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Research Article

In Vitro Bulbification of Five Lily Varieties: An Effective Method to Produce Quality Seeds and Flowers

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Lilies are one of the most important, beautiful, and economically valuable flowers in the world. *Lilium* is regarded as a popular floral trade cut flower, so viable protocols are needed to provide seed production, multiplication, and preservation. In vitro protocols allow for rapid large-scale production and rejuvenation of planting material, but to be a commercially viable multiplication method, the procedure must allow for rapid production of viable, true-to-type plants quickly. The objective was to evaluate the in vitro production of microbulbs of five lily varieties (*Lilium* "Champion Diamond," *Lilium* "Yellow Diamond," *Lilium* "Batavus," *Lilium* "Hyde Park," and *Lilium* sp.) using different concentrations of 6-benzylaminopurine (0, 0.5, 1.0, 1.5, 1.5, and 2.0 mg L^{-1} BAP) and to determine the commercial quality (flowering) of the plants grown from the seed obtained. Results from the micropropagation phase show *Lilium* "Batavus" and *Lilium* "Hyde Park" varieties had better in vitro responses, especially when grown with 1.0 and 1.5 mg L^{-1} BAP, respectively. Plants (of all varieties) grown from microbulbs showed positive growth and generally resulted in commercially viable flower production. Finally, the results of this study support the use of bulb scales as an alternative for obtaining vegetative seeds with high potential for lily cultivation.

1. Introduction

Lilies (*Lilium* sp.) are one of the most economically important cut flowers in the world. This flower belongs to the Liliaceae family, which includes about 250 genera and 3000 species spread throughout the world [1, 2].

Due to favorable agroclimatic conditions, lily cultivation is very popular in Peru, particularly in the Chachapoyas (Amazonas) and Caraz (Ancash) Highlands [3–5], making it a viable option for farmers to diversify their economy by adding the sale of one of the most popular cut flowers in floriculture to their current product portfolio. In view of this growth opportunity, Peruvian growers have been working to expand the range of varieties grown, which are now mainly limited to white inflorescence cultivars. However, due to the high import cost, the availability of bulbs (seeds) can be a limiting factor for lily growers in countries such as Peru, Argentina [6], and Colombia [1, 7]. In Peru, this is evidenced by the drastic decrease in the volume of imported bulbs, which fell from 2.7 million bulbs in 2018 (an estimated market value of U$S 752,531.93) to approximately 570000 bulbs in 2021 (estimated market value of U$S 168,000.53).

As a result, in the near environment, lilies are grown from the natural formation of new bulbs (obtained from previous harvests), which represents a potential source of pathogen transmission, also resulting in low-seed production and poor quality [1, 3, 6]. Due to this situation, there is significant room for growth in the bulb production market (driven by the demand for quality and healthy bulbs) since the import of planting material can represent up to 80% of cut flower production costs [6]. To this end, it is necessary to develop an innovative system aimed at the partial

substitution of lily bulb imports that mainly benefits small producers [6]. In this regard, in vitro culture techniques provide a key tool to achieve mass production [8], making them potentially one of the best methods for vegetative propagation of lilies [9]. In addition, these technologies provide alternatives to obtain pathogen-free plant material and rapidly produce genetically identical plants [10], satisfying the need for seeds. However, in vitro regeneration has been shown to depend on sucrose concentration [11–13], photoperiod and irradiance [11], scale position [14], and cytokinin and auxin concentration [8, 15, 16], among other factors. Therefore, there is a need to develop further studies that would optimize in vitro microbulb propagation. In addition, it is also economically important to evaluate the growth and marketability (flowering) characteristics of plants grown from in vitro produced microbulbs to ensure the ornamental quality of the plant.

In this context, the present investigation was carried out with the objective of identifying the adequate concentration of 6-benzylaminopurine to optimize microbulb production from bulb scales in five varieties of lilies. In addition, the growth and flowering of the lilies grown from microbulbs were evaluated.

