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In vitro germination and growth protocols of the ornamental *Lophophora williamsii* (Lem.) Coult. as a tool for protecting endangered wild populations

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ABSTRACT

Lophophora williamsii is an ornamental slow growth cactus highly appreciated by cacti growers and hobbyists. Its demand is often satisfied through illegal collection of wild plants and many populations are threatened with extinction. Thus, an efficient *in vitro* protocol without plant growth regulators will be of great interest for conservation purposes of this cactus. Eight different germination media, combining Murashige and Skoog medium (MS, full and half-strength), sucrose (20 and 30 g L^{-1}) and agar (8 and 10 g L^{-1}), were used to study germination rate, number of seedlings with areoles and initial seedling development. Germination rates among culture media only differed significantly in the first 14 days after sowing (DAS), reaching 67–75% at the end of the assay (49 DAS). Remarkable interactions among media components were detected, and 20 g L^{-1} sucrose and 8 g L^{-1} agar combination gave the highest performance for both size and number of areoles. Following germination assay, a growth assay was conducted during 105 days using three growth media (GrM) at different sucrose concentration (15, 30 and 45 g L^{-1}) to evaluate the increase in seedling size and number of areoles. Regardless of their initial size, 15 g L^{-1} sucrose provided the best results for both traits. Size increase was higher in the 4–5 mm seedling group, while increase in areoles was greater in 2-3 mm seedlings. It was possible to develop an *in vitro* protocol, in absence of plant growth regulators, which allows maximizing *L. williamsii* germination and growth during its first stages of development, which may increase the availability of plants in the market and avoid exhaustion of wild populations. Furthermore, plants grown *ex situ* could be reintroduced in endangered natural populations.

1. Introduction

Lophophora williamsii (Lem.) Coult., commonly known as 'peyote', is a small (5–12 cm in diameter) blue-green, button-like, spineless and slow growth cactus with napiform root (Fig. 1) whose wild populations are distributed in Mexican highlands and in the arid regions of Southwestern United States (Anderson, 1996). This plant has been used during centuries in several rituals and ceremonies by Indian Tribes (McLaughlin, 1973; Borchers et al., 2000; Halpern et al., 2005) due to its content in alkaloids (of which the major one is mescaline) with psychotropic activity (Casado et al., 2008). In addition to its ancestral ethnobotanical use, *L. williamsii* has always aroused a great interest among cacti lovers and collectors. In fact, its growing demand in the market (as in other Cactaceae) has been often satisfied through illegal collection of wild individuals (Anderson et al., 1994; Sajeva et al., 2013), in part due to the fact that its growth from seeds is very slow.

The plundering of wild plants, added to other problems related to human activity, such as agricultural and urban expansions, introduction of exotic grasses, use of herbicides and pesticides among others, has led to many cacti being threatened with extinction (Taylor 1997; Sanchez-Martinez et al., 2009), especially in those places close to urban areas where access to natural populations is easier. This high pressure on ecosystems compromises the viability of certain populations and could result in an irremediable loss of unique genetic pools. In this way, genus *Lophophora* is protected by Mexican laws and is included in the Convention on International Trade in Endangered Species of Fauna and Flora (CITES) (Sajeva et al., 2013).

In vitro culture techniques may play a key role to accelerate the growth of *L. williamsii* plants, especially after sowing from seeds and during early stages of the development, when seedlings are more vulnerable. Furthermore, areoles are characteristic of Cactaceae, equivalent to the buds of other higher plants, and include two points of growth: one that leades to the thorns and another that originates the flowers and new buds (Ballester-Olmos, 1997). In this sense, as active growth organs, the number of areoles must considered for *in vitro* protocols as they represent the capacity of each plant for a higher

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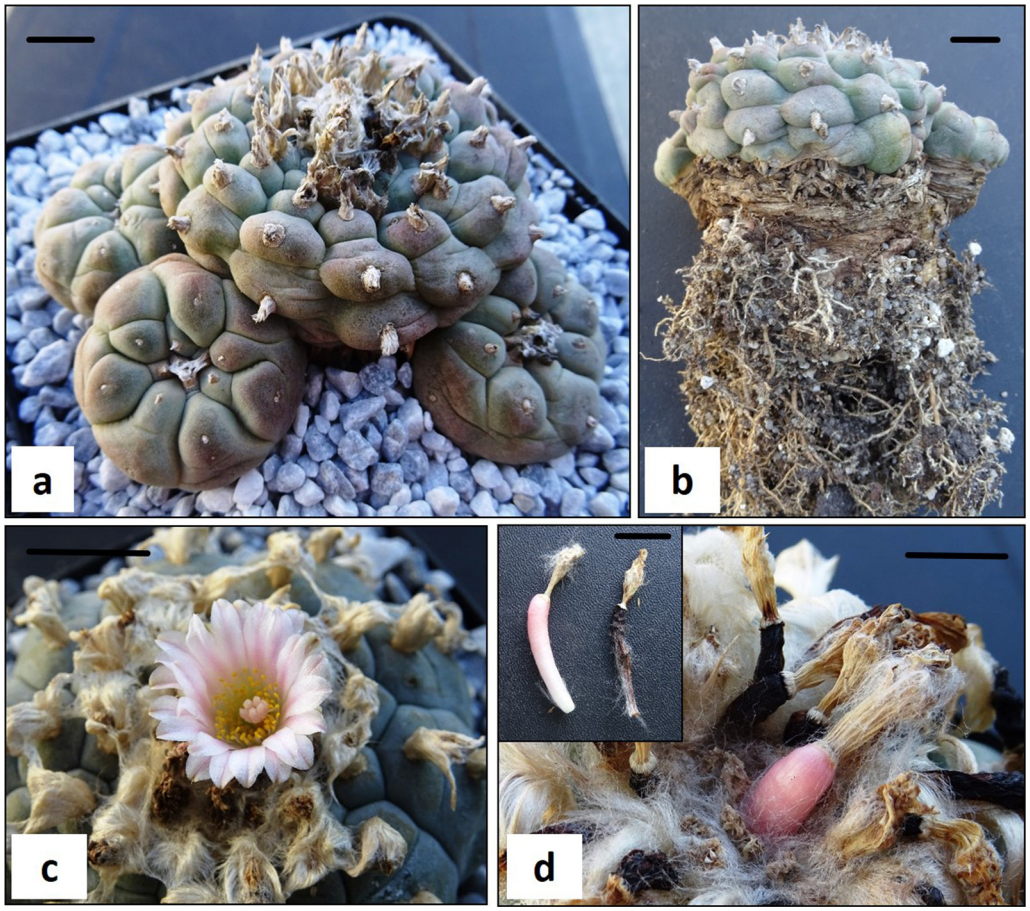

