

Droplet-vitrification methods for apical bud cryopreservation of yacon [*Smallanthus sonchifolius* (Poepp. and Endl.) H. Rob.]

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Abstract

This study aimed to develop a cryopreservation protocol for the long-term preservation of yacon [*Smallanthus sonchifolius* (Poepp. and Endl.)], an Andean crop with high fructooligosaccharide content in its tuberous roots. Initially, the cryopreservation protocol was developed using a yacon clone originated from Ecuador classified as ECU 41. Osmotic dehydration of apical buds (2–3 mm long) was carried out by assessing two plant vitrification solutions, PVS2 (15, 30, and 60 min) at 0°C and PVS3 (30, 45, 60, and 75 min) at 22°C. After cryopreservation, the apical buds were thawed and placed on MS medium \pm 0.1 mg l⁻¹ N⁶-benzyladenine (BA). The survival rates ranged from 37 to 90% within all treatments, with those subjected to PVS2 and PVS3 for 60 min showing the highest survival rates on MS medium without BA (87 and 90%, respectively). At 12 weeks post cryopreservation, these treatments also provided the highest regrowth rates, both reaching 73% of normally growing (shooting, rooting) plantlets. Survival rates on MS + 0.1 mg l⁻¹ BA regrowth medium reached up to 90%; however, regrowth into normally rooted plantlets did not exceed 67% post cryopreservation. The optimized protocols were then applied to 4 additional yacon clones originated from Bolivia and Peru, classified as BOL 22, BOL 23, PER 12, and PER 14. This resulted in survival and regeneration rates ranging between 79.7–94.1% and 66.3–75.4% respectively. Our study shows that optimal cryopreservation protocols for the long-term conservation of yacon can be based on both PVS2 and PVS3 vitrification solutions.

Key Message

An efficient PVS2 and PVS3 based cryopreservation protocol for yacon was developed, ensuring shoot tip survival of up to 94.1% and subsequent regrowth up to 75.4% after cryopreservation.

Introduction

Smallanthus sonchifolius [(Poepp. and Endl.) H. Robinson], commonly known as yacon or jicama, is a perennial crop of the Asteraceae family, originating in the Andean region (Malice & Baudoin 2009; Ojansivu et al. 2011). The plant is mainly cultivated for its succulent edible tuberous roots, rich in inulin-type fructooligosaccharides (FOS) (Pedreschi et al. 2003) and leaves, containing bioactive compounds, antimicrobial, antioxidant, and probiotic substances (Hudsara et al. 2014). Nowadays, yacon is widely known throughout the world for its multipurpose use and health benefits; hence, there has been a rapid increase in the interest in this crop worldwide, leading to the intensification of its cultivation (Lorenzoni et al. 2017; Ojansivu et al. 2011).

Yacon is a vegetatively propagated species (Ovesna et al. 2018) which produces a limited number of flowers and impoverished seed sets, causing conventional breeding and application of conservation methods, such as seed collections, difficult to perform (Seminario et al. 2003; Mansilla et al. 2010; Malice et al. 2010; Manrique et al. 2014).

Yacon and many other vegetatively propagated Andean root and tuber crops are constantly under threat of genetic erosion due to changes in food habits, the spread of intensive agricultural practices (Malice et al. 2010), and anthropogenic factors such as the selection of varieties for food purposes (Tapia and Estrella 2001; Malice and Baudoin 2009). Moreover, genetic variability within yacon species is considered to be relatively low (Grau and Slanis 1996; Svobodova et al. 2013; Ziarovska et al. 2019) which, in combination with the threats of genetic erosion, indicates the rising need to introduce new genotypes and conserve the already existing germplasm of yacon (Lorenzoni et al. 2017).

The need to develop an efficient, cost-effective, and reliable conservation method to reinforce the *ex situ* germplasm collections of yacon is therefore of immense importance (Izquierdo and Roca 1997). In the case of yacon, the germplasm is mainly maintained in the centre of origin, either on-farm by small farmers (Seminario et al. 2003) or *ex situ* under *in vitro* slow-growth conditions using basal MS medium (Murashige & Skoog 1962) or half-strength MS medium supplemented by mannitol or sorbitol and lower cultivation temperatures and light intensity (Panta et al. 1999; Skalova et al. 2013). The labour-intensive and medium-term nature of these methods create a rising need to develop long-term conservation techniques using more modern plant biotechnology tools, such as *in vitro* conservation by cryopreservation (Izquierdo and Roca 1997).

Cryopreservation presents a reliable and secure alternative for the long-term backup of field collections (Day et al. 2008; Reed 2008; Engelmann and Dussert 2013; Niino et al. 2015). This technique has been applied to various tuberous crops species, such as *Ipomoea batatas* L. (Park et al. 2015; Wilms et al. 2020), *Solanum tuberosum* L. (Panta et al. 2014; Wang et al. 2014; Vollmer et al. 2016), *Ullucus tuberosus* Cal., and *Oxalis tuberosa* Mol. (Sanchez et al. 2011), among others. Cryopreservation technique entails the cooling of plant tissues to an ultra-low temperature using liquid nitrogen (LN) intending to arrest metabolic activity while maintaining the vitality of the tissue (Panis et al. 2001; Walters et al. 2009; Kaviani 2011; Hammond et al. 2019). However, the high water content in plant tissue can result in undesirable events, such as intracellular ice nucleation and subsequent ice crystallization causing cell damage during the cooling and thawing processes; hence, there is a need to reduce the water content in the plant tissue before cryopreservation in LN (Dumet and Benson 2000; Muldrew et al. 2004; Uemura et al. 2009; Quain et al. 2012; Hammond et al. 2019). The use of plant vitrification solutions (PVSs) is considered as one of the best ways to circumvent the injuries associated with ice formation because it greatly reduces water content in plant tissues prior to LN exposure, thus avoiding damage to the membranes. The operation of PVSs can differ due to the presence of substances penetrating or not penetrating the cell membranes (Benson 2004; Panis and Lambardi 2005; Engelmann and Dussert 2013).

Within the present study, we aimed at assessing two PVSs based on PVS2 (Sakai et al. 1990) or PVS3 (Nishizawa et al. 1993) substances to determine their effect on the survival and regrowth of yacon apical buds after exposure to LN. We also aimed to determine the optimal medium for post cryopreservation regrowth by using full-strength MS medium with and without 0.1 mg l⁻¹ N⁶-benzyladenine (BA) and applying the optimized cryopreservation protocol on five yacon clones in total. Assessing the effects of

these procedures will contribute towards the general knowledge on cryopreservation of vegetatively propagated species and will lay the baseline for the long-term conservation of yacon germplasm.

