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In vitro Propagation Strategies to Improve Reinforcing Activity for Two Italian Endangered Species: *Lilium pomponium* L. and *Lilium martagon* L.

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Abstract

Lilium pomponium and *Lilium martagon* belong to the Liliaceae family and in Italy are submitted to a total protection by regional laws in Liguria and Piedmont. The ornamental value of these species is high and they are also used for breeding programs to obtain commercial cultivars. The micropropagation provides an adequate number of plants to be used as mother stock plants and to be re-introduced into the natural habitat. The size of the bulbs coming from *in vitro* culture is a crucial point to guarantee the good acclimatization of the plants and a suitable aerial part development after transfer to soil. The research was conducted to improve the *in vitro* growth of bulbs in number and dimensions useful for fast re-introduction. Experiments were carried out to evaluate factors typically conditioning bulb development and enlargement, such as medium salt composition, concentration of indole butyric acid, sucrose, photoperiod and temperature. For *L. martagon*, the multiplication rate, in term of bulb/explant, is best obtained with the use of WPM salts, with a temperature of 18°C and a sucrose concentration of 30 g/L; *L. pomponium* propagated better in the presence of MS salts supplemented with 30 g/L of sucrose and 0.5 mg/L of IBA, at 24°C with a photoperiod of 16 hours of light. Results show that in order to obtain large bulbs is preferable to use salts, at a temperature of 24°C, with high IBA and sucrose concentration. The *in vitro* growth in darkness in *L. pomponium* ensures higher bulbs weight; after 6 months of *ex vitro* growth, it was possible to obtain a minimum of 57% to a maximum of 84.5% of plant survival, in a very good growth conditions.

INTRODUCTION

Lilium pomponium L. and *Lilium martagon* L. belong to the genus *Lilium* (Liliaceae) that includes about 100 species [1,2], which are geographically distributed in the Northern hemisphere (North America, Europe and temperate Asia) [3], in particular between 10° and 60° of latitude [4]. Intragenic classification of *Lilium* is subject of considerable discussion and the number of section used differs depending on the authors [2], recently molecular markers have proved to be useful in developing an improved classification that showed that these two species are very distant in phylogenetic tree following the analysis with ITS sequence [2]. Only four species grow wild in Italy and all are included in the list of regional protection; among them, *L. martagon* and *L. pomponium* are submitted to a total protection by regional laws in Liguria (L.R. n°28/2009) and Piedmont (L.R. n°32/1982).

L. pomponium (Lesser Turk's-cap Lily) [1], is a Liguria-Provencal endemic plant belonging to Liriotypus region [2], in Italy it grows only in Western Liguria [5], in a few locations. It grows in xeric grasslands, rocky hillsides and Limestone Mountains on calcareous soil at an altitude of 1000–1950m [6,7]. It is a plant with high ornamental value and wild populations are threatened mainly by the activities of human disturbance and by grazing and excavation of wild boars, looking for the bulbs. This taxon is included in the category EN (endangered) in Italy by the IUCN [8], and it is cited in the "Atlante delle specie a rischio di estinzione" by Scoppola and Spampinato [8].

L. martagon L. (Turk's-cap lily) [1], belongs to the Martagon section [2,4,10], it is widely spread in Europe and western Asia [1,11], and in Italy widely present excluding Puglia, Basilicata, Calabria and the islands. It grows in mountain grasslands, glades and light woods, above all beech, of the Alps and Apennines on soil with pH basic and rich in humus at an altitude from 300 to 2000m. *L. martagon* has a very important ornamental value for the cut flower and potted plant industries for its beautiful flowers [12]. The species is threatened in Italy for anthropic pressure since there is an uncontrolled harvested of the flowers due to the high ornamental value; for this reason, it is also included in the red list of total regional protection in Liguria and Piedmont region (L.R. n. 28/2009).

Lilium genus produces flat seeds with a complete peripheral wing. The endosperm is generally firm to hard and semitransparent and is not starchy [13]. The embryo is classified as linear [13], in some species morphophysiological dormancy of the embryo may be possible [14]. *L. pomponium* shows an epigeal immediate germination, while for *L. martagon* is reported to have hypogeal delayed germination [15,3]. A recent study, verified that the germination is immediate [6,16]. A protocol for seed germination was studied some years ago for seed bank germplasm *ex situ* conservation [17].

