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ORIGINAL ARTICLE

The role of scale explants in the growth of regenerating lily bulblets in vitro

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Abstract

Lily scale-explants cultured in vitro regenerate adventitious bulblets at their base. Large scale-explants (6 × 18 mm; the basal side is 6 mm) yielded more (26%) bulblet growth than small ones (6 × 6 mm). The beneficial effect of the scale was also clear when bulblets excised from scale explants were transferred to fresh medium for additional growth. When a small piece of the original scale was left attached to these bulblets, growth increased by 33%. The growth of bulblets was highest in explants cut from the middle of a scale as opposed to the edge, and in explants cut from the basal half as opposed to the apical half. We examined the development of the scale-explants during the period of bulblet regeneration in vitro and the scale explants were physiologically very active as judged by the decrease in the amount of polysaccharides in the explant (ca.70%) and the increase in total amount of soluble sugars in the explant (ca. 40%). In the basal scale explants, the number of starch granules was far higher than in apical scale explants. During culture, the number of vascular bundles increased in basal and apical scale explants from 6 to 3.3 to 8 and 4 bundles, respectively.

Key Message

Explants excised from different parts of lily bulb scales exhibit a different growth in vitro dependent on their amount of storage starch and vascular bundle intensity.

Keywords Lily · Scale explant · in vitro · Starch granule · Vascular bundle

Abbreviations

NAA α-naphthaleneacetic acid
BA Benzyl adenine

Introduction

Lily is one of the most important ornamental crops worldwide. It is commercially used as a cut flower and as a flower bulb (Watanabe et al. 2022). Bulb scales are the common starting material in both in vitro and in vivo propagation of lily. In practice, the initial propagation cycles are carried out in tissue culture because of the speed and the phytopathological safety, while the final cycles are in the field because of the high costs involved in micropropagation. The amount of growth of in vitro lily bulblets after planting in soil depends on their initial size. Large bulblets emerge faster and more uniform and have a higher sprouting percentage (Yae et al. 2001). Moreover, large bulblets display better growth (Langens-Gerrits et al. 1997). In tulip, large bulblets regenerated in vitro also show better performance after transfer to soil compared to small bulblets (Hulscher et al. 1992). In addition, large bulblets are more often in the adult phase as compared to small bulblets that are usually juvenile (Langens-Gerrits et al. 2003a). Adult lily bulblets sprout with a stem and have far better growth than juvenile bulblets that sprout with a rosette. A third growth determining factor is the degree of dormancy, as bulblets in which dormancy is only partly broken show less growth (Langens-Gerrits et al. 2003c).

On the other hand, the growth of plantlets/tissues in vitro depends on the composition of the nutrient medium, but also on the uptake and transportation of

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medium components to the growing areas. In lily, uptake predominantly occurs through the cut surfaces of the scale explant since the waxy epidermis is relatively impermeable (Askari and De Klerk 2020). Next, the nutrients have to be transported from the site of uptake to the areas of use, in this case to the regenerating bulblets. Just as in vivo, vascular tissues are the pathway via which solutes are transported since the only alternative, translocation via diffusion is incredibly slow for long distances (more than a few mm, (Taiz et al. 2015).

Starch is the main component of lily bulbs and makes up between 53 and 69% of the bulb's dry weight (Li et al. 2020). In lily, the breakdown of starch is vital for bulblet formation and bulblet growth. It has been shown that starch hydrolysis increased in lily mother scales during bulblet formation and further developmental stages (Li et al. 2014). Also, in Lycoris, starch degradation in the mother scale plays a crucial role during bulblet initiation and development as this delivers the required energy (Li et al. 2014; Ren et al. 2017).

Here, we report on the influence of type and size of explants on the growth of lily bulblets during in vitro culture and show that increased soluble sugars content resulting from starch degradation plays a critical role besides the development of vascular bundles in lily scale explants during the in vitro culture period. This knowledge should result in procedures that significantly increase the size of lily bulblets and subsequently improve the performance of in vitro bulblets after planting in soil.