Materials And Methods

Plant material, establishment of *in vitro* culture, and plant propagation

The yacon accessions used for the experiments included one allooctoploid ($2n=8x=58$) clone from Ecuador classified as ECU 41 (white tuber flesh), two allooctoploids from Bolivia classified as BOL 22 (yellow flesh) and BOL 23 (white flesh with violet pigmentation), and two dodecaploids ($2n=12x=87$) from Peru classified as PER 12 (white flesh) and PER 14 (yellow flesh). The cryopreservation protocol was optimized using the accession ECU 41 and the optimized protocol was then applied to the other 4 clones. To establish the *in vitro* culture, apical shoots (top shoot of field plants comprising of the meristematic dome plus 2-4 primordial leaves) were collected from yacon plants maintained in the field collection of the Botanical Garden at the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague. The plant material was collected within the summer season at the peak of the vegetative growth of field plants (June-August). From the shoots, apical buds (comprising the meristematic dome plus 2 primordial leaves) were then excised under a binocular microscope. Thereafter they were surface-disinfected using 70% (v/v) ethanol for 1 min, and 2% (v/v) NaClO for 15 min. The sterile apical buds were then rinsed 3 times in sterile distilled water and transferred to MS medium, containing 1 mg l⁻¹ thiamine 30 g l⁻¹ sucrose, 100 mg l⁻¹ myo-inositol, and pH adjusted to 5.7. The medium was sterilized in an autoclave at 121 °C in 100 kPa for 20 min before being used.

The cultures were thereafter placed in a culture room and maintained under a 16/8 h light/dark regime at 25/23 °C. A photosynthetic photon flux density of 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was provided by cool-white fluorescent tubes. The *in vitro* plantlets were sub-cultured every 28 days using segments with axillary or apical meristems on the MS medium until sufficient plant material was obtained to carry out the experiment.

Microshoot preparation and pre-culture

Apical buds (ca. 0.5 cm) were excised from 2-3-week old *in vitro* plantlets. The leaves were removed, and the stem was cut off until an apical bud (2-3 mm long) comprising the meristematic dome plus 2 primordial leaves remained. The plant material was prepared under a binocular microscope. A total of 180 apical buds were excised which were then placed on pieces of sterile filter paper on solid MS medium containing 0.3 M sucrose, and placed in a culture room in dark conditions overnight as a pre-culture before exposure to the cryoprotective treatments. Ten apical buds were used as main control (pre-cultured apical buds on MS+0.3 M sucrose with no exposure to cryoprotectants or LN).

PVS2 and PVS3 treatments and cryopreservation

Pre-cultured apical buds were placed in 5 ml loading solution (LS) composed of MS medium supplemented with 2 M glycerol and 0.4 M sucrose and filter-sterilized before being used. These were

incubated for 20 min at room temperature (22 °C). Ten apical buds were used as LS control (apical buds only exposed to LS) and 20 were used for each PVS2 and PVS3 treatment time exposure. The apical buds belonging to each treatment (including the LS controls) were loaded in separated containers. The PVS2 solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO), dissolved in MS salts with 0.4 M (w/v) sucrose, pH adjusted to 5.8 (Sakai et al. 1990). The PVS3 solution consisted of 50% (w/v) glycerol and 50% (w/v) sucrose (Nishizawa et al. 1993).

To place the apical buds in the PVS2 solution, the LS was removed with the use of a Pasteur pipette, and replaced with 5 ml ice-cooled (0 °C) filter-sterilized PVS2 solution. The apical buds were maintained for either 15, 30, or 60 min on ice to determine the optimal PVS2 treatment time for yacon cryopreservation. At the end of each PVS2 treatment, 10 buds were randomly chosen and transferred to a PVS2 drop on a sterile aluminium foil strip (0.5 wide × 2 cm long). All manipulations of the strips were done in a Petri dish placed on ice to maintain a temperature of around 0 °C, following the method according to Panis et al. (2005).

Once on the ice, the aluminium foil strips containing the bud were then cryopreserved by rapidly being plunged in LN using forceps and were placed in cryotubes filled with LN for 1 hour. The remaining 10 apical buds were used as control. These were directly placed in recovery media (5 buds on MS medium and 5 buds on MS medium with 1 mg l⁻¹ BA) and were maintained in a cultivation room with minimal light exposure for 2 weeks and were then placed in 24 h light at 25 ± 1 °C cultivation conditions to use as control.

In the case of the PVS3 treatment, all steps of the experiments were carried out as in the case of the PVS2 treatments with slight differences; i) the experiments were carried out at room temperature (22 °C), ii) the apical buds were maintained in PVS3 for up to 75 min (30, 45, 60, or 75 min).

Both PVS2 and PVS3 experiments were carried out in 3 independent repetitions.

Explant thawing and unloading

In both PVSs (PVS2 and PVS3), 2 recovery media were used for apical buds after cryopreservation; MS medium without the addition of PGRs (plant growth regulators) and MS supplemented with 1 mg l⁻¹ BA, both containing 30 g l⁻¹ (w/v) sucrose, 100 mg l⁻¹ (w/v) myo-inositol, 1 mg l⁻¹ (w/v) thiamine, and pH adjusted to 5.7. Before being used, the medium was autoclaved at 121 °C in 100 kPa for 20 min.

The unloading solution for the thawing of PVS2 treated apical buds consisted of a filter-sterilized solution containing 1.2 M sucrose dissolved in MS medium (pH 5.8). The frozen apical buds on aluminium foil strips were taken out of LN and were rinsed in 10 ml unloading solution at room temperature for 15 min. The thawed meristems were then placed on a filter paper on top of a solid hormone-free MS medium containing 0.3 M sucrose and were placed in dark conditions for 1 day at room temperature (22 °C). Thereafter, they were transferred directly into the above-mentioned recovery media (5 buds in MS and 5 in MS + 0.1 mg l⁻¹ BA) and were again placed in dark conditions for an additional 6 days, after which they

were placed in a cultivation room and maintain in 24 h light at 25 ± 1 °C cultivation conditions. Regrowth of apical buds was determined up to 12 weeks following cryopreservation.

The PVS3 unloading solution consisted of a sterile liquid solution of water containing 0.3 M sucrose. A rapid rewarming (at 40 °C for 30 s) of the cryopreserved samples was carried out by quickly plunging the aluminium foil strips with frozen apical buds in the PVS3 unloading solution. The thawed apical buds were then placed directly in the above-mentioned recovery media (5 buds in MS and 5 in MS+ 0.1 mg l⁻¹ (w/v) BA). These were then placed in a cultivation room in dark conditions for 1 week, after which they were transferred to 24 h light at 25 ± 1 °C cultivation conditions.

For both PVS2 and PVS3 treatments, post-thaw survival was determined within the first 3 weeks and the regrowth of apical buds was determined every week up to 12 weeks following cryopreservation.

Survival and regrowth evaluation after cryopreservation

In both PVS2 and PVS3 treatments, apical bud survival was defined as apical buds remaining green up to 3 weeks after thawing, while white apical buds were considered as dead with no potential for regrowth.

Regrowth was defined as further development of apices into shoots up to 12 weeks after rewarming containing a minimum of 3 pairs of leaves, 2-3 well-developed roots (2-3 cm in length). Survival and regrowth rates were expressed relative to the total number of apical buds per treatment. During the regrowth phase, callus formation, hyperhydricity (HH), shoot + callus formation (live shoot with callus base), colour and fully grown normal (FGN) plantlets (fully regenerated plantlets with 2-3 pairs of leaves and 2-3 well-developed roots), full growth + callus (fully grown plant with callus base) were evaluated to determine the effect of PVS2 and PVS3 solutions or recovery media on the quality of apical bud regrowth post cryopreservation.