In order to counteract the biodiversity decline, over the last 30 years, has been reported a significant increase of plant collections in *ex situ* storage centers [18]. *In vitro* technology has been widely demonstrated as a powerful strategy for *ex situ* conservation of rare and endangered plants [19], in *Limonium* species, for example, the results demonstrated that the *ex vitro* plants produce more flowers in compare with the same native species [19]. *Ex situ* techniques are generally used to complement *in situ* methods, but in some cases they are the only possible conservation techniques when the species has propagation problems or when there is a very few original material as mother plant [20], thus imposing minimum impact on the endangered wild population [21]. Bulbous plants, like lilies, have proved to be ideal for *in vitro* culture, as their regeneration potential is usually high [22-24], to increase mass production of selected and/or virus-free plants, the *in vitro* culture is necessaries [25]. *In vitro* cultures of endangered species are the base of the germplasm banks which provide an adequate number of plants to be re-introduced into their natural habitat [26,27]. The interventions of re-introduction have become an essential component in the conservation of rare and endangered plant species. The success of these interventions which aim is to recreate an independent population able to reproduce and adapt to environmental changes strongly depends on the quality of the plant material [28-30]. High quality *in vitro* bulblets should grow rapidly and flower as fast as possible after transfer to soil. Fast growth occurred in large bulbs forming a stem with several leaves instead of one or two leaf-bearing scales [31]. Since stem formation occurred often in large bulblets, we assumed that the development of *in vitro* bulbs is an important factor to be studied in order to promote a rapid growth of plants after planting [31]. Preliminary experiments on three endemics Ligurian *Lilium* (*L. bulbiferum, L. martagon* and *L. pomponium*) have been reported in 2002 by Beruto et al. No reports are available for a complete analysis of the *in vitro* propagation system.

The present research was therefore conducted to improve the *in vitro* growth of bulbs in number and dimensions useful for fast re-introduction of these important endangered species. Experiments were carried out to evaluate factors that are typically conditioning bulb induction and growth such as medium salts composition [33], concentration of plant growth regulators [34,35] sucrose [34,36,37] and temperature [38].

MATERIALS AND METHODS

L. martagon seeds were collected in October 2010 in Val Nervia (Liguria region, Italy, altitude 1800 m); they were sterilized by sodium hypochlorite 1% of active chlorine for 20 minutes, washed twice with distilled and sterilized water and laid *in vitro* onto sterile filter paper soaked with distilled water or onto water-agar (0.7% agar) or onto MS [39], agarized base medium [16]. Distilled water (230 µS/cm) had pH 7.05 while MS base medium (4970 µS/cm) had pH 5.7 adjusted with NaOH/HCl.

L. pomponium seeds were collected in September 2008 (Monte Alto, Imperia) (Liguria region, Italy). 160 seeds of 320 totals were sterilized with 1% of NaOCl while the remaining ones were sown directly in Petri dishes containing two pieces of filter paper moistened with distilled water [6]. After germination, seedlings were transferred to MS medium added with indole butyric acid (IBA) 0.5 mg/L, pH 5.7 ± 1 and 0.8% agar. Media were autoclaved at 120 ± 1°C for 20 minutes.

Some *L. martagon* seedlings were excised and cotyledons and roots were removed. If not otherwise specified, culture conditions were set up at 24 ± 1°C temperature and 30 µE s^{-1} m^{-2} with 16h light as photoperiod.

Some seedlings were cloned in order to obtain a sufficient number of bulblets to perform *in vitro* trials. Three clonal lines for each *Lilium* species were selected for *in vitro* high proliferation aptitude; in order to set up the best *in vitro* performance, the addiction of two concentrations of IBA (0.5 and 1.5 mg/L) to the standard control medium for *Lilium* spp. (MS salts added with 30 g/L of sucrose) were compared to PGRs free medium.

We use as control a standard culture medium composed by: MS macroelements, 30 g/L of sucrose, 0,5 mg/L IBA, at 24°C. Then, the *in vitro* experiments were set up in glass vessels with 5 bulbs for each clone and each treatment, as complete randomized block considering the following variables: two environmental growth temperatures (18°C±1 and 24°C±1), two salt compositions as MS or WPM [40], and three sucrose concentrations (30, 45 and 90 g/L); only for *L. pomponium* bulbs a darkness constant treatment was applied in comparison to light condition (30 µE s^{-1} m^{-2} with 16 h light as photoperiod). In *L. pomponium* for every treatment 10 uniform bulbs were used divided in 2 replications of 5 bulb for jar; in *L. martagon* 6 uniform bulbs were used divided in 2 replications of 3 bulbs for jar. The differences between the salt composition of MS or of WPM consisted in: the first having total salts concentration of 43.02 g/L, N content of 60.01 mM and a NPK ratio of 1:0.02:0.33. The second with total salts concentration 23.59 g/L, rich proportionally in K and Ca (15.89 mM and 3.01 respectively) and a NPK ratio of 1:0.08:1.08.

