

## Brief Note

# Hydrogen peroxide in micropropagation of *Lilium*. A comparison with a traditional methodology

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**ABSTRACT:** The micropropagation of *Lilium longiflorum* requires adequate equipment which may not be afforded by small laboratories or producers. In this work we compared traditional methodology with a protocol that included easily available elements to sterilize materials and culture media, together with addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into the nutrient media as chemical sterilizer. A series of H<sub>2</sub>O<sub>2</sub> concentrations (0.005, 0.010, 0.015 and 0.020% p/v) was used to control contamination during *in vitro* establishment and subsequent cultivation; the explant organogenic response was also examined and compared to the traditional micropropagation technique. The level of culture contamination was within acceptable limits in all treatments, though it was higher in the H<sub>2</sub>O<sub>2</sub> treatments (40%) compared to the traditional methodology (20%). There were not significant differences in the number of bulblets per explant, and at the end of the multiplication phase, bulblets from 0.02% H<sub>2</sub>O<sub>2</sub> treatment had greater biomass than from other treatments, indicating a beneficial effect. These bulblets also had a higher relative growth ratio with respect to the traditional method when cultivated for an additional period and showed the highest average bulblet fresh weight. It is expected that this higher bulblet mass would result in better performance during *ex vitro* cultivation.

## Introduction

The *in vitro* production of bulbous species and particularly that of *Lilium* species has a lead position compared with other micropropagated plants (Pierik, 1990). Successful micropropagation requires to assure establishment of an axenic culture and preservation of plant material free from contamination during successive stages of *in vitro* cultivation. Otherwise, serious losses due to deficiency in the growth or death of the tissue explants might occur (Cassells, 1991). To obtain an axenic culture, it is important to perform an adequate a

disinfection procedure. In the case of *L. longiflorum*, explants are obtained from a bulb which has been grown in soil; hence, they harbor an elevated number of potentially harmful contaminants currently leading to losses ranging from 20 to 80% (Marinangeli and Curvetto, 1997a). Some microorganisms develop during the first phase of tissue culture while others may remain subliminal and their presence may not be detected until more advanced cultivation stages (Cassells, 1991). Autoclaves and air sterile cabinets are required in micropropagation. However, these tools are expensive and pose a situation hardly affordable for hobbyists or even small producers. This situation might be overcome through the use of available materials to sterilize either using fluent vapor or vapor under pressure as could be made with a steamer pan or a home pressure cooker pan, respectively.

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Addition of H<sub>2</sub>O<sub>2</sub> as a chemical sterilizer into the culture medium and during explant manipulation following aseptic techniques might preserve the medium and the explant free from contamination. In orchid, it was reported that H<sub>2</sub>O<sub>2</sub> has germicidal and fungicidal activities at low concentrations, without affecting *in vitro* seed germination and plantlet growth (Snow, 1985). Lack of cellular and tissue damage has been attributed to the activity of plant peroxidases that act against H<sub>2</sub>O<sub>2</sub> by transforming it into water and oxygen. It is well-known that many plant cells contain enzymes, including catalases and peroxidases that decompose the H<sub>2</sub>O<sub>2</sub> (Goodwin and Mercer, 1972), thus providing a protection mechanism to preserve cells from the deleterious effects of peroxides produced either by their own metabolism or from an external source.

Hydrogen peroxide was early used as chemical sterilizer by McAlpine (1947), who developed a medium culture for orchid seeds without the need of autoclaving. In the range 0.009-0.18%, H<sub>2</sub>O<sub>2</sub> inhibited the development of molds without affecting the germination of orchid seeds (Snow, 1985). Concentrations between 0.005 and 0.01% in orchid culture medium were effective as chemical sterilizer (Yanagawa *et al.*, 1995).

With the aim of developing a low cost protocol to micropropagate *L. longiflorum* for hobbyists and small producers, the effectiveness of H<sub>2</sub>O<sub>2</sub> to surface disinfect Easter lily scale explants was assessed to provide a successful means for *in vitro* establishment. The potential of H<sub>2</sub>O<sub>2</sub> to maintain media free from bacteria, molds, sporulated forms, and small multicellular organisms, thus allowing the normal evolution of the direct organogenesis pathway from scale explants was also assessed. Effects of H<sub>2</sub>O<sub>2</sub> on bulblet differentiation and bulblet growth during *in vitro* propagation was also examined.

Effects of different H<sub>2</sub>O<sub>2</sub> concentrations (0.005, 0.010, 0.015 and 0.020% w/v) during disinfection steps and in the culture media used to micropropagate *L. longiflorum* was evaluated *vis-à-vis* with traditional methodology (TM) (Stimart and Ascher, 1978; Marinangeli and Curvetto, 1997b) for determining levels of contamination, rates of explants producing bulblets, number of bulblets per explant, and bulblet biomass at the end of the multiplication stage and after a period of additional *in vitro* growth.

## Materials and Methods

Bulbs of *L. longiflorum* cv "Snow Queen" (size 12-14) coming from the field were stored at 4°C until use.