Fig. 1. *Lophophora williamsii* morphology: a) adult plant, b) napiform root, c) flowering and d) inmature fruit and mature dry fruit containing viable seeds. Black bar indicates 10 mm.

Table 1
Composition of*in vitro* media studied in both the germination and plant growth assays of *L. williamsii*.

Media	Murashige & Skoog	Sucrose (g L^{-1})	Agar (g L^{-1})
Germination Assay			
M1	full-strength	20	8
M2	full-strength	20	10
M3	full-strength	30	8
M4	full-strength	30	10
M5	half-strength	20	8
M6	half-strength	20	10
M7	half-strength	30	8
M8	half-strength	30	10
Growth Assay			
GrM-1	full-strength	15	8
GrM-2	full-strength	30	8
GrM-3	full-strength	45	8

multiplication. *In vitro* culture could help to obtain larger and flowering plants faster than by conventional seed reproduction, satisfying the demand of the market and thus reducing consequently the need to plunder wild plants. Also, *in vitro* culture could contribute to the *ex situ* conservation of plants and populations with the aim to reintroduce them in their habitat for restoring extinct or critically endangered natural populations.

In vitro plants propagation and micropropagation for conservation purposes requires efficient methods. In some cases, it has been reported that medium composition and its supplementation with plant growth regulators (PGRs) may alter morphological and physiological characters, even genetic stability, in the obtained plants (Lema-Ruminska and Kulus, 2014). Therefore, media without PGRs would be desirable as they are less prone to induct of somaclonal variations on material collected from the wild. In addition, media containing no PGRs are cheaper and easier to prepare than those formulations including PGR. Thus, in this study we compared different *in vitro* culture media in absence of PGRs, in order to detect those that maximize the development and growth of *L. williamsii* seedlings during the early stages after sowing. This would also be the first step to establish a *Lophophora* micropropagation protocol in absence of PGRs.

2. Material and methods

2.1. Seed disinfection

Seeds of a population of *L. williamsii* were kindly donated by Cactusloft (Valencia, Spain). Three hundred and twenty seeds were disinfected for 1 min in 70% etanol (v/v), followed by 25 min in 15% domestic bleach solution (v/v; 4% sodium hypochlorite), supplemented with 0.08% Tween-20 (v/v) and rinsed 3 times in distilled sterilized water under aseptic conditions under laminar flow cabinet conditions (model AH-100, Telstar, Terrassa, Spain).

2.2. Germination assay

Disinfected seeds were sown on different germination media (Table 1) in Petri dishes (10 seeds per dish, 4 dishes per medium, n = 4). A total of eight *in vitro* formulations were evaluated in this assay, which included all combinations of Murashige and Skoog medium (MS, at full-strength or half-strength, 1 × MS or 1/2 × MS, *i.e.*

Table 2
Germination assay: germination efficiency and percentage of *L. williamsii* seedlings with areoles for the eight *in vitro* media evaluated along 49 days after sowing (DAS).