Materials and methods

Standard tissue culture conditions

Field-grown bulbs (circumference 18–20 cm) of Lilium 'Santander' and Lilium 'Stargazer' were harvested, cold-treated to break dormancy and stored at − 1.0 °C until use as described (Askari et al. 2016). Scales were surface-sterilized for 30 min in 1% (w/v) NaClO, rinsed for 1, 3, and 10 min with sterile water (Askari and De Klerk 2018), then stored in sterile water (on average for 1–2 h) until use. Different types of explants were cut from lily scales, leaves, and petioles and placed on 15 ml medium in plastic culture tubes (35 mm diameter) or petri dishes (60 × 15 mm). The standard medium was composed of macro- and micro-elements (Murashige and Skoog 1962), 30 g l^{-1} sucrose, 0.4 mg l^{-1} thiamine, 100 mg l^{-1} myo-inositol, 7 g l^{-1} agar (Microagar), and 0.05 mg l^{-1} NAA (α-naphthaleneacetic acid). As standard growth conditions, the explants were cultured at 25 °C and 30 µmol m^{-2} s^{-1} (Philips TL 33) for 16 h per day.

Different types of explants

Three different types of explants: scale, leaf, and petioles, were excised from cv. Santander in vitro plantlets, and then cultured at standard growth conditions for 11 weeks on standard medium supplemented with 2 mg/l NAA and 2 mg/l BA (benzyl adenine). After 11 weeks, the fresh weight (FW) of lily bulblets and the regeneration percentage were scored. Also, scale explants of Santander and Stargazer of three different sizes (6 × 6, 6 × 12, and 6 × 18 mm) were cultured on standard medium and incubated at standard growth conditions. After 11 weeks the FW of lily bulblets was measured. To examine the role of lily scales in vitro, we cultured on standard medium, 11-week old Stargazer bulblets fully detached from scale explants and bulblets from which a large part but not all of the original scale explant had been cut off. After 6 weeks incubating at standard growth conditions, the FW of the bulblets was measured. Finally, scale explants excised from different positions of Santander and Stargazer scales (see Fig. 1) were also cultured on standard medium and kept at standard growth conditions. After 11 weeks FW of the regenerated bulblets was measured.

Quantification of soluble carbohydrates and polysaccharides

Explants were collected and stored at − 20 °C until analysis. Soluble sugars were extracted from 50 mg of powdered freeze-dried material in 8 ml 80% ethanol by heating at 70 °C for 7 h. The tubes were centrifuged at 4000×*g* for 10 min (Hereaus Multifuge 3 S), and then the supernatant, containing the soluble sugars, was decanted of into a new tube. The pellet was washed with 2 ml of 80% ethanol and, after centrifuging, this supernatant was combined with the first one. Both the pellets (reserve carbohydrates and structural sugars) and the supernatants (soluble sugars)

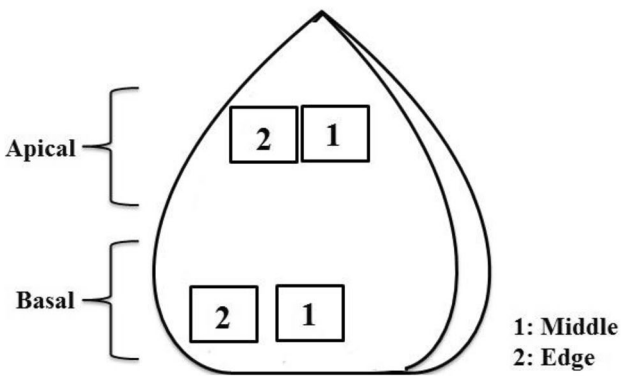


Fig. 1 Schematic drawing of the position of basal, apical, middle and edge explants on a lily bulb scale

were evaporated to dryness. Sugar content was determined with the Phenol-Sulphuric acid assay according to Dubois et al. (1956). Absorbance was read at 490 nm in a Shimadzu UV-1800 spectrophotometer. All treatments were represented by means of five measurements, prepared from mixed plant materials originating from 10 to 15 explants for each measurement. A calibration curve was constructed using standard glucose solutions containing 15 to 150 µg ml^{-1}.

Histological observations

Scale explants of Santander and Stargazer were fixed and rinsed in 5% glutaraldehyde in phosphate buffer at pH 6.8. They were then dehydrated in an ethanol series and embedded in Spurr's resin. Transverse sections were made using a Sorvall MT 5000 microtome mounted on glass slides and stained with toluidine blue to visualize the vascular bundles and with Lugol IKI solution to visualize starch. The sections were observed with an Axiophot light microscope (Zeiss, Oberkochen, Germany) and images were taken with an AxionCam ERc5S digital camera (Zeiss). Five photos taken from five different scale explants were analyzed with ImageJ 1.4 software to calculate the percentage of the starch granule area of the freshly cut scale explants and 12-week old scale explants.

