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Article

Light promotes *in vitro* bulbing of *Sprekelia formosissima*

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Abstract

Sprekelia formosissima is an ornamental geophyte that is propagated by bulb division since it takes up to four years to obtain it by seed. Micropropagation is an option to produce bulbs that grow and accumulate carbohydrates in a differentiated way under different light conditions and depending on the species. In 2022, the *in vitro* growth of *S. formosissima* bulbs with 0, 30, 60, 120, and 130 µmol m^{-2} s^{-1} of light was evaluated. The bulbs were grown in MS medium with 4% sucrose and a photoperiod of 16 h. At 80 days, number, color, chlorophyll and length of leaves, fresh and dry weight of leaves and bulbs, and width and length of bulbs were measured. Total soluble sugars, reducing sugars, sucrose, fructose in leaves and starch in bulbs were evaluated. To determine starch, 4 and 8 U of amyloglucosidase and #-amylase were tested, with 6, 12 and 24 h of digestion. The data were used to perform an Anova and a Tukey test ($p< 0.05$). With 130 µmol m^{-2} s^{-1}, the fresh and dry weight and diameter of the bulb, number of leaves, starch in bulbs, and chlorophyll a, b and total increased by 152, 433, 44, 87, and 251% and 55, 35, and 58 times, respectively, compared to the data recorded in the dark. The high intensity of light *in vitro* increases the biomass and starch in *S. formosissima* bulbs, which was estimated with 8 U of amyloglucosidase and α-amylase and 24 h of digestion.

Keywords:

carbohydrates, light intensity, micropropagation of geophytes.

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Introduction

Geophytic plants occupy an important place in ornamental horticulture; they are used as a cut flowers, potted plants, gardening, and landscaping (Zhao *et al*., 2022). The most important genera in the flower industry are *Tulipa*, *Lilium*, *Narcissus*, *Gladiolus*, *Hyacinthus*, *Crocus*, and *Iris*, as well as *Freesia*, *Ornithogalum*, *Hippeastrum*, *Allium*, and *Muscari*; however, the saturation of the market with these flowers and consumer demand for new varieties has stimulated interest in evaluating native flora as a source of ornamental crops (Kamenetsky, 2017).

One option is *Sprekelia formosissima* (L.) Herbert, which is known as the Aztec lily and is distributed from northern (Chihuahua) to southern Mexico (Oaxaca). It is a perennial herbaceous geophytic plant that has tunicate globose bulbs that produce a floral stem (rarely two or three) that can reach 75 cm, regularly with a flower of different shades of red up to 20 cm (Cruz-Duque *et al*., 2019).

The traditional propagation of *Sprekelia formosissima* is by bulb division since obtaining flowering plants from seed can take up to four years (Borys *et al*., 2005), and vegetative propagation is limited to the production of one or no shoots per year. For this reason, *in vitro* propagation techniques are an alternative for the mass propagation of this species, in which up to 96 bulbils can be obtained in six months from a single bulb (Cázarez *et al*., 2010).

Nevertheless, the challenge in micropropagating this species is bulbing (bulb formation and growth) since the bulbils produced *in vitro* that grow sufficiently reach an adult developmental stage sprout quickly and form stems, leaves, and flowers; conversely, smaller bulbs go through a juvenile state in which, in the case of lilies, they form only rosette leaves without producing flowers (De Klerk, 2012).

Plant growth after sprouting is related to the accumulation of starch and soluble sugars, such as sucrose, glucose, and fructose, during bulbing (Podwyszynska, 2012). The accumulation of carbohydrates in ornamental bulbous plants can be affected by environmental incubation variables such as light (intensity, quality, and photoperiod), which modulates the growth and development of plants grown *in vitro* (Yang *et al*., 2018).

