrose (0%; No sucrose, 3%; 30g/L, 6%; 60g/L and 9%; 90g/L) at pH 5.8 adjusted prior to autoclaving; at last autoclaved for 15 minutes.

3.3 Histological observations

Scale explants were fixed in 5% glutaraldehyde in phosphate buffer, pH 6.8, and rinsed in the same buffer. Then, they were dehydrated in an ethanol series and embedded in Spurr's resin. Transversal sections, made on a Sorvall MT 5000 microtome were mounted on glass slides, stained with Toluidin blue to visualize the vascular bundle and with Lugol's IKI solution to visualize starch grains degradation during lily bulblet regeneration in vitro in different scale explant.

3.4 Cold treatment

Lily scale explants was collected from bulblets regenerated *in vitro* at age of 20 weeks were placed on petridish contain filter paper with sterilize water to prevent drying. Then retain in refrigerator at 4^0C for 6 weeks and then scale explants were cultured on 15 ml per explant of MS-medium containing 0% and 3% sucrose.

3.5 Statistical analysis

Data was recorded after a 12-week period without subculture and population size varied between experiments. Results are expressed as mean ± standard deviation (SD). For all comparisons, statistical analysis was performed by a one-tailed paired t-test and probability values less than 0.05 were considered statistically significant.

4 Results

4.1 Effect of explant position on scale and sucrose concentration on regeneration percentage (%)

A lily bulb consists of a compressed stem (the basal plate), to which swollen petioles, the scales are attached (Langens-Gerrits, De Klerk, *et al.*, 2003). During the regeneration period of bulblets various factors influences the growth of bulblets. The effect of explant position on scale and sucrose concentration on regeneration was studied in both cultivars "Star Gazer" and "Santander" to observe the correlation between them. Higher regeneration percentages were observed from basal explants compare to apical explants but there is no such difference in among different sucrose concentrations and cultivars (Figure 3). Thus, explant position on scale influenced the regeneration percentage during regeneration *in vitro*.

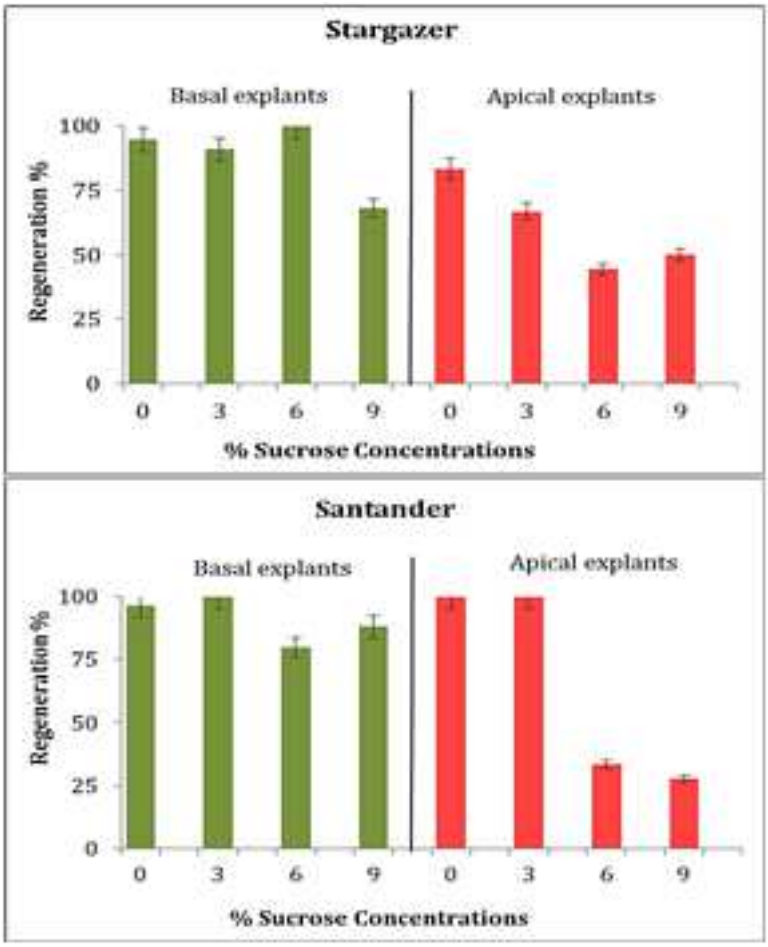

Figure 3. Influence of explant position on scale and sucrose concentration on regeneration percentage; (After 12 weeks *in vitro* culture, the number of sprouted explants was determined. Regeneration was calculated as percentage of the surviving explants with ±SD)

4.2 Effect of explant position on scale and sucrose concentrations on bulblets fresh weight (FW)

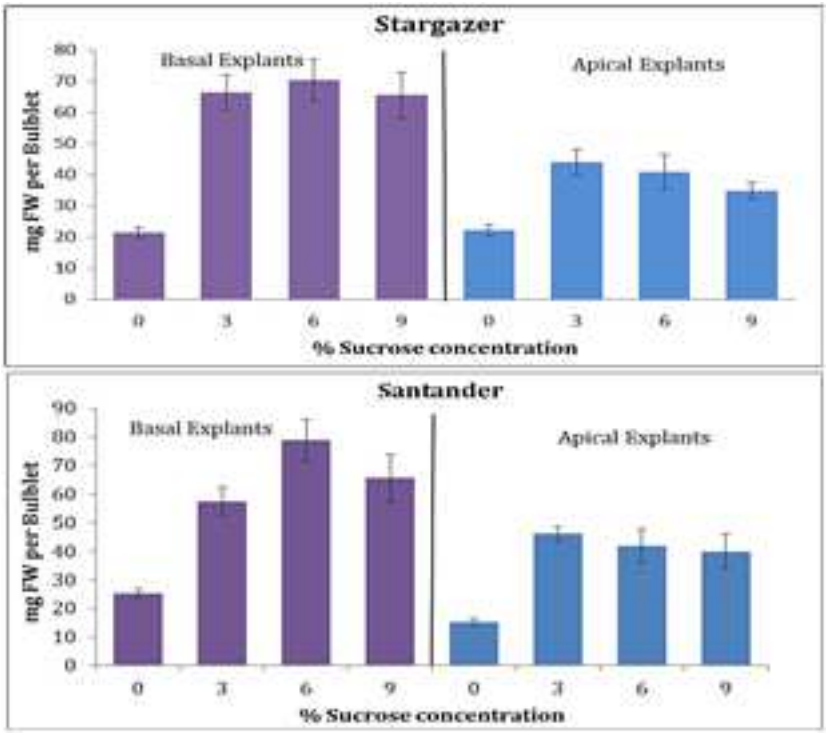

Figure 4. Effect of explant position on scale and sucrose concentration on bulblets growth; (Explants were cultured for 12 weeks under standard condition and the fresh weight of bulblets {mg} was determined. Each bar represents the average FW of bulblets ± SD.)

Bulblets regenerating on scale segments or explants could be categorized into two groups along with their position on the explant: Basal explant and Apical explant. After regeneration of bulblets from both basal and apical explants were taken into account and compared during the 12th week of culturing *in vitro*. Irrespective of cultivars, bulblets on basal explant appeared earlier than apical explant and size of bulblets was higher and accumulated more FW than apical bulblets (Figure 4).

The effect of sucrose concentration was studied in both cultivars and explants position on the scale to examine the correlation between Bulblets FW and sucrose concentrations. However, sucrose might exert its effect mainly during the period of regeneration specially induction period. Regardless of cultivars, there is a linear relation between bulblet FW and sucrose concentrations in terms of both basal and apical bulblets. The higher the sucrose concentrations the size of bulblets was higher and accumulated more FW. At all concentrations, bulblets from basal explant size were significantly higher and accumulated more FW compare to bulblets from apical explant (Figure 4).

Hence, irrespective of cultivars and sucrose concentrations, bulblets on basal explant were higher in size and accumulated more FW than apical bulblets. Thus, the experiments reveal that bulblets position on explant influenced size of bulblets and FW accumulation during the culture period. However, bulblets FW were positively affected by sucrose concentrations also as, sucrose is a major nutritional factor that influence bulblets growth.

4.3 Effect of explant position on scale and sucrose concentration on bulblet number

The influence of explant position on scale and sucrose concentration was evident from the fact that more bulblets regenerated at high than low sucrose concentrations (Figure 5). There was no stimulating effect on type of explants in both cultivars (Figure 5).