Applicability of the optimised cryopreservation protocol

The developed cryopreservation protocol using either PVS2 and PVS3 vitrification solutions (the best treatments of both) using the yacon clone ECU 41 was then applied to apical buds of the 4 other yacon clones (see chapter - plant material, establishment of *in vitro* culture, and plant propagation) to assess the efficiency of the optimized cryopreservation protocols. To carry out these experiments, a total of 160 apical buds (0.5 cm) were excised from 2-3-week old *in vitro* plantlets of each accession (40 belonged to each clone, of which 20 were for the PVS2 treatment and 20 for PVS3). After cryopreservation, the apical buds were placed on the recovery media optimized for clone ECU 41. All experiments were carried out in three independent repetitions.

Data collection and statistical analysis

The data collected throughout the experiment were transformed by arcsine square root transformation prior to analysis of variance (ANOVA). The least significant ($P < 0.05$) differences between mean values were estimated using Tukey's HSD test [STATISTICA 12.0, StatSoft, Inc. USA].

Results

Apical bud survival after cryopreservation using PVS2 and PVS3

The results from the LS control treatments (apical buds only exposed to LS for 20 min) showed that LS treatment did not have a significant influence on the survival of the apical buds before exposure to the PVS2 and PVS3 cryoprotectants and LN. In comparison to the control apical buds (no exposure to LS, PVS treatments, and LN) that showed 100% survival there was only a 1.7% decline in survival after 20 min LS (Fig. 1a, b).

Overall, apical bud survival rate after cryopreservation ranged from 37 to 90% for all treatments (Fig. 1c). After cryopreservation, the survival of apical bud for both PVS2 and PVS3 treatments in many cases showed no significant differences to those of control treatments with no exposure to LN (Fig. 1c).

In the case of PVS2, the longest treatment time duration (60 min) at 0 °C provided the highest apical bud survival percentage (83%) post cryopreservation (Fig. 1a, c). Shorter PVS2 treatment durations (15 min and 30 min) at 0 °C led to more reduced apical buds survival with only a 53% and 70% post-exposure to LN, respectively (Fig. 1a, c), and did not provide results as promising as PVS2 60 min post cryopreservation (Fig. 1a, c).

In the case of PVS3 treatments, 60 min treatment duration also proved to be most effective in comparison to other PVS3 treatments, providing the highest apical bud survival of 90% after cryopreservation. Shorter exposure of apical buds (30 min and 45 min) as well as longer (75 min) provided lower survival rates when compared to control treatments and PVS3 60 min, reaching a maximum of 77% (Fig. 1b, c).

When comparing the differences among treatments between the PVS2 and PVS3 treatments, there was no statistical difference in survival between PVS2 at 30 min and 60 min treatment durations and PVS3 at 45 min and 60 min treatment duration (Fig. 1c).

Effect of PVS2 and PVS3 on apical bud regrowth

Control treatments not exposed to LS, PVS2 or PVS3, and LN did not exhibit any damage (Fig. 2a). However, apical buds exposed to both PVS2 and PVS3 treatments and LN appeared to have surface damage, exhibiting a dark/black colour (Fig. 2b). This, however, did not affect further shoot development and regrowth.

After exposure to PVS2 or PVS3 treatments and cryopreservation, most surviving apical buds regenerated into FGN within 12 weeks. During the first two weeks on both types of recovery media, the cryopreserved apical buds started to regrow. During further cultivation (week 4±2 days) they formed new leaves (Fig. 2c), and by week 8 (±2 days) apical bud had formed into *in vitro* plantlets with 2-3 pairs of well-developed leaves (Fig. 2d). These plantlets had 0, 1, or only 2 underdeveloped roots. By week 12 (±2 days) the regenerated plantlets were well-rooted, had at least 3 pairs of leaves, and 2-3 well-developed roots (Fig.

2e). Only plantlets with these characteristics and no morphological abnormalities were considered as fully regenerated into FGN. Some surviving apical buds showed signs of HH: these were excluded from the group of FGN.

Most apical buds regenerated in the form of FGN post cryopreservation in both PVS2 and PVS3 treatments and on both recovery media (MS medium and on MS supplemented with 0.1 mg l⁻¹ BA) (Fig. 3). This type of regrowth was mostly observed on MS media within 12th week of cultivation period post cryopreservation (Fig. 3c). PVS2 and PVS3 applications for 60 min proved to be most effective in comparison to other PVSs treatments. Regrowth of plantlets in these treatments on MS medium reached 73% FGN respectively (Fig. 3c).

Shorter PVS2 treatment durations (15 min and 30 min) were not as effective on MS media as the longer treatment (60 min), reaching a maximum FGN regrowth of only 27 and 60%, respectively, after cryopreservation (Fig. 3c). The shorter PVS3 treatment time duration (30 min and 45 min), as well as the longer treatment (75 min), also provided lower regrowth percentages in comparison to 60 min PVS3 treatment reaching a maximum FGN regrowth of 67%, post cryopreservation on MS regrowth media (Fig. 3c). PVS3 60 min treatment also provided the highest regrowth rate in the form of FGN (67%) on MS medium supplemented by 0.1 mg l⁻¹ BA (Fig. 3b, d). The other PVS3 and the PVS2 treatments did not exceed 53% FGN regrowth on this media post cryopreservation (Fig. 3b).

Shoot hyperhydration (HH) represented the most frequent form of morphological abnormalities observed within the regenerating apical buds on both regrowth media in control treatments and those post cryopreservation (Fig. 3a, b, c, d). The hyperhydrated plantlets were characterized by having translucent leaves and stem, undeveloped trichomes, as well as leaf veins (Fig. 4a, b). The highest rate of HH was observed in plantlets on MS medium supplemented by 0.1 mg l⁻¹ BA (Fig. 3b, d). The phenomenon of HH on this medium was presented in regenerated plantlets from both PVS2 and PVS3 treatments before and after cryopreservation, in control apical buds, and apical buds regenerated post-exposure to the vitrification solutions and LN (Fig. 3b, d). The level of HH on medium supplemented with BA ranged from 5 to 48% in treatments before and after cryopreservation (Fig. 3b, d). On this medium, HH started appearing within the first week of regrowth. By the end of the regrowth period (8-12 weeks), the plantlets could not fully recover. Hyperhydration of plantlets on MS medium was also detected; however, it only ranged from 0 to 30% and was only observed in some treatments before and after exposure to LN (Fig. 3a, c).

Callus formation was also observed within the apical bud regrow phase, as well as fully grown plantlets with callus developed on the shoot base (FGN+callus) after cryopreservation. These forms of regrowth were recorded only in shoots cultivated on MS medium supplemented by 0.1 mg l⁻¹ BA.