Media	7 DAS	14 DAS	21 DAS	28 DAS	35 DAS	42 DAS	49 DAS
Germination efficiency (%)							
M1	0.0a[1]	42.5a	60.0a	60.0a	62.5a	67.5a	67.5a
M2	7.5abc	55.0ab	67.5a	70.0a	70.0a	70.0a	70.0a
M3	5.0ab	60.0b	65.0a	70.0a	70.0a	70.0a	75.0a
M4	12.5bc	57.5ab	70.0a	70.0a	70.0a	72.5a	75.0a
M5	12.5bc	55.0ab	62.5a	67.5a	70.0a	72.5a	72.5a
M6	17.5c	67.5b	67.5a	67.5a	67.5a	70.0a	70.0a
M7	12.5bc	52.5ab	55.0a	65.0a	65.0a	67.5a	67.5a
M8	0.0a	62.5b	67.5a	67.5a	70.0a	72.5a	77.5a
1/2 MS	10.6A	59.4A[2]	63.1A	66.9A	68.1A	70.6A	71.9A
MS	6.3A[2]	53.8A	65.6A	67.5A	68.1A	70.0A	71.9A
Sucrose 20 g L^{-1}	9.4A	55.0A	64.4A	66.3A	67.5A	70.0A	70.0A
Sucrose 30 g L^{-1}	7.5A	58.1A	64.4A	68.1A	68.8A	70.6A	73.8A
Agar 8 g L^{-1}	7.5A	52.5A	60.6A	65.6A	66.9A	69.4A	70.6A
Agar 10 g L^{-1}	9.4A	60.6A	68.1A	68.8A	69.4A	71.3A	73.1A
Seedlings with areoles (%)							
M1	0.0	0.0a	32.5ab	42.5ab	42.5ab	42.5ab	42.5a
M2	0.0	0.0a	35.0ab	50.0b	52.5ab	52.5ab	52.5a
M3	0.0	5.0ab	42.5ab	52.5b	55.0ab	60.0b	62.5a
M4	0.0	7.5ab	22.5a	25.0a	35.0a	37.5a	45.0a
M5	0.0	10.0b	45.0b	52.5b	62.5b	62.5b	65.0a
M6	0.0	12.5b	40.0ab	45.0ab	52.5ab	52.5ab	55.0a
M7	0.0	10.0b	30.0ab	40.0ab	45.0ab	47.5ab	50.0a
M8	0.0	0.0a	22.5a	32.5ab	42.5ab	47.5ab	50.0a
1/2 MS	0.0	8.1A[2]	34.4A	42.5A	50.6A	52.5A	55.0A
MS	0.0	3.1A	33.1A	42.5A	46.3A	48.1A	50.6A
Sucrose 20 g L^{-1}	0.0	5.6A	38.1A	47.5A	52.5A	52.5A	53.8A
Sucrose 30 g L^{-1}	0.0	5.6A	29.4A	37.5A	44.4A	48.1A	51.9A
Agar 8 g L^{-1}	0.0	6.3A	37.5A	46.9A	51.3A	53.1A	55.0A
Agar 10 g L^{-1}	0.0	5.0A	30.0A	38.1A	45.6A	47.5A	50.6A

[1] Values with different letters among *in vitro* media indicate significant differences according to the least significant difference test (LSD) at $P < 0.05$.
[2] Values with different capital letters indicate significant differences between levels of each component of *in vitro* media according to the least significant difference test (LSD) at $P < 0.05$.

at original or half concentration of commercial MS salts concentration, respectively), sucrose concentration (at 20 or 30 g L^{-1}), and agar concentration (at 8 or 10 g L^{-1}). The pH of all media were adjusted to 5.7 before autoclaving. Four replicates per medium were cultured for 49 days in a growth chamber at 25 ± 2 °C, 16 h day length and photosynthetic photon flux of 50 µmol $m^{-2}s^{-1}$.

Germination ratio, number of seedlings with areoles, number of areoles per seedling, and seedling size were recorded weekly. Germination was evaluated during 49 days after sowing (DAS) of *in vitro* culture. For the rest of the traits (*i.e.* seedlingss with areoles, areoles per seedling, and seedling size), only seedlingss germinated within the first 28 DAS were evaluated, as data from seedlings germinated later, due to their low size, could bias the analysis. The germination viability and the number of seedlings with areoles were measured and expressed in percentage. Data in these traits were transformed by arcsine square root (Little and Hills, 1978), but differences between the results obtained by transformed and non-transformed data were negligible. Thus results presented in this work were performed on the original non-transformed data.

2.3. Plant growth assay

After 49 DAS (germination assay), 197 healthy plants from the germination assay were subcultivated to analyze growth parameters. Seedlingss were distributed in three groups depending on their size and similar root development: i) seedlings with sizes between 2 and 3 mm (64 plants); ii) seedlings with sizes between 3 and 4 mm (100 plants); iii) seedlings with sizes between 4 and 5 mm (33 plants). Each size group was divided in three subgroups and each subgroup was cultivated in a different growth medium (GrM) (Table 1) for 15 weeks, maintaining the same pH and culture conditions described in the germination assay. Subculturing was performed monthly.

The number of areoles per seedling and seedling size were quantified by using a binocular microscope and recorded weekly.

2.4. Statistical analysis

Unifactorial and multifactorial (using MS concentration, sucrose, and agar as factors) ANOVA analyses were performed using Statgraphics X64 (Statpoint Technologies, The Plains, VA, USA). Culture media and media component effects were analysed in both assays.

3. Results and discussion

3.1. Germination assay

3.1.1. Germination viability

Significant differences were found among media in the first two weeks of the experiment in terms of germination rate (Table 2). From 21 DAS on, germination values among media did not differ statistically. Therefore, only the two first weeks were of interest to study the effect of media formulation. As a whole, although differences were not significant in all cases, our results suggest that most half-strength MS formulations may enhance early germination, especially at 7 DAS. Thus, with the only exception of M8, media M5, M7 and, particularly,

Table 3
Germination assay: seedling size and number of areoles per *L. williamsii* seedling for the eight *in vitro* media evaluated along 49 days after sowing (DAS).