In this experiment, the explants from both cultivars were exposed to various sucrose concentrations and thus bulblets number were positively affected by sucrose concentrations as sucrose is a major nutritional factor that exerts its effect during the actual period of induction of bulblets *in vitro* (Langens-Gerrits & Miller, 2003).

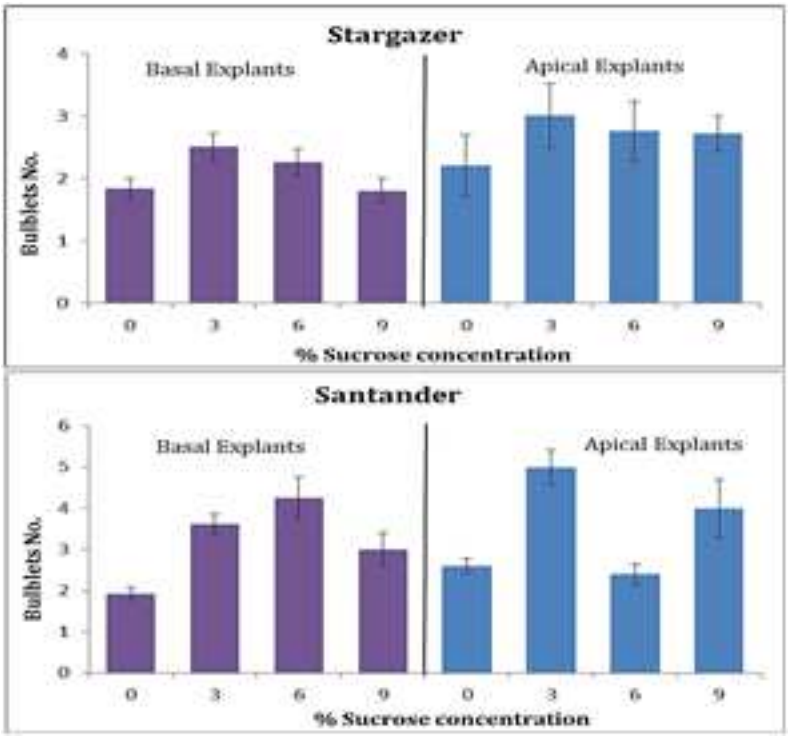

Figure 5. Influence of explant position on scale and sucrose concentration on bulblet number; (Explants were cultured for 12 weeks under standard condition and the number of bulblets was determined. Each bar represents the average number of bulblets ± SD.)

4.4 Effect of explant position on scale and sucrose concentration on leaf and root formation

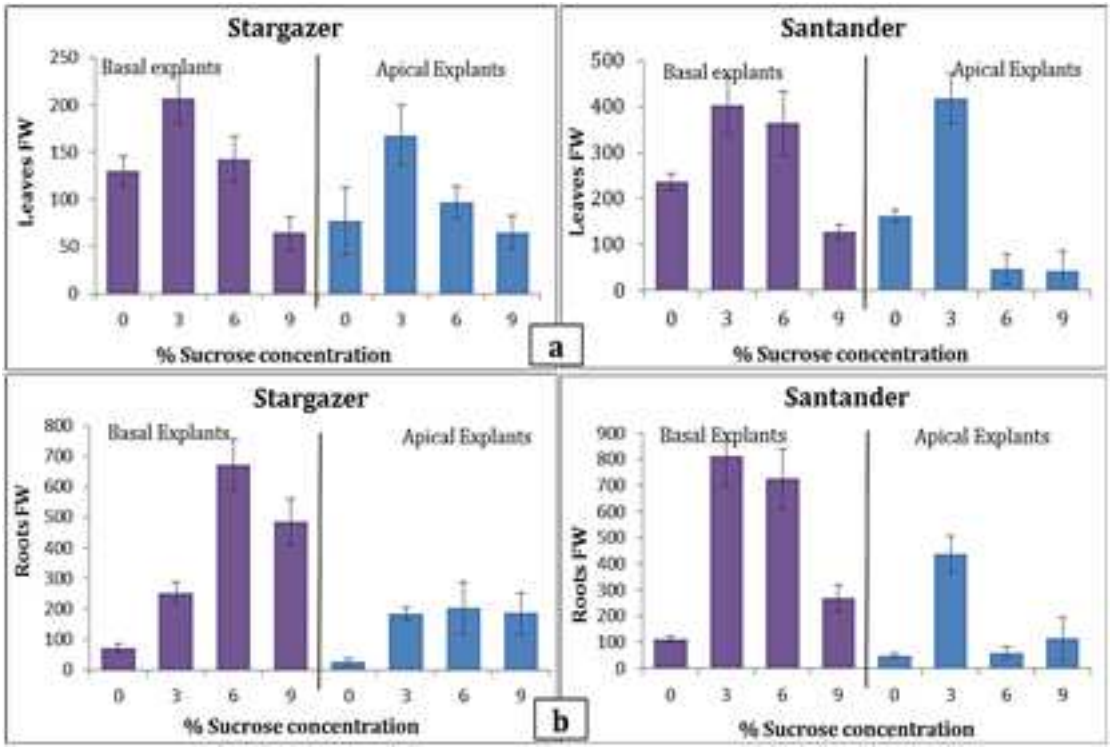

Figure 6. Effect of explant position on scale and sucrose concentration on leaf and root formation; a) Growth of leaves, b) Root growth; (Leaf and root FW {mg} was determined after explants were cultured for 12 weeks under standard condition *in vitro*. Each bar represents the average leaves and roots FW ± SD.)

The stimulating effect of explants position on scale and sucrose concentrations on bulb growth may be due to stimulation of leaf and root growth during regeneration *in vitro*. This was investigated by comparing leaf and root formation among different scale positions, sucrose concentrations and cultivars with bulblets growth (Figure 6) (Table 1).

Average leaves fresh weight (FW) per explant was higher on basal explants at 3 and 6 percent sucrose concentrations. However, cultivars itself also influence the leaves formation (Table 1). Then again, average roots fresh weight (FW) per explant was higher on basal explants with high sucrose concentrations. However, there is no cultivars influence on the roots formation (Table 1). Thus, explants positions on scale, sucrose concentrations and cultivars has influence on leaves and root formation and hence, bulblets growth.

Table 1. Influence of leaf and root formation on bulblets growth *in vitro*

	Leaves FW (mg)	Roots FW(mg)	Bulblet No.	Bulblet FW (mg)
0% Basal, Stargazer	129.9 ± 15.9	70.9 ± 14.3	1.8 ± 0.2	21.4 ± 1.7
3% Basal, Stargazer	206.8 ± 26.4	252.1 ± 35.3	2.5 ± 0.2	66.3 ± 5.6
6% Basal, Stargazer	143.0 ±24.5	673.7 ± 85.0	2.3 ± 0.2	70.3 ± 6.7
9% Basal, Stargazer	64.1 ± 17.7	485.7 ± 75.7	1.8 ± 0.2	65.5 ± 7.3
0% Apical, Stargazer	76.8 ± 35.9	26.2 ± 9.9	2.2 ± 0.5	22.1 ± 1.8
3% Apical, Stargazer	168.0 ± 32.3	184.8 ± 20.4	3.0 ± 0.5	43.9 ± 4.1
6% Apical, Stargazer	97.0 ± 16.9	202.8 ± 86.5	2.8 ± 0.5	40.8 ± 5.7
9% Apical, Stargazer	65.0 ± 18.0	186.1 ± 67.4	2.7 ± 0.3	34.8 ± 2.8
0% Basal, Santander	236.1 ± 17.4	111.4 ± 10.5	1.9 ± 0.2	25.3 ± 1.6
3% Basal, Santander	403.1 ± 62.8	813.7 ± 120.7	3.6 ± 0.2	57.4 ± 4.9
6% Basal, Santander	363.5 ± 70.7	724.9 ± 115.1	4.3 ± 0.5	78.9 ± 7.3
9% Basal, Santander	126.5 ± 17.9	268.5 ± 50.1	3.0 ± 0.4	65.6 ± 8.3
0% Apical, Santander	161.6 ± 14.8	46.2 ± 9.0	2.6 ± 0.2	15.2 ± 1.2
3% Apical, Santander	417.4 ± 58.4	437.4 ± 70.7	5.0 ± 0.4	46.0 ± 2.6
6% Apical, Santander	45.8 ± 32.9	59.8 ± 22.2	2.4 ± 0.2	41.8 ± 5.9
9% Apical, Santander	43.2 ± 23.2	113.6 ± 82.7	4.0 ± 0.7	40.0 ± 6.1