Applicability assessment of the optimal PVS2 and PVS3 treatments on various yacon clones

All control treatments (no exposure to LS, PVS treatments, and LN) showed 100% survival. The LS treatment also did not have a significant influence on the survival of the apical buds of the other 4 yacon

clones (BOL 22, BOL 23, PER 12, and PER 14) as was the case with the ECU 41 clone. Overall, the apical bud survival rate of the 4 yacon clones after cryopreservation ranged from 79.7 to 94.1% (Fig. 5a, b). In the case of PVS2 at 0 °C, the highest apical bud survival rate (90.2%) post cryopreservation was shown in the BOL 23 clone, followed by the PER 14 clone with 90%, PER 12 with 89.1%, and BOL 22 with 87.6% survival. When these results are compared to the ECU 41 clone (86.7%), the survival rates are not statistically significant (Fig. 5a). In the case of PVS3 60 min treatment duration, the BOL 22 clone provided the highest survival rate (94.1%) in comparison to all other clones (including ECU 41), followed by BOL 23 which had a survival rate of 88.9%, and PER 14 at 87.5%. The PER 12 accession provided the lowest survival rate in this treatment (79.7%) (Fig. 5b). When comparing the results among treatments between the PVS2 and PVS3 treatments, there was no significant difference in survival between the clones (Fig. 5a, b). The regeneration of surviving apical bud after exposure to the PVS2 or PVS3 treatment and cryopreservation was mainly in the form of FGN (Fig. 5a, b). This was determined within 12 weeks post thawing. All apical buds from each clone started germinating within the first two weeks on the MS recovery media, as was the case with the ECU 41 clone (± 2 days). Slight signs of morphological abnormalities, mainly in the form of HH, were detected in the regenerated apical buds reaching a maximum of 18.2% (Fig. 5c, d); however, the main form of regeneration in all clones was FGN, ranging from 75.4-62.3% (Fig. 5c, d).

Discussion

Effect of PVS2 and PVS3 on the apical bud survival of yacon

When developing a vitrification-based cryopreservation method for plant tissues, the composition of vitrification solution, duration and temperature of exposure to the solution, and cooling and rewarming rates are five key elements that influence the success of the cryopreservation protocol (Kim et al. 2006; Yin et al. 2014; Liu et al. 2017). Similarly, pre-culture and post thawing conditions of the plant tissue are considered crucial for successful long-term conservation (Niino et al. 2003; Harding et al. 2009). Within our study on yacon, we focused mainly on assessing the effects of PVS2 and PVS3 treatment duration and emphasized the post thawing conditions by assessing two different regrowth media before and after cryopreservation.

Our study has shown that both PVS2 and PVS3 vitrification solutions are effective for cryopreservation of yacon. Overall, PVS2 60 min and PVS3 60 min treatments showed the highest apical bud survival percentages in the optimization phase. These findings show that even though PVS2 and PVS3 treatments are different in their temperature of implementation (PVS2 at 0 °C and PVS3 at 22 °C) and composition (different types and concentrations of cryoprotectants), they seemed to have a similar cryoprotectant activity in yacon, reaching similar rates of dehydration. The similarities in the desiccation rates between both treatments at similar time duration may be due to the presence of DMSO in PVS2. This solvent is widely considered as the main penetrating colligative cryoprotectant which causes higher desiccation rates (Kim et al. 2009). PVS3 is composed of less penetrating substances (Kim et al. 2009), hence the desiccation rate of this treatment is slower. Therefore, the apical bud exposure of PVS2

treatments is implemented at 0 °C. This reduces or controls the dehydration rates of PVS2 during its application. Therefore, the presence of the penetrating substance DMSO in the PVS2 solution may be a possible justification as to why at 0 °C PVS2 60 min treatment duration reaches similar dehydration as those of PVS3 60 min implemented at 22 °C in yacon apical buds. The presence of DMSO not only accelerates the desiccation rates but may also lead to cytotoxicity due to its penetrating properties. The possible toxic effects of the solution may compromise the viability of the plant cell; hence, the treatment exposure time and application temperature are fundamental parameters which must always be optimized when using PVS2 treatment (Martinez et al. 1998; Perez et al. 1999; Kim et al. 2009; Bekheet et al. 2020).

In addition to DMSO, PVS2 also includes ethylene glycol. Both are more membrane permeable than the components in PVS3 (glycerol and sucrose), as reported by Volk et al. (2017) who assessed the osmotic responses of sweet potato using PVS2 and PVS3 vitrification solutions. After treatment with PVS2 and PVS3, the results of their study showed that, contrary to our findings in yacon, the optimal dehydration time for PVS3 and PVS2 is different with PVS3 treatments and requiring up to 3 hours. PVS2 and PVS3 are among the most often used PVSs and are applied to a wide range of plant species. However, the effectiveness of the protocol is very variable and strongly species-dependent and should, therefore, be optimized accordingly (Sakai and Engelmann 2007; Reed 2008). For example, cryopreservation protocols using the PVS2 method have been developed for 1,028 *Solanum* accessions at 50 min treatment time duration with a recovery rate ranging from 34 to 70% (Vollmer et al. 2016); for *Ribes aureum* cv. Red Lake at 20 min PVS2 treatment with a 30% recovery rate post cryopreservation (Reed et al. 2001); for *Lycopersicon esculentum* Mill. at 30 min PVS2 treatment duration and a recovery rate ranging from 60 and 70% (Coste et al. 2015); for *Vitis* sp. at 20 min treatment duration and recovery rates ranging from 43 to 59% (Bi et al. 2018); and for *Ullucus tuberosus* Cal. and *Oxalis tuberosa* Mol. at 60 min PVS2 treatment time duration and a recovery rate reaching 35 and 15%, respectively, after cryopreservation (Sanchez et al. 2011). The PVS3 method, on the other hand, has been optimized for species such as *Allium cepa* var. *Aggregatum* at 3 hours treatment duration, where this method resulted in 58% recovery rate (Wang et al. 2020), and *Lotus tenuis* INTA Pampa with at PVS3 treatment time duration of 1 hour and recovery rate post cryopreservation, reaching 79% (Espasandin et al. 2019). In the case of yacon, we found that both solutions proved to be effective at the same treatment time duration (60 min) with up to a 73% recovery rate post cryopreservation.

As in the present study, a comparison of PVS2 and PVS3 vitrification solution was executed by Wang et al. (2020), who tested both treatments for apical bud cryopreservation of shallot (*Allium cepa* var. *Aggregatum*). The findings also showed that in the case of PVS2, 40-60 min at 0 °C provided the highest rate of apical bud survival (40%) among the treatments. This corroborates our findings in yacon, where PVS2 60 min also provided the highest apical bud survival rates; however, in the case of yacon, the rates were higher with an 83% survival (PVS2 60 min treatment). In the case of PVS3, Wang et al. (2020) reported that this vitrification solution was most effective in the survival of shallot after a 3-hour treatment duration at room temperature, with a 72% survival rate. In contrast, our findings showed that the PVS2 60 min treatment in yacon was just as effective as the PVS3 applied for 60 min with a 90% survival rate, showing that both solutions can successfully be used for the cryopreservation of this

species. Similar findings were also reported by Da Silva Cordeiro et al. (2015) for *Cleome rosea* Vahl, where both PVS2 and PVS3 methods proved to be effective for this species post cryopreservation, reaching high survival rates of 97 and 70%, respectively.

