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ORIGINAL ARTICLE

Efficient slow-growth conservation and assessment of clonal fidelity of *Ullucus tuberosus* Caldas microshoots

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Abstract

Slow-growth is a biotechnological tool for medium-term conservation of plant germplasm under in vitro conditions. In the present study, we assessed six culture media supplements and two cultivation temperatures to develop a reliable in vitro conservation protocol for ulluco (*Ullucus tuberosus* Caldas), an Andean tuber crop rich in carbohydrates and vitamin C. One-nodal segments of ulluco were cultivated on half-strength Murashige and Skoog (MS) medium supplemented with mannitol (10–30 g l^{-1}), sorbitol (10–30 g l^{-1}), sucrose (10–120 g l^{-1}), chlorcholinchlorid (CCC; 300–700 mg l^{-1}), abscisic acid (ABA; 1–3 mg l^{-1}), or maleic hydrazide (MH; 0.1–0.5 mg l^{-1}). In vitro cultures were maintained at either 5 °C or 17 °C cultivation temperature. After 18-month cultivation, based on growth characteristics, survival percentage and form of survival (plant, live shoot + microtuber, microtuber + dead shoot), four treatments were selected: 30 g l^{-1} mannitol|17 °C, 30 g l^{-1} sorbitol|17 °C, 0.3 mg l^{-1} MH|17 °C and 60 g l^{-1} sucrose|17 °C. The plantlets from these treatments were tested for regrowth ability on half-strength MS medium. After 28 days of cultivation, survival percentage and morphological characteristics were evaluated. Plantlets originating from conservation medium supplemented with 30 g l^{-1} mannitol showed the fastest regrowth and provided overall superior characteristics over shoots from other treatments. These plants were subjected to inter simple sequence repeat analysis, which gave rise to monomorphic patterns indicating no detected genetic variation between plants recovered after conservation on this medium and proving the reliability of this conservation protocol.

Key message

Slow-growth of ulluco plantlets at 17 °C and the use of mannitol as a culture medium supplement, represents a reliable system of conservation with minimal risk of somaclonal variation.

Keywords ISSR · In vitro conservation · Mannitol · Minimal growth · Ulluco

Introduction

Ullucus tuberosus Caldas is a crop from the Basellaceae family and native to the Andean mountain region (Malice and Baudoin 2009). It is mainly cultivated for its edible storage tubers rich in carbohydrates and vitamin C, which are a staple food for local communities. Ulluco is one of the most commercially viable Andean crops, second only to potato (*Solanum tuberosum* L.) (Campos et al. 2018; Manrique et al. 2017). It is a vegetatively propagated crop with a high morphological and genetic diversity. Most ulluco genotypes are diploid (2n = 24), although triploids (2n = 36) and tetraploids (2n = 48) have also been found (Mendez et al. 1994).

Vegetatively propagated species such as ulluco, are under threat of genetic erosion due to the spread of

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intensive agricultural practices, changes in food habits, and impoverished seed sets (Malice et al. 2009; Gulati 2018). In the past, there was a rapid decline of Andean tuber genetic diversity, driven by anthropogenic factors such as selection of varieties for food purposes, marginalization of the species, or intensive cultivation of monocultures of crops introduced to Latin America (Tapia and Estrella 2001; King and Gershoff 1987; Malice and Baudoin 2009).

Such conditions make traditional on-farm ulluco conservation difficult to perform. Moreover, reduced ability to produce viable seeds of this species (Pietila and Jokela 1990) is a major constraint facing ex situ conservation in the form of seed collections. The need to use advanced biotechnological approaches such as medium to long-term in vitro conservation methods is, therefore, justified as an alternative to preserve the species' genetic resources and biodiversity in the Andean region.

Effective medium-term in vitro conservation can serve to maintain the vitality of the species, free from pest and diseases, and provide plant material immediately available at a lower cost than in field preservation (Tyagi et al. 2006; Kulus 2018). Physical factors such as reduced illumination, low temperature, growth in small culture vessels, and short photoperiod, along with chemical factors such as supplementation of culture media with different chemical compounds (Holobiu et al. 2018), including abscisic acid (ABA; Banasiak and Snyman 2015), mannitol, sorbitol, sucrose (Nasiruddin and Islam 2018; Munoz et al. 2019; Goncalves and Romano 2007), and maleic hydrazide (MH; Tyagi et al. 2006), can be applied to conserve plants by the slow-growth method. The potential of this conservation strategy in ulluco has been suggested by Hammond et al. (2017) in the primary study on short-term application of osmotic agents on ulluco in vitro cultures. Despite the numerous advantages of slow-growth conservation such as an increase of the time between consecutive subcultures, it can be associated with somaclonal variation, a phenomenon occurring randomly during in vitro cultivation (Krishna et al. 2016).

Explant source, the duration of in vitro culture, physical parameters, as well as chemical agents used for in vitro cultivation are all potential determinants of somaclonal variation (Bordallo et al. 2004). Thus, the detection of potential somaclonal variation of the in vitro plantlets needs to be carried out after conservation, most commonly by molecular markers (Koc et al. 2014; Bradai et al. 2019).

This study aims to develop an efficient minimal-growth conservation protocol for ulluco by applying chemical agents such as osmotics (mannitol, sorbitol, and sucrose) and plant growth inhibitors: chlorocholine chloride (CCC), abscisic acid (ABA) and maleic hydrazide (MH), at various concentrations and storage temperatures (5 °C and 17 °C), to assess regrowth of plantlets after 18-month conservation, and to evaluate plant genetic fidelity using inter simple sequence repeat (ISSR) markers.

Materials and methods

Plant material, establishment of in vitro culture, and plantlet multiplication for the experiment

The initial plant material used in this study was a diploid clone of ulluco (2n = 2x = 24), classified according to the morphological descriptors proposed by the International Potato Center (CIP) (Manrique et al. 2017) as morphotype No. 156. This plant material displays a combination of pale orange and reddish purple colour of tuber surface, and it is maintained at the Botanical Garden of the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague.