Media	7 DAS	14 DAS	21 DAS	28 DAS	35 DAS	42 DAS	49 DAS
Seedling size (mm)							
M1	0.0	2.7a[1]	3.4c	3.6e	3.9e	4.0e	4.1e
M2	0.0	2.6ab	2.5a	2.5ab	2.8ab	2.8ab	2.9ab
M3	0.0	2.6ab	2.6a	2.9bcd	3.1bc	3.2bc	3.3bc
M4	0.0	2.4a	2.4a	2.4a	2.5a	2.6a	2.6a
M5	0.0	3.4c	3.4c	3.3de	3.7de	3.8de	3.9de
M6	0.0	2.6ab	2.7ab	2.8abc	2.9ab	2.9ab	2.9abc
M7	0.0	3.0b	3.3bc	3.3cde	3.4cd	3.5cd	3.5cd
M8	0.0	2.4a	2.4a	2.5ab	2.7ab	2.7ab	2.8ab
1/2 MS	0.0	2.9B[2]	3.0B	3.0A	3.2A	3.2A	3.3A
MS	0.0	2.6A	2.7A	2.8A	3.1A	3.1A	3.2A
Sucrose 20 g L^{-1}	0.0	2.8A	3.0A	3.1A	3.3B	3.4B	3.4B
Sucrose 30 g L^{-1}	0.0	2.6	2.7A	2.8A	2.9A	3.0A	3.0A
Agar 8 g L^{-1}	0.0	2.9B	3.2B	3.3B	3.5B	3.6B	3.7B
Agar 10 g L^{-1}	0.0	2.5A	2.5A	2.6A	2.7A	2.8A	2.8A
Areoles per seedling							
M1	0.0	0.00a	1.13a	2.81d	3.91d	4.53e	5.19e
M2	0.0	0.00a	1.23ab	1.49a	1.85ab	2.26ab	2.80ab
M3	0.0	0.50ab	1.04a	1.91abc	2.39b	3.33cd	3.83cd
M4	0.0	0.50ab	1.08a	1.56ab	1.46a	1.88a	1.94a
M5	0.0	0.75b	1.49b	2.67d	3.08c	4.09de	4.64de
M6	0.0	0.75b	1.25ab	2.09bc	2.29b	2.88bc	3.34bc
M7	0.0	1.00b	1.23ab	2.33cd	3.06c	3.56cd	3.77cd
M8	0.0	0.00a	1.13a	1.81abc	1.80ab	2.62abc	2.84b
1/2 MS	0.0	0.63B	1.27B	2.23A	2.56A	3.29A	3.65A
MS	0.0	0.25A	1.05A	1.94A	2.40A	3.00A	3.44A
Sucrose 20 g L^{-1}	0.0	0.38A	1.27A	2.27A	2.78B	3.44B	3.99B
Sucrose 30 g L^{-1}	0.0	0.50A	1.12A	1.90A	2.18A	2.85A	3.10A
Agar 8 g L^{-1}	0.0	0.56A	1.22A	2.43B	3.11B	3.88B	4.36B
Agar 10 g L^{-1}	0.0	0.31A	1.10A	1.74A	1.85A	2.41A	2.73A

[1] Values with different letters among *in vitro* media indicate significant differences according to the least significant difference test (LSD) at $P < 0.05$.
[2] Values with different capital letters indicate significant differences between levels of each component of *in vitro* media according to the least significant difference test (LSD) at $P < 0.05$.

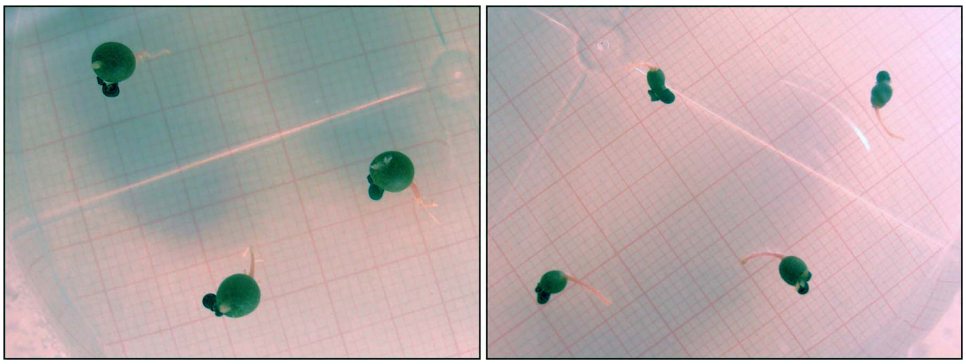

Fig. 2. Comparative radicle development at 21 days after sowing in media with different agar concentration: M1 (8 g L^{-1}) (left) *vs*. M8 (10 g L^{-1}) (right). Each small square in the grid indicates a size of 1 × 1 mm.

M6 provided ≥ germination rates at 7 DAS in comparison to full-strength MS media (Table 2). Thus, M6 followed by M4, M5 and M7, had the highest percentage of germination (> 10%) at 7 DAS, while M6, with 67% of germination efficiency, was also found the best formulation at 14 DAS, followed to a lesser extent by the rest of media (42–62%) (Table 2). In this regard, full-strength MS is capable to cause a one-week delay in germination compared to half-strength MS in some species like *Pilosocereus robinii* and *Astrophytum asterias* (Quiala et al., 2009; Lema-Ruminska and Kulus, 2012). Even in the case of inmature embryos from other species grown *in vitro*, like *Capsella* and *Capsicum*, it has been reported that full-strenght MS level could be slightly toxic for them and decrease their efficiency rates (Monnier, 1995; Manzur et al., 2013).

After 49 DAS, the percentage of germination observed ranged between 67.5% (M1 and M7) to 77.5% (M8), similar to many other *in vitro* germination rates in cacti, as detected in *Ariocarpus kotschoubeyanus* and *Cereus hildmannianus* (Moebius-Goldammer et al., 2003; Langer and Mergener, 2013). Furthermore, our results revealed that germination rates in the different media reached similar values (nonsignificant differences) at the end of the experiment (Table 2). This fact indicates that the seed population used as a starting material was homogeneous for this trait and differences in germination observed at the earlier stages have been solely caused by the different media formulation.