According to Sevgin and Karatas (2022), the intensity of light used in the micropropagation of geophytic species ranges from 30 to 40 µmol m^{-2} s^{-1}, although other studies indicate that darkness promotes bulb formation *in vitro*, as it simulates natural underground growing conditions (Ulrich *et al*., 1999; Rice *et al*., 2011). In *Narcissus tazetta* L., Rahimi *et al*. (2020) studied the effect of incubation with a photoperiod of 16/8 h light/darkness and darkness, and they reported that treatment with light (40 µmol m^{-2} s^{-1}) led to a greater number of bulbs (13.5 ±1.44), number (6.2 ±0.87) and length (0.86 ±0.46) of leaves.

Likewise, darkness completely inhibited bulb formation in *Eucomis zambesiaca* and the photoperiod of 8 h of light promoted their formation (Cheesman *et al*., 2010). In contrast, in *Hyacinthus orientalis* (Kim *et al*., 1981) and *Lilium longiflorum* (Kumar *et al*., 2006), there was a higher number of shoots under dark conditions. These data show that the effects of light and darkness on the *in vitro* production of bulbs in geophytic plants should be studied in each species to determine the optimal conditions for the production of quality bulbs.

Therefore, this research aimed to study the effect of light intensity on 1) the *in vitro* growth of *Sprekelia formosissima* bulbs; and 2) the concentration of starch, total soluble sugars, reducing sugars, sucrose, and fructose in the bulbs.

Materials and methods

Plant material

The seeds of *Sprekelia formosissima* were collected in the locality of Tejerías in Uruapan, Michoacán (19° 23’ 89” north latitude and 102° 00’ 22.82” west longitude). These were immersed in solution with 1 mg L^{-1} of Tecto® (thiabendazole 60%) and 60 drops of Microdin® (ionized silver

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0.35%) for 15 min, then rinsed three times with sterile water. Disinfection was carried out with a solution of sodium hypochlorite (2.1%) for 15 min and three rinses with sterile water. Afterward, the seeds were germinated in Murashige and Skoog (1962) medium with 100 mg L^{-1} myo-inositol, 0.4 mg L^{-1} thiamine, 6 g L^{-1} agar (basal medium) plus 3% sucrose.

The pH of the medium was adjusted to 5.8, the incubation of the cultures was at 25 °C, with a photoperiod of 16/8 h light/darkness and photosynthetically active radiation of 60 µmol m^{-2} s^{-1}, with 75 W white, fluorescent light lamps. Seedlings with bulbils were obtained at eight weeks.

Treatments and experimental design to evaluate the effect of light intensity

The apical part of the bulbils was removed by means of a transverse cut, and the basal part was made a longitudinal cut to promote the propagation of new bulbils. These explants were grown in 100 ml bottles with 25 ml Murashige and Skoog medium with 4% sucrose, 2 mg L^{-1} benzyladenine (BA), and 2 ml L^{-1} plant preservative mixture (PPM) with 0.135% 5-chloro-2-methyl-3(2H)-isothiazolone and 0.0412% 2-methyl-3(2H)-isothiazolone.

At 60 days, bulbils of 0.2 to 0.3 cm in diameter were selected, placed in basal medium and incubated in darkness and with different light intensities (30, 60, 120, and 130 µmol m^{-2} s^{-1}) with a photoperiod of 16/8 h darkness/light. The experimental design used was completely randomized and the experimental unit consisted of a bottle with two bulbils of *S. formosissima*.

At 80 days after the experiment was established (dae), the concentration of chlorophyll, color, number of leaves and length of the longest leaf were evaluated. In bulbs, width, length, and starch content were measured. In bulbs and leaves, fresh weight, dry weight, and soluble carbohydrate content (total soluble, reducing, sucrose and fructose) were recorded.

To measure chlorophyll, the leaves were placed in 5 ml of N, N dimethylformamide in darkness for 72 h at 4 °C, then 1 ml of the solvent was taken and the absorbance was measured in a spectrophotometer (Jenway 6305) at 664 and 647 nm (Porra *et al*., 1989); these data and the functions of chlorophyll a= 12.7 (A_{664})-2.79 (A_{647}), chlorophyll b= 20.70(A_{647})-4.62 (A_{664}), and total chlorophyll= 17.9 (A_{647}) - 8.08 (A_{664}) were used to calculate the chlorophyll concentration in mg L^{-1} and with the sample weight, in mg g^{-1} fresh weight.