To establish in vitro culture, nodal segments were collected from clonally propagated ulluco plants grown under greenhouse conditions and were surface-disinfected using 70% ethanol for 1 min, and 1% NaClO for 10 min. The nodal segments were then rinsed three times in sterile distilled water and transferred to half-strength MS medium (Murashige and Skoog 1962), containing 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamine, and pH adjusted to 5.7. Prior to being used, the medium was autoclaved at 121 °C in 100 kPa for 20 min. The in vitro cultures were maintained in a culture room at 25/23 °C under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of approximately 35 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. After sprouting of shoots from meristems, the plantlets were regularly sub-cultured every 28 days using segments with axillary or apical meristems on the same medium, until sufficient plant material for the experiment was obtained.

Slow growth treatments for microshoot conservation

For the medium-term conservation of ulluco, nodal segments about 2 cm in length containing one axillary bud were used. To standardize the experiment, all segments were collected from the same position on in vitro plantlets; the second nodal segment from the top of the shoot was always selected. Slow-growth storage of ulluco was optimized using half-strength MS medium containing 30 g l^{-1} sucrose (except for sucrose treatments, where specific sucrose concentrations were used), and supplemented with mannitol at a concentration of 10, 20 and 30 g l^{-1}, sorbitol at 10, 20 and 30 g l^{-1}, sucrose at 10, 20, 60, 90 and 120 g l^{-1}, CCC at 300, 500 and 700 mg l^{-1}, ABA at 1, 2 and 3 mg l^{-1}, or MH at a concentration of 0.1, 0.3 and 0.5 mg l^{-1}. Half-strength MS medium without any plant growth regulators

was used as the control. Culture media with pH adjusted to 5.7 were autoclaved at 121 °C and 100 kPa for 20 min. ABA, CCC and MH were filter sterilized and added to media after autoclaving. 20 ml of culture medium was always dispensed into test tubes (25 × 150 mm). In each treatment, 40 explants were used out of which 20 were placed in constant cultivation temperatures of 5 °C and twenty at 17 °C. The experiment was conducted in two repetitions. The in vitro cultures were maintained in a culture room under a 16/8 h light/dark regime, and at a photosynthetic photon flux density of approximately 35 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Plantlet height was measured once a month for a total of 18 months. The total number of meristems including apical meristems per plant was counted at the end of the conservation period.

After an 18-month conservation, the survival form of the plants was evaluated (on visual 0–3 scale, 0 = dead shoot, 1 = dead/dried shoot + live microtuber (MT), 2 = live shoot + live MT, 3 = only live shoot without MT), as well as the survival percentage (as sum of grade 1–3). Dying/drying stem in combination with MT development was regarded as a plant senescence sign and potentially less suitable for further ulluco conservation. In this experiment, microtubers were not considered for ulluco conservation, due to their size variability, different numbers of vegetative buds, and potential dormancy.

Post-storage regrowth of microshoots

After 18 months of ulluco conservation, the regrowth potential of surviving microshoots was tested. For post-storage regrowth, four conservation treatments were selected based on the following criteria: survival percentage after conservation (preferably with the highest shoot and/or shoot + MT survival rate), and treatments with superior morphological characteristics; i.e., mannitol|17 °C, sorbitol|17 °C, sucrose|17 °C and MH|17 °C, all of them being represented by one concentration (30 g l^{-1}, 30 g l^{-1}, 60 g l^{-1} and 0.3 mg l^{-1}, respectively). As a control treatment, plantlets from the half-strength MS medium|17 °C were used for post-storage regrowth.

Apical shoots were used from microplants that survived in form of either shoots or shoots + MT. Shoots about 2 cm in length collected from these plantlets were transferred to half-strength MS medium containing 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, and 1 mg l^{-1} thiamine (pH of 5.7). The cultures were maintained in an incubator at 25/23 °C under a 16/8 h light/dark regime for 28 days. Thereafter, survival percentage, plant height (cm), number of shoots, meristems and roots were evaluated. Based on the results from the post storage regrowth experiment, plantlets from the best treatment (30 g l^{-1} mannitol) were selected for ex vitro transfer and genetic fidelity assessment.

Ex vitro transfer to greenhouse conditions

Twenty rooted plantlets after post-storage regrowth from a selected treatment, 30 g l^{-1} mannitol, and control plantlets were transferred to a greenhouse. Microplants approximately 3–5 cm high, with a minimum of six leaves and a minimum of two well-developed roots (min. 2 cm long), were selected for ex vitro transfer. They were removed from flasks, the roots were rinsed with tap water to remove adhering medium and the plantlets were transferred into flower pots (0.4 dm^3) containing a sterile mixture of garden substrate and perlite (1:1 v/v) and covered with plastic perforated foil to maintain a microclimate with approximately 85% humidity. A temperature of 25/19 °C (day/night) was maintained in the greenhouse. The plastic covers were gradually removed from the pots after 2–3 weeks of cultivation. The survival percentage of plants was evaluated after an 8-week period.

DNA isolation and ISSR analysis

Leaves of 10 plants from 30 g l^{-1} mannitol treatment, collected in the greenhouse after plant acclimation were used for molecular analysis. As a control, the initial mother plant/field control was used. Additionally, samples from 10 control plants obtained after post-storage regrowth, and after ex vitro transfer, were also analyzed.