As 60 min treatment time duration applied for PVS2 at 0 °C and PVS3 at 22 °C proved to be the overall best treatments in the optimization phase using the ECU 41 clone, the treatments were then applied to the other yacon clones (BOL 22, BOL23, PER 12, and PER14). Both treatments provided high survival rates, ranging from 86.7%-90.2% in the case of PVS2 and 79.7%-94.1% in the case of PVS3, with no significant differences in survival rates within clones and the PVS treatments, showing that both methods are applicable as cryoprotectants for yacon cryopreservation. Similarly, Senula et al. (2018) tested both PVS2 and PVS3 in various *Mentha* species and recommended both vitrification solutions as cryoprotectants for all the species under study. Nevertheless, in contrast to our results, better shoot regrowth was reported using PVS3. Ellis et al. (2006) conducted research on garlic (*Allium sativum* L.); their study showed that both PVS2 and PVS3 treatments can be applied to various accessions of garlic with both ensuring more than 40% survival and regrowth without any further modification of the treatment, as is the case with yacon. This shows that, even though PVS treatments are strongly variable and species-dependent, optimization and assessment is of paramount importance as it can determine whether or not the cryoprotectants are applicable to one, more, or all accessions of the targeted species.

Regrowth of yacon post cryopreservation

In our study, the most frequent type of regrowth post cryopreservation for all clones was in the form of FGN; however, morphological abnormalities such as HH were also detected in control treatments as well as after cryopreservation. Hyperhydration was mainly observed on MS medium supplemented by 0.1 mg l⁻¹ BA, regardless of exposure to LN and PVS treatments. This shows that the form of regrowth was mainly influenced by the composition of recovery media rather than the vitrification solutions. Therefore, only MS without 0.1 mg l⁻¹ BA was used for apical bud regrowth post cryopreservation when applying the optimal PVS2 and PVS3 treatment time duration to the other yacon clones (BOL 22, BOL23, PER 12 and PER14). In the optimization phase using the ECU 41 clone, we also found that the vitrification solution influenced the overall survival of yacon apical bud post cryopreservation because the variation in treatment duration caused different rates of dehydration. Hence the best PVS2 and PVS3 dehydration treatment (60 min for both) were applied to the other yacon clones. Da Silva Cordeiro et al. (2015) also investigated the effects of PVS2 and PVS3 as vitrification solutions for the cryopreservation of *Cleome rosea*, and MS medium supplemented various concentration of BA (0-0.5 mg l⁻¹) as recovery medium after cryopreservation. They showed that for both PVSs the highest recovery rate (100%) post cryopreservation was obtained on media supplemented either with 0.10 or 0.5 mg l⁻¹ BA, while MS media without BA proved to be inefficient for post cryopreservation recovery and only provided a maximum regrowth of 33%. These results are not in accordance with our study; we found that MS medium without the presence of BA proved to be optimal for the regrowth of yacon apical buds post cryopreservation providing a 73% FGN regrowth rate in both PVS2 and PVS3 60 min treatment. Da Silva Cordeiro et al. (2015) also found that *Cleome rosea* apical buds cultivated on MS media supplemented with BA formed

morphological abnormalities, such as callus at the base of the regenerated plantlets after cryopreservation, which corroborates with our study in yacon.

Comparable findings to the results obtained within our study were also reported in *Alnus glutinosa* (L.) Gaertn. A 50% post cryopreservation regrowth rate was obtained on MS regrowth medium supplemented with BA; however, the apical buds that did not regenerate into FGN plantlets produced non-proliferating calli (San Jose et al. 2014). Similar results were also reported by Lambardi et al. (2000) in poplar (*Populus alba* L.), where the addition of 1.5 mg l⁻¹ BA in MS regrowth medium led to callus formation and mainly HH of plantlets after cryopreservation. In the mentioned study, MS medium without supplements proved to be more effective for apical bud regrowth. Marco-Medina et al. (2010) also tested PVS2 at 0 °C for cryopreservation and MS with and without BA supplement as recovery media for *Thymus moroderi* Pau ex Martínez. In their study, it was found that PVS2 at 0 °C for 30 min in combination with one-week cultivation of apical buds on MS medium supplemented with 0.06 mg l⁻¹ BA led to the highest recovery rates after cryopreservation. However, prolonged cultivation of plantlets on recovery media and supplementation of media with BA concentrations above 0.2 mg l⁻¹ BA resulted in high rates of HH. On the other hand, they found that MS without the addition of BA was not as effective, showing that the use of low concentration of BA could boost the post cryopreservation recovery rates in the case of *T. moroderi*. This shows that the regrowth media influences the quality of the recovered plantlets post cryopreservation and the composition of culture medium must be optimized for each species.

Conclusion

The present study aimed at assessing the efficiency of PVS2 and PVS3 osmotic dehydration methods to develop a long-term preservation protocol for yacon, as well as to determine the optimal medium for post cryopreservation regrowth using full-strength MS media with or without 0.1 mg l⁻¹ BA. The results in the optimization phase using the ECU 41 clone proved the feasibility of both PVS2 and PVS3 plant vitrification techniques for the long-term storage of yacon. With 60 min treatment time duration at difference temperatures (at 0 °C PVS2 and 22 °C PVS3) in combination with MS medium without 0.1 mg l⁻¹ BA supplementation as recovery media being optimal for yacon cryopreservation providing the highest rate of apical bud regrowth post thawing with minimal morphological abnormalities. When applied to the other 4 yacon clones originated from Bolivia and Peru (BOL 22, BOL 23, PER 12, and PER 14), both methods were effective in ensuring high survival and recovery rates of these clones post cryopreservation. Plantlets regenerated from cryopreserved apical buds maintained their *in vitro* multiplication capacity and overall quality, demonstrating the efficiency of the assessed PVS cryopreservation protocols. Despite comparable results of these two PVS treatments, the use of PVS3 treatment (unlike PVS2) does not contain DMSO, which may lead to cytotoxicity and thus can be considered as most suitable. The high survival and regrowth rates of these 5 yacon clones after cryopreservation using both methods provide technical support for a promising alternative to safeguard the yacon accessions currently being preserved by traditional methods using cryo-banking.

Declarations

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Conflicts of interest/Competing interests The authors declare no conflicts of interest.

Availability of data and material Data not applicable

Code availability Data not applicable

Authors' contributions SDHH performed the cryopreservation experiments and wrote the draft of the manuscript, IV conceived the idea and reviewed the manuscript, JZ developed the experimental design and did the data processing, BP and MF contributed to the experimental design, interpretation of the data, and revised the paper. All authors read and approved the final manuscript.

Ethics approval Not applicable

Consent to participate All authors voluntarily agreed to participate in this research study.

Consent for publication All authors of this manuscript declare a consensus with publishing the manuscript in PCTOC.