At the beginning and at the end of the experiments bulb weight (g) was recorded in order to calculate the Relative Growth Rate (RGR index) according to the following formula: $\ln FW_{final}$-$\ln FW_{initial}$ ×100/days of culture [41]. After 150 days, data of diameter (cm), weight (g), percentage of bulbs that produced secondary bulbs, multiplicaton rate (number of secondary bulbs *per* principal bulb), and weight and diameter of secondary bulblets were collected and evaluated. Data were analyzed grouping all the common treatments for each one.

Since *L. martagon* bulb number was not sufficient for the acclimatization trial, only 20 principal bulbs (diameter > 1.6 cm) were transferred in greenhouse in order to create a stock of plant material to be used in re-introduction program. *L. pomponium* bulbs were instead transferred in December 2012 to the greenhouse for acclimatization in plastic plateau containing

commercial potting soil-perlite (30:70, v:v). The humidity was guaranteed by a mist system (10 s water spray every 40 min) at 15°C ± 3 temperature and 80% relative humidity (R.H.) for the first 30 days. The material was then maintained in the greenhouse with irrigation two times a week. Survival percentage (%), bulb diameter (cm) and weight (g) were recorded after 180 days. Survived bulbs were transferred in 11 cm diameter pots with commercial substrate drained with pumice and maintained fewer than 50% shading nets. Bulbs diameter (cm) and weight average (g) were recorded after two years from transplant.

All the recorded data were statistically analyzed with mean ± standard error (SE) and using the statistic program COSTAT; the analysis of variance was performed at $p \leq 0.05$ and the averages were compared with the Student Newman Keuls test (SNK).

RESULTS

Lilium martagon

In *Lilium martagon,* the addition of IBA to the multiplication medium had a significant effect on the diameter and the weight of the principal bulbs respect to the PGR free medium. Since there was not any statistically differences between the two levels 0.5 or 1.5 mg/L of IBA, it was confirmed that the multiplication medium for *Lilium*, used as a control and applied for the further experiments, was represented by MS salts + 30 g/L of sucrose + IBA 0.5 mg/L, even if the RGR was higher at 1.5 mg/L of IBA (0.82) in comparison to the lower concentration of 0.5 mg/L (RGR 0.78) but the multiplication rate was slightly higher.

It was possible to detect that the control temperature applied (24°C) induced bulbs more heavy and with higher diameter compared to 18°C; these results were statistically confirmed. The same behavior was achieved with the salt composition MS in comparison to WPM. The increasing sucrose concentration permitted to reach 1.71 cm and 1.88 g (diameter and weight, respectively) at 90 g/L sucrose. Lower concentration (30 g/L sucrose – the control dose) induced statistically lower weight and shorter diameter.

100% of production of secondary bulbs was recorded when they were grown onto WPM salts and at lowest sucrose concentration (control medium: 30 g/L). The same trend occurred for the multiplication rate at 30 g/L of sucrose (2.7). About the dimensions (diameter and weight) of the secondary bulbs, it was confirmed, as already described for the principal bulbs growth, that the weight increased at increasing of concentration of sucrose, in presence of MS salts, at 24°C of temperature and at 0.5 mg/L IBA (Table 1).

In Figure 1 it is possible to appreciate the plant material at different phase of culture of *L. martagon*. As shown in Figure 2a, any principal bulbs of *L. martagon* showed a diameter smaller than 0.8 cm at any treatments evaluated; in the medium containing the highest level of IBA, the percentage of bulbs, with diameter at least of 1.6 cm was higher than in the other conditions; the same occurred at 90 mg/L of sucrose and at 24°C. This was a prerequisite to ensure a successfully acclimatization. For the secondary bulbs (Figure 2b) the critical level of diameter of 0.8 cm was more evident in particular using 1.5 mg/L. The 20 bulbs transferred to the greenhouse were alive after 60 days from transfer to soil and it is possible to assume that they are a suitable material that could be used in further re-introduction program.

Lilium pomponium

As described for *L. martagon*, the two levels of IBA ensured a better response related to the PGRs free medium and, also for this species, the level of IBA 0.5 mg/L was then considered as standard control in the further trials.

Any other factor had a significant effect on the diameter of principal and secondary bulbs even if the best value was achieved by culturing the principal bulbs at 24°C (1.17 g), onto substrate containing MS salts (1.21 g) at 90 g/L of sucrose (1.54 g) and 1.5 mg/L of IBA (1.59 g) and the same trend was observed for the secondary bulbs.