Statistics

The data were scored 6 and 11 weeks after culturing of different explants in various experiments. Data were first tested for normality test of residuals (Shapiro–Wilk test). The means are the average of 5–100 measurements per treatment in various experiments. In the figures, the means are shown ± SE. The means were evaluated with a t-test.

Results

Growth of lily bulblets regenerating from various types of explants

To examine the capacity of lily bulblets regeneration in vitro on different lily organs we used tissue-culture grown bulblets consisting of scales which bore a leaf blade and a petiole as the source of scale, leaf and petiole explants. The bulblet regeneration percentage of scale explants was 88%. In leaf and petiole explants, a much lower regeneration percentage was achieved, viz., 12% and 18%, respectively (Fig. 2a). Scale explants produced bigger and heavier bulblets weighing 51 mg/bulblet compared to the leaf and petiole explants weighing 33 and 38 mg/bulblet, respectively (Fig. 2b).

Results of the experiment on the effect of the size of explants on bulblets growth showed that the FW of bulblets increased with the size of scale explants. The FW of bulblets regenerated on 6 × 18 mm scale explants was 66 mg/bulblet and the FWs of bulblets regenerated on 6 × 6 mm and 6 × 12 mm scale explants were 49 mg/bulblet and 53 mg/bulblet, respectively (Fig. 2c). Thus, bigger scale explants improved lily bulblet growth by 26% compared to smaller scale explants.

To study the positive effect of the scale explants in more detail, we excised 11-week-old lily bulblets from scale explants leaving a small piece of scale attached to the bulblets or removing the original scale explant completely. They were cultured on standard medium for 6 more weeks.

The FW of lily bulblets with a small piece of explant was 400 mg/bulblet and the FW of lily bulblets from which the original scale explant had been removed completely was 300 mg/bulblet (Fig. 2d). The initial average FW of 11-week old bulblets at the beginning of each experiment was 100 mg/bulblet for both explant types. We also examined in vitro lily bulblet growth on scale explants excised from various positions of the bulb scale (Fig. 1). Explants excised from the middle of a scale produced bigger bulblets compared to the explants cut from the edge (ca. 40–50% bigger) and explants cut from the basal part of a scale produced bigger bulblets compared to the explants cut from the apical portion (ca. 40–50% bigger, Fig. 2e, f) in both cv. Santander and Stargazer.

Determination of starch, soluble carbohydrate and polysaccharide content in lily scale explants during in vitro culture period

Increased growth of the bulblets on basal scale explants compared to apical scale explants raised the question of how much carbohydrate, and mainly starch reserve is present in these two types of scale explant tissues at the beginning and at the end of the growth period. Thus, to assess starch distribution in the tissue, we visualized starch grains under the microscope and evaluated starch content by measuring the area covered by starch granules. The surface area covered with starch granules was clearly larger in the basal scale explants as compared to the apical scale explants in both varieties (Fig. 3a, c) and decreased with time for both explant types (Fig. 3b, d). When quantifying the area it was 31% and 44% in basal scale explants and 15% and 19% in the apical scale explants at the beginning of the tissue culture period in cv. Santander and Stargazer, respectively. The surface area covered with starch granules went down to 8% and 9% in basal scale explants and 7% and 5% in the apical scale explants at the end of the tissue culture period in Santander and Stargazer, respectively (Fig. 3e, f). The surface area covered with starch granules decreased by 74% and 79% in basal and 53% and 74% in apical explants after 12 weeks in Santander and Stargazer, respectively. In general, the surface