Middle scales (Marinangeli *et al.*, 2003) were excised from bulbs and carefully washed with tap water. Scale and explant disinfection were performed following either traditional methodology (Stimart and Ascher, 1978) or using H<sub>2</sub>O<sub>2</sub> at different concentrations (0.005, 0.010, 0.015 and 0.02% w/v). Basal medium for *in vitro* culture consisted of MS pH 5.7 (Murashige and Skoog, 1962), supplemented with 100 mg L<sup>-1</sup> inositol, 0.40 mg L<sup>-1</sup> thiamine, 0.10 mg L<sup>-1</sup> pyridoxine, 0.50 mg L<sup>-1</sup> nicotinic acid, 0.03 mg L<sup>-1</sup> naphthalene acetic acid (NAA), sucrose 30 g L<sup>-1</sup> and 3 g L<sup>-1</sup> Phytigel (Sigma). Twenty five explants were used per treatment.

The traditional methodology (TM) used in our laboratory is based on the use of scale segments as primary explants (Stimart and Ascher, 1978). In addition, microbulblets were subjected to an additional growth in a medium containing elevated sucrose content to reach a higher biomass and scale number (Marinangeli and Curvetto, 1997b).

Taking into account the previous considerations, the establishment of the axenic culture and subsequent multiplication starting from segments of bulb scales and an additional period of growth of the newly regenerated microbulblets were performed after conducting common disinfection procedures. Scale disinfection was conducted as follows: scales were successively treated with ethanol 70% for 1 min, sodium hypochlorite solution (1.6 g L<sup>-1</sup> active chlorine) containing 2 drops of Tween 20 per liter for 20 minutes, and washed three times with sterile distilled water. In a sterile airflow cabinet, explant sections (ca. 2 mm) were obtained by transversally slicing the bulb scales; slides were placed into culture tubes (10 X 150 mm) containing 5 mL autoclave-sterilized culture medium.

For H<sub>2</sub>O<sub>2</sub> treatment, the culture medium was placed into 250 mL bottles and sterilized in a domestic 10 L pressure pot as follows: the pot was considered purged after the first escape of water vapor; then, the bottles remained under sterilizing heat for 40 min. When the temperature in the culture medium decreased to near 50°C, H<sub>2</sub>O<sub>2</sub> was added from a 30% stock solution (w/v) to finally obtain 0.005%, 0.010%, 0.015% and 0.020% (w/v) H<sub>2</sub>O<sub>2</sub> solutions, which were dispensed in 5 mL aliquots into culture tubes previously sterilized in a pressure pot for 30 minutes.

To obtain the explants, scales were placed in Petri dishes - previously sterilized by fluent vapor - that contained a 0.03% H<sub>2</sub>O<sub>2</sub> solution, then cut into ca. 2 mm transverse sections, and placed on the nutrient medium. During this operation, scales remained immersed in to the H<sub>2</sub>O<sub>2</sub> solution, which was frequently changed for fresh one. This work was performed on a lab counter using

basic aseptic technique similar to that used in handling cultivation of bacteriological materials in a standard microbiology laboratory, i.e. without using an equipment to supply sterile air.

All explants were cultivated at 20-25°C in darkness. The number of contaminated explants (those showing evidence of fungal or bacterial contamination by eye inspection), and the appearance of organogenic response were weekly recorded. At the end of the cultivation period (6 weeks), the percentage of explants producing microbulbets, the number of explants harboring bulbets, and their fresh weight were obtained. Bulbets obtained were cultivated during 12 weeks in a bulbet-growing medium (Marinangeli and Curvetto, 1997b), which contained 90 g L<sup>-1</sup> sucrose and 2X MS; H<sub>2</sub>O<sub>2</sub> concentrations were maintained for each treatment. At the end of

this additional growth period, the relative bulbet growth was calculated as the fresh biomass increase in relation to the corresponding biomass at the beginning of the growth period.

The experimental design was fully randomized and results were evaluated with one-way ANOVA test followed by a Dunnett's test to separate means.

**Results and Discussion**

The level of contamination was in the range of 40-68% in the cultures corresponding to H<sub>2</sub>O<sub>2</sub> treatments. The lowest mean value (40%) was observed with 0.02% H<sub>2</sub>O<sub>2</sub>; 20% contamination resulted when the traditional protocol was used (Table I). Even though the traditional

**TABLE I.**

**Comparison of two micropropagation techniques for *Lilium longiflorum*.**

Traditional methodology viz-a-viz *in vitro* culture technique using different H<sub>2</sub>O<sub>2</sub> concentrations as sterilizer, during an 18 week cultivation period. Contamination percentage, percentage of explants with bulbets, number of bulbets per explant, bulbet biomass in the multiplication phase and at the end of the growing phase, and relative bulbet growth are shown. Mean values +/- SE; ns, \* and \*\*: no significant differences; significant differences at P<0.05 and P<0.01, respectively, of H<sub>2</sub>O<sub>2</sub> treatments in relation to the traditional methodology by Dunnett's test.