2. Materials and Methods

2.1. Bulb Selection and Preparation. Healthy bulbs (6–8 cm diameter) of four lily hybrid varieties (*Lilium* "Champion Diamond," *Lilium* "Yellow Diamond," *Lilium* "Batavus," and *Lilium* "Hyde Park") and one local variety with white inflorescence (*Lilium* sp.) were collected at the "Asociación de Productores de Azucenas Cruz de Mayo" (6° 15′ 05″ S; 77° 50′ 26″ W), located in the province of Chachapoyas, Amazonas region (Peru). Bulbs were washed, dried, and stored at 8°C for six weeks to promote rapid and uniform production of microbulbs.

After cold treatment, the scales were removed from the bulbs and immersed in a fungicide solution for 20 minutes (2 mg L^{-1} Rizolex®; manufactured by Sumitomo Chemical Co. Japan). The surface of the scales was then sterilized in 70% (v/v) ethanol for 15–20 seconds, 1.5% household bleach (obtained from Clorox® with manufacturer's indicated concentration of 4% sodium hypochlorite) for 10 minutes, and 2% (v/v) mercuric chloride (Sigma-Aldrich, St Louis, MO) for 10 minutes. Three rinses with sterile water were performed at the end of each sterilization process.

2.2. Phase 1: Microbulb Formation. Basal segments of sterile scales were isolated and used as explants. Explants were seeded in Magenta™ containers containing 30 mL of Murashige and Skoog medium [17] supplemented with 150 mg L^{-1} ascorbic acid, 6% sucrose (w/v), 0.01% myoinositol (w/v), 0.7% agar (w/v), and 1 mg L^{-1} naphthaleneacetic acid (NAA). Four concentrations of 6-benzylaminopurine (0, 0.5, 1.0, 1.0, 1.5, and 2.0 mg L^{-1} BAP) were evaluated for microbulb induction. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C and 1.5 atm for 20 min. Explants were grown in growth room at 24 ± 1°C with a photoperiod of 16 h and a light intensity of 3000 lux provided by cool white fluorescent lamps. All the reagents used in the micropropagation phase were obtained from Sigma-Aldrich®.

At four weeks, the percentage of explants producing microbulbs (defined as bulb-shaped structure) was recorded, while at 90 days, the number of microbulbs per explant, the number of scales per microbulb, and the diameter of microbulbs were measured.

2.3. Phase 2: Hardening, Acclimatization, and Flowering. In the hardening stage, microbulbs (>2 g and 0.5 cm in diameter) derived from in vitro induction were immersed in benomyl solution (1 mg L^{-1}) for 1 minute and then in 300 ppm indole butyric acid (Sigma-Aldrich, St. Louis, MO) for 10 seconds. Subsequently, they were transferred to plug trays containing peat + perlite (2 : 1), covered with a transparent polyethylene sheet and placed in a growth room at 24 ± 1°C with a 12 h photoperiod. During the first two weeks, the film was gradually removed.

For the acclimatization stage, 10 microbulbs for each variety were transferred to pots containing peat + coconut fiber (2 : 1 ratio; pH: 4.02; organic matter: 12.87%; bulk density: 0.18 g/cm^3; porosity: 74%), then placed in a microtunnel (arranged under 70% shade), and maintained at a temperature between 22 and 25°C (regulated with a mist irrigation system) and relative humidity above 80%. After 45 days, the percentage of acclimatization (survival), plant height (cm), and the number of leaves per plant were recorded.

At the flowering stage (between the eighth and ninth month after planting), plant height (from the soil line to the top of the inflorescence), number of flower buds per inflorescence, flower bud diameter, flower bud length, flower tube length, tepal length and width, and flower diameter were measured. The floral tube (i.e., the trumpet-shaped section of a flower) was measured from the base of the tepal to the entrance of the trumpet-shaped part. The parameters were measured on the first fully expanded flower of the inflorescence. At the end of flowering, the weight, equatorial and polar diameter of the main bulb, and the number of secondary bulbs were recorded.

