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ORIGINAL ARTICLE

Long-term preservation of *Lotus tenuis* adventitious buds

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Abstract
Encapsulation-dehydration, encapsulation-vitrification, and vitrification were tested for cryopreservation of *Lotus tenuis* (Fagaceae) adventitious buds clusters (ABCs) obtained by a direct regeneration system from leaves cultures. Among them, the PVS3-based vitrification procedure was found to be useful for survival and regrowth of the preserved explants. For vitrification, the ABCs were dehydrated in a solution containing 2 M glycerol + 0.4 M sucrose for 25 min at room temperature, submerged in PVS3 solution for 1 h at 0 °C, then immersed in liquid nitrogen for 48 h and rapidly rewarmed. Afterword, the explants were unloaded in MS liquid medium with 1.2 M sucrose for 30 min. The washed tissues were dried superficially on filter paper and cultured in semisolid hormone-free MS medium containing 0.1 M sucrose. All cultures were maintained at 25 °C in the dark for 10 days and transferred to the light conditions. With this procedure, 79 ± 5.3% survival and more than 80% of the plantlets displaying a phenotype similar to the non-treated control after acclimatization. The data settled from ISSR showed no genetic dissimilarities between in vitro regenerants derived from cryopreserved tissues and the non-treated plants. Thus, our results indicate that the use of vitrification-based PVS3 solution offers a simple, accurate, and appropriate procedure for the cryopreservation of *L. tenuis* adventitious buds.

Keywords Cryopreservation · PVS3 · Vitrification · ISSR markers

Introduction

The genus *Lotus* (Fabaceae) comprises approximately 120–130 species mostly native to Europe (Ferreira and Pedrosa-Harand 2014; Escaray et al. 2012). The most investigated species of the genus belongs to the *L. corniculatus* group since it is widely used as forage and soil bioremediatory. Among them, *Lotus tenuis* is naturalized in a large area of the Flooding Pampa (Buenos Aires, Argentina) with saline and poorly drained soils where cattle production is sustained almost exclusively on the native grassland (Montes 1988). In this situation, *L. tenuis* has great economic importance for farmers since it is a source of a nutritious forage in nutrient deficient soils which are inadequate for agricultural purposes (Ferraro et al. 2010; Escaray et al. 2012). The dispersion and adaptation of *L. tenuis* to this disadvantageous environments could be explained by the phenotypic plasticity and genetic variations giving place to the existence of several ecotypes (Stoffella et al. 1998). Many agronomics and organoleptic traits distinguish the different ecotype which is using in the breeding program to produce seeds for monoculture pastures. In this context, we previously developed a protocol for mass propagation and transformation of *L. tenuis* (Espasandin et al. 2010) to improve the tolerance to drought (Espasandin et al. 2014) and salinity (Espasandin et al. 2017). The possibility to rescue wild ecotypes, propagate selected genotypes, generate transgenic plants for the incorporation of pest, diseases and stress tolerance traits, creates the need to have a procedure that allows the conservation of genetic resources.

Cryopreservation is considered a useful tool for the long-term storage of plant germplasm, requiring minimum space and maintenance (Sakai and Engelmann 2007; Matsumoto

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2017). Additional advantages are the preservation of cultures in the disease-free state without genetic alteration or modification. Currently, several techniques are available for plant cryopreservation: among them, the standard controlled rate of cooling and the advanced cryopreservation techniques which comprise a fast cooling stage (Withers and Engelmann 1998). Both include the vitrification-based procedures and the encapsulation-dehydration method based on synthetic seed technology. Vitrification involves the application of cryoprotectant solutions that increase cell viscosity to a critical point at which water forms a meta-stable glass on exposure to ultra-low temperatures (Fahy 1986; Sakai and Engelmann 2007; Sakai et al. 2008) and the encapsulation-dehydration comprises the removal of cell water through osmotic and evaporative desiccation to achieve the same state (Hirai and Sakai 1999). The implementation of these vitrification-based techniques has extended the applicability of cryopreservation to a wide range of plant species (Kaczmarczyk et al. 2012). In the case of the genus *Lotus*, a controlled-rate cooling procedure is available for the cryopreservation of cell line from *Lotus japonicus* (Ogawa et al. 2012).

Here, we developed a vitrification-based standard procedure for the cryopreservation of *L. tenuis* adventitious buds. This protocol was performed by evaluating the effect of twelve vitrification solutions on the survival and regrowth of the explants after the liquid nitrogen (LN) exposure. We also assessed the genetic stability and diversity of plants regenerated from cryopreserved adventitious buds using ISSR markers. To the best of our knowledge, this is the first protocol for the cryopreservation of *L. tenuis*.

Materials and methods

Plant material

The cryopreservation experiments were carried out with adventitious bud clusters (ABCs) obtained following the regeneration protocol previously reported (Espasandin et al. 2010). Leaf segments excised from leaves of the first to fourth nodes of 30-day-old in vitro seedlings of *L. tenuis* cv. INTA Pampa was cultured on the semisolid (agar 6.5 g L^{-1}) induction medium composed of full strength Murashige and Skoog (MS 1962) medium with sucrose (0.1 M), naphthaleneacetic acid (NAA; 0.5 µM), and 6-benzylaminopurine (BA; 22 µM). The pH was adjusted to 5.8 before autoclaving (121 °C, 1 atm for 20 min). The cultures were incubated in a growth room at 27 ± 2 °C with a 14 h photoperiod (120 µmol m^{-2} s^{-1} PPFD from white LEDs lamps).