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Figures

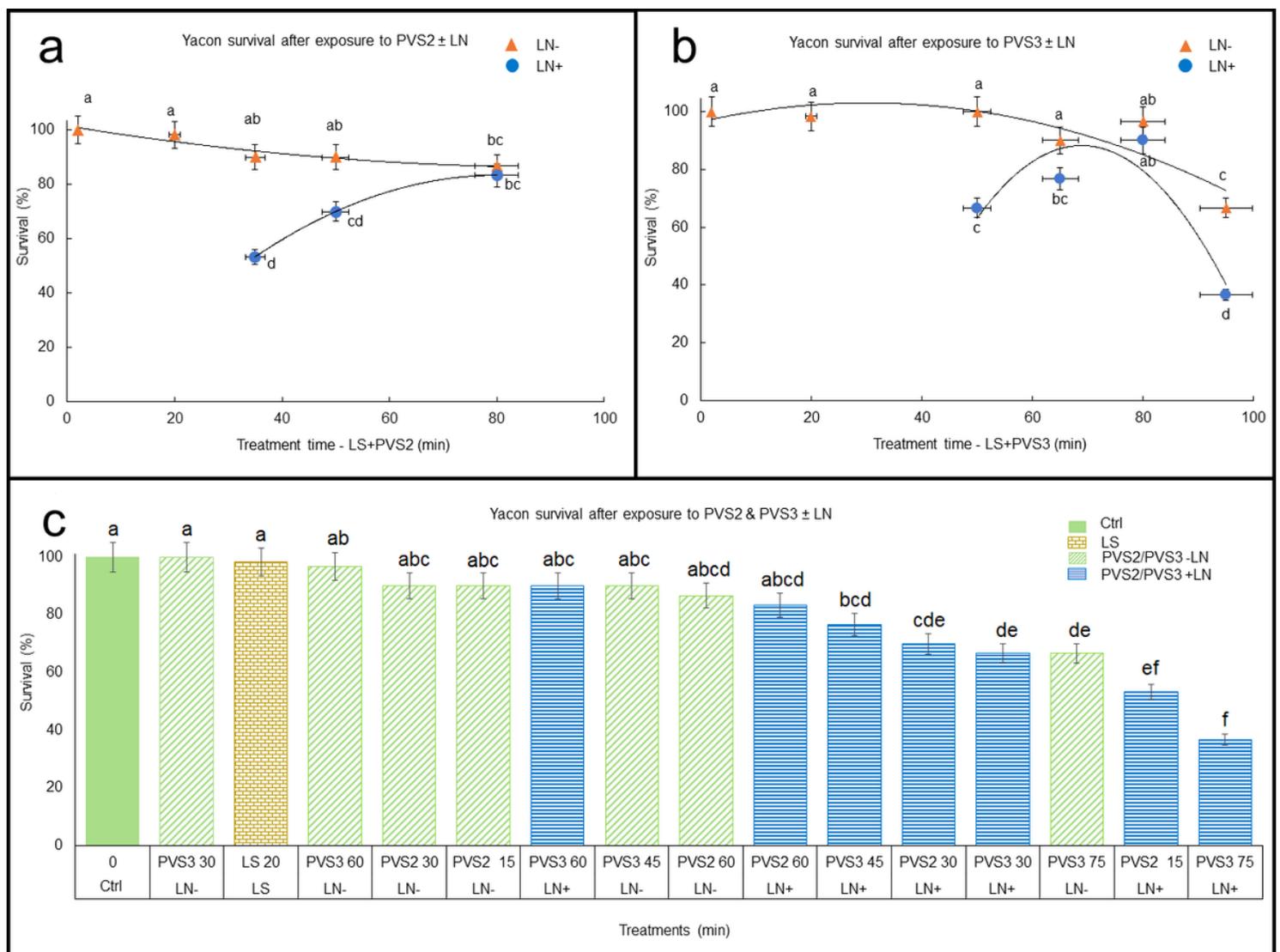


Figure 1

Survival of yacon apical buds post-exposure to LN and vitrification solutions. a. Influence of PVS2 on survival. b. Influence of PVS3 on survival. c. Comparison of both PVS2 and PVS3 treatments. Apical buds -LN as controls and +LN as cryopreserved. Differences in mean values of survival with different letters are statistically significant ($p < 0.05$) in each treatment. Ctrl) general control with no exposure to vitrification solutions and LN, LS) loading solution after 20 min, -LN) apical buds exposed to LS and either PVS2 or PVS3 vitrification solutions but not to LN, +LN) apical buds exposed to LS and either PVS2 or PVS3 vitrification solutions and LN. Values represent the mean of three repetitions.

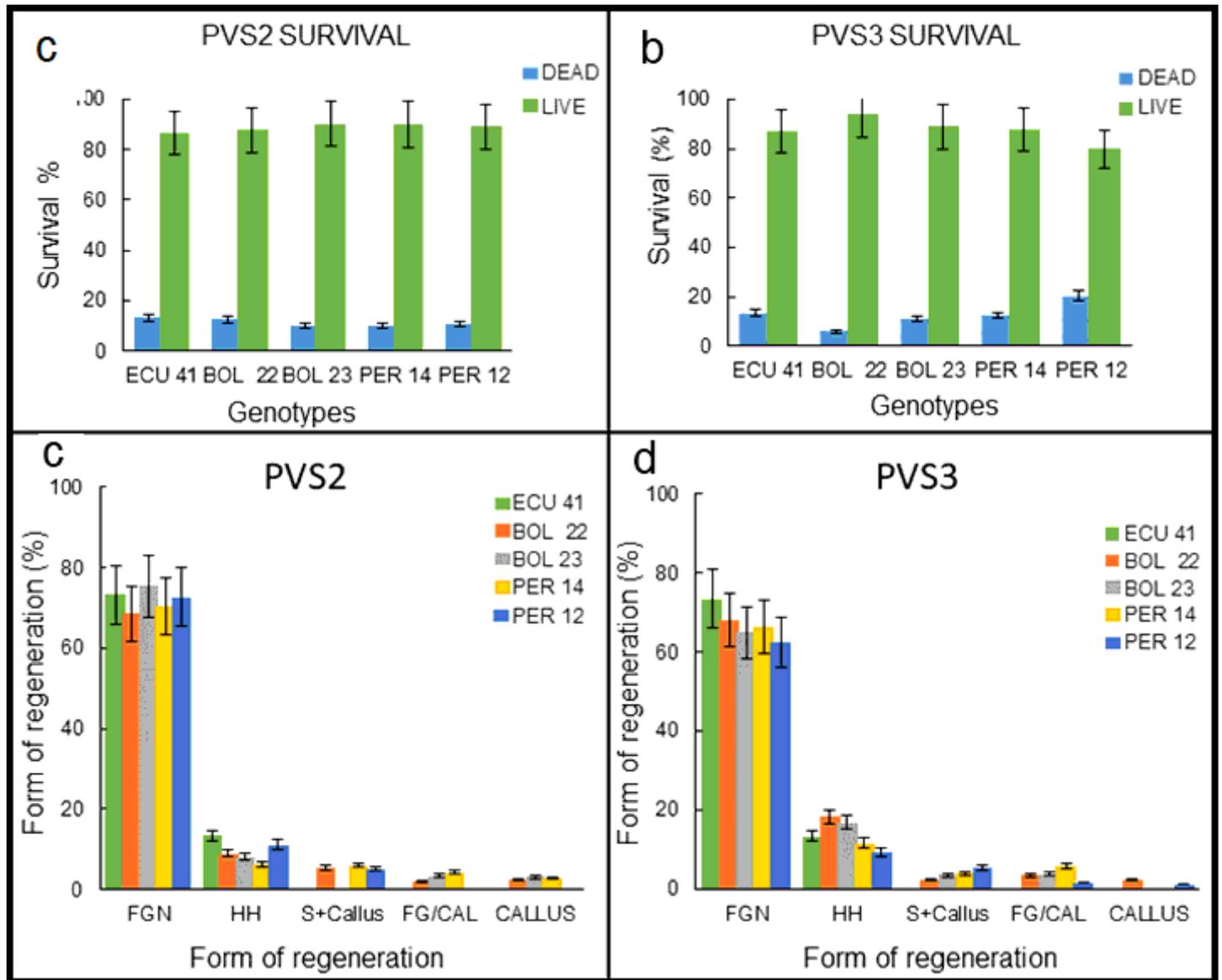


Figure 2

Yacon apical buds regrowth after cryopreservation a. Excised yacon apical buds with no exposure to LN. b. Excised yacon apical buds after exposure to LN. c. apical bud regrowth after 4 weeks of cultivation post cryopreservation on MS medium. d. Yacon plantlet 8 weeks after cultivation post cryopreservation. e. Yacon shoot tip regrowth 12 weeks post cryopreservation.