For the color, the Royal Horticultural Society color charts (RHSCC) sixth edition were used, with which a scale was established with consecutive values from 1 to 27, which correspond to the number of colors registered, from white (NN155D) to green with different shades from light to dark (N189A) (Figure 1).

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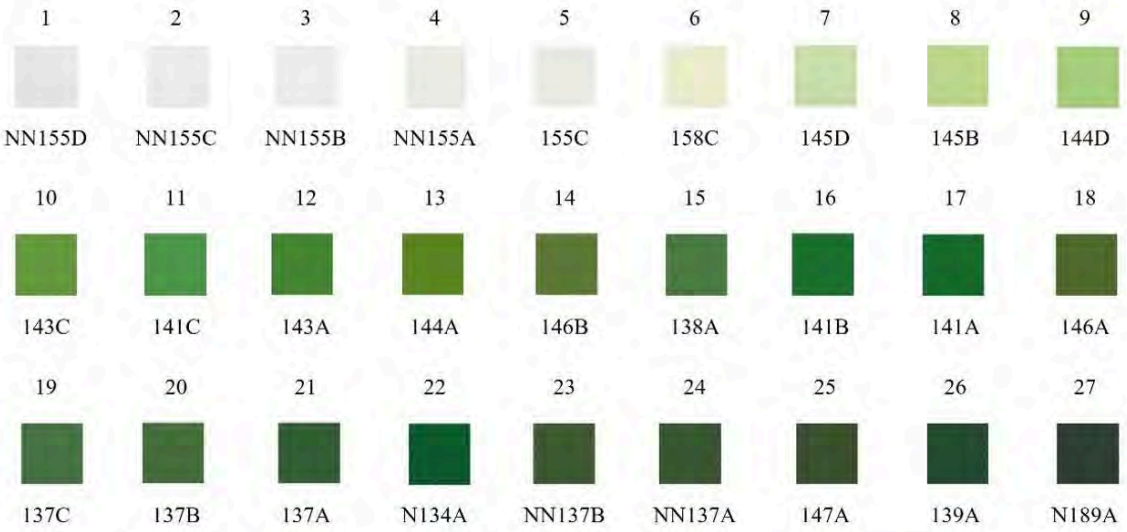
Figure 1.Assigned consecutive number and name of the Royal Horticultural Society colors recorded on leaves of *Sprekelia formosissima* propagated *in vitro* with different light intensities.



To determine soluble carbohydrates, a sample of approximately 50 mg of frozen pulverized tissue was used, to which 500 µl of 80% ethanol was added, then the sample was heated to 80 °C for 30 min with stirring every 10 min and centrifuged at 13 000 rpm for 10 min to separate and recover the supernatant from the sediment; this procedure was repeated three times after the addition of 80% ethanol.

From the supernatant, total soluble sugars were determined by the phenol-sulfuric acid method (Rover *et al*., 2013), reducing sugars by Somogyi-Nelson (Maldonade *et al*., 2013), and sucrose and fructose by the method proposed by Seliwanoff (Quesada, 2007). Chlorophyll determination was performed with three replications per treatment, non-structural carbohydrates with four, number and length of longest leaf with ten, and for the rest of the variables, seven replications per treatment were measured.

Standardization of the technique for the enzymatic determination of starch in *Sprekelia formosissima* bulbs

Before evaluating the effect of light intensity on starch content in *Sprekelia formosissima* bulbs, a trial was performed to establish the optimal concentration of #-amylase, amyloglucosidase, and digestion time to estimate the concentration of this polysaccharide. To determine the starch, the sediment described in the determination of soluble carbohydrates was used; in this case, the washing with 80% ethanol was performed three times more.

After 80% ethanol washing, the sediment was dried at 80 °C and 200 µl of sterile distilled water was added; the sample was heated to 95 °C for 45 min to gelatinize the starch, and then 300 µl of a 0.2 M sodium acetate buffer solution (pH 5.4) containing *Bacillus licheniformis* #-amylase (EC 3.2.1.1) and *Aspergillus niger* amyloglucosidase (EC 3.2.1.3) was added.