Fourty day-old leaf segment containing six to eight visible adventitious buds (ABCs) were employed for the following experiments.

Encapsulation-dehydration

The detailed protocol is described in González-Arnao and Engelmann (2013). Briefly, the ABCs were included into a sterile 3% (w/v) calcium alginate solution prepared with MS medium containing 0.1 M sucrose. Drops of this solution comprising ABC explants were poured in 0.1 M $CaCl_2$. After 20 min, the beads were transferred to MS liquid medium supplemented with increasing sucrose concentration (0.25, 0.5, and 0.75 M) and maintained under continuous shaking (90 r.p.m.) for 24 h in each solution. Then, the beads were placed either on blotting paper under the sterile laminar airflow or into a desiccator with silica gel at room temperature until the water content decreased to 20%. The beads were subjected to the dehydration procedure for 4 and 6 h, respectively. Afterward, the dehydrated beads were placed in cryovials (ten beads/cryovial) and immersed in LN for 48 h. Rewarming of cryovials were carried out in a water bath at 40 ± 2 °C for 3 min. Afterward, the beads were transferred to a successive decreasing of sucrose medium concentration (0.75, 0.5, and 0.25 M) under continuous agitation (90 r.p.m.) for 24 h in each solution. Finally, the beads were cultured on a semisolid MS medium with 0.1 M sucrose and devoid of plant growth regulators. The treated ABCs were maintained at 25 °C in the dark for 10 days before being transferred to light conditions.

Encapsulation-vitrification

The explants were encapsulated in calcium alginate beads and dehydrated in the loading solution (2 M glycerol and 0.4 M sucrose in MS medium) for 25 min at room temperature. Subsequently, ABCs were transferred to the ice-cooled PVS3 solution (Nishizawa et al. 1993) for 60 min and immersed in LN for 48 h. After a rapid rewarming in a water bath at 40 ± 2 °C for 3 min, the explants were washed in MS medium with 1.2 M sucrose for 30 min at room temperature and cultured following the protocol described above.

Vitrification

ABCs were dehydrated in a loading solution containing 2 M glycerol and 0.4 M sucrose in MS medium for 25 min at room temperature in 5 mL cryovials. Then, the loaded ABCs were exposed to different cryoprotectants mixtures (CMs) composed by various proportions of dimethyl-sulfoxide (DMSO), glycerol, ethylene glycol (EG), and sucrose (Table 1) at 0 °C for 1 h. Dehydrated ABCs immersed in the CM were directly transferred to LN for 48 h. Afterward, the cryovials containing ABCs were rewarmed in a water-bath at 40 ± 2 °C for 3 min. Then, the CM solution was removed

Table 1 Composition of the cryoprotectant mixtures solutions for vitrification method

Cryoprotectant solution (CM)	Components (% w v^{-1})				
	DMSO	EG	Glycerol	Sucrose	MS
CM0	–	–	–	–	100
CM1	5	15	30	15	35
CM2 (PVS2)	15	15	30	15	25
CM3 (PVS3)	–	–	50	50	–
CM4 (PVS4)	–	20	35	20	25
CM5	15	–	15	15	55
CM6	–	15	15	15	55
CM7	–	5	15	15	65
CM8	15	5	30	15	35
CM9	–	–	40	40	20
CM10	–	5	30	20	45
CM11	–	10	40	40	10
CM12	5	–	15	15	65

PVS2 (Sakai et al. 1990), PVS3 (Nishizawa et al. 1993), PVS4 (Sakai 2000). The cryoprotectant mixtures were dissolved in MS liquid medium

DMSO dimethyl-sulfoxide, *EG* ethylene glycol

and replaced by unloading solution (1.2 M sucrose in MS medium) for 30 min at room temperature. The washed tissues were dried superficially on filter paper under the sterile airflow cabinet and cultured in semisolid hormone-free MS medium plus 0.1 M sucrose for shoot elongation. The treated ABCs were maintained at 25 °C in the dark for 10 days before being transferred to light. The survival rate and the mean number of adventitious buds regenerated per explant were determined after 15 and 45 days of culture under light conditions, respectively. Samples that received all treatments except LN exposure served as the treated control (− LN).

Survival and regrowth evaluation

After 3 week from the treatments, survival rate (explants remaining green) and regrowth expressed as the mean number of shoots per explant were assessed.