3.1.2. Number of seedlings with areoles

There were no significant differences between MS levels (full-

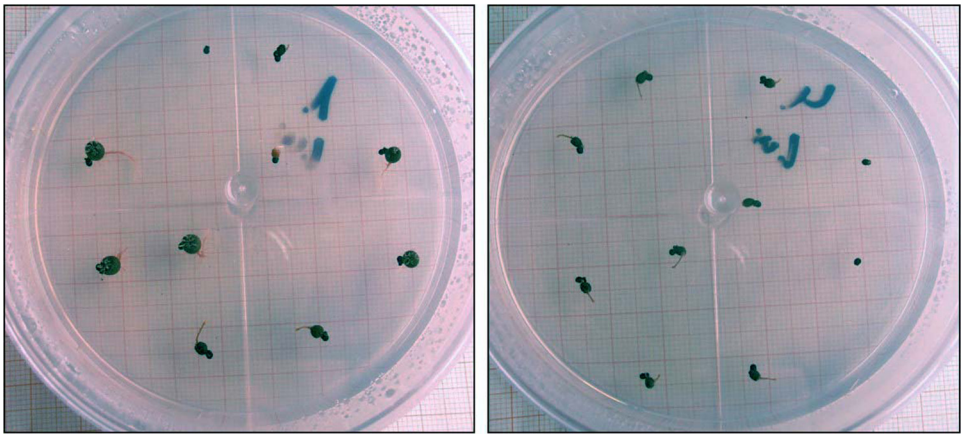

Fig. 3. Comparative initial seedling development at 49 days after sowing: medium 1 (left) *vs*. medium 4 (right). Each small square in the grid indicates a size of 1 × 1 mm.

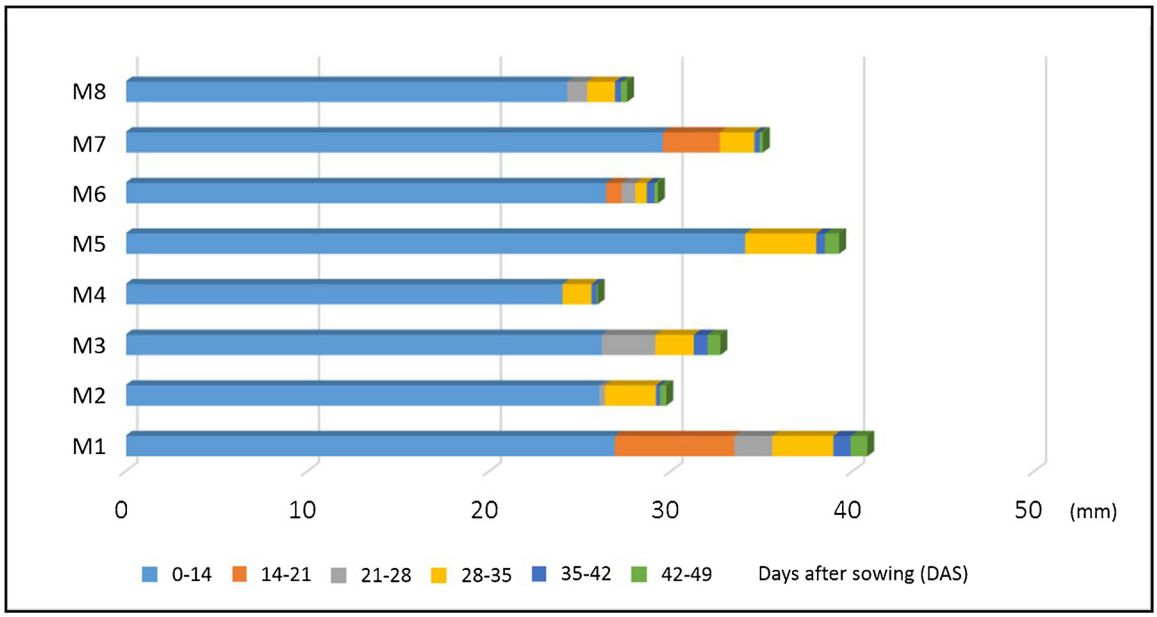

Fig. 4. Comparative seedlings sizes per week in each media. Each color represents the increase of size (in mm) during each time interval (days after sowing, DAS).

strength MS *vs*. half-strength MS), sucrose levels (20 g L^{-1} *vs*. 30 g L^{-1}) or agar levels (8 g L^{-1} *vs*. 10 g L^{-1}) along the experiment for the percentage of seedlings with areoles, which suggests that there is no clear effect of each of these factors for this trait (Table 2). By contrast, significant differences were found among the different media formulations, indicating a considerable interaction among the components of the media (Table 2). In this regard, media which enabled an earlier germination (*i.e.* M4, M5, M6 and M7) also favored an earlier emergence of areoles until 14 DAS (Table 2).

Regarding the values corresponding to the most advanced stages (from 21 DAS on), we found that M3 and particularly M5, both with agar concentration of 8 g L^{-1}, displayed the highest percentages of plants with areoles (Table 2). On the contrary, media such as M1 and M7, also containing 8 g L^{-1} of agar, were among the worst in the experiment. These findings suggest the occurrence of strong interaction between MS and sucrose levels at 8 g L^{-1} agar to explain such differences (Table 2). Thus, the simultaneous combination of high levels of both MS and sucrose (M3) or alternatively low levels of both (M5) are required for a good response on this trait, while the contrary is true for combinations of high levels of MS and low levels of sucrose *or vice versa* in M1 and M7, respectively.