A factorial treatment arrangement with six replications was used to test two enzyme concentrations (4 and 8 U of each enzyme) and three reaction incubation times at 37 °C (6, 12, and 24 h); subsequently, the amount of hydrolyzed glucose was measured by the Somogyi-Nelson method (Maldonade *et al*., 2013) to estimate the starch content in the sample.

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Statistical analysis

The normality and homogeneity of variances of the data was corroborated with the Shapiro-Wilks and Levene tests, respectively. The following variables were transformed: chlorophyll a and total chlorophyll (z^2), fresh and dry weight and longest leaf length [log(z + 1)], fresh weight [log {z + √(z + 1)}] and dry weight of the bulb (√z), fructose, total soluble sugars in bulb [log{z + √ (z^2 + 1)}] and reducing sugars in leaves and bulbs (1/z). The data obtained were used to perform an analysis of variance ($p \leq 0.05$) and Tukey's mean test ($p \leq 0.05$) with the (2023) SAS® On Demand for Academics program.

Results and discussion

Growth and accumulation of biomass in bulbs

Contrary to what happens in lilies that are grown in darkness, *in vitro* bulb growth and the number of leaves formed in *S. formosissima* increased with the highest light intensity tested in this experiment (130 µmol m^{-2} s^{-1}) compared to bulbs grown in darkness, which accumulated the least amount of biomass and generated fewer leaves (Table 1).

Table 1. Effect of light intensity on the growth of *S. formosissima* seedlings propagated *in vitro*.

Light intensity (µmol m^{-2} s^{-1})	Bulbs			No. of leaves
	Diameter (cm)	Fresh weight (mg)	Dry weight (mg)	
0	0.27 b	47.21 b	6.21 b	1.2 b
30	0.32 ab	64.5 ab	12.35 b	1.75 ab
60	0.37 ab	71.57 ab	13.78 b	1.8 ab
120	0.31 ab	65.93 ab	16.07 b	1.95 ab
130	0.39 a	119.36 a	33.14 a	2.25 a
HSD (0.05)	0.11	61.95	15.51	0.82

Means with equal letters are not statistically different (Tukey, $p \leq 0.05$). HSD= honest significant difference.

Plants grown *in vitro* are generally photomixotrophic; they use sucrose from the culture medium as a carbon source, although they can satisfy part of their demand by photosynthetic fixation of CO_2 available in the containers where they are grown (Badr *et al*., 2011), which is why the increase in biomass in *S. formosissima* bulbs recorded with the highest intensity of light can be explained by the formation of a greater number of leaves, which, under these conditions, supply the photosynthates accumulated in the bulbs, which accumulate carbohydrates from the source organs and the culture medium.

In *Narcissus papyraceus* cv. Shirazi, the bulbs store nutrients and increase their diameter (11.43 ±0.93 mm) and length (19.68 ±0.52 mm) under conditions of high light intensity (108 µmol m^{-2} s^{-1}) and 90 g L^{-1} sucrose (Hosseini *et al*., 2013). In 'Siberia' lily bulbs grown *in vitro*, there was an increase in bulb diameter of 443.7% when bulb differentiation and filling was induced in the dark and 245.33% with a photoperiod of 16/8 h light/dark, compared to the initial diameter of the explants, the number and length of roots per bulb was 14.53 and 7.31 roots of 3.68 and 0.79 cm, respectively (Zhang and Jia, 2014).

S. formosissima bulbs grown in the dark developed, on average, two roots with a length of 0.8 cm in the longest root; in contrast, the 'Siberia' lilies bulbs produced a greater number of roots with greater length per bulb under these conditions, which allowed a greater absorption of nutrients from the culture medium and the increase in the size of the bulb in the dark.