In *L. pomponium*, the adjunctive factor tested, darkness induced the growth of the principal bulbs; the weight was higher in dark condition (1.41 g) and this was confirmed by RGR. Oppositely, the presence of light (16/8) induced the highest number of secondary bulbs. (2.7 at 16/8 and1.9 in dark) (Table 2).

After 6 months, it was showed that the best acclimatization performances, in term of survival, diameter and weight of bulbs were achieved at higher concentration of sucrose and the higher temperature tested *in vitro*. No differences were observed for the others factors (Table 3).

After 2 years of culture, it was observed that diameter and weight were still influenced by *in vitro* treatment concerning the photoperiod and temperature applied: the best performances occurred with bulbs cultivated *in vitro* in light at 18°C (Figure 3).

In Figure 4, it is possible to appreciate principal step of the *in vitro* culture that ensure the production of functional bulbs after 2 years of *in vivo* cultivation. It was observed that bulbs with diameter bigger than at least 0.8 cm acclimatized with high facility. In Figure 5a, it is evident that most of the principal bulbs had a range size between 0.8 and 1.6 cm of diameter, considering at all treatments; the percentage of secondary bulbs with smaller size (< 0.8 cm) was higher than 70%.

DISCUSSION AND CONCLUSION

For *L. martagon* the best material, suitable for further *in vitro* experiments was achieved by seeds sterilized with 1% free chlorine and germinated on water-agar substrate in agreement with the data of Mascarello et al., [16]. The excision of cotyledon and seed coat did not affect seedling viability but induced a suitable growth with the development of true leaves and lateral bulblets in short time. The explants showed slow growth with lower multiplication rate of secondary bulblets, but they were morphologically complete and produced entire plants that were transferred to soil without any acclimatization problems.

L. pomponium seeds showed double dormancy removable with 30 days at 24°C followed by 60 days at 4°C [6], three seedlings were selected and *in vitro* cultured showing a good proliferative aptitude.

In vitro media salts composition is one of the most important factors that influence the *in vitro* culture [33,42]. In some species

Table 1: IBA concentrations, salts macro element, temperature and sucrose concentration effects on *L. martagon* bulbs *in vitro* growth. (mean ± standard error) For each factor and each parameter, different letters indicate values which differ at p<0.05, Student Newman Keuls test.

		Diameter of principal bulb (cm)	Weight of principal bulb (g)	RGR	Production (principal bulbs that produce secondary bulbs) (%)	Multiplication rate (n. of secondary bulbs/ principal bulb)	Diameter of secondary bulbs (cm)	Weight of secondary bulbs (g)
IBA (mg/L)	**0**	1.22 ± 0.10 a	0.52 ± 0.10 a	-	0	0	-	-
	0.5	1.67 ± 0.09 b	1.67 ± 0.14 b	0.78	55.0	1.1 ± 0.3 a	0.77 ± 0.05 b	0.47 ± 0.06 b
	1.5	1.82 ± 0.11 b	1.92 ± 0.18 b	0.82	55.0	0.9 ± 0.2 a	0.53 ± 0.04 a	0.20 ± 0.03 a
Salts composition	**MS**	1.65 ± 0.07 a	1.56 ± 0.12 b	0.72	56.2	1.2 ± 0.2 a	0.67 ± 0.03 a	0.27 ± 0.03 b
	WPM	1.44 ± 0.10 a	0.61 ± 0.09 a	0.22	100	2.2 ± 0.4 b	0.68 ± 0.05 a	0.16 ± 0.02 a
Temperature (°C)	**18**	1.32 ± 0.07 a	0.56 ± 0.07 a	0.15	84.2	2.3 ± 0.4 b	0.68 ± 0.03 a	0.14 ± 0.02 a
	24	1.75 ± 0.07 b	1.79 ± 0.11 b	0.80	55.0	0.9 ± 0.2 a	0.66 ± 0.04 a	0.35 ± 0.04 b
Sucrose (g/L)	**30**	1.38 ± 0.09 a	0.63 ± 0.08 a	0.32	100	2.7 ± 0.7 c	0.70 ± 0.04 a	0.13 ± 0.02 a
	45	1.63 ± 0.09 b	1.35 ± 0.16 b	0.60	62.1	1.5 ± 0.3 b	0.64 ± 0.03 a	0.25 ± 0.03 b
	90	1.71 ± 0.10 b	1.88 ± 0.17 c	0.86	50.0	0.6 ± 0.2 a	0.69 ± 0.08 a	0.44 ± 0.10 c