2.4. Experimental Design and Data Analysis. In the in vitro propagation stage, the experiment tested two factors (five varieties of lily x five concentrations of BAP), while in the acclimatization and flowering stage, only one factor (five varieties of lily). All trials were conducted under a completely randomized design, each treatment consisting of 10 replicates. Data were subjected to analysis of variance and measurements were compared and pooled using Tukey's test ($p \leq 0.05$). The analysis was performed with the statistical package InfoStat version 2017.

3. Results

3.1. Phase 1: Microbulb Formation. The formation of the first microbulb was observed about two weeks after explant establishment. *Lilium* "Batavus" explants had the fastest

microbulb formation (about 8 days), while *Lilium* sp. took longer (15–18 days) to observe the formation of this vegetative structure.

Lilium "Champion Diamond," *Lilium* "Hyde Park," and *Lilium* sp. explants treated with 1.5 or 2.0 mg L^{-1} BAP showed high efficiency in microbulb formation, with 100% regeneration (Figure 1(a)). In contrast, in *Lilium* "Yellow Diamond" and *Lilium* "Batavus," high concentrations of BAP (>1.0 mg L^{-1} BAP) had a negative effect. However, it is worth noting that, in this study, at least one of the BAP concentrations improved the percentage of microbulb induction compared to explants grown on a medium without growth regulator (Figure 1(e)).

Figure 1(b) shows that explants of *Lilium* "Batavus" and *Lilium* "Hyde Park" produced more than 4 microbulbs when grown on medium supplemented with 1.0 and 1.5 mg L^{-1} BAP, respectively. On the other hand, explants (of all varieties) grown at concentrations different from those mentioned above produced a lower number of microbulbs.

Lilium "Hyde Park" microbulbs treated with 0.5, 1.0, and 2.0 mg L^{-1} BAP had the highest number of scales (>4 scales) and diameter (0.87–0.93 cm) (Figures 1(c) and 1(d), respectively). The lowest number of scales was found in microbulbs of *Lilium* "Yellow Diamond" and *Lilium* "Batavus," which were grown on a medium without a growth regulator and under the concentration of 1.5 mg L^{-1} of BAP, respectively (Figure 1(c)). It also can be mentioned that microbulb diameter is a variable that is related to the number of scales present.

3.2. Acclimatization and Flowering. Microbulbs developed and transformed into vigorous plants throughout their acclimatization to ex vitro conditions. During the second week, all samples showed good growth and development, as well as 100% acclimation survival. Furthermore, with the exception of *Lilium* sp. plants, which averaged 4 leaves and a height of 5 cm in the first 45 days, all other plants grew between 10 and 13 cm and had good leaf formation, with an average of 10 leaves (data not shown).

The data obtained show positive results in the morphological parameters evaluated in lily plants grown from microbulbs (Tables 1 and 2). *Lilium* "Hyde Park plants reached the greatest height (113.90 ± 9.32 cm) and produced the greatest number of leaves (79.00 ± 9.70). *Lilium* "Yellow Diamond" variety produced bulbs with the highest fresh weight (21.28 ± 6.10 g) and equatorial diameter (3.74 ± 0.39 cm), as well as the highest number of secondary bulbs (9.60 ± 1.67). Plants of the five lily varieties showed no significant differences in stem diameter or main bulb polar diameter (Table 1).

All lily plants flowered between the eighth and tenth months after planting and produced flowers with attractive characteristics (Table 2 and Figures 2(a)–2(e)). *Lilium* "Batavus" plants produced the largest number of flower buds (5.20 ± 1.92), while on the other hand, *Lilium* sp. plants produced only one larger flower bud (10.35 ± 1.05 cm), indicating that their tepals were also longer (12.23 ± 1.62 cm). In the case of the hybrid varieties, it was observed that the floral tepals were wider (between 4.85 and 5.23 cm) than in the local variety. Flower bud diameter (between 2.47 and 3.03 cm), flower tube length (between 8.12 and 9.18 cm), and flower diameter (between 13.32 and 16.38 cm) of the five lily varieties were not significantly different. In this study, all flower buds formed on the lily plants were fully opened.