The leaves that grew from the bulbs of *S. formosissima* grown *in vitro* presented an intense green color in the treatments with more light and a marked discoloration when the seedlings grew in the

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dark (Figure 2), under these conditions, the lowest concentrations of chlorophyll a and b and total in leaves were recorded with values 55, 35, and 58 times lower than those recorded with 130 µmol m^{-2} s^{-1}, respectively (Table 2).

Figure 6. Leaf color in *S. formosissima* seedlings from bulbs propagated *in vitro* with different light intensities. The bar in the figure represented 1 cm.

Table 2. Effect of light intensity on chlorophyll concentration in leaves of *S. formosissima* cultured *in vitro*.

Light intensity (µmol m^{-2} s^{-1})	Chlorophyll a	Chlorophyll b	Total chlorophyll
		(mg g^{-1} fresh weight)	
0	0.013 c	0.006 c	0.016 c
30	0.373 b	0.106 bc	0.48 bc
60	0.523 ab	0.133 ab	0.656 ab
120	0.58 ab	0.133 ab	0.72 ab
130	0.713 a	0.21 a	0.923 a
HSD (0.05)	0.306	0.1	0.4

Means with equal letters are not statistically different (Tukey, $p \leq 0.05$). HSD= honest significant difference.

The results in experiments with different light conditions show that the highest concentration of chlorophyll (the main pigment associated with photosynthesis) is related to the accumulation of fresh and dry matter in plant explants *in vitro* (Batista *et al*., 2018), as occurred in *S. formosissima*, where the highest accumulation of biomass was recorded with the highest light intensity.

It has also been documented that plants exposed to high light intensity can produce high concentrations of chlorophyll and carotenoids as a protective mechanism against photooxidation (Alvarenga *et al*., 2015) since if the excess energy absorbed by the photosynthetic apparatus is not dissipated quickly, it can result in photoinhibition and damage to the photosynthetic reaction center (Takahashi and Murata, 2008).

The accumulation of chlorophyll in *S. formosissima* leaves recorded in this work with the light intensity of 130 µmol m^{-2} s^{-1} could be a protective mechanism, as suggested by Alvarenga *et al*. (2015), who grew *Achillea millefolium* (a rhizome-forming Asteraceae) *in vitro* with five light intensities, 13, 27, 35, 47 and 69 µmol m^{-2} s^{-1} and in which they recorded the highest concentration of chlorophyll a, b and total (1.18, 0.63, and 1.81 mg g^{-1} fresh weight) with the highest light intensity, but the highest vegetative growth (number of roots, shoot length, and accumulation of dry matter) with 27 µmol m-2 s-1.

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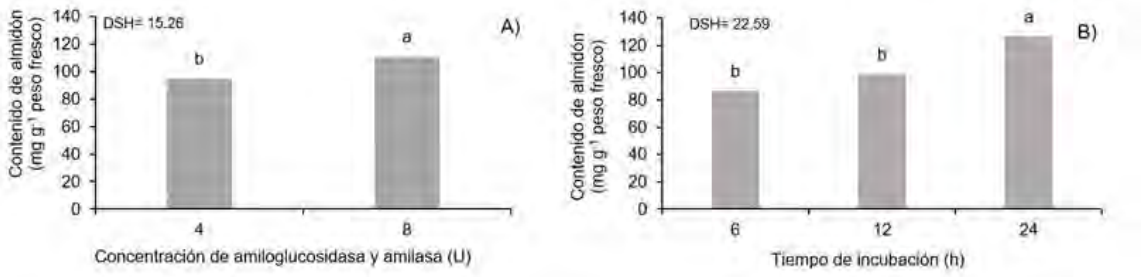
In contrast, the greater accumulation of biomass in *S. formosissima* bulbs with 130 µmol m^{-2} s^{-1} of light tested in this experiment can be explained by the increase in the number of leaves, chlorophyll, and photosynthesis, which allowed mobilizing and accumulating in these organs a greater amount of photosynthates generated in the leaves compared to the bulbs in the dark that obtained carbon only from sucrose in the medium or bulbs with lower light intensities that obtained a lower amount of carbon by fixing CO_2.