(* 0, 3, 6 and 9%, sucrose concentrations; Basal and Apical, Explants position on scale; Cultivars, Stargazer and Santander)

4.5 Effect of explant position on scale and sucrose concentrations on explants fresh weight (FW)

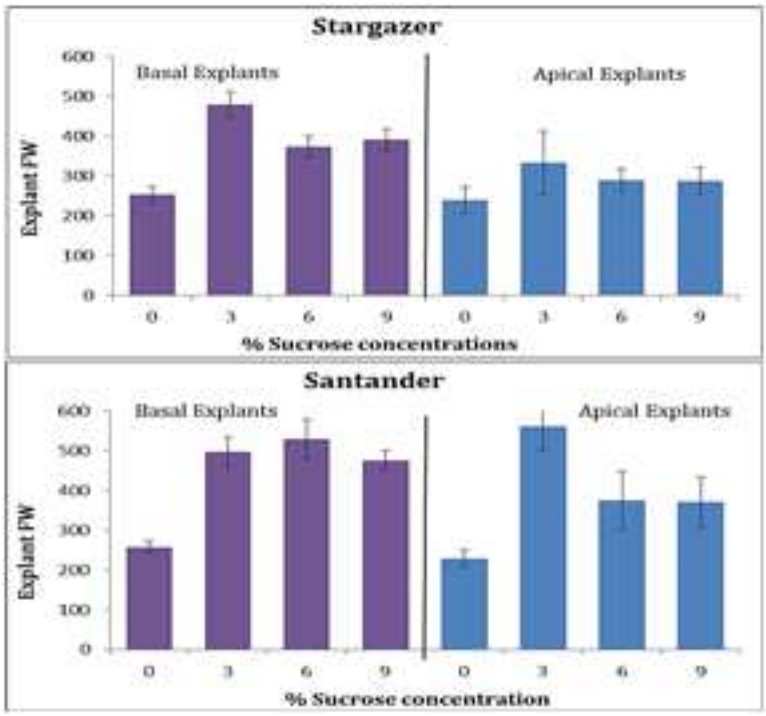

Figure 7. Influence of explant position on scale and sucrose concentration on explants growth; (After 12 weeks cultured under standard condition *in vitro* explants FW {mg} was determined. Each bar represents the average FW of explants ± SD.)

After 12 weeks culture *in vitro*, the size or FW of the explants themselves also increased during culture period (Figure 7). Apparently, the explants were a sink that accumulated nutrients from the medium and thus show that bulblets weight was positively affected by explants increased weight with sucrose concentrations (Table 2). Apparently, there was no influence on explants FW due to positions on scale and cultivars.

Table 2. Influence of explants growth on bulblets *in vitro*

	Explants FW (mg)	Bulblet No.	Bulblet FW (mg)
0% Basal, Stargazer	253.6 ± 19.7	1.8 ± 0.2	21.4 ± 1.7
3% Basal, Stargazer	479.6 ± 33.5	2.5 ± 0.2	66.3 ± 5.6
6% Basal, Stargazer	373.6 ± 25.8	2.3 ± 0.2	70.3 ± 6.7
9% Basal, Stargazer	390.9 ±26.5	1.8 ± 0.2	65.5 ± 7.3
0% Apical, Stargazer	239.4 ± 33.6	2.2 ± 0.5	22.1 ± 1.8
3% Apical, Stargazer	333.2 ± 78.2	3.0 ± 0.5	43.9 ± 4.1
6% Apical, Stargazer	289.3 ± 29.0	2.8 ± 0.5	40.8 ± 5.7
9% Apical, Stargazer	287.0 ± 35.0	2.7 ± 0.3	34.8 ± 2.8
0% Basal, Santander	257.1 ± 16.5	1.9 ± 0.2	25.3 ± 1.6
3% Basal, Santander	496.1 ± 37.4	3.6 ± 0.2	57.4 ± 4.9
6% Basal, Santander	528.4 ± 48.6	4.3 ± 0.5	78.9 ± 7.3
9% Basal, Santander	475.3 ± 23.9	3.0 ± 0.4	65.6 ± 8.3
0% Apical, Santander	228.0 ± 21.6	2.6 ± 0.2	15.2 ± 1.2
3% Apical, Santander	560.8 ± 58.7	5.0 ± 0.4	46.0 ± 2.6
6% Apical, Santander	374.4 ± 73.7	2.4 ± 0.2	41.8 ± 5.9
9% Apical, Santander	370.4 ± 63.0	4.0 ± 0.7	40.0 ± 6.1

(* 0, 3, 6 and 9%, sucrose concentrations; Basal and Apical, Explants position on scale; Cultivars, Stargazer and Santander)

4.6 Contribution of starch in relation to explant position on scale and sucrose concentration on bulblets growth

According to Langens-Gerrits *et al.*, 2003; sucrose accumulated and stored in the explants and used for bulb growth later during culture *in vitro*. The growth of bulblets and explants in relation to explant position on scale and sucrose concentrations, led to the question, how much carbohydrate (mainly starch) reserve present in tissue. The reserves of starch, in freshly cut explants and after bulblet regeneration and growth at 12 week are shown in Figure 8. The uptake of sucrose during culture period *in vitro* was evident form the experiment as there is clear difference between fresh cut and after 12 weeks cultured explants (Figure 8). Freshly cut explants were covered with densely black colored starch grains and after uptake during bulblets growth it is sparsely scattered less starch grains in the tissue. Hence, the turnover strongly clears the contribution that starch grains were taken up and used during regeneration and growth of bulblets.