In vitro rooting and plantlets acclimatization

The elongated shoots (≥ 10 mm in length) were rooted by pre-treatment in an aqueous solution of indolebutyric acid (IBA; 500 µM) for 2 h, transferred to MS lacking phytohormones (Espasandin et al. 2010), and cultured in the same environmental conditions as mentioned before. The rooted shoots were set in 200-ml pots filled with a mixture of sterile soil and sand (1:1 v/v) plus 0.5 g of controlled-release micro-fertilizer (Osmocote®; N, P, K; 9, 45, 15, 180-day-release). Plantlets were grown for 6–8 weeks under a day/night air temperature of 25–27 °C/20–22 °C and 14 h photoperiod (345 mol m^{-2} s^{-1} photosynthetic photon flux density) provided by cool-white fluorescent lamps. Finally, the rusticated plants were transferred to 4-L pots under greenhouse conditions.

Genetic stability assessment of plants regenerated from cryopreserved adventitious buds

Genomic DNA was isolated from leaves of ten plants regenerated from cryopreserved adventitious buds (+ LN_S1 to S10) and ten plants regenerated from non-treated control (NT_S1 to S10) using a modified CTAB method (Yamamoto et al. 2000). A total of eight primers were used for inter-simple sequence repeat (ISSR) amplification. The ISSR reaction was performed as described by Brugnoli et al. (2013). Amplification products were separated by electrophoresis in 2% agarose gels in 1 × TAE buffer at 70 V. The DNA amplification profiles obtained for each plant was introduced in a binary-data matrix. ISSR markers were scored for the presence (1) and absence (0) of homologous DNA bands. Fragments with the same molecular size were considered as analogous amplicons representing the same locus. The resulting binary-data matrix was analyzed using the InfoGen software package (Balzarini and Di Rienzo 2013). Genetic stability among samples was measured by the Jaccard's dissimilarity coefficient (1 − S). A dendrogram was created based on the matrix of distance using the unweighted pair group method with arithmetic averages (UPGMA).

Data collection and statistical analysis

The experiment was performed in triplicate with ten ABCs per treatment. The experiment was repeated three times. Data were expressed as mean with their standard error (SEM). The survival rate and the number of shoots regenerated per explant were determined after 15 and 45 days of incubation, respectively. Statistical tests were performed with the help of Graph Pad software version 7.0 (San Diego, CA, USA) and InfoStat (Córdoba, Argentina). As controls, explants subjected to the same procedure but without immersion in LN were included. For the genetic stability assessment plantlets from non-treated and cryopreserved ABCs were used.

Results

Encapsulation-dehydration and encapsulation-vitrification

Figure 1 shows the effect of air drying and silica gel on the beads water content from ABCs explants subjected to

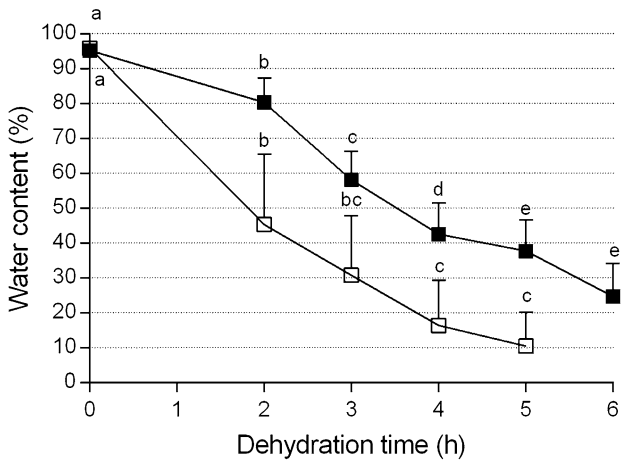

Fig. 1 Effect of dehydration time on adventitious buds' beads water content exposed to either sterile laminar airflow (empty squares) or silica gel (full squares). Encapsulated buds were precultured on MS liquid medium supplemented with increasing sucrose concentration and maintained under continuous shaking for 24 h in each solution and dehydrated in laminar flow for 0–5 h or silica gel for 0–6 h. The water content of the beads was measured on a fresh weight basis. Data are presented as mean ± SEM. Different letters indicate statistical differences at $P < 0.05$ (Tukey multiple comparison test)

the encapsulation-dehydration technique. The initial water content of the precultured ABC beads was 96.8 ± 0.2% on a fresh weight basis (after preculture in increasing concentrations of sucrose during 72 h). Water content decreased to 16.3 ± 5.8% after 4 h of dehydration under laminar airflow. The reduction in water content was slower when the beads were exposed to silica gel reaching 24.7 ± 3.9% after 6 h of dehydration. Although more than 85% of the explants overcome the dehydration procedure, no survival of encapsulated beads after the LN treatment was observed.

Likewise, 88 ± 4.6% of the ABCs encapsulated in calcium alginate beads and dipped in the ice-cooled PVS3 solution (encapsulation-vitrification method) survived to the cryoprotection procedures however they were not able to tolerate the ultra-low temperatures.

The recovered non-cryopreserved explants from both procedures grew strongly without symptoms of malformation visible and provided multiple shoots that later became plants.