Genomic DNA from fresh leaves was isolated by the DNA extraction method with cetyltrimethylammonium bromide (CTAB) (Doyle and Doyle 1989) with a few drops of polyvinylpyrrolidone (PVP). The extracted DNA was used for ISSR analysis with twelve selected ISSR primers (Integrated DNA Technologies, Belgium; Table 2). Polymerase chain reaction (PCR) amplification was done on the individual samples in two repetitions. The DNA samples were used in 50 ng $µl^{-1}$ concentration. The PCR amplification was done in a total reaction volume 20 µl containing 2 µl template DNA, 0.5 µl of primer at a concentration of 0.1 mM, 10 PPP Master Mix (Top-Bio, Czech Republic), 0.2 µl bovine serum albumin (BSA) of concentration 20 mg ml^{-1} (Thermo Scientific, Lithuania), and 7.3 µl PCR water. Amplifications were carried out in cycler (Bio-Rad, USA) with modification of the annealing temperature depending on which primer was being used for ISSR (Table 2). The specifications for PCR cycling were initial denaturation for 5 min at 94 °C, then 40 cycles of 1 min each at 94 °C, 1 min at specific annealing temperature, 2 min at 72 °C for extension, and then a final extension cycle for 10 min at 72 °C. The PCR amplification of the DNA was done in duplicates. Electrophoresis was done with 7 µl of the amplified DNA on 2% agarose with the final concentration of ethidium bromide (EtBr) in the gel 0.5 µg ml^{-1}. The gels were run for 3–3.5 h at 60 V and 120 mA. Bands were visualized on the gel under UV light (Cleaver Scientific, UK). The size of amplicons

was estimated using Thermo Scientific Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific, Lithuania).

ISSR fragments were scored for the presence (1) or absence (0) of bands in the gel profile. Only clear and distinct bands were evaluated.

Statistical analysis

Data from the slow-growth experiment and post-storage evaluation were analyzed using analysis of variance (ANOVA) and the least significant ($P < 0.05$) differences between mean values were estimated using Tukey's HSD test [STATISTICA 12.0, StatSoft, Inc., USA].

Results

Effect of culture medium supplements on ulluco plantlet conservation at 5 °C

Conservation at 5 °C led to extremely reduced plant growth in all tested treatments. The differences in plantlet height (1.6–5.3 cm) (Fig. 1a–f) and number of meristems between plantlets (0–7 meristems per plant) (Fig. 2) from various treatments were relatively small. Overall, shoots produced during the conservation period were thin and leaves were reduced or absent. During cultivation, usually one MT per individual plant developed within 5–8 months in all treatments, which was followed by drying of the stem and, therefore, by the end of the conservation period, survival in the form of MTs on a dried shoot prevailed in the experiment, including plantlets from control treatment. Survival in the form of a shoot without MT was not recorded in any of the treatments maintained at 5 °C. Overall, survival percentage after 18-month plant cultivation varied from 0 to 100% between treatments (Fig. 3a).

Mannitol and sorbitol (10–30 g l^{-1}) treatments showed similar tendencies in plant survival; at the end of the conservation period, survival percentage of plants was 100%. All plants cultivated on media with both osmotic agents survived only in MT form. In both cases, MT formation at lower concentrations (10–20 g l^{-1}) started within the sixth month of conservation, while at higher osmotic concentration; plantlets developed MTs 2 months later. In all cases, the shoot started drying within 2 months after MT formation, with the exception of treatment with 30 g l^{-1} mannitol, where shoots started dying approximately 3 months after MT development.

Supplementation of culture medium with sucrose showed a positive effect on conservation, providing high survival rate at concentrations of 30, 60 and 90 g l^{-1}, with 100%, 90% and 80% survival respectively; this was not only in MT formation, but also with a partial survival of shoots with developing MTs. The highest (120 g l^{-1}) and lowest (10 g l^{-1}) sucrose concentration tested had a reducing effect on ulluco plant survival, and led to survival exclusively in MT form (Fig. 3a).

MH (0.1–0.5 mg l^{-1}) addition in culture medium displayed a negative effect on ulluco plants at 5 °C, leading to poor growth and providing 0% survival in all treatments after the conservation period. During in vitro cultivation, the plants did not produce MTs and grew only in the form of shoots. At higher MH concentration (0.5 mg l^{-1}), shoots started dying within the first 8 months of conservation while shoots on media with lower MH concentrations (0.1–0.3 mg l^{-1}) started drying 3 months later.

In all CCC treatments (300–700 mg l^{-1}) the plants survived after 18 months only in the form of a dried shoot with MTs, and the survival did not exceed 30%. A similar negative effect on ulluco conservation showed ABA supplementation (1–3 mg l^{-1}) into culture medium, leading to poor growth of the plants and their early dying at all concentrations tested.

Considering the fact that most microshoots under 5 °C conservation temperature displayed dwarfed growth, had minimal numbers of meristems, and after development of microtubers tended to turn brown and dry, showing low potential of these treatments for further conservation, they were not considered optimal for ulluco plant maintenance.

Effect of culture medium supplements on ulluco plantlet conservation at 17 °C

Ulluco conservation at 17 °C, on the other hand, led to more vigorous shoot growth (height ca. 4.0–11.6 cm) (Fig. 1a–f) and a higher number of developed meristems (4–21 meristems per plant) (Fig. 2) in comparison to plantlets maintained at a cultivation temperature of 5 °C. Plantlets of all treatments, including the control, conserved at 17 °C generally displayed higher percentage of survival in the form of plantlets without MT formation, or of living shoots with MTs. In treatments where MTs developed, this process usually began in the ninth month of conservation and the main shoot started dying 2 or 3 months later.