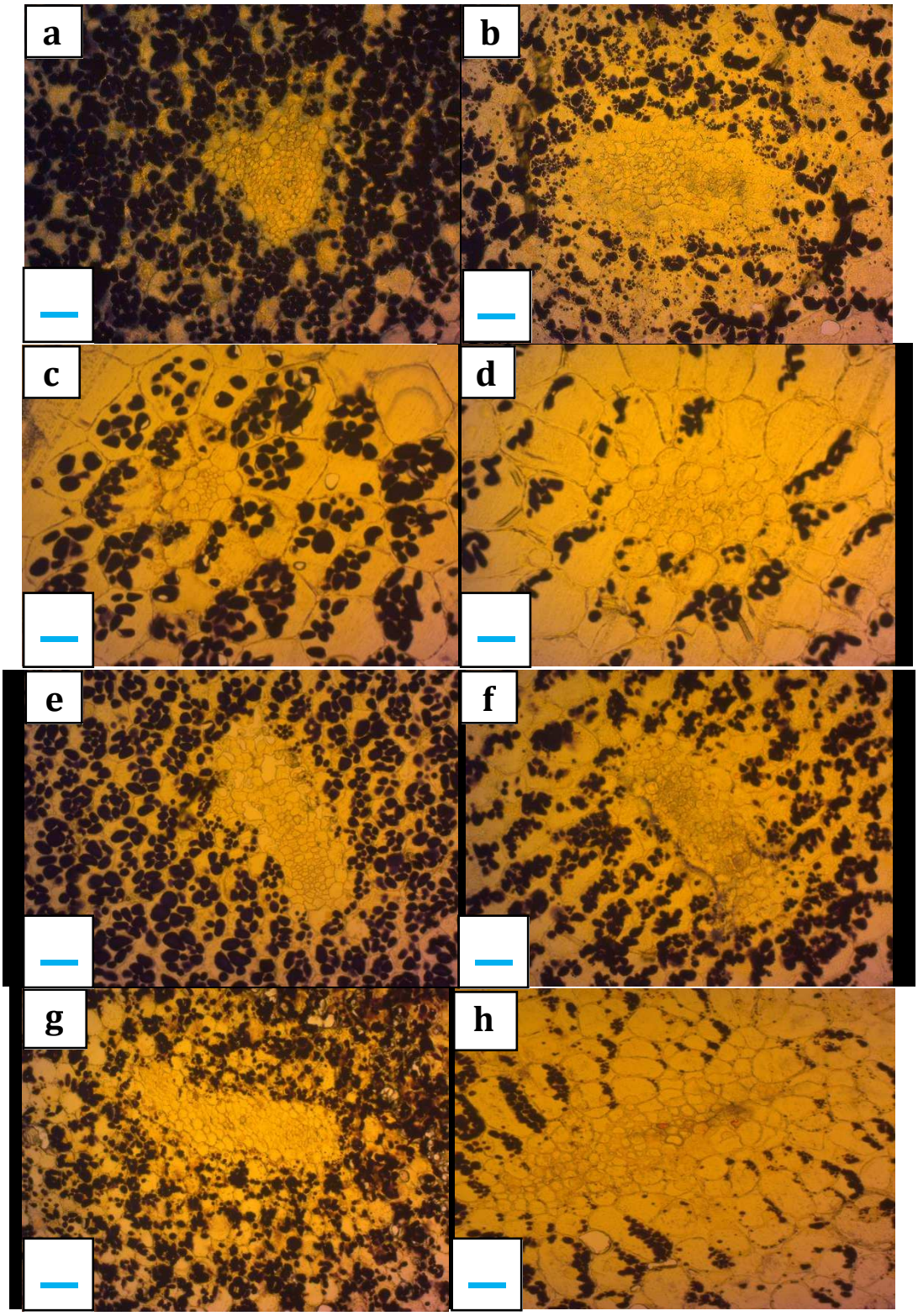

Figure 8. Distribution of starch in relation to explants position on scale and cultivars on bulblets growth; a) Freshly cut basal explants of Stargazer; b) Basal explants of stargazer after 12 weeks cultured in vitro; c) Freshly cut apical explants of Stargazer; d) Apical explants of stargazer after 12 weeks cultured in vitro; e) Freshly cut basal explants of Santander; f) Basal explants of Santander after 12 weeks cultured *in vitro;* g) Freshly cut apical explants of Santander; h) Apical explants of Santander after 12weeks cultured in vitro; Bar — 5 µm in a, b, e, f, g and 25 µm c, d, h.

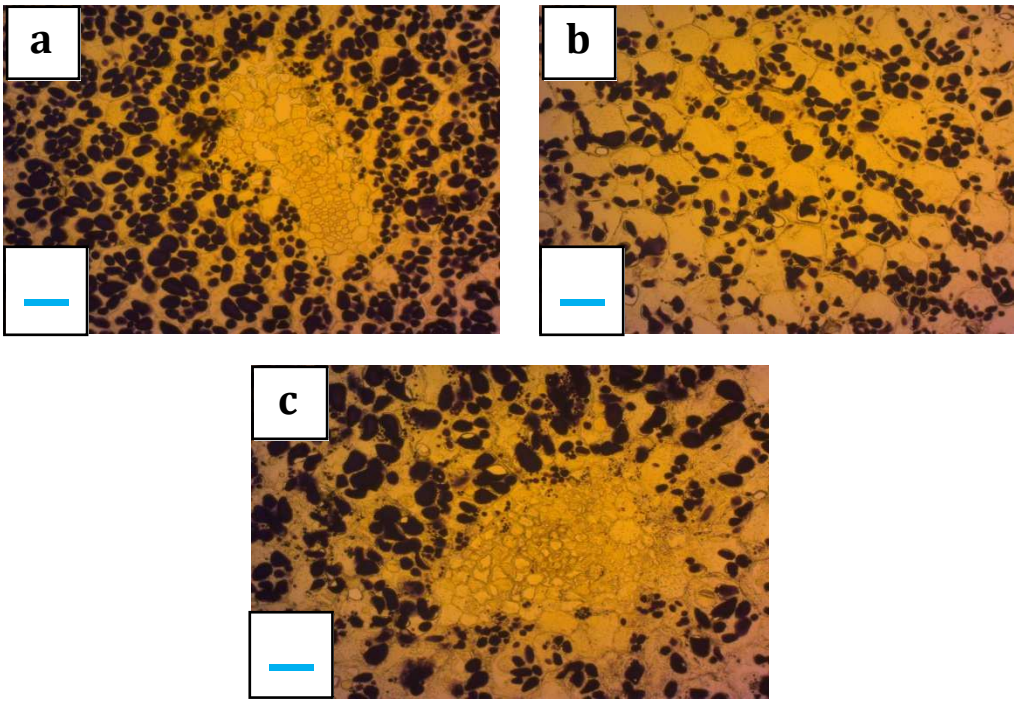

Figure 9. Distribution of starch in relation to sucrose concentrations on bulblets growth; a) Freshly cut basal explants of Stargazer; b) After 12 weeks cultured *in vitro* with 3% sucrose; c) After 12 weeks cultured *in vitro* with 9% sucrose; Bar — 5 µm in a, b and c.

4.7 Relation between bulblets FW and explants starch content

The effect of starch concentration on uptake was studied after 12 weeks culture *in vitro*. During the whole culture period starch from the medium was accumulated by the explant and there were differences in tissue between apical and basal explants regardless the cultivar. Bulblets growth was always higher with the amount of starch and the contribution of medium was stable during culture period but it was higher on basal explants than apical ones (Figure 8 and 9). Thus, histological observation shows that bulblets use explants reserves for growth which explants accumulated from medium.

Vascular bundles inside explants tissue raised the question whether the uptake of sucrose inside the explants correlates with regeneration and bulblets growth activity. For this reason, in both types freshly cut explants and 12 week old in vitro cultured explants were histologically observed and compared. The increase of size and number of vascular bundle after culture for 12 weeks *in vitro* indicates that there is definite relationship between starches in explants tissue and explants growth and bulblets FW (Figure 10) (Table 3 and 4).

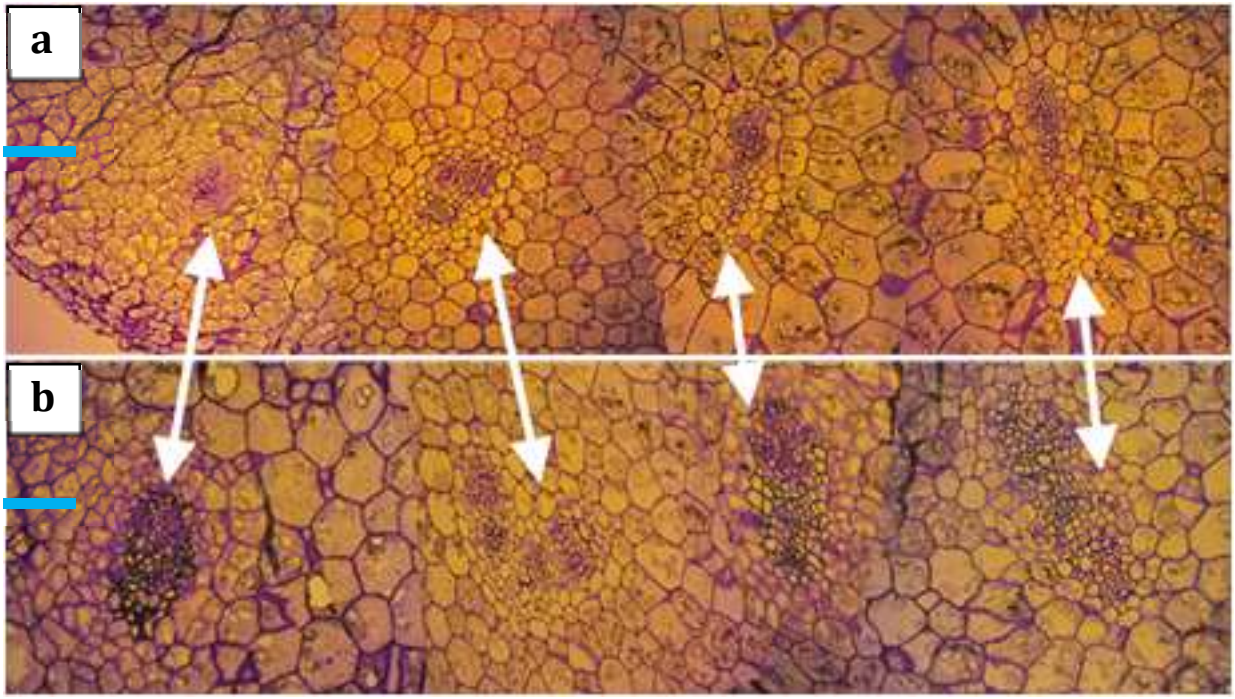

Figure 10. Growth of vascular bundles in relation to starch uptake from medium; a) vascular tissue in freshly cut basal explants of Stargazer; b) Increased and number size of vascular bundle after 12 weeks cultured *in vitro* with 3% sucrose; Bar — 25 µm in both and b.