In addition, it should be noted that in general M4 and M8 displayed a lower efficiency than the other media from 7 to 42 DAS, with significant differences in many cases, particularly M4 (Table 2). Probably their high osmotic gradient, due to high sucrose and agar levels, is the main reason for the poor performance observed in these formulations (Pérez-Molphe-Balch et al., 1998). In this respect, many authors have reported in a broad range of species that isolated mature embryos cultivated *in vitro* had better response and good growth in media at low sugar contents (Fischer and Neuhaus, 1995; Monnier, 1995; Bhojwani and Razdan, 1996; Manzur et al., 2013).

Finally, we found that from 35 to 42 DAS on, the percentage of plants with areoles did not increase significantly in most of the media, reaching their highest values at this stage (Table 2). This is correlated with the corresponding germination rates, which also reached their highest values at 21–28 DAS, indicating that all germinated seedlings develop areoles in all media about 2-3 weeks after germination.

3.1.3. Initial seedling development

The average size of seedlings germinated in M5 was greater than those observed in the other media until 21 DAS. However, M1 offered the highest performance, even higher than M5, in the later stages (Table 3). Also, for all the media studied most growth was detected during the first 14 DAS. Therefore, M5 provided the fastest initial

Table 4
Growth assay: evolution of seedling size and mean number of areoles per *L. williamsii* seedling in germinated seedlings, grouped by initial sizes, after 105 days of *in vitro* growth culture in three different growth media (GrM at 15, 30 and 45 g sucrose L^{-1}).

	Size (mm)						Areoles per seedling					
	Initial		Final		Increase		Initial		Final		Increase	
2–3 mm seedlings												
GrM-1	2,5	a[1]	4,4	b	1,9	b	1,32	a	9,11	b	7,79	b
GrM-2	2,5	a	3,6	a	1,1	a	1,42	a	6,84	a	5,42	a
GrM-3	2,5	a	3,5	a	1,0	a	1,92	b	6,62	a	4,69	a
3–4 mm seedlings												
GrM-1	3,1	a	4,4	b	1,3	b	3,09	a	9,03	a	5,94	b
GrM-2	3,1	a	3,9	a	0,8	a	3,68	b	8,26	a	4,58	a
GrM-3	3,2	a	4,0	a	0,8	a	3,90	b	8,30	a	4,40	a
4–5 mm seedlings												
GrM-1	4,3	a	6,5	b	2,3	b	6,33	a	12,83	a	6,50	b
GrM-2	4,3	a	5,8	a	1,5	ab	6,80	a	12,80	a	6,00	ab
GrM-3	4,4	a	5,6	a	1,1	a	6,73	a	11,36	a	4,64	a

[1] Values with different letters among *in vitro* media within each size group indicate significant differences according to the least significant difference test (LSD) at $P < 0.05$.

growth, while M1 enabled a more sustained trend. In terms of number of areoles per seedling, a similar behaviour was found. Thus, as a whole, M5 followed by M2, M6 and M7, resulted in a higher number of areoles per seedling until 21 DAS (Table 3). However, from 28 DAS to the end of the germination assay, M1 had the highest values, followed by M5. Both media had the same levels of sucrose and agar, suggesting that the combination of sucrose 20 g L^{-1} and agar 8 g L^{-1} favors both traits.

Considering the global effect of each factor, it seems that both sucrose and agar concentration affected to a greater extent seedling growth and number of areoles per seedling. In the case of the former, levels of 20 g L^{-1} increased on average both traits, particularly at the later stages, *i.e.* from 35 DAS on (Table 3). Considering the latter, seedlings grown in media with agar concentration at 8 g L^{-1} showed on average a faster growth and number of areoles since the beginning of the assay, in comparison to those observed in media with agar at 10 g L^{-1}. In fact, it was observed that media formulations including agar at 10 g L^{-1} hindered radicle penetration just after germination (Fig. 2), causing developmental delay with respect to those grown with 8 g L^{-1}. Furthermore, high concentration of gelling agents may decrease the amount of available water (Pérez-Molphe-Balch et al., 2002;Garcia-Osuna and Benavides-Mendoza et al., 2011), affecting adversely seedling development. These findings may also explain why media M4 and M8, with the highest sucrose and agar levels, showed the poorest values in general, particularly in the most advanced stages (*i.e.* after 28 DAS) (Table 3, Fig. 3).

Finally, half-strength MS media appeared to be more adequate on average during the first 21 days, while no significant differences were found at more advanced stages (Table 3). These results could explain the differences between M1 and M5, which only differed in MS concentration. Thus, half-strength MS in M5 favoured early growth of seedlings, while full-strength MS provided a better performance in the most advanced stages as was observed in M1 (Fig. 4). Therefore, similarly to germination efficiency, it looks like that full-strength MS may have a negative interaction with plantlets at the earliest stages of development, while it has no effect or even positive effect on later stages. Many studies have reported that 1/2 × MS, or even 1/4 × MS, provides a better effect not only in germination but also in subsequent early development than full MS (Gland-Zwerger, 1995; Xu et al., 2007; Manzur et al., 2013; Koné et al., 2015). This is probably due to a deleterious effect of some of the salts present in the MS formulation (Monnier, 1995). By contrast, full strength MS is advised for more advanced seedling stages as, once seedlings increase their size and become photosynthetically active, their requirements in micro and macronutrients are higher (Stewart and Kane, 2006; Paul et al., 2012).