Vitrification

A principal component analysis (PCA) displays differences in the survival rate and regrowth of ABCs treated with different cryoprotectant mixtures. The first principal component (PC1) explains 58.3% of the variation and allows separating the cryoprotectant mixtures solutions while the PC2 explains 19.2% of the total variation in the data (Fig. 2). In this context, the ABCs survival rate to freezing is promoted by the CMs 1, 3, 4, 8, 9, and 11. CM3 is the treatment with the highest correlation in CP1, and together with the cryoprotectant mixtures CM9 and CM11 constituent the treatments most positively correlated with the adventitious buds survival rate and the number of shoots per explant. In contrast;

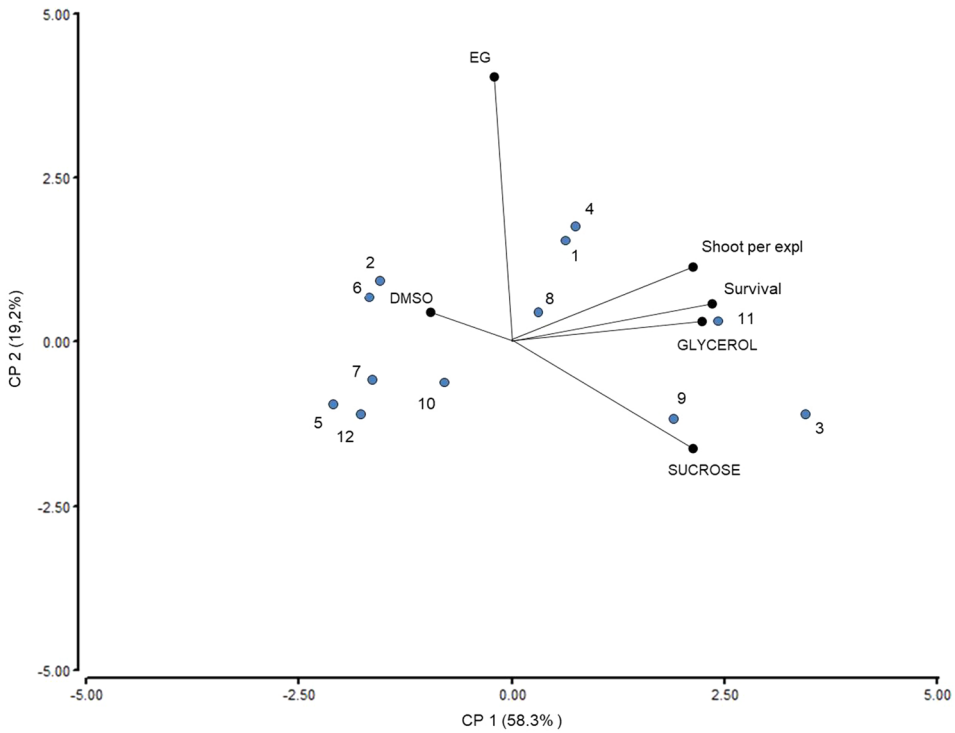

Fig. 2 Biplot of principal component analysis of osmoprotectants mixtures, bud's survival rate, and regrowth of cryopreserved ABCs. *CP1* principal component 1, *CP2* principal component 2

these variables were negatively affected by the CMs 0, 2, 5, 6, 7, 10, and 12. Likewise, it was observed that EG showed no specific relationship across the treatments since the vector related to EG showed a distance from all treatments on the biplot. Glycerol and sucrose were the closely related groups while DMSO was negatively correlated with both variables.

The effects of CMs exposure on survival and regrowth after thawing are shown in Table 2. Non-cryopreserved *L. tenuis* ABCs showed tolerance to all CMs tested as survival ranged from 85 ± 5.6 to 92 ± 3.6%. By contrast, survival of cryopreserved buds was influenced by the CM composition and the proportion of the different cryoprotectants, varying between 46.7 ± 7.6 and 79 ± 5.3%. The best results were obtained with the CM3 (PVS3) solution containing glycerol and sucrose equally (Table 1). The variance respect to the CM1, CM4, CM8, and CM9 solutions was statistically significant according to the *t* test ($P < 0.01$). In addition, the shoots number developed per recovered explant was affected by the composition of the cryoprotectant mixture. Although, we noticed a positive response to the CM1, CM4, and CM9 solutions, the survival and regrowth of the recovery treated explants significantly decreased after immersion in LN. Finally, we observed a null relationship of DMSO or EG, and a moderate to strong relationship of sucrose and glycerol with the survival rate (Fig. 3). Likewise, we noticed a moderate correlation of sucrose and glycerol with the mean number of shoots differentiated per explants ranged from 0.56 to 0.72, respectively (all $P < 0.0001$).

All cryopreserved ABCs growth actively after recovery and show a phenotype comparable with the non treated control. Subsequently, more than 80% of the elongated shoots rooted and produced healthy plants after acclimatization.