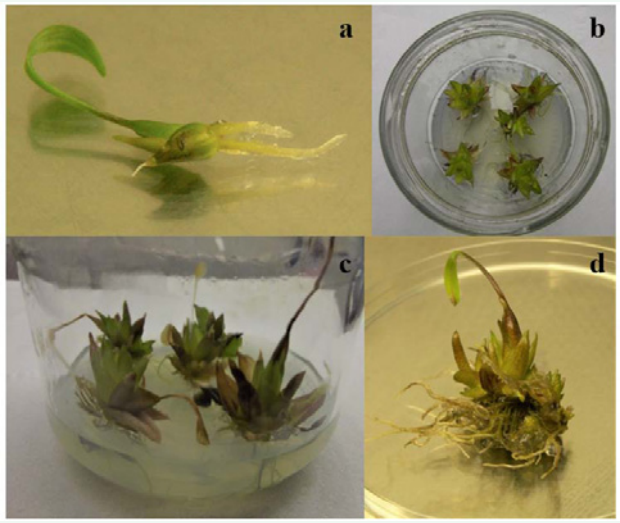

Figure 1 Different step of *in vitro* culture of *L. martagon.* a) Bulb as starting material for the *in vitro* trials. b) and c) Multiplication phase. d) Secondary bulblets arising from the principal bulb.

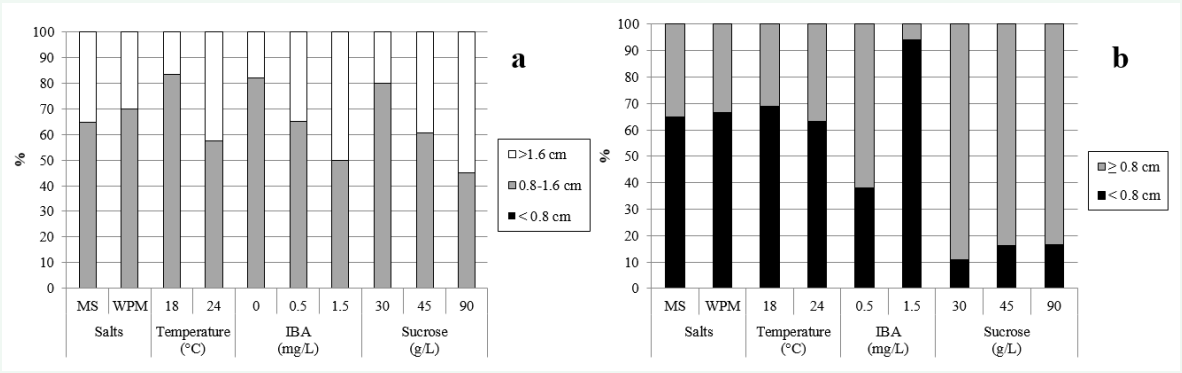

Figure 2 Class of *Lilium martagon* bulbs. a) Principal bulbs; b) Secondary bulbs.

Table 2: IBA concentrations, salts macro element, temperature and sucrose concentration, photoperiod effects on *L. pomponium* bulbs *in vitro* growth. (mean ± standard error) For each factor and each parameter, different letters indicate values which differ at p<0.05, Student Newman Keuls test.

		Diameter of principal bulb (cm)	Weight of principal bulb (g)	RGR	Production (principal bulbs that produce secondary bulbs) (%)	Multiplication rate (n. of secondary bulbs/ principal bulb)	Diameter of secondary bulbs (cm)	Weight of secondary bulbs(g)
IBA (mg/L)	0	0.94 ± 0.05 a	0.38 ± 0.04 a	-	0	0	-	-
	0.5	1.21 ± 0.05 b	1.27 ± 0.10 b	0.80	80.7	2.2 ± 0.2 a	0.63 ± 0.02 a	0.29 ± 0.03 a
	1.5	1.20 ± 0.04 b	1.59 ± 0.14 b	1.16	87.9	3.1 ± 0.3 b	0.58 ± 0.01 a	0.24 ± 0.02 a
Salts composition	MS	1.20 ± 0.02 a	1.21 ± 0.07 b	1.05	86.0	2.9 ± 0.2 b	0.59 ± 0.01 a	0.21 ± 0.01 b
	WPM	1.18 ± 0.03 a	0.52 ± 0.03 a	0.49	77.7	1.8 ± 0.1 a	0.58 ± 0.01 a	0.14 ± 0.01 a
Temperature (°C)	18	1.17 ± 0.04 a	0.58 ± 0.03 a	0.53	78.2	2.4 ± 0.2 a	0.54 ± 0.01 a	0.13 ± 0.01 a
	24	1.20 ± 0.02 a	1.17 ± 0.07 b	1.03	85.9	2.6 ± 0.1 a	0.60 ± 0.01 a	0.22 ± 0.01 b
Sucrose (g/L)	30	1.14 ± 0.03 a	0.49 ± 0.03 a	0.50	88.7	2.8 ± 0.2 b	0.56 ± 0.01 a	0.13 ± 0.01 a
	45	1.19 ± 0.03 a	1.06 ± 0.08 b	0.96	83.6	2.6 ± 0.2 ab	0.58 ± 0.01 a	0.19 ± 0.01 a
	90	1.24 ± 0.04 a	1.54 ± 0.12 c	1.05	78.7	2.3 ± 0.3 a	0.64 ± 0.02 a	0.31 ± 0.02 b
Photoperiod (light/dark) (h)	16/8	1.21 ± 0.02 a	0.95 ± 0.06 a	0.79	83.8	2.7 ± 0.1 b	0.59 ± 0.01 a	0.19 ± 0.01 a
	0/24	1.12 ± 0.05 a	1.41 ± 0.13 b	1.13	83.3	1.9 ± 0.2 a	0.55 ± 0.02 a	0.26 ± 0.02 a