In mannitol treatments, the height of the microshoots decreased with osmotic concentration (10–30 g l^{-1}), while meristem number increased. Application of 30 g l^{-1} mannitol resulted in the highest survival percentage in the form of shoots without formation of MT from all treatments in the experiment, namely 70%. Thus, shoots after 18-month conservation on this medium displayed relatively slow growth, had numerous meristems, mostly did not initiate MT formation and their total survival reached 90%. Similar tendencies were also recorded for sorbitol, where the highest concentration tested (30 g l^{-1}) also led both to a high total survival rate and a high proportion of plantlets surviving

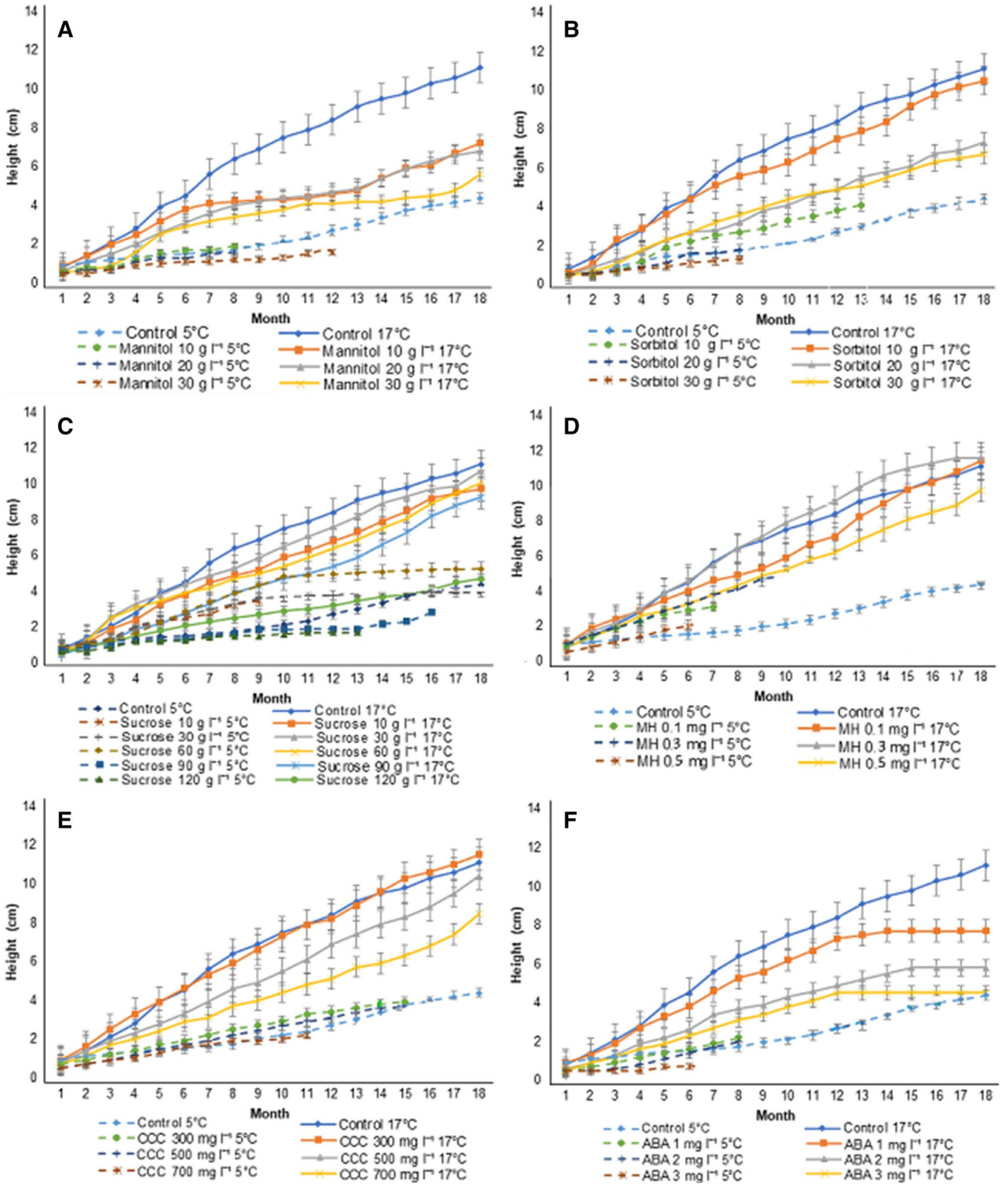

Fig. 1 Effect of culture medium supplements and culture temperature on plant height during 18-month conservation period. **a** Mannitol at 17 °C and 5 °C. **b** Sorbitol at 17 °C and 5 °C. **c** Sucrose at 17 °C and 5 °C. **d** MH at 17 °C and 5 °C. **e** CCC 17 °C and 5 °C. **f** ABA at 17 °C and 5 °C. All values are presented as mean ± standard deviation (SD)

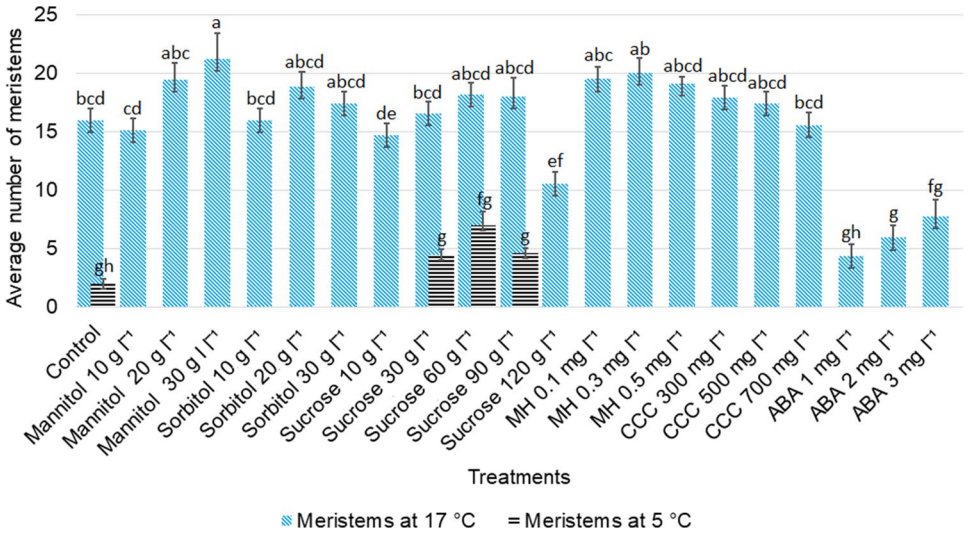

Fig. 2 Effect of culture medium supplements and conservation temperatures on average number of plant meristems per explant after 18-month conservation period. All values are presented as mean ± standard deviation (SD)