	Traditional methodology	H <sub>2</sub> O <sub>2</sub> concentration in culture media			
		0.005%	0.010%	0.015%	0.020%
Contamination (%)	20	52.5	67.5	45	40
Explant with bulbets (%)	87.5	89.5	92.3	95.4	100
Number of bulbets per explant	2.0 ± 0.2	1.7 ± 0.2 ns	1.5 ± 0.1 ns	1.8 ± 0.2 ns	1.8 ± 0.2 ns
Bulbet fresh weight in the multiplication phase (mg) <sup>(1)</sup>	42.1 ± 4.3	40.4 ± 5.6 ns	41.1 ± 6.4 ns	41.4 ± 6.9 ns	53.5 ± 3.7 *
Bulbet fresh weight in the growing phase (mg) <sup>(2)</sup>	160.8 ± 5.0	142.7 ± 12.3 ns	184.2 ± 25.4 ns	173.4 ± 19.4 ns	276.8 ± 18.4 **
Relative bulbet growth at the end of the growing period (g/g) <sup>(2)</sup>	2.9 ± 0.2	4.4 ± 0.5 *	4.0 ± 0.6 *	4.4 ± 0.4 *	5.0 ± 0.5 **

<sup>(1)</sup> 6-week multiplication period, in MS medium containing 30 g L<sup>-1</sup> sucrose.

<sup>(2)</sup> 12-week growth period, in MS medium containing 90 g L<sup>-1</sup> sucrose.

protocol was better than the H<sub>2</sub>O<sub>2</sub> one, the latter might be still considered useful for tissue culture establishment because of its low cost and also because of the additional benefits provided by H<sub>2</sub>O<sub>2</sub>, which will be discussed later. Bulbs came from the field and consequently carried soil contaminants on their surface. In the presence of H<sub>2</sub>O<sub>2</sub> only mold was observed with an apparent absence of bacterial growth. Fungi also may display peroxidase activity (Miles and Chang, 1997), which might be effective in degrading H<sub>2</sub>O<sub>2</sub> at those concentrations. A H<sub>2</sub>O<sub>2</sub> bacteriostatic effect causing a delay in the growth of bacterial competitors during early stages of infection was reported (Rafael *et al.*, 2001).

After 6 weeks of cultivation, no significant differences were observed among the H<sub>2</sub>O<sub>2</sub> treatments with the traditional method of micropropagation in terms of the number of bulblets per explant. Bulblet biomass obtained in the 0.02% H<sub>2</sub>O<sub>2</sub> treatment (53 mg) was significantly higher ( $P < 0.05$ ) than the biomass obtained with the traditional methodology (42 mg).

The increase of H<sub>2</sub>O<sub>2</sub> in the culture media increased the number of explants with bulblets, but not the number of bulblets per explant ( $P > 0.5$ ) (Table I). In fact, the bulblet production per explant was ca. 2 bulblets, a result that is in good agreement with that previously reported by Stimart and Ascher (1978) and Marinangeli and Curvetto (1997a) for *L. longiflorum* micropropagation.

The average bulblet fresh weight (277 mg) obtained after 12 weeks of cultivation in nutrient medium containing high sucrose levels and 0.02% H<sub>2</sub>O<sub>2</sub> was significantly higher ( $p < 0.01$ ) than the value obtained with the traditional methodology (161 mg). Therefore, 0.02% H<sub>2</sub>O<sub>2</sub> had a promotive effect on bulblet growth under the conditions specified above. Moreover, the bulblets corresponding to the 0.02% H<sub>2</sub>O<sub>2</sub> treatment presented a higher relative growth (5 g/g initial weight) than that observed with the traditional method (2.94 g/g initial weight),  $p < 0.01$  (Table I). Higher bulblet size might represent an advantage, since epigeous type plants are originated from well-sized bulblets and this type of bulblet will normally advance the commercial bulb size one year or at least will reach higher size in the next growing season (Marinangeli and Curvetto, 1998; 1999). This would represent an advantage, since the time factor is important when carrying these bulblets into field production.

## Conclusions

These results obtained under the optimized conditions given in this work, suggest that H<sub>2</sub>O<sub>2</sub> might be

used as a low cost alternative method to traditional micropropagation techniques by hobbyists or small lily producers to micropropagate *L. longiflorum*. H<sub>2</sub>O<sub>2</sub> up to 0.02% has no deleterious effects on bulblet differentiation, but it does have a stimulatory effect on bulblet growth. The higher bulblet mass obtained after the 18 week cultivation period in the case of the 0.02% H<sub>2</sub>O<sub>2</sub> treatment would eventually result in a better plantlet performance growth *ex-vitro*. Further studies are currently being conducted to test the use of H<sub>2</sub>O<sub>2</sub> in the traditional methodology for *in vitro* propagation of *L. longiflorum* to explore additional benefits on bulblet growth.

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