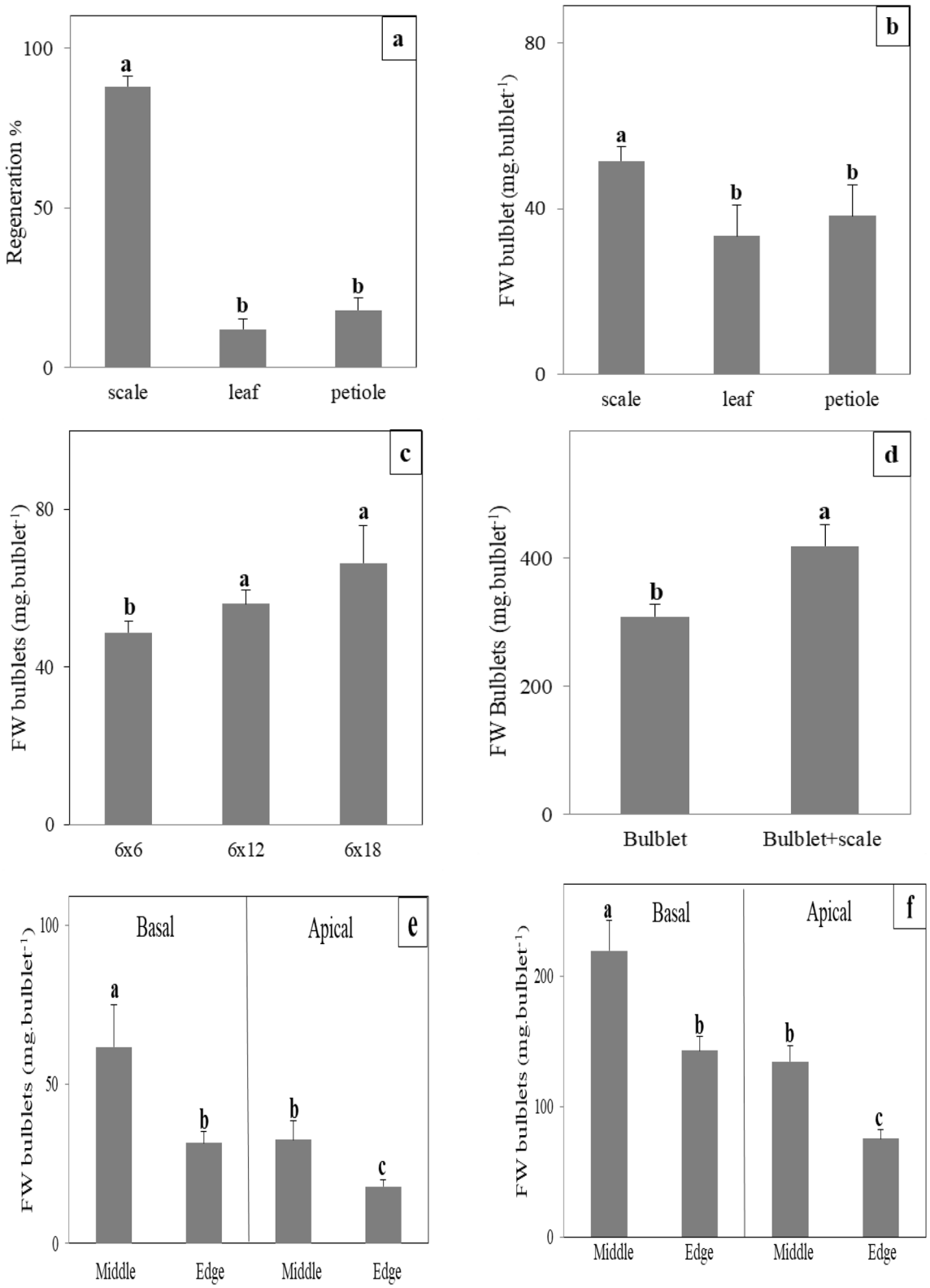


Fig. 2 Regeneration percentage (**a**); FW of lily bulblets regenerated on scale, leaf and petiole explants in vitro (**b**) in Santander. Effect of the size of the explants on FW of regenerated bulblets (**c**); Effect of the presence of scale explants on growth of excised bulblets (**d**) in Santander; effect of scale explant position either basal (middle and edge) or apical (middle and edge) on lily bulblet growth in vitro; cv. Santander (**e**), cv. Stargazer (**f**)