4. Discussion

4.1. Microbulb Formation. Lilies have high regeneration potential, with bulb scale tissue having the highest capacity to produce new bulbs [13, 18]. Therefore, they have been designated as the explant of excellence for in vitro culture of *Lilium* [8].

The use of BAP in the culture medium improved the ability to form microbulbs but did not stimulate root development, a result that was observed in explants grown without a growth regulator. This response could be related to the presence of endogenous auxins, such as naphthaleneacetic acid (NAA), which help in the induction of rooting [19]. The use of BAP stimulates cell division and new shoot formation [20], although it may have an inhibitory effect on root formation. Han et al. [21] reported a similar response, stating that when BAP is used, numerous bulbs are obtained, but root formation is inhibited.

In that context, the combination of auxins and cytokinin's at concentrations below 2 mg L^{-1} has been reported to improve regeneration efficiency and bulb formation [2, 8, 22]. However, factors such as explant type and sucrose concentrations [23, 24] and the presence of reserve elements, endogenous growth regulators, and genetic characteristics of the plant material must be taken into account [16]. In other words, the sum of all these factors could explain why each type of lily has such diverse responsiveness.

The highest number of microbulbs (4 per explant) occurred in explants of *Lilium* "Batavus" and *Lilium* "Hyde Park" treated with 1.0 and 1.5 mg L^{-1} of BAP, respectively. Similar results were reported by Kapoor et al. [25] who, using 2.0 mg L^{-1} NAA + 1.5 mg L^{-1} BAP, recorded 4 microbulbs per scale. On the other hand, the combination of NAA and BAP at concentrations lower than 1 mg L^{-1} resulted in the formation of only 2 microbulbs per explant [16, 26].

In this study, both scale number and microbulb diameter were higher in *Lilium* "Hyde Park" explants established at concentrations of 0.5, 1.0, and 2.0 mg L^{-1} BAP. Regarding the number of scales, the results are higher than those reported by Parić et al. [18], who with a treatment of 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} IBA obtained microbulbs consisting of 2 scales. Meanwhile, Youssef et al. [27] reported the formation of up to 8 scales per microbulb in explants grown in 1.0 mg L^{-1} of BAP + 0.2 mg L^{-1} of NAA. Regarding microbulb diameter, the results are lower than the report of researchers such as Youssef et al. [27] and Akçal and Kahraman [28], as they describe microbulbs of 1.68 and 1.32 cm in diameter, respectively.

These studies show that while all cells in a plant have the potential to regenerate the plant, the degree of difficulty in expressing totipotency varies between different lily varieties and even between different cells within the same plant [29].