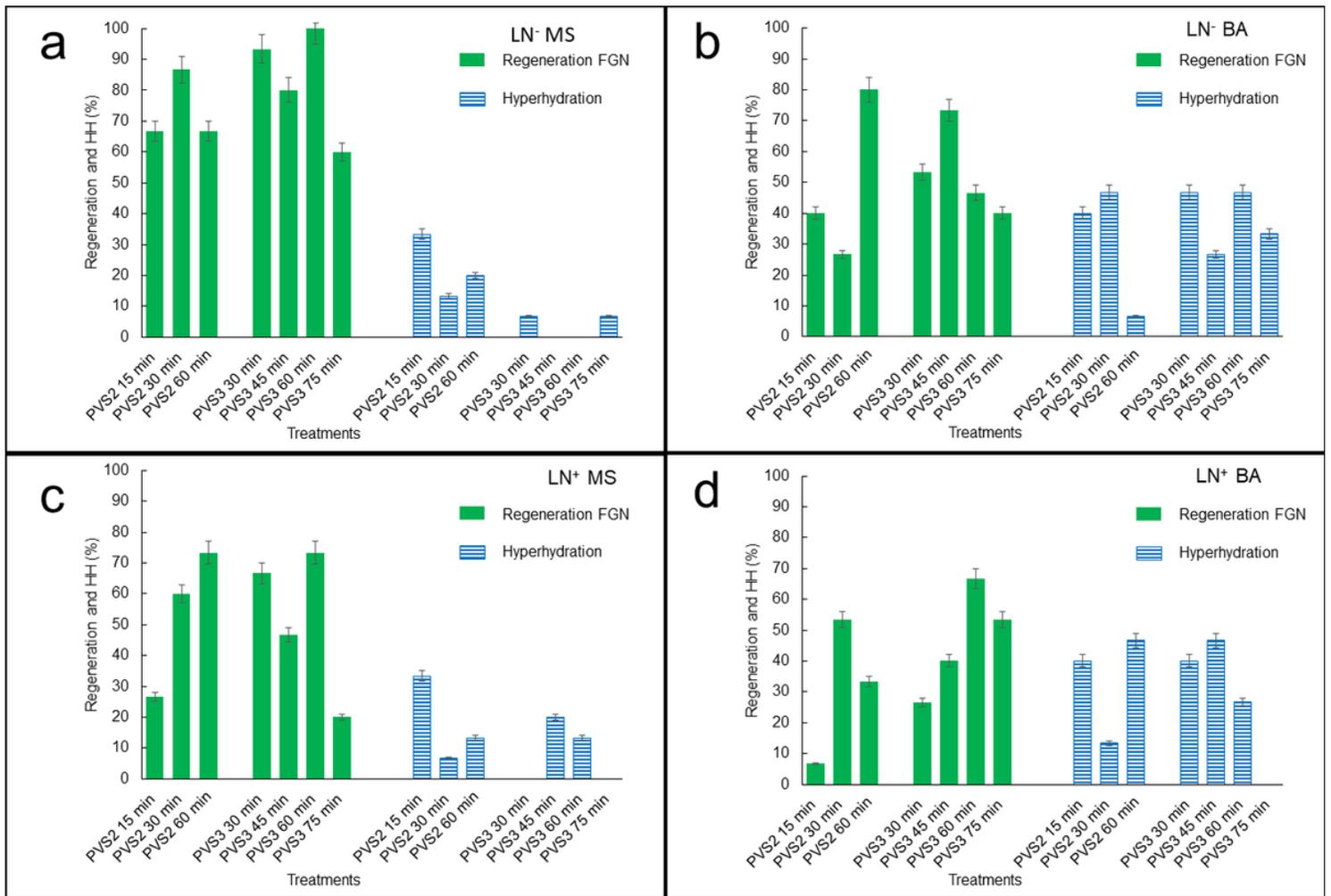


Figure 3

Effect of PVS2 and PVS3 treatments and recovery media on yacon apical bud regrowth and hyperhydration occurrence before and after cryopreservation. a. Control apical buds exposed to PVS2 and PVS3 and their recovery on MS medium with no exposure to LN. b. Control apical buds treated with PVS2 and PVS3 and their recovery on MS+0.1 mg l-1 BA medium with no exposure to LN. c. Apical buds treated with PVS2 and PVS3 on MS medium and exposed to LN. d. Apical buds treated with PVS2 and PVS3 on MS+0.1 mg l-1 BA medium and exposed to LN. All values are presented as mean \pm standard deviation (SD). LN+) Treatments exposed to LN, LN-) control treatments not exposed to LN, LN) liquid nitrogen.