Table 3: Acclimatization data of *L. pomponium* bulbs after 6 months. (mean ± standard error) For each factor and parameter, different letters indicate values which differ at p<0.05, Student Newman Keuls test.

	Salts macroelement		Temperature (°C)		IBA (mg/L)		Sucrose (g/L)			Photoperiod (light/dark hours)	
	MS	WPM	18	24	0.5	1.5	30	45	90	0/24	16/8
Bulbs survival (%)	79.4 ± 3.6 a	72.3 ± 11.4 a	57.4 ± 3.2 a	84.4 ± 2.9 b	80.0 ± 5.5 a	82.2 ± 3.5 a	70.1 ± 7.0 a	78.1 ± 6.5 ab	84.5 ± 2.8 b	81.0 ± 2.9 a	75.3 ± 5.3 a
Bulbs diameter (cm)	1.17 ± 0.02 a	1.02 ± 0.03 a	1.04 ± 0.02 a	1.17 ± 0.02 a	1.26 ± 0.02 b	1.16 ± 0.02 a	0.99 ± 0.02 a	1.15 ± 0.02 a	1.21 ± 0.02 a	1.24 ± 0.02 a	1.10 ± 0.02 a
Bulbs weight (g)	1.83 ± 0.06 a	1.79 ± 0.15 a	1.97 ± 0.13 a	1.77 ± 0.07 a	1.93 ± 0.09 a	1.81 ± 0.11 a	1.46 ± 0.11 a	1.87 ± 0.09 b	1.96 ± 0.10 b	1.83 ± 0.10 a	1.82 ± 0.07 a

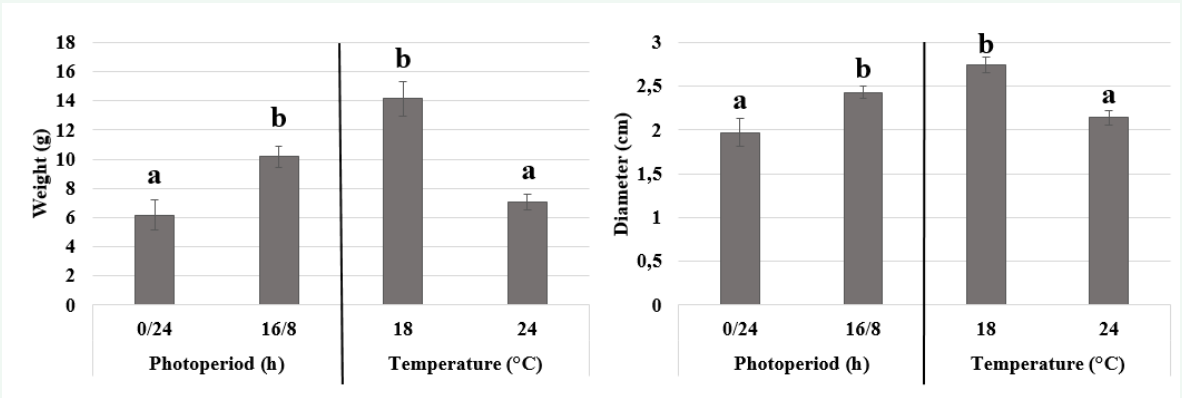

Figure 3 *L. pomponium*: bulb weight and diameter after two years from acclimatization related to photoperiod and temperature applied during *in vitro* culture. For each factor, different letters indicate values which differ at p<0.05, Student Newman Keuls test.