Table 3. The number of Vascular bundles in basal and apical scale explants

	Week 0	SD (±)	Week 12	SD (±)	t-test
Basal scale explant	6.0	0.2	8.0	0.2	0.00
Apical scale explant	3.3	0.2	4.0	0.0	0.01

(* SD (±); Standard Deviation, In column t-test; P value < 0.05 means Significant and > 0.05 NS)

Table 4. The number of vascular bundles in basal scale explants cultured on different concentrations of sucrose in week 0 and 12

	Week 0	SD (±)	Week 12	SD (±)	t-test
0% sucrose concentration	4.8	0.3	5.0	0.0	0.20
3% sucrose concentration	4.8	0.4	6.4	0.2	0.00
9% sucrose concentration	4.8	0.3	6.5	0.3	0.00

(* SD (±); Standard Deviation, In column t-test; P value < 0.05 means Significant and > 0.05 NS)

4.8 Effect of cold treatment on regeneration percentage (%)

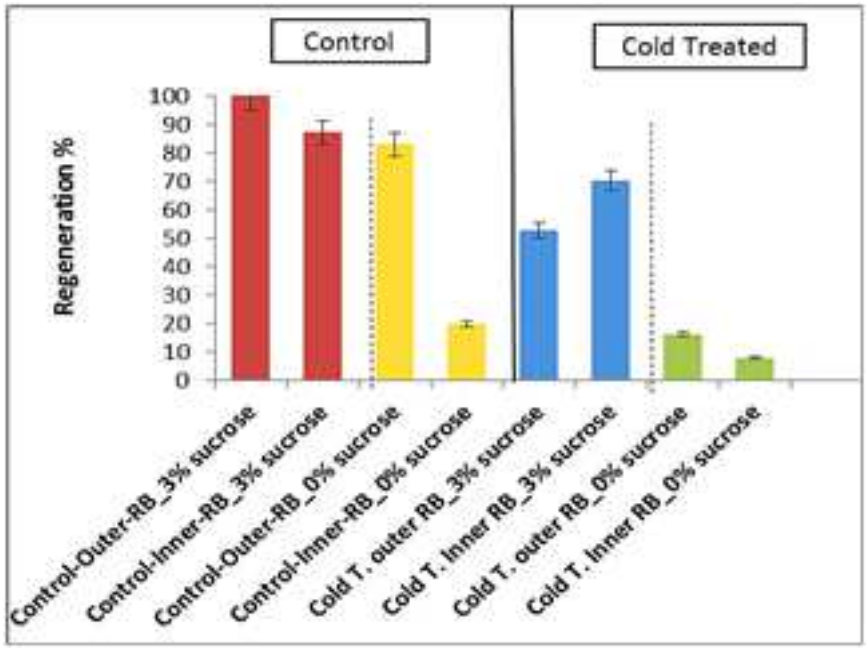

Figure 11. Influence of cold treatment on regeneration percentage; (After 6 weeks at 4^0C explants were cultured 12 weeks *in vitro*, the number of sprouted explants was determined. Regeneration was calculated as percentage of the surviving explants with ±SD)

20 weeks old regenerated bulblets of Star Gazer were taken and cultured *in vitro* for 12 weeks with cold treatment and control and inner and outer scales. The maximum percentage of regeneration was in control or non-cold-treated scales with sucrose and outer scales (Figure 11). Thus, temperature, sucrose and scale position all three has influence regeneration of lily.

4.9 Effect of cold treatment on bulblets growth

The effect of cold treatment on bulblets growth was studied by comparing bulblets size and numbers with control and cold-treated scales culture *in vitro* for 12 weeks (Figure 12). Bulblets regenerated *in vitro* after cold treatment was very small in size compared to control regardless of outer and inner scales. In relation to sucrose bulblets FW or size was higher with sucrose regardless of control and cold treatment and outer and inner scales. In contrast, we also found unchanged results for bulblets number (Figure 12).

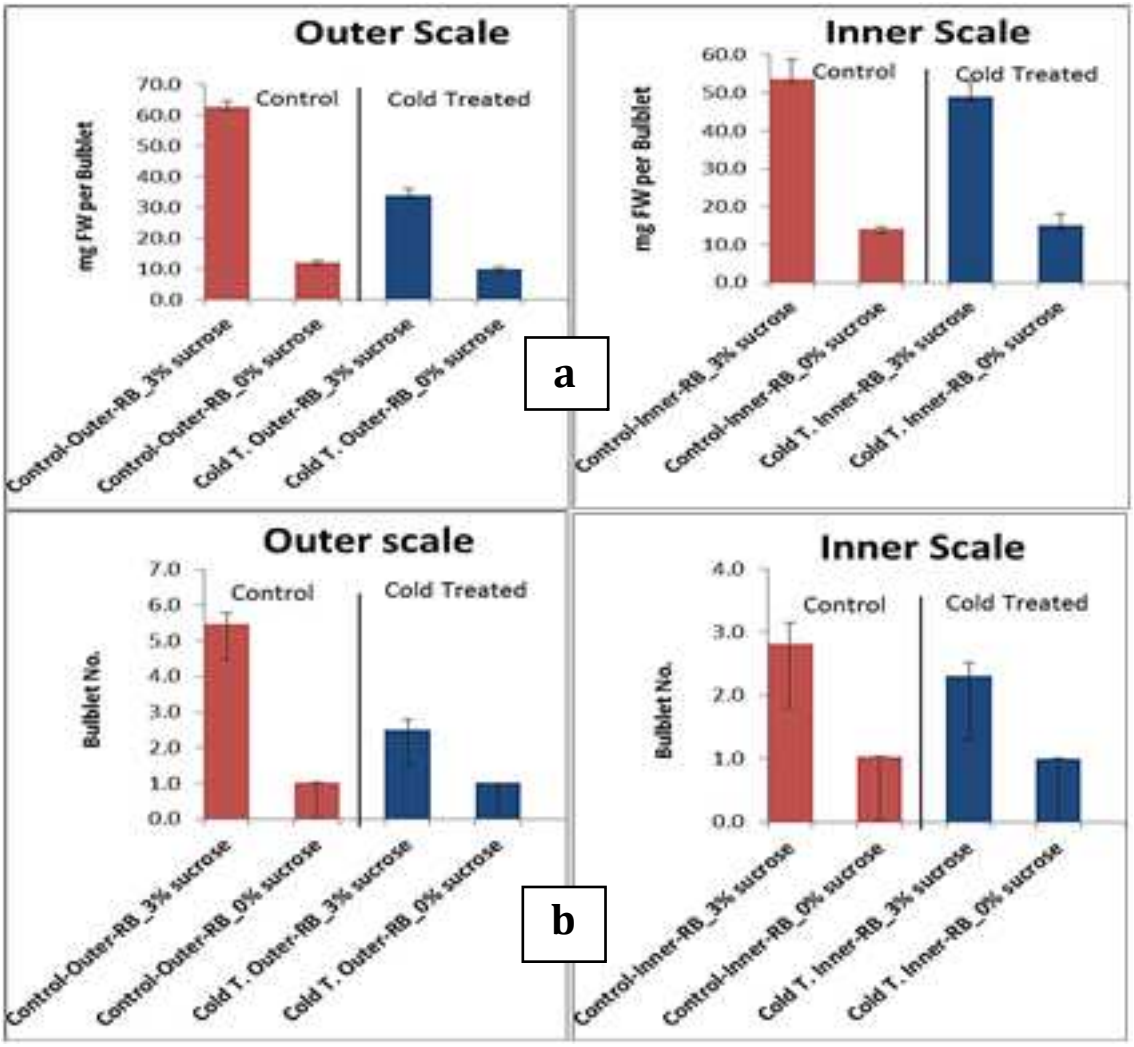

Figure 12. Influence of cold treatment on bulblets growth; a) Size of bulblets (mg); b) Number of bulblets; (Each bar represents the average number ± SD)

4.10 Effect of cold treatment on leaf and root formation

This was investigated by comparing leaves and roots FW between control and cold-treated scales cultured *in vitro* for 12 weeks (Figure 13). Average leaf FW per explant was higher in control explants with sucrose regardless of outer and inner scales. However, leaves FW of outer scales is much higher in control compare to cold-treated scales with sucrose but similar without sucrose. Alternatively, leaves FW of inner scales was approximately similar in control and cold-treated scales. In contrast, we also found there were unchanged results for roots FW (Figure 13).