in the form of just shoots when compared to lower sorbitol concentrations. Supplementation of MH (0.3–0.5 mg l^{-1}) in medium at 17 °C provided the best results in terms of total survival among all treatments at higher tested temperature. These conditions generally decelerated plantlet senescence and MT formation, resulting in a maximum survival in the form of shoots without MTs, especially at a concentration of 0.3 mg l^{-1}. Sucrose added to a medium at concentrations of 10–60 g l^{-1} gradually increased the plantlet survival percentage, as well as the number of meristems formed, while plantlet height among treatments was relatively comparable. In contrast, further increasing sucrose concentrations (120 g l^{-1}) negatively affected all measured parameters. The microshoots cultivated on a medium with CCC produced MTs at higher rates and, by the end of conservation period, plant stems with MTs tended to dry, especially at 500 and 700 mg l^{-1} CCC concentrations. Application of ABA (1–3 mg l^{-1}) in culture medium provided low plant survival, not exceeding 40%. The plantlets did not produce tubers and they grew exclusively in the form of shoots.

Based on the results obtained from the microshoot storage experiment, four promising treatments for ulluco conservation were selected. Taking into consideration total survival percentage and preferably high proportion of plants surviving in the form of shoots or living shoots with MTs, shoots cultivated on media supplemented with 30 g l^{-1} mannitol, 30 g l^{-1} sorbitol, 0.3 mg l^{-1} MH and 60 g l^{-1} sucrose, all cultivated at 17 °C, were tested for regrowth ability.

Recovery of plantlets after conservation and ex vitro transfer

After 7 days of cultivation on recovery medium, the shoots collected from the medium supplemented with 30 g l^{-1} mannitol started to regrow first among all treatments and, within 28 days, they produced the highest number of meristems (approx. 16.33 meristems per plant). Plantlets from this treatment also grew significantly faster (approx. plant height 5.67 cm), produced a higher number of roots (approx. 6.38 roots per explant), and had a survival rate of 100% at the end of the recovery period. Plantlets from other treatments started to regrow on recovery medium 12 days (± 2 days) after their transfer to this medium. The survival percentage also reached 100% in these treatments, except for plantlets conserved on a medium with the addition of 0.3 mg l^{-1} MH, where just 87.5% plantlets survived. Relatively low regrowth capacity, as well as slow growth of plantlets from this treatment resulted in excluding this variant from further experiments. Plantlets conserved on media supplemented with 30 g l^{-1} sorbitol and 60 g l^{-1} sucrose also did not reach optimal results obtained in plantlets from the medium with mannitol (Table 1; Fig. 4a). Therefore, the conservation medium with 30 g l^{-1} mannitol was evaluated as the most suitable for ulluco conservation and subsequent plantlet regrowth, and the plantlets from this medium were used for ex vitro transfer.

Shoots of this treatment rooted well on the recovery medium, and they were transferred to the greenhouse. Three weeks later, the plants began to regrow and, within further cultivation, they produced new leaves. No morphological abnormality, was observed during greenhouse cultivation (Fig. 4b). The plants acclimated well and after 8 weeks of cultivation, 100% of plants survived.

ISSR analysis

Genetic stability of these plants was assessed using ISSR analysis. A total number of 1659 amplified products were produced by the 12 ISSR markers and 79 loci were generated. Fragment sizes ranged from 200 to 1600 bp. The