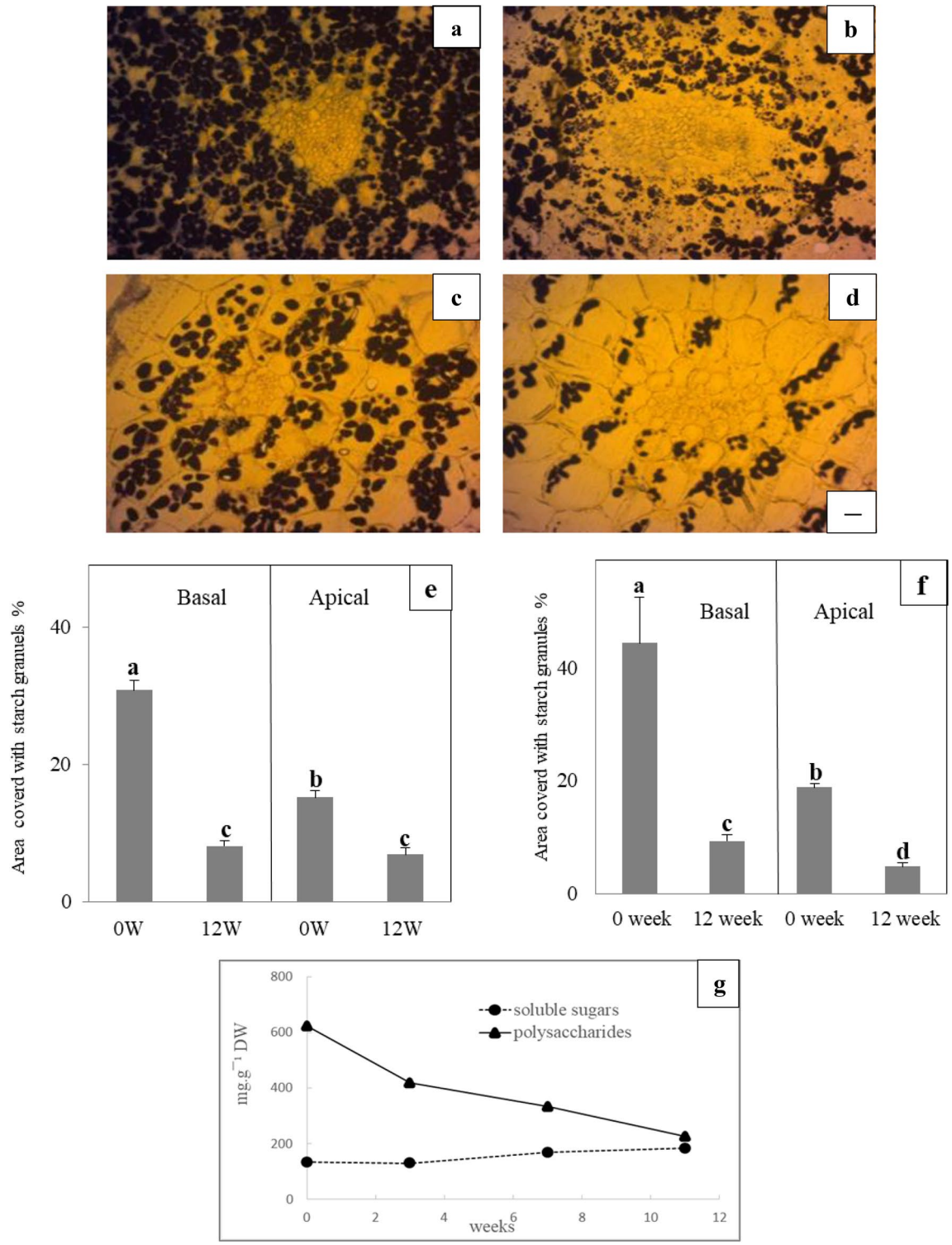


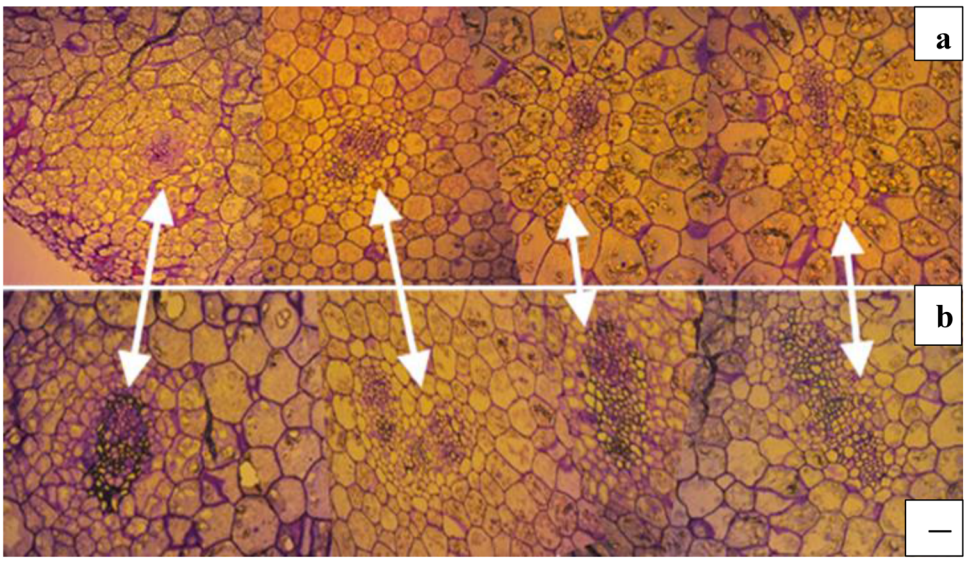
Fig. 3 Distribution of starch in scale explants in relation to their position on bulb scale. **a** Freshly cut basal explants; **b** Basal explants after 12 weeks cultured in vitro; **c** Freshly cut apical explants; **d** Apical explants after 12 weeks cultured in vitro. Bar (——) = 5 µm and surface area covered with starch granules in basal and apical scale explants at the start of culture (0 weeks) and after 12 weeks of culture in cv. Santander (**e**) and cv. Stargazer (**f**). Amount of soluble sugars and polysaccharides present in the scale explant during in vitro culture period (**g**)

area covered with starch granules in the basal scale explants was ca. 2 times larger than in the apical scale explants.

We also evaluated the amount of polysaccharides and soluble sugars in the scale explants during the growth period. The amount of polysaccharides in the scale explants decreased ca.70% during the in vitro culture period. This could indicate a breakdown of reserves during the in vitro period which is faster in the beginning and also at the end of the culturing period. The total amount of soluble sugars in the scale explants increased slightly with time (ca. 40%). There is a relationship between polysaccharide (notably