Table 2 Effects of different cryoprotectant mixtures (CM) on survival rate and regrowth of the non-cryopreserved (− LN) and cryopreserved adventitious buds (+ LN) of *Lotus tenuis* by vitrification

CM	Survival rate (%)		Shoots number/explant	
	− LN	+ LN	− LN	+ LN
CM0	85.5 ± 8.8	0***	27.0 ± 6.8	0***
CM1	92.0 ± 3.6	51.0 ± 5.5***	23.7 ± 6.2	12.3 ± 2.9*
CM2	86.0 ± 4.0	0***	22.5 ± 6.0	0***
CM3	88.7 ± 5.0	79.0 ± 5.3 ns	24.2 ± 6.8	12.2 ± 2.4 ns
CM4	91.3 ± 4.2	46.7 ± 7.6***	26.0 ± 5.6	7.9 ± 1.8**
CM5	85.7 ± 7.0	0***	25.2 ± 6.6	0***
CM6	85.0 ± 5.6	0***	25.0 ± 5.9	0***
CM7	87.0 ± 2.0	0***	24.2 ± 5.7	0***
CM8	91.0 ± 6.2	55.6 ± 11.9*	24.5 ± 6.4	9.8 ± 2**
CM9	87.0 ± 9.2	52.0 ± 6.2**	25.0 ± 4.0	8.1 ± 1.9**
CM10	89.3 ± 7.0	0***	27.3 ± 1.5	0***
CM11	89.3 ± 4.5	62.9 ± 12.7*	23.4 ± 4.9	11.8 ± 1.6 ns
CM12	90.0 ± 5.0	0***	24.0 ± 3.6	0***

Values are means ± SEM (n = 30)

Asterisks mark statistically significant difference (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ determined by Student's *t* test) among − LN and + LN for each CM solutions

ns not statistical significance

ISSR analysis

The primers tested generated 62 bands, which scored between 500 and 5000 bp in length. All primers produced clear and reproducible patterns (Fig. 4). A total of 62 fragments were amplified, of which 23 (37%) were monomorphic bands while the residual were polymorphic resulting in a total polymorphic index of 63%. The polymorphic information content (PIC) for the selected markers varied from 0.13 to 0.34 and the markers were therefore classified as highly informative (Table 3). The genetic distances among the regenerated plants measured by the Jaccard's dissimilarity coefficient (1 − S) ranged

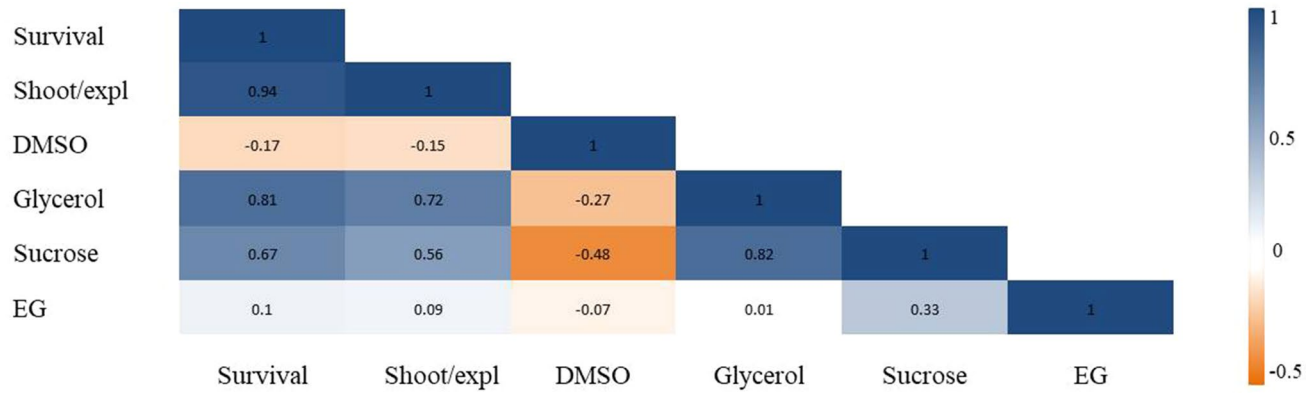

Fig. 3 Heatmap correlation matrix between cryoprotectants concentrations and survival rate and regrowth (number of shoots per explant) of cryopreserved adventitious buds. Pearson's correlation coefficient ($P < 0.0001$). Blue color intensity indicates increasing sample correlation, whereas orange color intensity indicates decreasing sample correlation