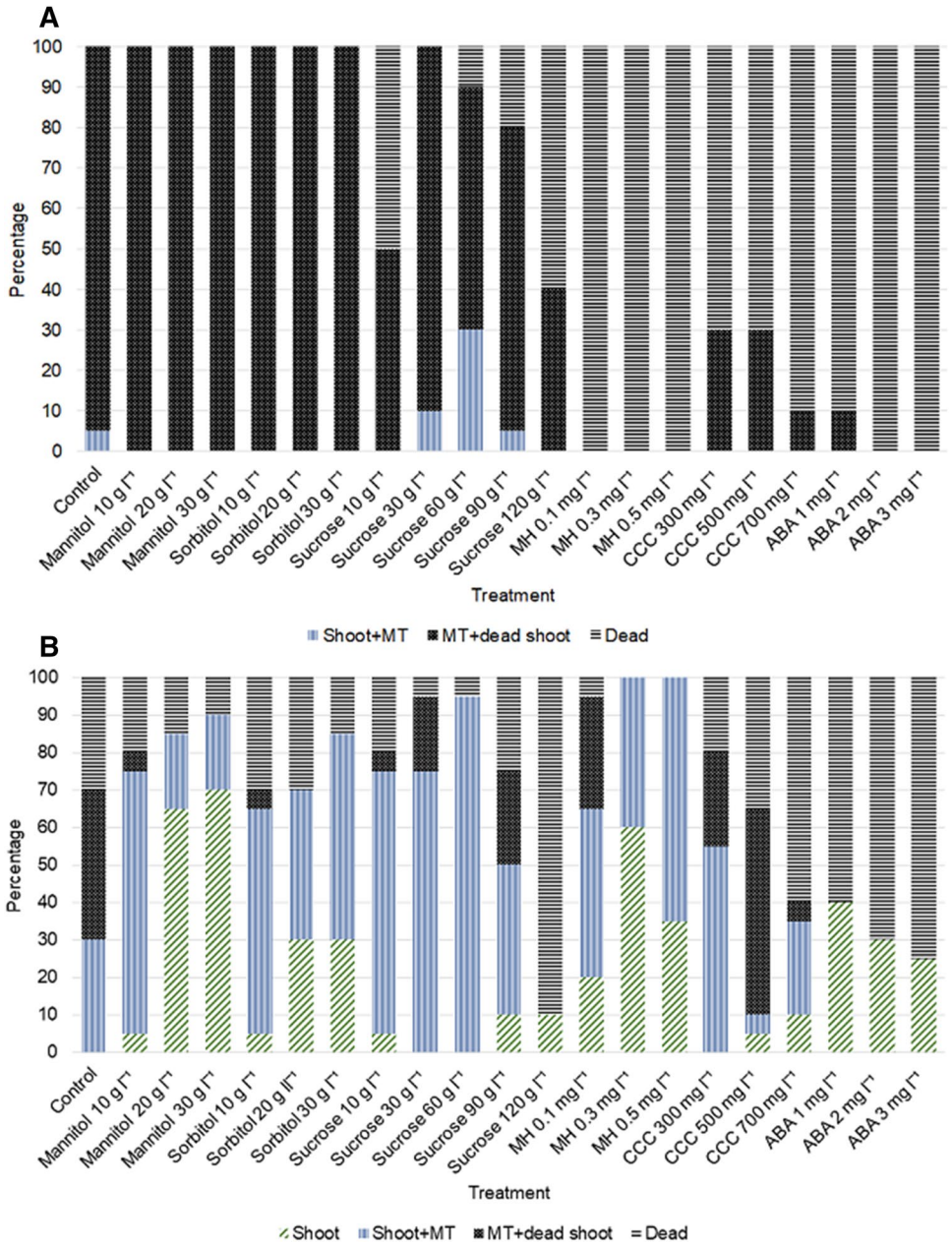

Fig. 3 Effect of culture medium supplements and conservation temperature on plant survival after 18-month conservation period. **a** Plant survival at 5 °C. **b** Plant survival at 17 °C (*MT* microtuber)

Table 1 Effect of culture medium supplements on in vitro microshoot regrowth after 28 days of cultivation on recovery medium

Treatment	Height (cm) (mean ± SE)	No of meristems (mean ± SE)	No of roots (mean ± SE)
Control	3.12 ± 0.44b	14.00 ± 1.57ab	4.63 ± 0.95ab
Mannitol 30 g l^{-1}	5.67 ± 0.34a	16.33 ± 0.91a	6.38 ± 0.74a
Sucrose 60 g l^{-1}	3.32 ± 0.34b	12.31 ± 0.68b	3.42 ± 0.72b
Sorbitol 30 g l^{-1}	3.12 ± 0.35b	14.29 ± 1.13ab	4.76 ± 0.77ab
Maleic hydrazide 0.3 mg l^{-1}	2.80 ± 0.39b	13.42 ± 1.18ab	2.42 ± 0.84b

Mean values in a column, followed by different letters, were significantly different according to the Tukey's HSD test ($P \leq 0.05$)