starch) breakdown and soluble sugar content in lily scale explants during the in vitro culture period; an increase in polysaccharide/starch breakdown leads to an increase in soluble sugar amounts (Fig. 3g).

Vascular bundle numbers and size increase during lily bulblets growth in both basal and apical scale explants

In both types of scale explants (0 and 12 weeks old), vascular bundles were histologically analyzed. In freshly cut scale explants, the number of vascular bundles in basal scale explants (6 vascular bundles) (Fig. 4a) was higher than apical explants (3.3 vascular bundles). After 12 weeks, the number of vascular bundles increased in basal (Fig. 4b) and apical scale explants to 8 and 4 vascular bundles, respectively. The area of vascular bundles after 12 weeks was two to three times larger than in freshly cut scale explants in both basal and apical scale explants (Fig. 4c).



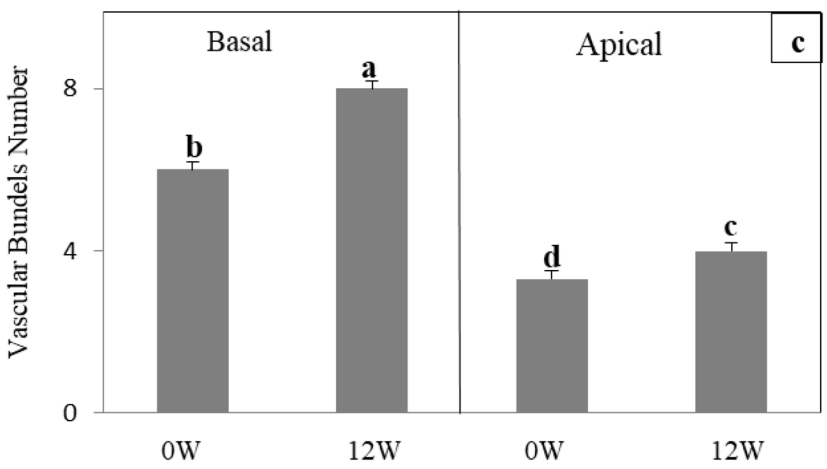


Fig. 4 Increase in the number and the size of vascular bundles in edge basal scale explants; **a** vascular tissue in freshly cut edge basal explants of Stargazer; **b** the vascular bundles number and size after 12 weeks in the same scale explants; Bar (——)=25 µm in (**a**, **b**); **c** Vascular bundles number in edge basal and apical lily scale explants at 0 and 12 weeks