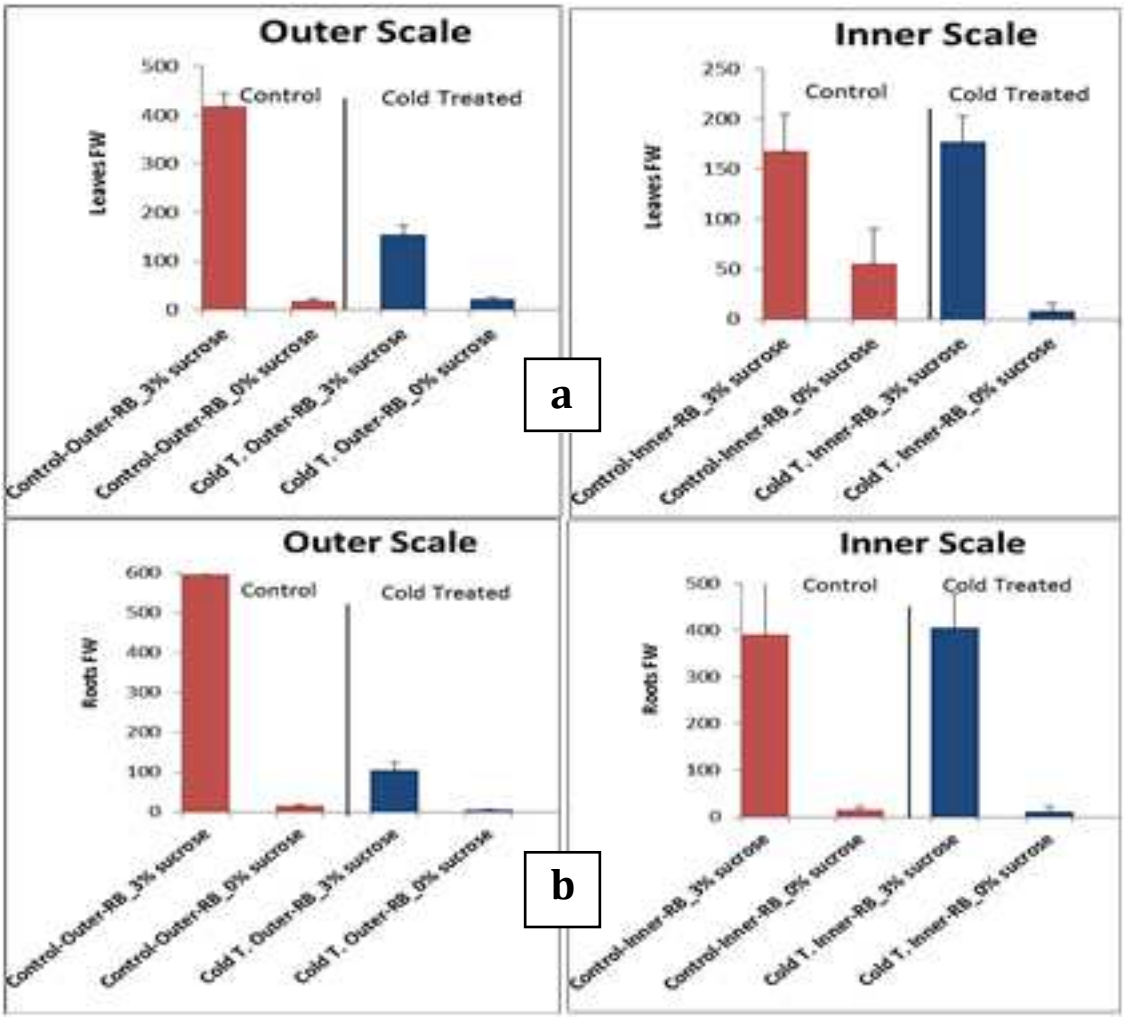

Figure 13. Effect of cold treatment on leaves and root formation; a) Growth of leaves, b) Roots growth; (Leaves and roots FW {mg} was determined after explants were cultured for 12 weeks under standard condition *in vitro*. Each bar represents the average leaves and roots FW with ± SD.)

4.11 Effect of cold treatment on scale explants fresh weight

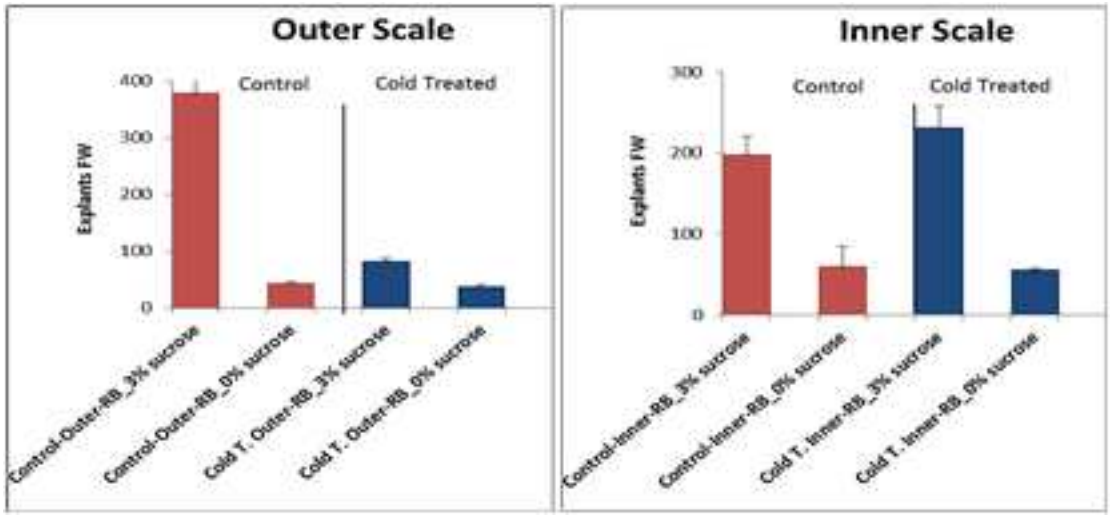

Figure 14. Effect cold treatment on explants growth; (After 12 weeks cultured *in vitro,* explants FW {mg} was determined. Each bar represents the average FW of explants with ± SD.)

After 12 weeks culture *in vitro*, the size or FW of the explants themselves also increased during culture period (Figure 14). However, scale explants FW of outer scales is much higher in control compare to cold-treated scales with sucrose but similar without sucrose. Alternatively, scale explants FW of inner scales were approximately similar in control and cold-treated scales.

4.12 Combined effect of cold treatment and sucrose in bulblets growth

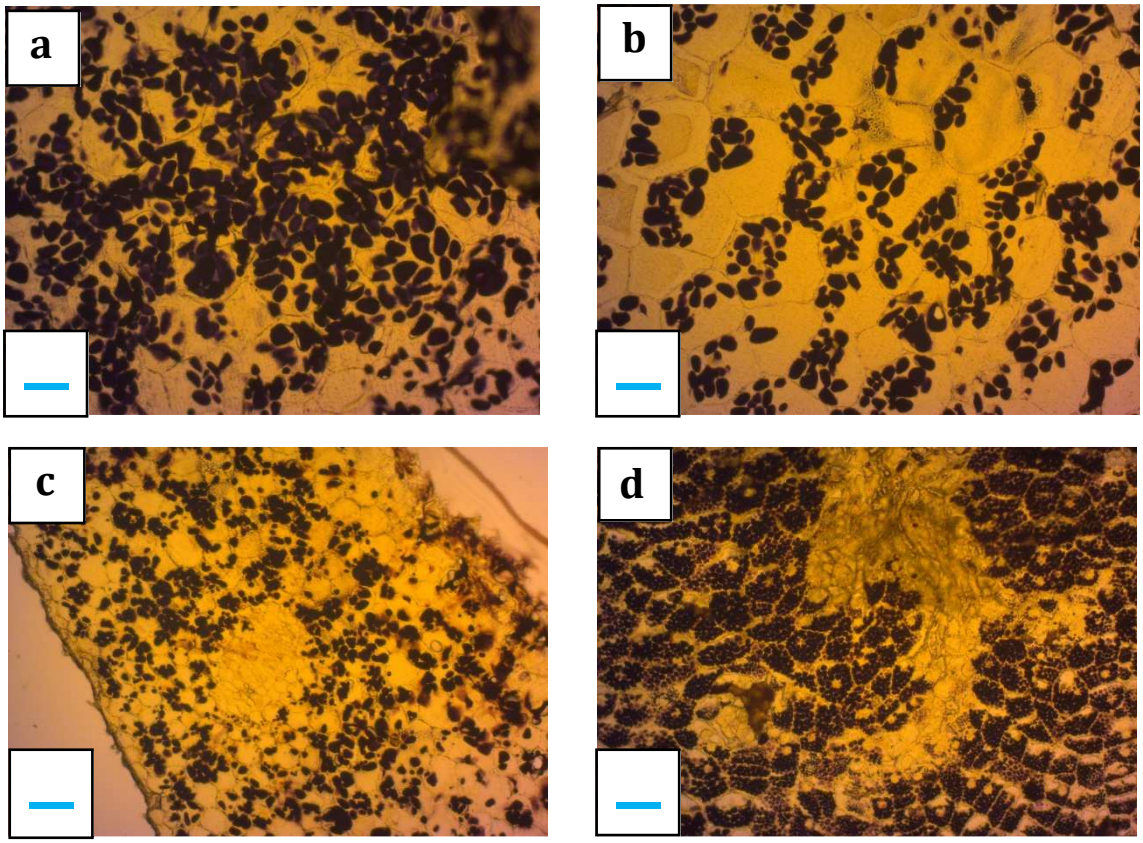

Figure 15. Distribution of starch in relation to cold treatment on bulblets growth; a) Freshly cut control explants of Stargazer; b) Control explants after 12 weeks cultured in vitro without sucrose; c) Freshly cut cold-treated explants of Stargazer; b) Cold-treated explants after 12 weeks cultured in vitro with sucrose; Bar — 25 µm in a, b and 5 µm in c, d.