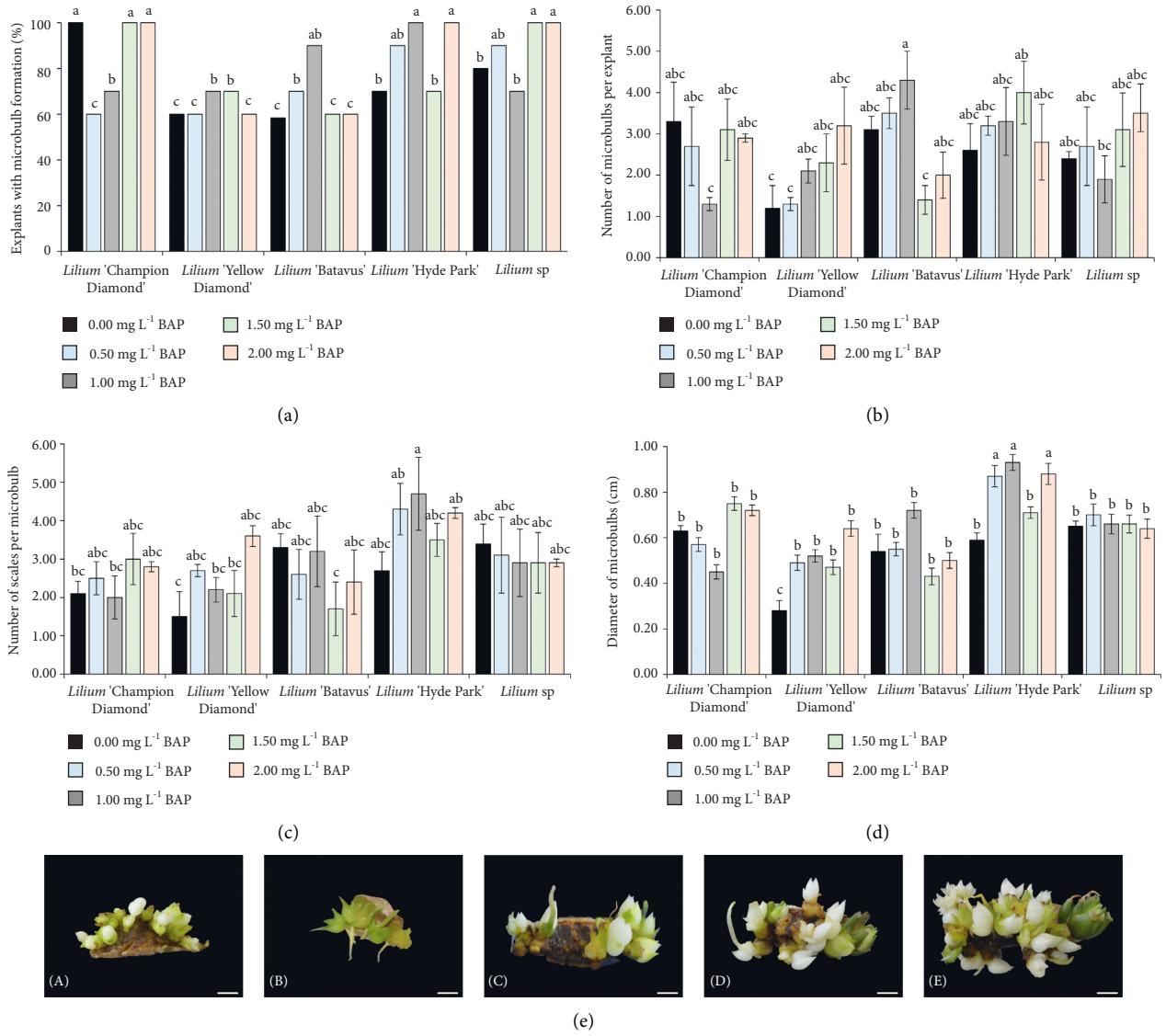

Figure 1: Effects of different concentrations of benzylaminopurine on microbulb regeneration from bulb scales. The panels are (a) percentage of explants with microbulb formation, (b) number of microbulbs per explant, (c) number of scales per microbulb, (d) microbulb diameter, and (e) microbulb regeneration in five lily cultivars. (A) Lilium "Yellow Diamond," (B) Lilium sp., (C) Lilium "Champion Diamond," (D) Lilium "Hyde Park," and (E) Lilium "Batavus." Scale = 50 mm.

Table 1: Comparison of morphological (growth) characteristics of five varieties of lilies grown from in vitro regeneration of microbulbs.