SE standard error

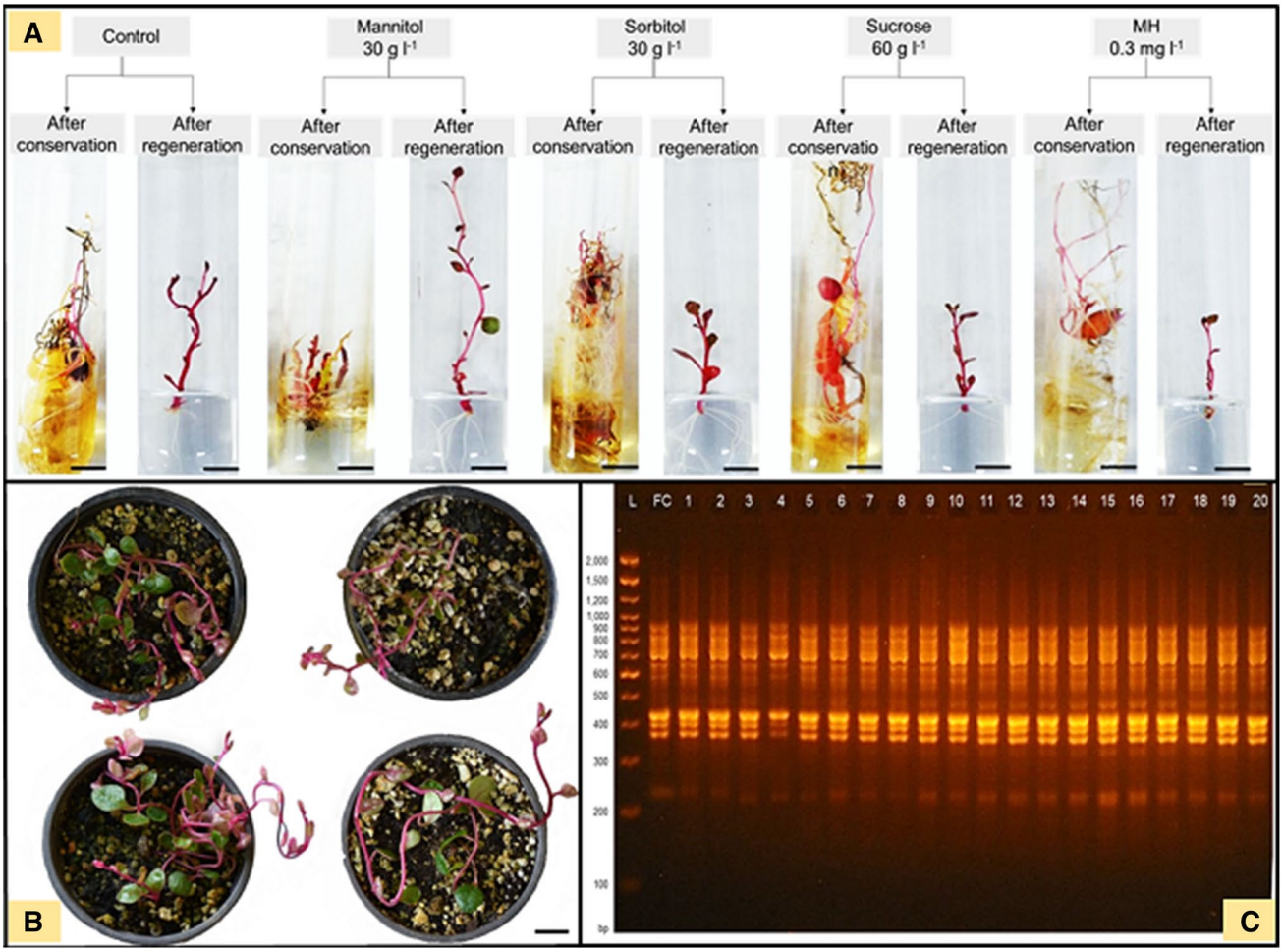

Fig. 4 Microshoots of *Ullucus tuberosus* after conservation, post-storage regrowth, plants after ex vitro transfer, and molecular analysis. **a** Microshoots of *Ullucus tuberosus* after 18-month conservation on half-strength MS medium supplemented with 30 g l^{-1} mannitol, 30 g l^{-1} sorbitol, 60 g l^{-1} sucrose, 0.3 mg l^{-1} MH cultivated at 17 °C (on the left) and plantlets after 28-day post-storage regrowth on half-strength MS medium (on the right) (bar = 1 cm). **b** Ulluco plants 8 weeks after ex vitro transfer to greenhouse (bar = 2 cm). **c** ISSR profile of ulluco field control (initial plant material), control plants from the experiment (1–10) and plants conserved on medium supplemented with 30 g l^{-1} mannitol at 17 °C (11–20), using primer 'UCB836' (*L* ladder, *FC* field control, *bp* base pair)

number of bands per primer varied from 5 to 9, with an average of 6.6 (Table 2). All plants included in the molecular analysis had identical ISSR fingerprints and gave rise to monomorphic patterns. A representative monomorphic amplification pattern obtained with ISSR primer 'UCB836' is shown in Fig. 4c.

Discussion

Slow growth is one of the most successful in vitro techniques for medium-term germplasm conservation (Peng et al. 2015), enabling plant storage for a prolonged period without plant manipulation, while maintaining plant resources readily available (Cruz-Cruz et al. 2013). In our study, the effect of cultivation temperature and culture medium supplements on ulluco survival and plant re-growth after 18-month conservation was investigated.

Effect of cultivation temperature

Cold during storage has a significant impact on a tissue condition and it is known to increase the survival time of in vitro cultures (Peng et al. 2015; Kaminska et al. 2018a). In our study, a significant reduction of plant growth at 5 °C was confirmed. However, overall, every growth inhibitor treatment group displayed superior results at 17 °C compared to 5 °C, despite ulluco's mountainous origin and its adaptation to lower temperatures. Many studies on slow-growth conservation show that optimal temperature is strongly species dependent and very variable; for example, shoot cultures of *Prunus canescens* Vilm. et Bois × *P. cerasus* L. were

Table 2 List of ISSR primers, annealing temperatures, numbers and sizes of amplified fragments

No.	Primer code UCB	Sequence (5′–3′)[a]	Annealing temperature (°C)	Total no. of bands amplified	No. of loci	No of polymorphic loci	Range of amplifications (pb)
1	UCB807	$(AG)_8T$	52	105	5	0	300–1150
2	UCB809	$(AG)_8G$	52	168	8	0	200–950
3	UCB810	$(GA)_8T$	50.8	105	5	0	500–1000
4	UCB812	$(GA)_8A$	51.5	147	7	0	200–900
5	UCB813	$(CT)_8T$	50.8	105	5	0	350–1150
6	UCB826	$(AC)_8C$	49.1	147	7	0	450–1200
7	UCB828	$(TG)_8A$	47.1	105	5	0	950– 1500
8	UCB834	$(AG)_8YT$	49.1	147	7	0	500–1500
9	UCB836	$(AG)_8YA$	47.1	189	9	0	300–1600
10	UCB856	$(AC)_8YA$	49.1	147	7	0	450–1200
11	UCB859	$(TG)_8RC$	46.4	168	8	0	300–1500
12	UCB866	$(CTC)_6$	46	126	6	0	800–1200
Total				1659	79	0	

[a]Single letter abbreviation for mixed-base positions: Y = (C, T), R = (A, G)

successfully maintained for 16 months at 4 °C (Ozudogru et al. 2017), *Tetrastigma hemsleyanum* Diels et Gilg ex Diels microplants survived for 10 months at 10 °C (Peng et al. 2015), while shoot cultures of *Vanilla planifolia* Jacks. were conserved at 24 °C for 6 months (Bello-Bello et al. 2015). This comparison shows that it is highly advisable to experimentally optimize conservation protocols using a range of various temperatures.

Effect of culture medium supplements on ulluco conservation

Among medium supplements, the osmotically active compound mannitol at concentration 30 g l^{-1}, in combination with 17 °C conservation temperature, led to a high plant survival percentage with a major rate of plants surviving in the form of shoots without microtubers, indicating the potential of this treatment for use in an even longer conservation period than 18 months. Development of microtubers on shoots was associated with senescence as it was observed that this phase is followed by early drying of the plant and, therefore, survival in the form of a shoot without microtuber was considered superior. Mannitol addition to the medium led to reduced plant growth, confirming that mannitol, perceived by cells, acts as a chemical signal, suppressing growth (Steinitz 1999) while being metabolically inert (Yaseen et al. 2013). Our results corroborate those reported by Hammond et al. (2017) in the primary study where 4-month application of 30 g l^{-1} mannitol in culture medium suppressed the growth of ulluco microshoots. Similarly, Ozudogru et al. (2017) reported in cherry rootstock 'Gisela 5' that mannitol inclusion in the storage medium was effective at reducing the metabolic activity of the shoot cultures during the storage period. However, in *Cannabis sativa* L., mannitol considerably decreased the survival percentage as well as the plant re-growth ability of nodal cultures (Lata et al. 2012), proving not to be a universal medium supplement for the conservation of all species.