The responses of regenerated bulblets to the cold treatment and sucrose suggest that dormancy and slowdown or stoppage of bulb growth processes together. To investigate this point in more detail, explants were histologically observed. The results indicate that the starch content of cold-treated explants were fully equivalent before and after culture *in vitro* (Figure 15). This suggests that cold treatment may play a role in the natural process of induce dormancy. This result reflects on bulblets growth also. However, after dormancy breaking with high temperature (25^0C) they perform better in

soil (Langens-Gerrits & Miller, 2003). In contrast, we also found unchanged result for vascular bundle (Figure 16) (Table 5). There is a minor increase in sizes of vascular bundles and nearly no increase in numbers. This also suggests that cold treatment may play a role in the natural process of induce dormancy.

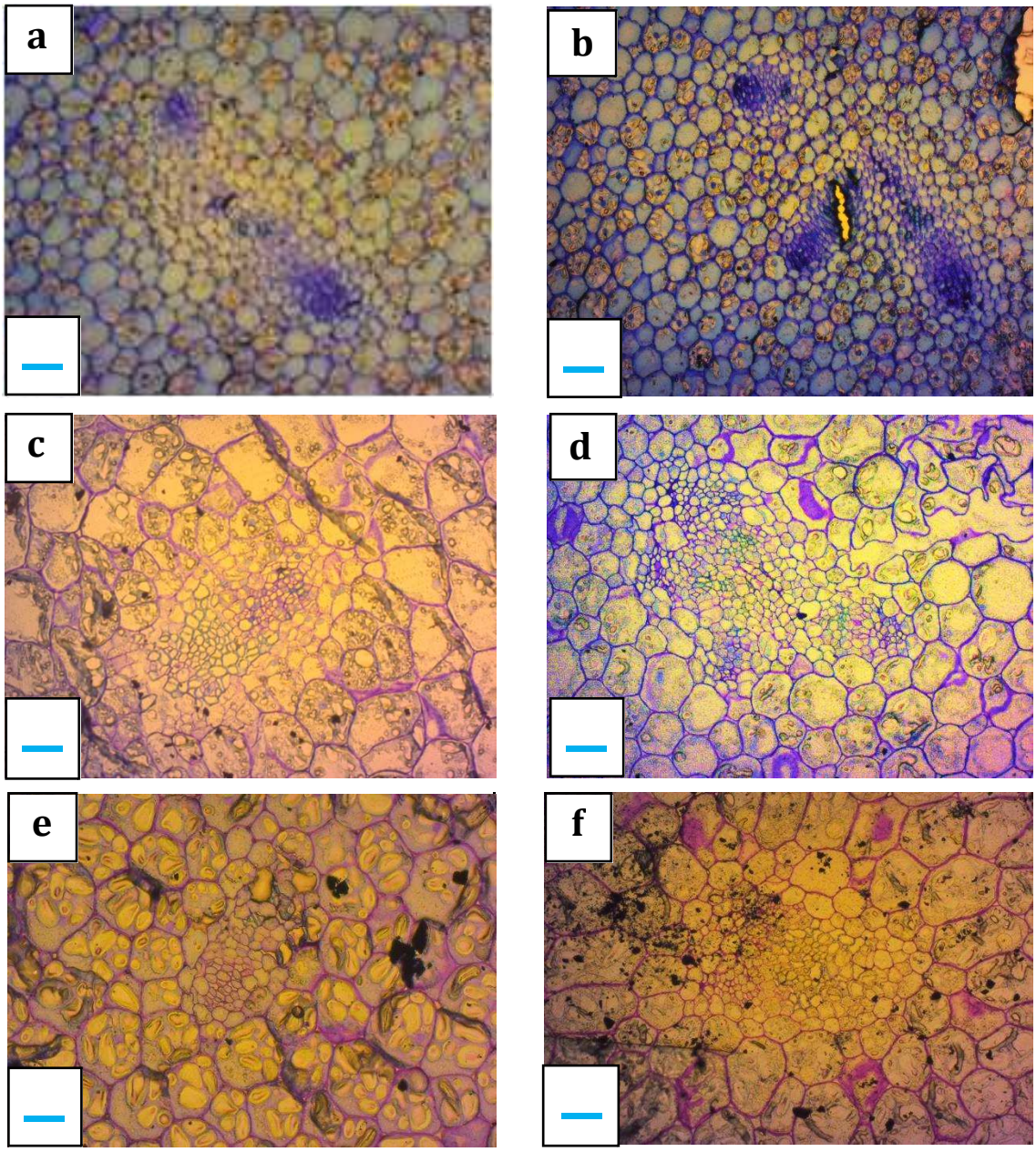

Figure 16. Growth of vascular bundle in relation to cold treatment and starch uptake from medium; a) vascular tissue in freshly cut cold-treated explants of Stargazer; b) Increased size and number of vascular bundle in cold-treated explant after 12 weeks cultured *in vitro* with 3% sucrose; c) vascular tissue in freshly cut control explants of Stargazer; d) Increased size of vascular bundle in control explant after 12 weeks cultured *in vitro* with 3% sucrose; e) vascular tissue in freshly cut explants of Stargazer; f) Increased size of vascular bundle after 12 weeks cultured *in vitro* with 0% sucrose; Bar — 5 µm in a, b and 25 µm in c, d, e, f.

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Table 5. The number of vascular bundle in cold-treated and non-cold-treated regenerated, outer scale explants

	Week 0	SD (±)	Week 12	SD (±)	t-test
Cold-treated explants	3.3	0.3	3.5	0.3	0.20
Non cold-treated explants	3.4	0.2	4.8	0.4	0.00

(* SD (±); Standard Deviation, In column t-test; P value < 0.05 means Significant and > 0.05 NS)

4.13 Effect of cold treatment on starch mobilization

The slowdown or stoppage effect of cold treatment on regeneration and bulblets growth may be due to immobilization of starch to be used for development of photosynthetic apparatus (leaves) after culture *in vitro*. Histological observations were done in comparison with freshly cut scales after 12 weeks culture *in vitro*. The results indicate that starch content mainly changed in non-cold-treated scales and coincides with sucrose content in medium (Figure 15). Nearly no or very small change in starch content occurred in cold-treated scales during culture *in vitro*. The change in starch content is similar in both outer and inner scales. Thus, starch mobilization contributes significantly to the growth of bulblets.

5 Discussion

The regeneration of lily bulblets on scale explants tissue was studied *in vitro*. The main factors determining the regeneration of bulblets are explants position on scale and sucrose concentrations in the medium. Explants position on scale is the main factor that positively affected regeneration (Figure 3). Higher regeneration percentages were observed from basal explants compare to apical explants. Changing of other components, such as sucrose concentrations and cultivars had very little effects. Often, the number of regenerating organs varies with the regeneration zone (Pierik and Van Post, 1975) or with size of an explant (Langens-Gerrits, Kuijpers, *et al.*, 2003 and Van Aartrijk and Blom-Barnhoorn, 1983). This indicates that, internal transport of sucrose from the apical to the basal side play the vital role and hence, influences bulblets growth on basal side of explants. However, sucrose taken up from the medium is mainly stored in the explant; a smaller percentage is used for regeneration and bulb growth (Langens-Gerrits *et al.*, 2003).