the salts composition variation in the culture medium is decisive for the explants survival during acclimatization [43]. This evidence is in accordance with the results obtained for the two tested species, since the use of MS salt promotes a better effect on the weight of the principal bulb, closely related to acclimatization performance. Among the macro-elements present in the culture media, N and K, and their ratio, play a critical role in the main bulb growth and in the secondary bulblets formation. According to [44], low N concentrations (15 mM) and an NKP ratio in its favor (1:0.07:0.7), as in MS salt composition allow the best *in vitro* average size bulbs production, as in our trials in which MS salt (1:0.02:0.33) induced a big size of principal bulbs, but the excess of N in the culture medium decreases plantlets acclimatization 45. In opposite, only in *Lilium martagon*, the high level of K in

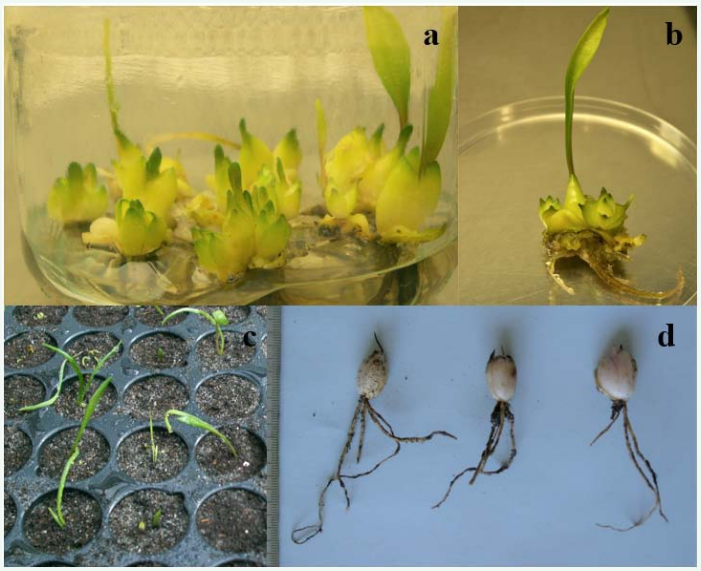

Figure 4 Different step of the culture of *L. pomponium.* a) *In vitro* multiplication phase. b) Secondary bulblets arising from the principal bulb. c) Acclimatization phase of the principal bulbs in greenhouse. d) Principal rooted bulbs 2 years after acclimatization.

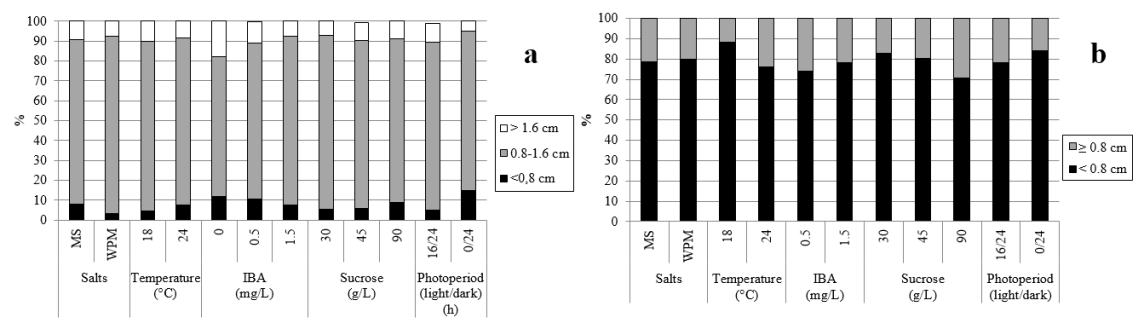

Figure 5 Class of *Lilium pomponium* bulbs. a) Principal bulbs; b) Secondary bulbs.

the NPK ratio of WPM salts (1:0.08:1.08) could play a role in the production and number of secondary bulbs arising from the principal ones, reaching a value of 100% and 2.2, respectively. In Hyacinth *in vitro*, bulb growth is directly proportional to the amount of N adsorbed [46], as well as *in vivo* onion [47], but the increase of the amount of K, and then the shift of the relationship toward this element *in vivo* improves the plants quality and the size and the weight of the bulbs of *Lilium* [48,49] and of other species with underground organs [50]. According to Varshney et al. [48], for both *Lilium* species, it was confirmed that the weight of principal and secondary bulbs cultivated onto MS salts was heavier, due to the higher nitrogen concentration of MS salts. Then the answer, therefore, is species-specific and also dependent on cultural conditions.