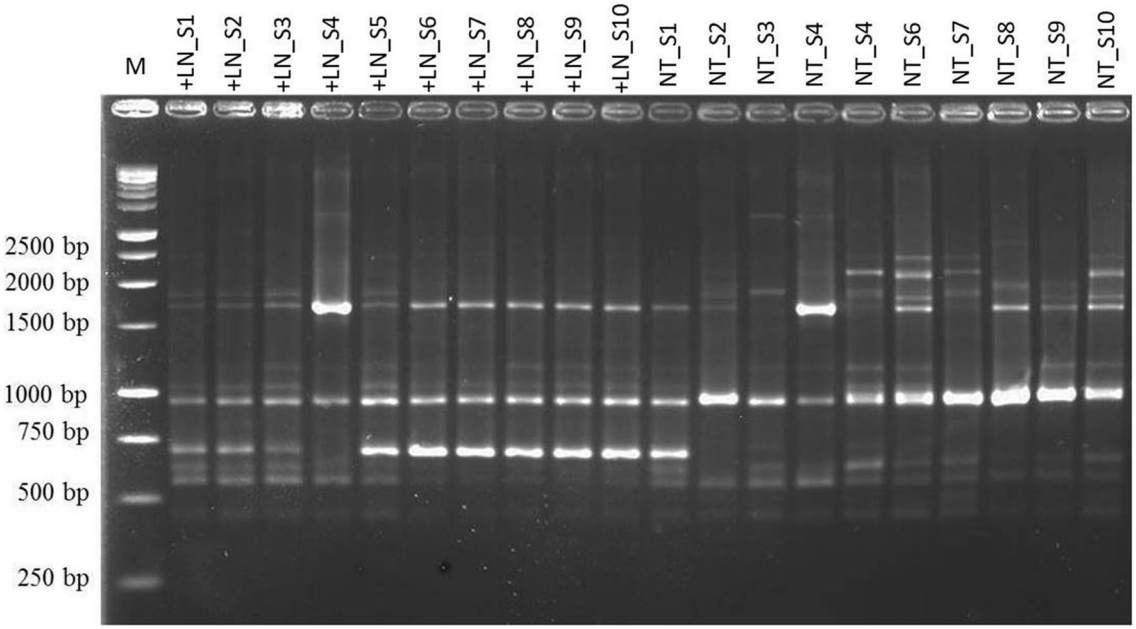

Fig. 4 Agarose gel electrophoresis of ISSR fragments of *Lotus tenuis* plants from non-treated (NT) and cryopreserved (+LN) adventitious buds. Agarose gel electrophoresis of ISSR fragments generated from primer (AC)8T. Lane M: molecular marker (1 kb); lanes +LN_S1 to S10: cryopreserved ABCs; lanes NT_S1 to S10: non-treated ABCs

Table 3 ISSR molecular characterization of non-treated and cryopreserved regenerated shoots

Primer repeat 5′→3′	BP	BM	BT	PIC ± SE	PI (%)
(GA)8T	7	2	9	0.32 ± 0.02	77
(CT)8G	2	1	3	0.13 ± 0.02	66
(TC)8A	3	3	6	0.30 ± 0.02	50
(GT)8TC	12	7	19	0.28 ± 0.03	63
(GA)8C	6	0	6	0.23 ± 0.02	100
(CA)8G	2	4	6	0.34 ± 0.003	33
(AGAC)4GC	3	3	6	0.32 ± 0.003	50
(AC)8T	4	3	7	0.23 ± 0.02	57
Total	39	23	62	–	63

BT number of total amplified bands, *BP* number of polymorphic bands, *PI* polymorphism index, *PIC* polymorphism information content

from 0.02 to 0.41. Furthermore, the comparison made by pairs of plants revealed more than 70% of similarity without variation between the non-treated and the cryopreserved treatments (Table 4). The dendrogram of ISSR markers (Fig. 5) determine a total genetic distance between all plants lower than 0.4 and separates the samples tested into three clusters that revealed the genetic distance less than 0.3 and where each cluster was formed without distinction between non-treated control and cryopreserved samples (Fig. 5; Table 4).

Discussion

We tested three different protocols to cryopreserve *L. tenuis* adventitious buds. Hereof, in encapsulation-dehydration and encapsulation-vitrification, before being directly immersed in LN, the ABCs were encapsulated in calcium alginate beads, osmoprotected with sugar solutions and then dehydrated in sterile airflow/silica gel or dehydrated with loading solution and loaded with the PVS3 solution. Even though these protocols resulted in a high survival of the treated control explants neither of them was able to overcome the stress after exposure en LN triggering necrosis and death of the explants. Thus, based on the vitrification procedure (Karlsson and Toner 1996), the ABCs were dehydrated by exposition to the loading and cryoprotectant solutions combined with fast warming and washed after freezing. We observed that explants treated with specific cryoprotectant solutions survived to the cryogenic temperatures. In this regards, several variations on solution composition were developed for plant cells (Volk et al. 2006) giving place to the glycerol-based plant vitrification solution (PVS) series which were included in the present experiment. Thus, PVS2 has been successfully used for different explants of *Allium* (Niwata 1995; Volk and Walters 2006), *Mentha* (Volk and Walters 2006) *Citrus* (Volk et al. 2017), *Saccharum* (Ferreira Nogueira et al. 2013), *Vitis* (Markovic et al. 2013), among others. PVS3 has been employed with *Wasabi* (Matsumoto et al. 1995), *Chrysanthemum* (Kim et al. 2009), *Prunus* (Ruzic

Table 4 Matrix-based on the Jaccard's (1 − S) dissimilarity coefficient of *L. tenuis* plants from non-treated (NT) and cryopreserved (+LN) adventitious buds