Discussion on explants position on scale:

In our study, bulblets on basal explants were always larger than those on apical explants, even with different sucrose concentrations (Figure 4). Contribution of sucrose to bulblets growth is shown also by the observation that an increase in sucrose concentration stimulates bulblet growth (Figure 4). Regardless of cultivars, bulblets grew larger on basal explants also. This suggests that some components in explant tissue that support bulb growth cannot be synthesized from medium compounds in sufficient amounts. Sucrose taken up in the explant was mainly recovered at the basal side of the explant, where regeneration occurs (Langens-Gerrits, Kuijpers, *et al.*, 2003). This indicates that uptake mainly occurs at that side of the explant. Internal transport of sucrose from the apical to the basal side may also play a role. In contrast, when lilies are propagated by culturing complete scales in a moist environment ('scaling'), starch mobilization proceeds from the apical to the basal region (Miller, 1990). Hence, the better bulb growth on basal explants reflects the higher contribution of explant reserves. Thus, also put forward that culture of scale explants on a high sucrose concentration *in vitro* for larger bulblets.

Discussion on sucrose concentration:

As bulb growth was stimulated by a high sucrose concentration. The contribution of the medium and the explant reserves to bulb growth were histologically observed in basal and apical explants using different sucrose concentrations (Figure 8 and 9). Bulblets regenerating from scale segments *in vitro* use two nutrient sources for growth: the explant and the culture medium. Sucrose was mainly taken up through the cut surfaces *(Langens-Gerrits, Kuijpers, et al.,* 2003). Preliminary data showed that on basal scale explants, larger bulblets regenerated than on apical ones (Figure 4). We hypothesize that bulblet formation on basal scale explants is mainly due to reserve starch content than that on apical explants. To investigate this, we determined the contribution of the starch in the medium and in the scale explants to bulblets growth in histological observation. There is a strong relation between bulblets growth and starch content in explants tissue. Furthermore, according to Langens-Gerrits *et al.,* 2003; "Sucrose taken up in the explant was mainly recovered at the basal side of the explant, where regeneration occurs". This indicates that, internal transport of sucrose from the apical to the basal side play the vital role. Hence, starch content stimulates bulblets growth. If elements other than starch influence bulblets growth, which is not reflected when changes in cultivars *in vitro*. Such as, in case of both cultivars result is identical. The positive effect of sucrose on bulblets size and stem formation in lily *in vitro* has been described earlier (Takayama *et al.,* 1982).

Discussion on cold treatment:

The effect of low temperature on regeneration and further bulblets growth was investigated in 20 weeks old regenerated bulblets of Star Gazer. The maximum percentage of regeneration was in control or non-cold-treated scales with sucrose and outer scales (Figure 11). Plants develop dormancy to survive adverse climatic conditions (Vegis, 1964 and Villiers, 1975). Dormancy in flower bulbs is regarded as a period during which there are no apparent external morphological changes (Langens-Gerrits *et al.,* 2001). Most lilies develop dormancy to survive a cold winter; their bulbs sprout and grow during spring and summer. On the other hand, dormancy possibly will decrease propagation rates (De Klerk and Langen-Gerrits, 1996).

The effect of cold treatment on bulblets growth was studied by comparing bulblets size and numbers with control and cold-treated scales culture *in vitro* for 12 weeks (Figure 11 and 12). Bulblets regenerated *in vitro* after cold treatment was very small in size compare to control regardless of outer and inner scales. A cold treatment of six weeks leads to the responses of regenerated bulblets to the cold treatment and sucrose suggest that dormancy and slowdown or stoppage of bulb growth processes together. That slowdown or stoppage effect of cold treatment on regeneration and bulblets growth may be due to immobilization of starch to be used for development of photosynthetic apparatus (leaves) after culture *in vitro*. Mobilization of carbohydrates during the low temperature may not only decrease propagation rates, but also bulblets growth (De Klerk and Langen-Gerrits, 1996).

In the study, results indicate that starch content mainly changed in non-cold-treated scales and coincides with sucrose content in medium (Figure 15). Nearly no or very small change in starch content occurred in cold-treated scales during culture *in vitro*. Result also shows that, in relation to sucrose bulblets size and numbers was higher with sucrose regardless of control and cold treatment and outer and inner scales (Figure 12). That means low temperature slow down bulblets growth *in vitro* during culture period due to rapid immobilization of carbohydrate reserves. After the cold treatment only little amount of the starch in explants are broken down (Figure 15). Possibly, after a longer cold treatment, negative effects of the cold start to dominate. Hence, sprouting and emergence cannot be stimulated any further.

Discussion on influence of leaf formation:

Leaf emergence is influenced by explants positions on scale and sucrose concentrations (Table 1) (Figure 13). Lily bulblets are regenerated from bulb scale segments in vitro and then produce efficiently photosynthesizing leaves so that bulblets increase rapidly in size. After leaf emergence, subsequent growth of the bulblets is preceded by a source to sink transition in the bulb (Langens-Gerrits *et al.*, 2003). Hence, it is significant to know that leaves emergence and growth has positive influence on bulblets growth *in vitro*.

Discussion on explants growth:

In our study, after 12 weeks culture *in vitro*, the size or FW of the explants themselves also increased during culture period (Figure 7 and 14). Vascular bundles inside explants tissue raised the question whether the uptake of sucrose inside the explants correlates with regeneration and bulblets growth activity. Explants were histologically observed and compared (Figure 10 and 16). The increase of size and number of vascular bundle after culture for 12 weeks *in vitro* indicates that there is definite relationship between starches contain in explants tissue and explants growth and bulblets FW (Table 2). Plant vascular systems are usually composed of phloem and xylem. In the plant body the vascular tissues are formed from embryonic tissues, called vascular meristems, whose cells retain the ability to divide and continually multiply. This continuous development of new vascular tissues enables regeneration of the plant and its adaptation to interruptions and changes in the environment (Aloni, 1987). This occurs around wounds also. In the intact plant, xylem differentiates only in the presence of phloem, though phloem often develops in the absence of xylem. In the young organs of intact plants, the phloem always differentiates before the xylem. This pattern of vascular development is also true in tissue culture conditions as well as in vascular regeneration around a wound (Aloni, 1987).

On the other hand, Wetmore & Rier, 1964 have shown that in tissue culture of Syringa, application of a sugar together with the auxin promote vascular differentiation. Wright & Northcote, 1972 also found that in callus of sycamore trees, the vascular differentiation occurred when any sugar promoting good growth was used. Hence, it can be concluded that sucrose and auxin (NAA) act as stimulus to increase size and number of vascular bundle (vascular differentiation) in lily scale explants regenerated *in vitro* in this study.

6 Conclusion

In conclusion, main factors determining growth of bulblets *in vitro* prove to be explants position on scale and sucrose concentration in the medium. On a basal explant, large size bulblets regenerate and bulblets growth stimulated by sucrose concentration in the medium. This phenomenon point out that internal transport of sucrose inside lily scales from apical to basal side plays a vital role. Furthermore, these two factors act together, as these effects are similar on both cultivars; Star Gazer and Santander.

Furthermore, Bulblets growth also correlates with the uses of starch granules inside explants tissue and in the medium. Mobilization of carbohydrate reserves (mainly starch) plays an important role in growth and development of the photosynthetic apparatus (leaves) and then which influence bulblets growth. From the histological observation it is clear that, bulblets use explants reserves for growth which explants accumulated from the medium. Hence, the turnover of starch granules inside explants tissue were uptaken and used during regeneration and growth of bulblets *in vitro*.

On the other hand, the response of regenerated bulblets to the cold treatment and sucrose suggest that dormancy and slowdown or stoppage of bulb growth processes together. This slowdown or stoppage effect of cold treatment on sprouting and bulblets growth is due to immobilization of starch to be used for development of photosynthetic apparatus (leaves) after culture *in vitro*. Histological observation of this findings indicate that, nearly unchanged starch content of cold-treated explants during culture *in vitro* play a similar role as the natural process of induce dormancy and later bulblets growth.

Plant vascular systems are usually formed from vascular meristems and have the ability to frequent divide and multiply and this occurs around wounds also in tissue culture. The increase of size and number of vascular bundles inside explants tissue *in vitro* put forward that there is definite relationship between starch reserve and explants growth and bulblets FW. However, application of a sugar together with the auxin promotes vascular differentiation. Therefore, it can be concluded that sucrose and auxin (NAA) act as stimulus to increase size and number of vascular bundle (vascular differentiation) in lily scale explants regenerated *in vitro*.

From horticultural point of view, it is advisable to culture lily scale explants on high sucrose concentration during regeneration of bulblets *in vitro*. Physiological factor such as basal explants influence sprouting and later bulblets growth. The role of increased size of explants, vascular tissues and number of vascular bundle forms an interesting subject for further research as they revealed a positive influence on bulblets growth in this study.

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