The temperature has a strong effect on the growth of *in vitro* bulblets of *Lilium* species [51-54] and on the growth after *in vitro* treatment [38].

During *in vitro* culture of *L. pomponium* and *L. martagon*, high temperature caused to reach bigger bulbs, in terms of diameter and weight. It has been reported that decrease of temperature up to 15°C induces an increase of fresh weight and bulblets rooting compared to a temperature of 20°C and 26°C [54], but reduces the growth speed of the first leaf [38]. The same trend was recorded in *L. pomponium*, after 2 years of cultivation (Figure 2), reaching high value of diameter and weight at 18°C in comparison with 24°C. Furthermore, a temperature of 25°C induced a high level of dormancy in *L. speciosum* [55]. It seems that the temperature effect may be related to the amount of sucrose in the culture medium; in tulip the lowering of the temperature from 25°C to 20°C and the increasing of the sugar content in the medium induce the bulb development [56].

It is well known that the addition of auxin in the *in vitro* culture medium of geophyte promotes the regeneration of bulbs [27]. In a similar geophyte endemism of Alpi Marittime (*Leucojum nicaeense*), IBA was used in the media and showed a positive effect on weight, multiplication rate and rooting of bulbs [57]. Also in *L. pomponium* and *L. martagon*, the highest IBA concentration used play an important role on the principal bulb weigh but, once induced, secondary bulblets can be easily propagated at lower concentration.

Several authors [58,59,60] demonstrated that the level of energy reserves accumulated by the seedling in the last stages of growth before transplantation determines the next *in vivo* growth rate. The sucrose is involved in the development of bulbs [56], and the sucrose quantity in the media have effect on *Lilium* bulblets formation and bulblets diameter and weight [31,61-63]. Furthermore in lily, a high level of sucrose and nitrogen strongly promoted the bulblets weight [64]. The increase in concentration to 6% caused to almost doubles in relative mass compared to 4% [37], and passing to 8-9%, there was an increase of the weight and the diameter of the main bulb [65], but it reduced the number of roots [36]. The increase of the sucrose concentration in the medium had different effects on the production of secondary bulblets. According to Kumar et al. [36], the sucrose induced an increase in the production of secondary bulblets, while according to Zhang and Jia [65], reducing the multiplication rate. Also on *L. pomponium* and *L. martagon*, high sucrose concentration positively influenced the growth of the bulbs but contemporary inhibit the multiplication rate. The effect of sucrose was also related to the acclimatization phase for *L. pomponium*: the increasing level of sucrose, from 30 g/L to 90 g/L, gave the best results in term percentage of survival, diameter and weight of bulbs.

There are studies on the influence of darkness on lily micro-propagation [65-67]. According to Mei-Lan et al. [67], PPF (Photosynthetic Photon Flux) enrichment conditions (photoautotrophic conditions) was beneficial for the production of high-quality bulblets in lily and promote growth with better anatomical and physiological characteristics for acclimatization than conventional heterotrophic and photomixotrophic plantlets [68]. According to the previous results, the presence of continuous darkness reduces the production of secondary bulblets, but increases the weight and the diameter of the main bulbs and inhibits the rooting [36]. In presence of constant darkness, however, there is a considerable reduction of the content of soluble carbohydrates and starch in the bulb [65]. In our work, *in vitro* darkness condition caused higher weight value of principal and secondary bulbs of *L. pomponium* respect to the light condition but reduced the diameter and the multiplication rate. After 2 years *in vivo*, it was observed that the weight of the bulbs was higher in case of light, indicating high growth efficiency.

The *in vitro* culture is a very important technique to preserve the endemism; the effect of the treatment applied during *in vitro* culture affected the subsequent growth *in vivo*. In order to obtain material with the best quality to be acclimatized *ex vitro* our suggestion is to use MS salts, maintain at 24°C, using a medium IBA concentration (0.5 mg/L) and high sucrose concentration (90 g/L), better in darkness (in particular for *L. pomponium*). If the purpose is to obtain a large multiplication rate, in *L. martagon* is better to use WPM salts composition, 18°C 1 mg/L of IBA and a lower sucrose concentration (30 g/L); for *L. pomponium* bulbs, instead, MS salts, 24°C, 0.5 mg/L of IBA, 30 g/L of sucrose in photoperiod 16/8 condition.

The protocols supported by the results reported for the first time in this paper can be used in reinforcing programs of these endangered species for biodiversity preservation.

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