	NT										+LN									
	S1	S10	S2	S3	S4	S5	S6	S7	S8	S9	S1	S10	S2	S3	S4	S5	S6	S7	S8	S9
NT_S1	0																			
NT_S10	0.27	0																		
NT_S2	0.32	0.32	0																	
NT_S3	0.32	0.32	0.31	0																
NT_S4	0.31	0.38	0.28	0.27	0															
NT_S5	0.27	0.27	0.31	0.29	0.29	0														
NT_S6	0.36	0.06	0.34	0.4	0.38	0.27	0													
NT_S7	0.23	0.27	0.29	0.32	0.29	0.22	0.3	0												
NT_S8	0.26	0.27	0.4	0.3	0.31	0.15	0.28	0.26	0											
NT_S9	0.32	0.27	0.29	0.3	0.3	0.23	0.3	0.24	0.23	0										
+LN_S1	0.33	0.35	0.27	0.31	0.31	0.3	0.35	0.33	0.36	0.29	0									
+LN_S10	0.21	0.26	0.35	0.33	0.33	0.25	0.25	0.22	0.23	0.31	0.33	0								
+LN_S2	0.3	0.37	0.27	0.28	0.27	0.27	0.33	0.35	0.34	0.32	0.09	0.36	0							
+LN_S3	0.2	0.36	0.27	0.28	0.27	0.31	0.35	0.3	0.29	0.28	0.19	0.29	0.17	0						
+LN_S4	0.25	0.36	0.3	0.3	0.06	0.29	0.37	0.33	0.32	0.33	0.35	0.34	0.3	0.29	0					
+LN_S5	0.37	0.27	0.28	0.34	0.38	0.26	0.24	0.27	0.29	0.23	0.16	0.33	0.16	0.2	0.41	0				
+LN_S6	0.23	0.28	0.34	0.33	0.32	0.27	0.29	0.22	0.25	0.31	0.34	0.06	0.37	0.31	0.33	0.34	0			
+LN_S7	0.2	0.25	0.33	0.33	0.32	0.29	0.26	0.23	0.19	0.29	0.37	0.02	0.36	0.27	0.34	0.31	0.08	0		
+LN_S8	0.22	0.25	0.35	0.32	0.32	0.27	0.23	0.22	0.24	0.32	0.36	0	0.34	0.27	0.33	0.32	0.05	0.03	0	
+LN_S9	0.21	0.26	0.33	0.33	0.3	0.26	0.31	0.23	0.24	0.31	0.35	0.07	0.39	0.29	0.32	0.35	0.02	0.09	0.07	0

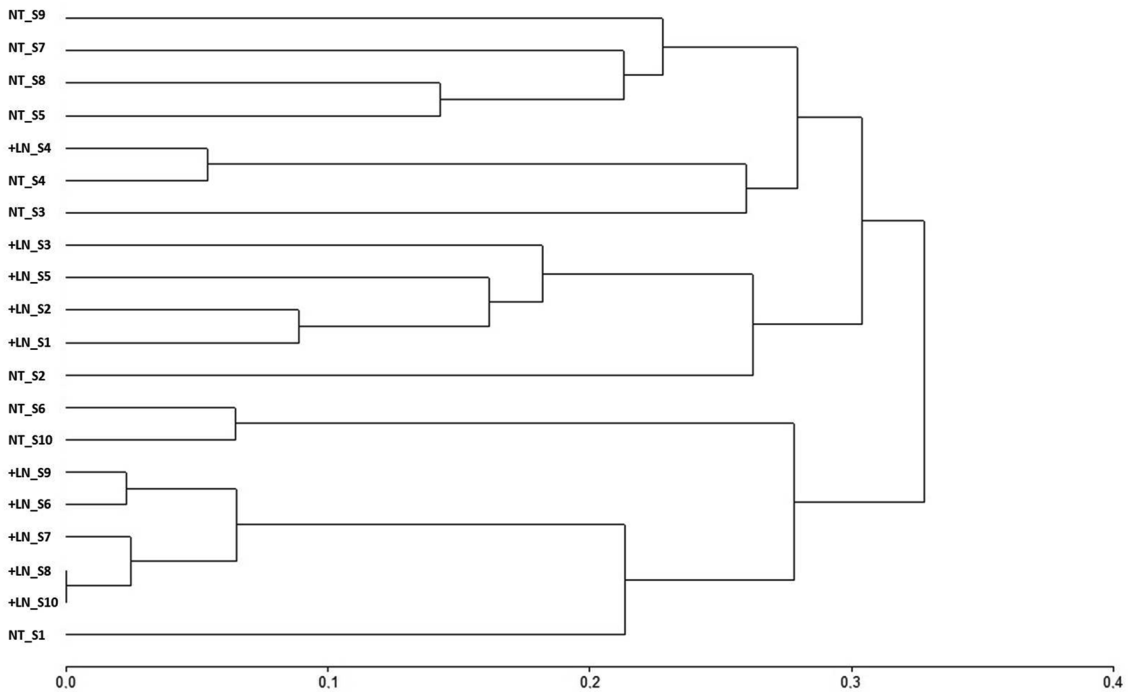

Fig. 5 UPGMA dendrogram derived using Jaccard's coefficient of similarity, demonstrating genetic similarity among plants regenerated from cryopreserved (+LN_S1 to 10) and non-treated (NT_S1 to 10) ABCs