In our study, comparable to mannitol, sorbitol at 17 °C provided promising results for ulluco conservation and subsequent plant regrowth, though mannitol provided both slightly better plant survival and higher rate of plantlets surviving without microtuber formation. Comparison between mannitol and sorbitol for conservation was also carried out in potato (Gopal and Chauhan 2010), resulting in 58% plant survival after 18 months with 40 g l^{-1} sorbitol compared to various mannitol treatments where only 30–48% plantlets survived. In *Asparagus racemosus* Willd. (Thakur et al. 2015) shoot culture, 100% plantlets survived after a 6-month conservation period using 20 g l^{-1} sorbitol or mannitol as osmotics, both in combination with a reduced concentration of sucrose (15 g l^{-1}).

The effect on ulluco conservation of various sucrose concentrations in culture media was also investigated in our study, resulting in a high survival rate after the conservation at 17 °C using only 30 or 60 g l^{-1}; however, all plantlets within these treatments developed microtubers, indicating the end of vegetation period was approaching. In general, hydrolysis of sucrose in the culture medium produces high concentrations of reducing sugars (glucose and fructose), speeding up cell division and consequently leading to an increase of the volume of cultured tissues (Gurel and Gulsen 1998). This indicates that increasing sucrose concentration to some point might be beneficial for plant growth. However,

hydrolysis of sucrose may cause lowering of the pH in the medium, limiting uptake of nutrients by the plantlets (Rahman et al. 2010; Leifert et al. 1992). Deceleration of plant growth in our study could therefore be associated with sucrose addition at higher than optimal concentration for plant growth. This could explain less intensive growth after the addition of 90 g l^{-1} sucrose and an extreme growth inhibiting effect of 120 g l^{-1} sucrose, resulting in the death of the microshoots. Our results correspond to those of Teixeira da Silva (2004), where the effect of various levels of sucrose on morphogenic response in *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. was investigated and it was found that a sucrose concentration higher than 60 g l^{-1} was inhibitory to shoot development.

MH as a culture medium supplement efficiently slowed down ulluco plant growth and provided the highest total survival among all treatments at 17 °C. These results confirm that MH acts as a growth regulator, inhibiting cell division by mitotic disruption (Venezian et al. 2017), and as an anti-auxin and anti-gibberellin (Hoffman and Parups 1964). Probably due to its inhibiting activity (Hu et al. 2016), the regrowth ability of plantlets after ulluco conservation on MH-medium was relatively low. By contrast, in *Tetrastigma hemsleyanum* microplants (Peng et al. 2015), 0.2 mg l^{-1} MH was successfully used for 10-month conservation, and microplants preserved on MH recovered normally. In the mentioned study, growth inhibitors ABA and CCC were tested for *T. hemsleyanum* conservation as well, providing lower survival percentages compared to MH treatments, and browning of plantlets in the case of ABA and leaf etiolation after CCC-conservation (Peng et al. 2015). Our study corroborates these results, as CCC and ABA supplementation led to low survival percentages and weak plant growth with low number of meristems, especially for ABA treatments.

Effect of ulluco slow-growth conservation on genetic fidelity

Tuberous crops are usually maintained in gene banks as in vitro microplants under disease-free tissue culture conditions (Rajasekharan and Sahijram 2015). Vegetative propagation ensures their genetic integrity; however, some changes may occur as a result of somaclonal variation among in vitro plantlets (Al-Qurainy et al. 2018). Thus, in our study, plants after conservation were subjected to ISSR analysis, which detected no genetic variation among them.

Genetic stability assessment using molecular markers has recently been considered as a crucial approach to evaluate true-to-typeness of meristem-derived microplants after their conservation; for example, genetic fidelity of *Tetrastigma hemsleyanum* plantlets after 10 month conservation was confirmed using ISSR and sequence-related amplified polymorphism (SRAP) markers (Peng et al. 2015), in *Curcuma longa* L. periodical ISSR and random amplified polymorphic DNA (RAPD) monitoring of plantlets conserved for 7 years with 90 day sub-cultures, revealed no variation (Nayak et al. 2011), *Taraxacum pieninicum* Pawl. microshoots recovered from artificial seeds containing shoot tips, and conserved for 12 months also displayed no genetic variation as confirmed by RAPD (Kaminska et al. 2018b), while in *Pistacia lentiscus* L. plantlets, after 6 month conservation at 4 °C, a genetic instability with a mean of 0.722 similarity using inter retrotransposon amplified polymorphism (IRAP) marker system and 0.741 using amplified fragment length polymorphism (AFLP) was detected (Koc et al. 2014).

Conclusion

This study shows that of the tested growth inhibitors and osmotic agents added to half-strength MS medium, for *Ullucus tuberosus* in vitro conservation in form of microshoots, the most suitable is mannitol at 30 g l^{-1} concentration. When shoot cultures are maintained on this medium at 17 °C and 16/8 light/dark period they can be stored for 18 months. With regard to minimal microtuber development on shoots, microplants under these cultivation conditions have the potential to be stored for even longer periods. This protocol represents a reliable conservation system, suitable for gene banks maintaining ulluco plantlets under slow-growth conditions, with minimal risk of somaclonal variation occurrence.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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