Discussion

The growth of bulblets on different explant types

In lily, in vitro bulblet regeneration can occur from a number of different plant tissues, like flower organs, leaf petiole, leaves and bulb scales (Bhandari et al. 2019; Youssef et al. 2019; Htwe et al. 2021; Askari et al. 2022). Our results showed that the regeneration percentage of leaf and petiole explants was very low compared to the scale explants (Fig. 2a) and the FW of bulblets regenerated on the scale explants was higher compared to leaf and petiole explants (Fig. 2b). Scale explants were superior explants for micropropagation of lily compared to leaf and petiole explants. The reason why scale explants perform better is probably due to the fact that they are more resistant to cutting stresses and might thus be better adapted to stresses related to in vitro culture conditions. Moreover, the scale explants contain lots of storage reserves (starch/polysaccharides) so that the excised explants depend less on the medium and on transport of sugars from the medium. Previous research (Langens-Gerrits et al. 2003b) showed that large explants regenerated larger bulblets than small explants. A large explant has a large contact area with the medium and can therefore take up more nutrients from it. The explants act as a sink (growing organ) and as a source (nutrient source for the growing bulblets) simultaneously (Langens-Gerrits et al. 1997). The bigger explants have more vascular bundles to provide the new bulblets with nutrients. Bulblets that have regenerated from large explants are often adult while those from small explants are usually juvenile. These bulblets with a different ontogenetic age sprout with a stem or with a rosette, respectively (Langens-Gerrits et al. 2003a). Adult bulblets grow much faster after planting probably because the stem carries many more leaves than present in a rosette (Langens-Gerrits et al. 2003a). In tissue culture of other crops, such as rose (Marcelis-van Acker and Scholten 1995) and mung bean (Gulati and Jaiwal 1992) also bigger regenerating organs were produced on bigger explants.

The presence of scale explants promotes the growth of lily bulblets in vitro

In lily tissue culture, regenerating bulblet growth depends on carbohydrates from starch degradation of scale explant as well as sucrose from the medium (Langens-Gerrits et al. 2003b). The presence of a small piece of scale explant still attached to the bulblets also improved the growth of excised lily bulblets and reveals a major role of the scale explants possibly as some kind of pump. But the starch in the explant also seems to play a role indicated by the breakdown of the starch reserves during 12 weeks of culture. Measurement of starch degradation after 12 weeks of culture of scale explants showed that ca. 21% and 26% of starch granules were still available in basal and ca. 26% and 45% in apical scale explants in cvs. Stargazer and Santander, respectively. In addition, measurement of starch granules in scale explants cultured on medium without sucrose after 12 weeks showed that most starch granules were degraded during bulblet regeneration on medium without sucrose (data not shown). The largest sink activity (µg sucrose taken up from the medium per mg FW) was found in scale explants and it was constant during tissue culture but sink activity decreased in bulblets regenerated in vitro with time (Langens-Gerrits et al. 2003b). These results confirm the major role of the scale explants on the growth of lily bulblets in vitro.

Effect of position of the scale explants on lily bulblets growth in vitro and visualization of starch granules and vascular bundles in basal and apical scale explants

The position of scale explants influenced regenerating bulblets growth. As shown in Fig. 2e and f, basal and middle explants regenerated bigger bulblets compared to apical and edge explants. In the basal part of lily bulb scales cells are younger (Rafiq et al. 2021), so the basal scale explants are more vigorous to regenerate bulblets than the apical part. Previous research did find that the lower part of a bulb scale was most suitable for multiple shoot differentiation and rapid growth of bulblets for in vitro propagation of lily (Chunlin et al. 2004). In tulip, basal scale explants produced more callus lumps and shoots compared to apical scale explants (Koster 1993). The FW of bulblets, bulblet regeneration percentage and the number of bulblets regenerated in vitro was higher on basal scale explants of hyacinth compared to apical scale explants (Pierik and Woets 1971). Sucrose taken up in the explants is mainly recovered at the basal side of the explants, where regeneration occurs (Langens-Gerrits et al. 2003b). When lilies are propagated ex vitro by keeping complete scales in a moist environment, starch mobilization proceeds from the apical to the basal region of bulb scales (Miller 1990). The increased bulb growth on the basal explants reflects the higher contribution of explant reserves during bulblet growth in vitro, supported by the larger density of starch granules visualized here in basal scale explants compared to the apical scale explants. In the basal explants, the area of degraded starch granules during lily bulblets growth was ca. twofold higher than in apical scale explants. Furthermore, the amount of polysaccharides in the scale explant reduced by ca. 70% during in vitro culture period