Varieties of lily	Plant height (cm)	Diameter of stem (cm)	No. of leaves per plant	Bulb fresh weight (g)	Equatorial diameter of bulb (cm)	Polar diameter of bulb (cm)	No. of bulbs
Lilium "Batavus"	84.20 ± 8.84^{b}	0.77 ± 0.16^{ns}	76.00 ± 18.53^{a}	14.99 ± 3.04^{ab}	3.32 ± 0.41^{ab}	3.05 ± 0.30^{ns}	8.60 ± 2.07^{a}
Lilium "Champion Diamond"	56.60 ± 9.58^{c}	1.14 ± 0.51^{ns}	42.80 ± 18.91^{b}	7.54 ± 2.18^{b}	2.02 ± 0.85^{c}	2.73 ± 1.05^{ns}	3.20 ± 1.30^{bc}
Lilium "Hyde Park"	113.90 ± 9.32^{a}	0.68 ± 0.17^{ns}	79.00 ± 9.70^{a}	9.88 ± 1.71^{b}	2.76 ± 0.15^{bc}	2.55 ± 0.23^{ns}	6.20 ± 2.59^{ab}
Lilium "Yellow Diamond"	84.60 ± 25.79^{b}	0.66 ± 0.15^{ns}	66.60 ± 24.89^{ab}	21.28 ± 6.10^{a}	3.74 ± 0.39^{a}	3.24 ± 0.20^{ns}	9.60 ± 1.67^{a}
Lilium sp.	46.20 ± 5.59^{c}	0.59 ± 0.07^{ns}	35.40 ± 6.27^{b}	5.85 ± 3.01^{b}	2.29 ± 0.40^{c}	2.22 ± 0.54^{ns}	2.40 ± 1.14^{c}

Means (± standard deviation) with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$).

Table 2: Comparison of the marketing (flowering) characteristics of five varieties of lilies grown from in vitro regeneration of microbulbs.

Varieties of lily	Number of flower buds per plant	Flower bud diameter (cm)	Flower bud length (cm)	Length of floral tube (cm)	Diameter of flower (cm)	Tepal length (cm)	Tepal width (cm)
Lilium "Batavus"	5.20 ± 1.92^{a}	2.72 ± 0.18^{ns}	8.57 ± 0.74^{b}	8.12 ± 0.51^{ns}	16.27 ± 1.86^{ns}	9.89 ± 0.61^{b}	5.13 ± 0.57^{a}
Lilium "Champion Diamond"	1.80 ± 0.84^{b}	2.87 ± 0.47^{ns}	8.64 ± 1.01^{b}	8.99 ± 0.58^{ns}	16.38 ± 1.46^{ns}	9.58 ± 1.05^{b}	5.23 ± 0.57^{a}
Lilium "Hyde Park"	3.20 ± 1.10^{ab}	3.03 ± 0.32^{ns}	9.46 ± 0.58^{ab}	8.50 ± 0.28^{ns}	15.45 ± 2.78^{ns}	10.44 ± 0.39^{b}	4.85 ± 0.43^{a}
Lilium "Yellow Diamond"	3.20 ± 1.92^{ab}	2.78 ± 0.17^{ns}	9.82 ± 0.51^{ab}	8.49 ± 0.80^{ns}	14.53 ± 0.54^{ns}	10.98 ± 0.48^{ab}	4.91 ± 0.16^{a}
Lilium sp.	1.00 ± 0.00^{b}	2.47 ± 0.52^{ns}	10.35 ± 1.05^{a}	9.18 ± 1.99^{ns}	13.32 ± 1.56^{ns}	12.23 ± 1.62^{a}	3.93 ± 0.10^{b}

Means (± standard deviation) with the same letter in the column do not differ significantly from each other (Tukey, $p \leq 0.05$).

Figure 2: Lilies flowering on plants grown from microbulbs. (a) *Lilium* "Batavus." (b) *Lilium* "Yellow Diamond." (c) *Lilium* "Champion Diamond." (d) *Lilium* sp. (e) *Lilium* "Hyde Park."

Similarly, Akçal and Kahraman [28] and Naing et al. [30] stated that the success of scale regeneration and subsequent scale formation depends largely on the explant and the presence of growth regulators. Deswiniyanti and Dwi-payani-Lestari [16] emphasized the importance of endogenous phytohormone content and the balance of bulb scales to regenerate new vegetative structures. In general, the use of phytohormones (e.g., AIA and BAP) has shown positive effects on shoot proliferation from bulb scales in different *Lilium* varieties [8, 21]. However, reports also pointed out that an increase in sucrose concentration during in vitro culture (>60 g L^{-1}) allows better formation and growth of microbulbs [27, 31]. This improvement in microbulb size, caused by an increase in sucrose concentration in the culture medium, could be attributed to a significant improvement in total carbohydrate and starch accumulation [27, 32].