These results show that the response to high light intensity is different between species and that only plants adapted to high radiation in their natural environment have a higher photosynthetic rate and growth, under these *in vitro* conditions (Cavallaro *et al*., 2022).

Standardization of enzyme concentration (α-amylase and amyloglucosidase) and *in vitro* digestion time to estimate starch in *Sprekelia formosissima* bulbs.

The interaction between enzyme concentration and digestion time of starch was not significant ($p \leq 0.05$), although independently, the highest degree of starch hydrolysis in *S. formosissima* bulbils was recorded with 8 U of both enzymes, α-amylase and amyloglucosidase, with 110.71 mg g^{-1} fresh weight and 24 h of digestion, with 125.87 mg g^{-1} fresh weight (Figure 3). This amount is 16 and 1.3 times higher than the amount of α-amylase and amyloglucosidase suggested by Smith and Zeeman (2006) to determine starch content in plant tissue with four hours of incubation at 37 °C.

Figure 3. Enzyme concentration and incubation time to estimate starch in *in vitro* propagated *Sprekelia formosissima* bulbs. A) effect of enzyme concentration (amyloglucosidase and α-amylase); and B) digesti on time at 37 °C. Means with equal letters are not statistically different (Tukey, $p \leq 0.05$). HSD= honest significant difference.



The mixture of both enzymes was efficient for starch digestion in *S. formosissima* bulbs, although some research suggests using only one enzyme, which seems to be inadequate based on the results of corn and potato starch digestion where different digestion times were tested with amyloglucosidase alone (14 U) and with the enzymes #-amylase and amyloglucosidase together (25 and 14 U, respectively), starch hydrolysis was faster with both enzymes and at 24 h, almost all corn starch was hydrolyzed to glucose while one-third of potato starch remained unhydrolyzed (Zhang *et al*., 2013).

In *Lilium lancifolium*, the starch content in mature bulbs and newly formed bulbils was determined with 50 U of α-amylase with a digestion time of 1, 2, 4, 8, 12, 24, 48, and 72 h. The starch in the bulbils showed less resistance to digestion with the highest percentage of starch (40%) recorded at 24 h compared to bulbs in which five times less starch was recorded at the same time.

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These variations in enzyme concentration and digestion time in the determination of starch content in plant tissue are due to differences in the characteristics of this polysaccharide, including granule size, amylose-amylopectin ratio, and tissue age (Yu *et al*., 2015).

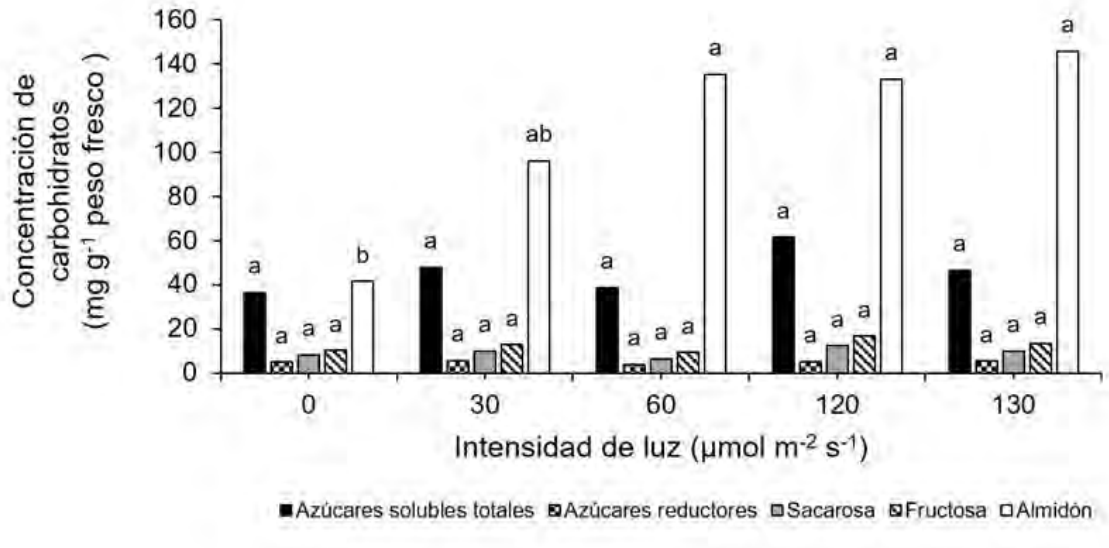
In the bulbils of *Lilium lancifolium*, the size of the starch granule is small, and it has a concave-convex shape with holes that provide it with a higher area-to-volume ratio that makes enzymatic digestion more efficient compared to the starch granules in bulbs that are larger and spherical or oval in shape. The starch granules present in the tissue of different species usually differ in size, structure, thermal properties, and digestion (Yu *et al*., 2015).