and simultaneously the amount of soluble sugars increased by ca. 40% (Fig. 3g). In field-grown lily bulbs, the carbohydrate as well as biomass content of the scales decreased after planting, and the degradation of the reserves stored in the outer bulb scale was higher compared to the inner bulb scale (Addai and Scott 2011). The presence of more starch is probably not sufficient to improve bulblet growth if the transport system of nutrients was the same. We observed that in basal scale explants the number of vascular bundles is almost twice than in apical scale explants. In tissue culture, remobilization of starch from Guzmania 'Hilda' leaves occurred when sucrose had been depleted from media (Lembrechts et al. 2017). On the other hand, the amount of starch increased in the leaves while the amount of initial sucrose in the media was higher. in vitro plantlets consume starch reserves to sustain growth when other carbohydrates are not or only limited available (Lembrechts et al. 2017). Similarly, in in vitro tobacco leaves the breakdown of starch reserve has been reported after depletion of sucrose from the media (Tichá et al. 1998). The middle explants in both basal and apical lily scale bulbs include the main vascular bundles and have therefore more and wider vascular bundles. In addition, the explants taken from the middle part are thicker compared to the edge explants. The average FW of middle-scale explants and edge explants of the same size (7 × 7 mm) was 250 mg/explant and 150 mg/explant, respectively. The middle scale explants have therefore more storage reserves compared to the edge scale explants. Both types of explants grow considerably during the tissue culture period. This result also shows that bulblets regenerated on edge scale explants used less storage material from the scale explants compared to the middle scale explant as, in the beginning, even the FW of middle-scale explant was higher than edge scale explants while at the end the difference between the FW of edge scale explants and middle scale explants was not statistically significant. This indicates that the vascular bundles play an important role during lily bulblet growth in vitro due to translocation of medium component and transport of scale storage organs to the bulblets regenerated in vitro. Development of the vascular bundle number and size during bulblet regeneration in vitro occurred in both basal and apical scale explants. In callus culture, redifferentiation of parenchyma cells leads to vascular bundle development, which is controlled by IAA. Low auxin concentration promoted phloem development and higher auxin concentration triggered xylem development (Aloni 1980). Recently new methods introduced for study of vascular bundle development in tissue culture indicated that, in leaf culture, the mesophyll cells re-differentiated to procambial tissues first after which xylem appears from these cells (Kondo et al. 2015; Saito et al. 2017). In plants, development of new vascular tissue enables regeneration of the plant and its adaptation to interruptions and changes in the environment (Aloni 1987). So, an increase in the number and the size of vascular bundles during lily bulblets growth in vitro is relevant to the growth of lily scale explants and may be a logical response of scale explants to change its growth patterns for adaptation to the new growth conditions. In cassava tuberous root, there is a relation between vascular cambium formation and starch storage metabolism in which starch storage metabolism was activated only after the formation of the vascular cambium (Rüscher et al. 2021). On the other hand, in lily, starch is the fundamental component in the shoot-to-bulblet transition (Wu et al. 2021). Probably, vascular bundle development in vitro also enhances starch storage metabolism in scale tissues and subsequently improve lily bulblet growth in vitro. In tulip, transition of scale explant vascular bundles to meristematic centers occurred after 10 days and callus lumps at the upper side of scale explants appearing after 30 days originated from meristematic cells of vascular bundles (Koster 1993).

Conclusions

This study shows that characteristics of the scale explants play a major role in the growth of bulblets that regenerate from the scales. Scale explants are the best explants for micropropagation of lily bulblets in vitro. The size of scale explants at the beginning of tissue culture is a useful tool to produce bigger bulblets during lily in vitro micropropagation. It is related to more starch granules that are degraded during the period of bulblet regeneration and more and wider vascular bundles to transfer the nutrients to the bulblets regenerated in vitro. In addition, the significant difference between the growth of lily bulblets on basal and apical scale explants also indicated that the number of starch granules and the intensity of vascular bundles play a major role in lily bulblet growth in vitro.

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Data availability The data will be available on request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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