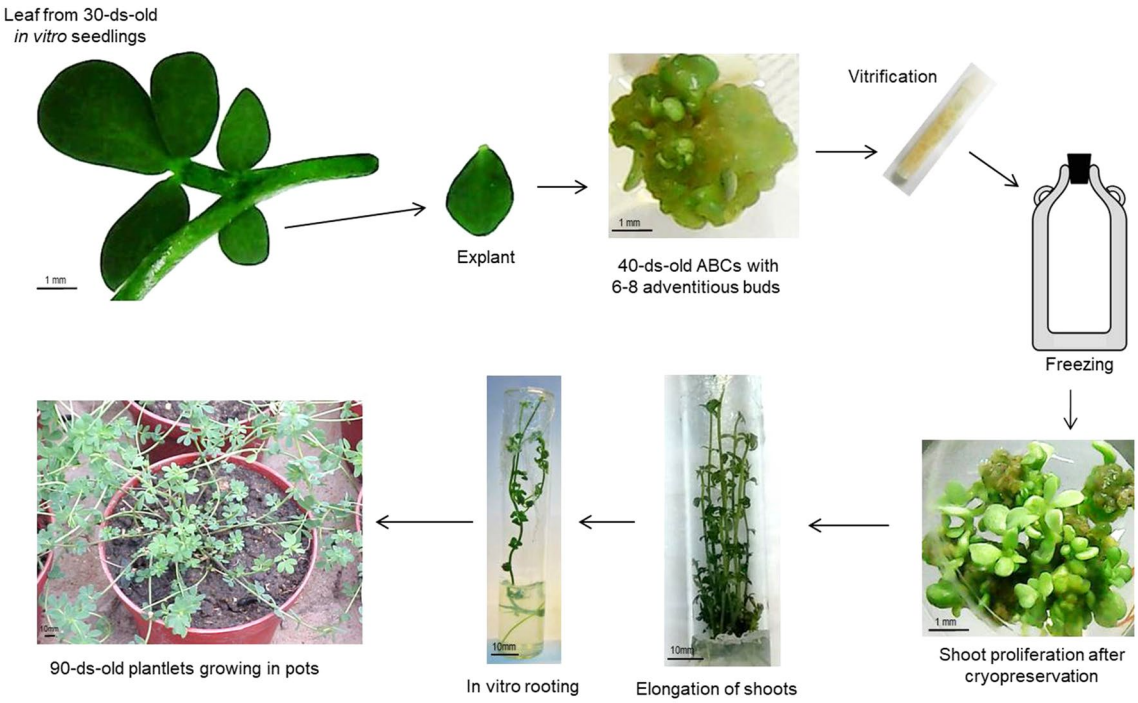

Fig. 6 Cryopreservation of adventitious bud clusters of *Lotus tenuis* by PVS3-based vitrification technique and regeneration of plantlets. The adventitious buds regenerated from leaf explants were dehydrated in a loading solution, immersed in the cryoprotectant mixtures (vitrification solutions), and stored in liquid nitrogen. After rewarming the cryopreserved ABCs were acclimated under darkness for 10 days and transferred to light conditions during the elongation and rooting phases. The genetic stability of the plantlets was confirmed by ISSR markers. *ABCs* adventitious bud clusters

et al. 2013) and *Allium* (Makowska et al. 1999) shoot tips. PVS4 has been used for the cryopreservation of in vitro-propagated protocorms of *Caladenia* (Watanawikkit et al. 2012). Also, we have included different modifications of the original PVS solutions, varying either the proportion between their components or its concentrations. Among them, PVS3-based solutions resulted in a significant survival rate of cryopreserved *L. tenuis* ABCs. Therefore, the lower survival and regrowth of cryopreserved samples dehydrated with a more dilute equivalent solution (CM9) might be caused by insufficient cryoprotection against freezing injury. In fact, glycerol and sucrose present in the PVS3 solution act as an osmotic agent protecting the cells from oxidative damages and preserving the stability of cell membranes by the interaction of glycerol with the lipid fraction (Dumet and Benson 2000). In addition, Ogawa et al. (2012) reported a simple protocol for the cryopreservation of cell lines from *L. japonicus* by using a cryoprotectant mixture of glycerol, sucrose, and proline. We observed that the exposure to PVS2 and variants in the concentration tested were not adequate to protect cells from damage during freezing. Kim et al. (2009) attributed the low efficiency of PVS2-based solutions to preserve shoot tips of *Allium* and *Chrysanthemum* to their toxicity and the insufficient protection of samples from freeze–thaw events and concluded that PVS3 and variants should be used when tissues are susceptible to chemical toxicity and tolerant to osmotic stress.

Finally, considering that the genetic stability of the preserved material is essential for the establishment of any germplasm bank, we confirmed the stability of the cryopreserved shoots using the ISSR markers. ISSR showed raised monomorphic banding patterns and revealed a low polymorphism index among the non-treated and cryopreserved regenerated plants suggesting that the vitrification-based cryopreservation protocol not affect the genetic stability of *L. tenuis* germplasm. Finally, we observed a natural genetic variation within the cultivar by which our work also shows a series of potential ISSR primers that could be used to determine genetic variation in the domesticated population of *L. tenuis*.

In conclusion, the PVS3-vitrification protocol (Fig. 6) has the potential to facilitate long-term preservation of *L. tenuis* providing high-efficiency shoot proliferation from the cryopreserved ABCs and genetic stability of the plantlets.

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Author contributions FDE, OAR, and PAS conceived and designed the experiments. FDE, EAB, PGA, and LPA performed the research. FDE and PAS wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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