Therefore, we can say that the regeneration efficiency of lily microbulbs and other similar species during tissue culture is related to determining the balance of several factors.

4.2. Acclimatization and Flowering. A key to successful micropropagation is to obtain high acclimation rates and ensure that seedlings obtain adequate development in a nonsterile environment [10]. However, it is well known that the final outcome will be determined by the interaction of multiple factors, including the seedling's encounter with environmental conditions that may cause stress during transplanting [33]. In this study, it was observed that a simple activity such as covering the pots with polyethylene sheeting and maintaining adequate watering frequencies allowed the seedlings to remain unaffected by the ex vitro conditions, resulting in the survival of all seedlings and growth of up to 13 cm in the first 45 days. In this regard, Daneshvar-Royandazagh [2] and Saetiew and Umamanit [34] concluded that keeping seedlings in a moist environment during their first days of growth is critical to prevent losses.

Experiments where bulblets produced in vitro were directly sown in the field have shown that bulblets emerge more slowly due to their small size [35]. Because of this, some recent studies have revealed that adding paclobutrazol and increasing the amount of sucrose to the culture medium allows microbulbs to improve their size through increased accumulation of carbohydrates and starch [27, 36]. Also, it has been described that transferring seedling with well-developed shoots and roots is ideal to aid rapid adaptation and growth during acclimation [37]. Through these improvements in the micropropagation process, it is possible to improve the growth of microbulbs, which would shorten the time required for bulb fattening (growth in soil) prior to flower stalk formation. In flower production, variables such as color, size, number of flowers per plant, flower architecture, and flower longevity have been identified as the most important characteristics for cut flower marketing [38]. In this study, lily plants of the five varieties were observed to have similar flower quality (flower bud diameter, flower tube length, flower diameter, and stem diameter), each with vibrant color and shelf life of 7–12 days. In addition, both tepal length and width were found to be consistent with the flowering characteristics of lily cv. Mona Lisa was treated with silver nanoparticles [39].

According to Auzaque-Rodriguez et al. [7], the commercial quality of flowers can be divided into four categories according to the number of buds: extra (4 or more buds; >65 cm plant height), super *3 (3 buds; 55–64 cm plant height), super *2 (2 buds; 55–64 cm plant height), and national (1 or more buds; <55 cm plant height). In that sense, it can be said that the flowers of the *Lilium* "Batavus" variety achieved extra quality, while the *Lilium* "Hyde Park" and *Lilium* "Yellow Diamond" varieties achieved super *3 quality; these categories are being the most suitable for commercialization. The commercial quality of the aforementioned varieties is comparable to that reported for lilies cv. Mona Lisa (4.0) [39], cv. Eldivo (3.81), and Fangio (3.88) [40].

On the other hand, it should be noted that bulb vernalization [7], fertilization management [41, 42], and substrate selection [43] are all important factors in maintaining lily quality and yield. In addition, these factors may contribute to an increase in bulb weight and number of scales, which would reflect better reproductive potential [39], potentially resulting in a significant reduction in seed import volume [26].

5. Conclusions

The results of this study confirm that BAP stimulates microbulb production. In trials with *Lilium* "Champion Diamond," *Lilium* "Hyde Park," and *Lilium* sp., it was observed that explants grown with 1.5 and/or 2.0 mg L^{-1} of BAP achieved high efficiency in microbulb formation with a regeneration rate of 100%. These findings support the use of bulb scale segments as a viable method of seed production. Furthermore, this study has shown that microbulb production and seeding are feasible because it leads to the development of plants with floral traits within commercial standards. The method presented here could boost floriculture by reducing lily production costs due to bulb importation because micropropagation makes mass production of good quality lilies feasible.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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