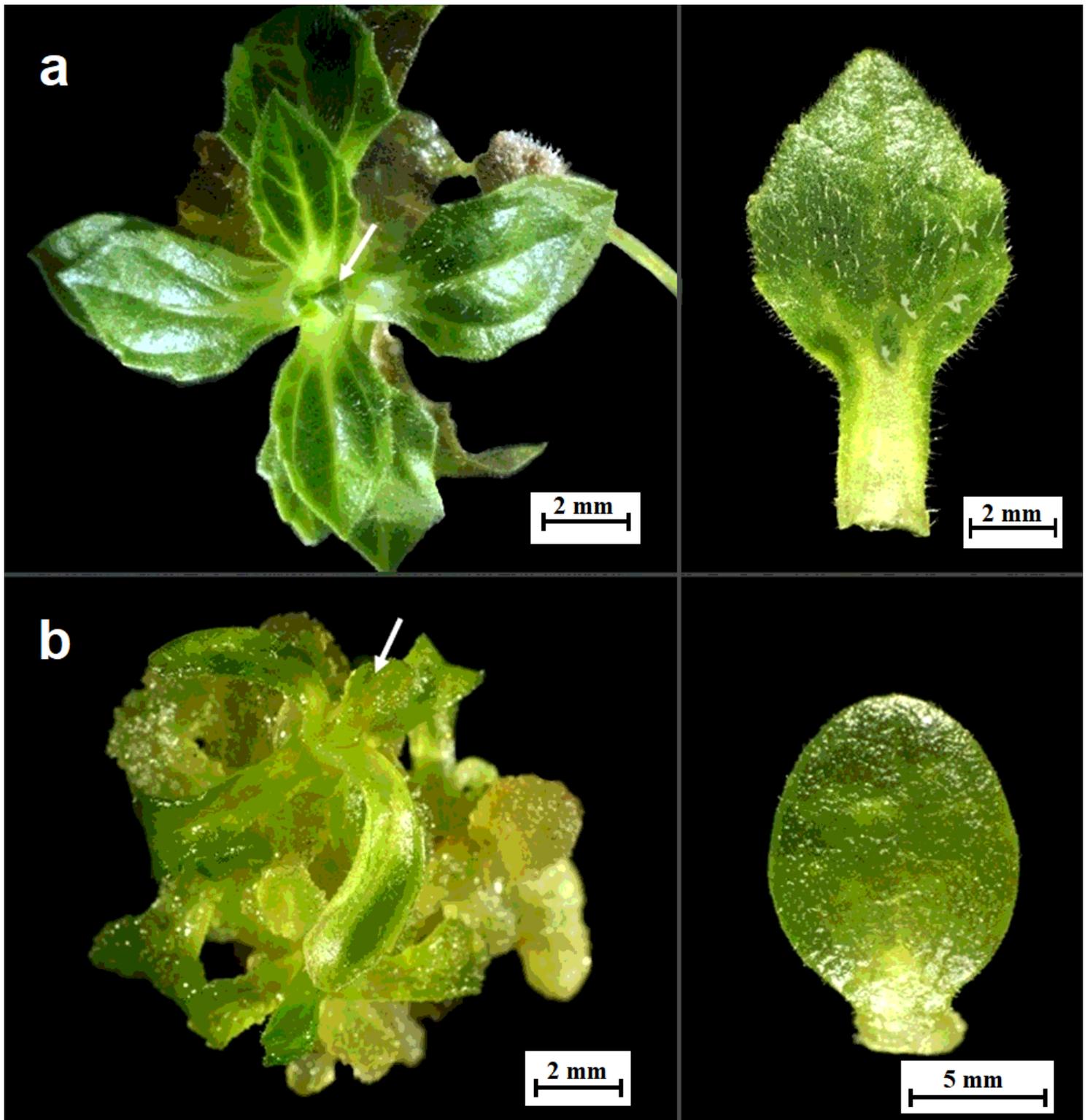


Figure 4

Comparison of HH occurrence in regenerated yacon plantlets on regrowth media post cryopreservation. a. Plantlets on MS medium with morphological abnormalities - magnified leaves. b. Yacon plantlets on regrowth MS medium containing 0.1 mg l⁻¹ BA and showing signs of HH (translucent leaves and stem, undeveloped trichomes, and leaf vein). Arrows indicate the position of the magnified leaves on the right.

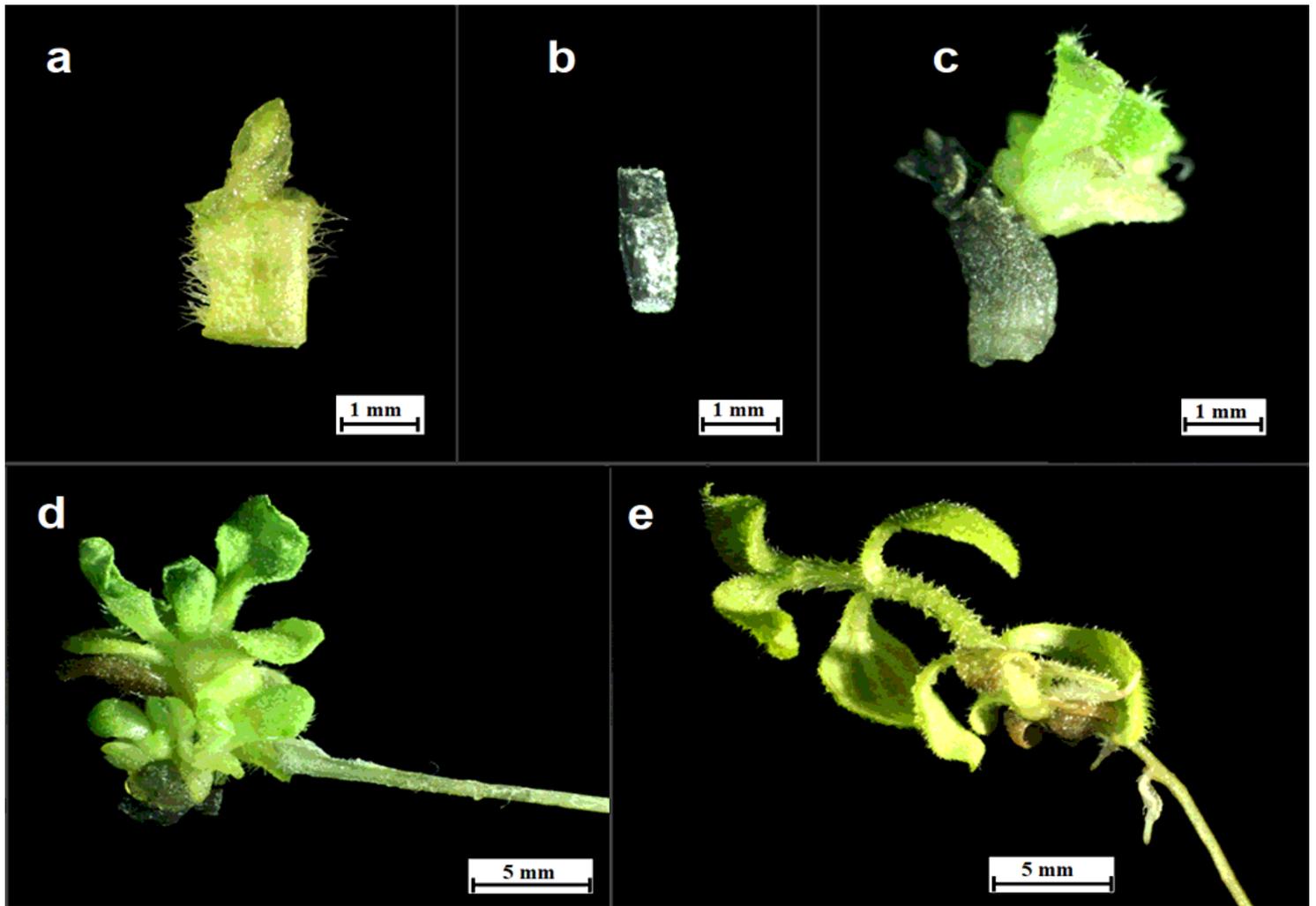


Figure 5

Survival and regeneration of apical buds excised from five yacon clones post cryopreservation using the optimal PVS2 and PVS3 treatment time duration and regrowth media. a. influence of PVS2 on survival. b. influence of PVS3 on survival. c. apical buds form of regeneration on recovery media after exposure to PVS2 and LN. d. apical buds form of regeneration on recovery media after exposure to PVS3 and LN. Ctrl) general control with no exposure to vitrification solutions and LN, LS) after 20 min loading solution. FGN) a fully regenerated plant with well-developed roots and leaves, HH) hyperhydration, S+Callus) live shoot with callus base, FG/CAL) fully grown plantlet with callus base and callus) regrowth of shoot only in the form of callus.