3.2. Growth assay

As observed in the germination assay, seedlings from M1 showed a more prominent development, largely due to the positive interaction between its factors. As the concentration of agar was determinant and the presence of full-strength MS displayed positive interactions, we decided to evaluate the influence of sucrose on the further development of germinated seedlings. Thus, media based on full-strength MS and 8 g L^{-1} of agar were supplemented with three different sucrose concentrations (Table 1), which encompassed a range larger than initially studied in the germination assay, keeping 30 g L^{-1} as an intermediate concentration (*i.e.* 15, 30 and 45 g L^{-1}). Also, seedlings were evaluated separately on the basis of their initial size/diameter: 2–3 mm, 3–4 mm and 4–5 mm.

3.2.1. Seedling size

There were no significant differences between the initial sizes of seedlings within each size group. In fact, differences were ≤0.1 mm (Table 4). This confirms a precise selection of seedlings for the growth assay as they were similar in size for the three treatments within each group.

In all size groups it was observed that seedlings grown in GrM-1 showed both a larger final size and increase in size at the end of the experiment than those grown in GrM-2 or GrM-3 after 105 days of cultivation (Table 4, Fig. 5). These findings suggest that, regardless the initial size of seedlings, relatively low levels of sucrose provided by GrM-1 (15 g L^{-1}) are better for seedlings in terms of growth. By contrast, higher sucrose concentrations (≥30 g L^{-1}) had comparatively a deleterious effect on growth (Table 4). These results are in agreement with those from the germination assay, on which the early growth of seedlings after germination was considerably higher under low sucrose levels (20 g L^{-1} *vs.* 30 g L^{-1}) (Table 3). Therefore, from germination on, *L. williamsii* seedlings evolve better under low sucrose levels (15–20 g L^{-1}). Probably, excessive levels of sucrose (> 30 g L^{-1}) in some culture media caused unfavourable osmotic gradient that limits the development of seedlings in this species. In this regard, several authors reported that only early immature embryos require high levels of sucrose as source of energy and for osmotic reasons, while both immature advanced and mature embryos (equivalent to our *L. williamsii* seeds and seedlings) have a good response to moderate levels of sucrose in terms of germination efficiency and further growth (Monnier, 1995; Bhojwani and Razdan, 1996; Manzur et al., 2013).

Moreover, as a whole, we found that those seedlings with initial size of 4–5 mm had a higher size increase than 2–3 mm and 3–4 mm seedlings (Table 4). These results could be related to the greater initial vigour and the presence of more developed root systems in larger seedlings compared to smaller seedlings. Consequently, these results suggest that subcultivating *L. williamsii* seedlings of 4–5 mm are more appropriate for growing purposes in order to boost their development in multiplication protocols.

Furthermore, no morphological variations, hyperhydration, or *calli* occurrence were detected during the growth (neither in the germination assay nor in the growth assay). In this way the plants obtained at the end of the assay displayed the usual appearance observed in seedlings grown *ex vitro*. These results suggest that media used during seedlings growth do not alter their genetic integrity, with seedlings having a normal morphology. Therefore, plants from seeds harvested in nature and grown with our *in vitro* protocols could be subsequently used to repopulate threatened populations without evidence of modification of their genetic structure.