In addition, the degree of hydrolysis of starch depends on the conservation of its structure and is higher if it is subjected to heating and stirring, which allows the granules to be broken, prior to the enzymatic reaction with #-amylase and amyloglucosidase (Zhang *et al*., 2013), as was done in this trial. The results found in this work and the data reported in the determination of starch in the reserve organs of *Lilium lancifolium*; Yu *et al*. (2015) suggest that the size of the starch granules in the bulbils of *S. formosissima* propagated *in vitro* is small in this young tissue, which allowed a more efficient digestion of starch in the longest digestion period of 24 h with 8 U of #-amylase and amyloglucosidase.

Non-structural carbohydrates in leaves and bulbs

Starch content increased by 225, 221, and 251% in bulbs grown in 60, 120, and 130 µmol m^{-2} s^{-1} light, respectively, compared to bulbs grown in the dark (Figure 4). The synthesis of reserve carbohydrates, including starch, is highly related to the last stage in the formation of storage organs in geophytic plants, which is characterized by the growth of these organs (Podwyszynska, 2012) and the storage of starch in large quantities, which ensures a carbon source and energy supply during unfavorable conditions and rapid sprouting and reproductive growth in favorable environmental conditions (Ranwala and Miller, 2008).

Figure 4. Effect of light intensity on non-structural carbohydrate content in *in vitro* propagated *S. formosissima* bulbs. Means with equal letters are not statistically different (Tukey, $p \leq 0.05$) with honest significant difference (HSD) for total soluble sugars= 33.06; reducing sugars= 3.3; sucrose= 6.38; fructose= 9.38; starch= 68.96.



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In this experiment, the increase in starch accumulation in *S. formosissima* bulbs *in vitro* due to the effect of higher light intensity coincides with that reported in *Lachenalia* 'Rupert' bulbs, in which white (30 µmol m^{-2} s^{-1}) and blue light promoted starch accumulation, while darkness and red light decreased the concentration of this carbohydrate, with concentrations of 4.7 to 105 mg g^{-1} dry weight (Bach *et al*., 2015).

These results suggest that knowing the appropriate maximum light intensity in micropropagation can be used as a strategy to increase the starch content and size of the *S. formosissima* bulbils obtained by this technique.

Conclusions

Unlike the *in vitro* growth of other well-characterized geophyte species, our results indicate that emulating the dark conditions in which the bulbs normally develop does not determine the mobilization of carbohydrates to these organs in *S. formosissima*, which results in the decrease in biomass. On the other hand, it was observed that the development of photosynthetic tissues in the presence of light was more favorable for obtaining bulbs with higher biomass and higher reserve content.

Although it was observed that soluble sugars in the bulb did not fluctuate in concentration at the different levels of light exposure, starch accumulation increased significantly in bulbs as light intensity increased. This suggests that the accumulation of biomass and starch in *S. formosissima* bulbs grown *in vitro* is strongly regulated by the development of photosynthetic organs and their demand-source transition.

Other determinants besides light, such as nutritional condition or root development, need to be studied. However, observing that high intensities of light *in vitro* promote the growth and storage of starch in reserve organs can be used as a strategy to induce bulbing in *S. formosissima*.

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Light promotes *in vitro* bulbing of *Sprekelia formosissima*

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