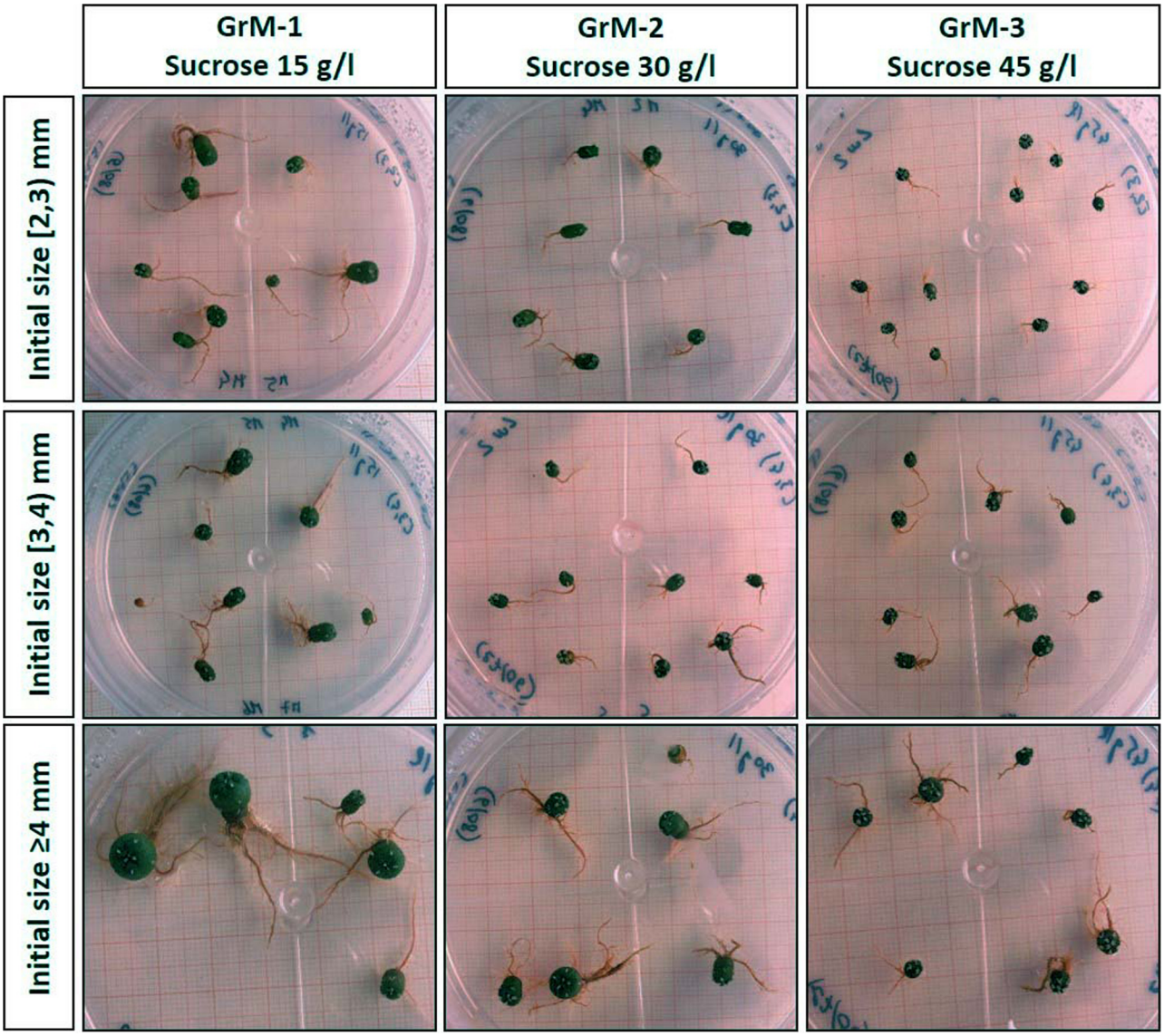

Fig. 5. Comparison of final size at the end of the growth assay (105 days after subcultivation) for each combination of initial size x growing media. Each small square in the grid indicates a size of 1 × 1 mm.

3.2.2. Number of areoles

Firstly, as can be observed on Table 4, the initial number of areoles was highly correlated to the initial size of seedlings. Thus, 2–3 mm seedlings had 1–2 areoles, while seedlings with 3–4 mm and 4–5 mm seedlings had 3-4 and 6-7 areoles, respectively. Nevertheless, little differences were found among seedlings of the same size within 2–3 mm and 3–4 mm groups. This was probably due to: a) seedlings for this assay were merely selected from germination assay based on size, without considering the number of areoles (random effect), and/or b) plants of the same size came from different germination media (initial media effect). Therefore, some differences in this trait could appeared randomly at the beginning within the same size group.

Nevertheless, despite some initial little differences within each group, remarkable effects of growth media were found for this trait. Thus, in the same way than for size, medium GrM-1 enabled a greater increase in the number of areoles than any of the other two media, regardless the initial size of the seedlings (Table 4). However, it was observed that within the 4–5 mm group, there were no significant differences between the increase in the number of areoles displayed by seedlings grown in GrM-1 and GrM-2, even considering the five best seedlings of each group (data not shown).

In addition, seedlings from the smallest initial size, *i.e.* the 2–3 mm group displayed in general a similar or higher increase in the number of areoles than those from higher initial sizes. It was observed that the appearance of the areoles was especially intense after germination (until seedlings reached 2–3 mm of diameter) and the distance between areoles changed during growth (data not shown), being closer in the early stages of seedling development (2–3 mm group) and separating as seedlings increased their volume (3–4 mm and 4–5 mm groups). These observations may explain the lower increase in the number of areoles observed in the 3–4 mm and 4–5 mm groups compared to the 2–3 mm group, even more considering that the distance between areoles in adult plants can also vary from 13 to 25 mm depending on the culture or environmental factors and it is closely related with hydration of the plant (Calhoun, 2012; Lodé, 2015).

Probably, this greater increase in the number of areoles observed in the 2–3 group can be expected in seedlings whose initial size and volume is smaller as an ancestral mechanism of protection against adverse environmental conditions and/or presence of herbivores in more susceptible seedlings (Ballester-Olmos, 1997; Hewitt, 2014). In fact, very often *L. williamsii* genotypes develop a delicate transient spinescence in the first areoles, while they lack pilosity (which appears later at more

advanced stages) (Kunte and Subik, 2003; Hunt, 2006). This is most clearly observed in *L. jourdaniana* (Haberm.) plantlets, on which these small spines are kept even for years.

4. Conclusions

On the basis of our findings, we consider that the best protocol for *L. williamsii* propagation would involve a seed germination and initial seedling growth in an *in vitro* medium including half-strength MS, 8 g L^{-1} agar and 20 g L^{-1} sucrose (M5). Then, after 14–21 DAS, seedlings should be subcultivated in a full-strength MS, keeping initial agar and sucrose concentrations (M1) to accelerate seedling growth. Once seedlings reach 4 mm in diameter, they should be subcultivated to a growth medium formulated with full-strength MS, 15 g L^{-1} of sucrose and 8 g L^{-1} of agar and keep them there until reaching the optimum size for rustication.

In the present work it has been possible to establish an *in vitro* germination and growth protocol for *L. williamsii* seedlings that allows maximizing their initial development, without the use of plant growth regulators. No morphological variations or *calli* appearance were found in the seedlings during our culture assays. In this sense, apparently these media without PGRs do not compromise the genetic or morphological integrity of the cultivated seedlings. In consequence, these seedlings could be later reintroduced into the habitat of threatened populations for repopulation purposes without altering the genetic structure of the population. Furthermore, this *in vitro* germination and growth protocol PGRs free is also the first step to establish massive propagation techniques focused on maintaining certain clones with ornamental interest.

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