Plant Cell, Tissue and Organ Culture (PCTOC) (2018) 134:193–204
https://doi.org/10.1007/s11240-018-1412-y

ORIGINAL ARTICLE

# In vitro regeneration of adventitious buds from leaf explants and their subsequent cryopreservation in highbush blueberry

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Received: 18 January 2018 / Accepted: 22 March 2018 / Published online: 26 March 2018


## Abstract

We report a new cryopreservation method for highbush blueberry (*Vaccinium corymbosum*) using small leaf squares-bearing adventitious buds. Leaf explants were cultured on adventitious bud regeneration medium composed of Woody Plant Medium (WPM) supplemented with 20 µM zeatin. After 21 days of adventitious bud regeneration, small leaf squares (SLSs, 2 × 3 mm), each bearing multiple adventitious buds, were cut from the leaf explant, precultured on WPM containing 0.3 M sucrose for 24 h and were treated for 30 min with a loading solution composed of WPM containing 1.0 M sucrose and 2 M glycerol, followed by exposure to plant vitrification solution 2 at 0 °C for 40 min. Each of dehydrated SLS was then transferred onto an aluminum foil with small holes and PVS2 was dropped until it covered the SLS, prior to a direct immersion in liquid nitrogen. Cryopreserved SLSs were re-warmed in WPM containing 1.2 M sucrose for 20 min at room temperature, followed by post-thaw culture for recovery. With this procedure, more than 23 adventitious buds were produced in each leaf explant, and 100% of SLSs were able to survive and resume shoot regrowth, with more than six shoots per SLS obtained following cryopreservation in three highbush blueberry cultivars. In 'Misty', the morphology of plantlets regenerated from cryopreserved SLSs was identical to that of the in vitro-derived ones. No polymorphic bands were detected using inter-simple sequence repeat markers and random amplified polymorphic DNA in plantlets of 'Misty' recovered from cryopreservation. The use of SLSs-bearing adventitious buds for cryopreservation reported in the present study eliminates shoot tip excision. This cryopreservation method can be considered an efficient cryopreservation of plant shoot tips, and has potential applications to other plant species.

**Keywords** Blueberry · Long-term preservation · Droplet-vitrification · Genetic stability · Leaf explant · Recovery

## Abbreviations

| | |
|---|---|
| ABRM | Adventitious bud regeneration medium |
| AD | Apical dome |
| ISSR | Inter-simple sequence repeats |
| LN | Liquid nitrogen |
| LP | Leaf primordium |
| PVS2 | Plant vitrification solution 2 |
| RAPD | Random amplified polymorphic DNA |
| SLS | Small leaf squares |
| SMM | Shoot maintenance medium |
| WPM | Woody plant medium |
| ZT | Zeatin |

Communicated by Sergio J. Ochatt.

Hai-Yan Chen and Jing Liu contributed equally to the present study.

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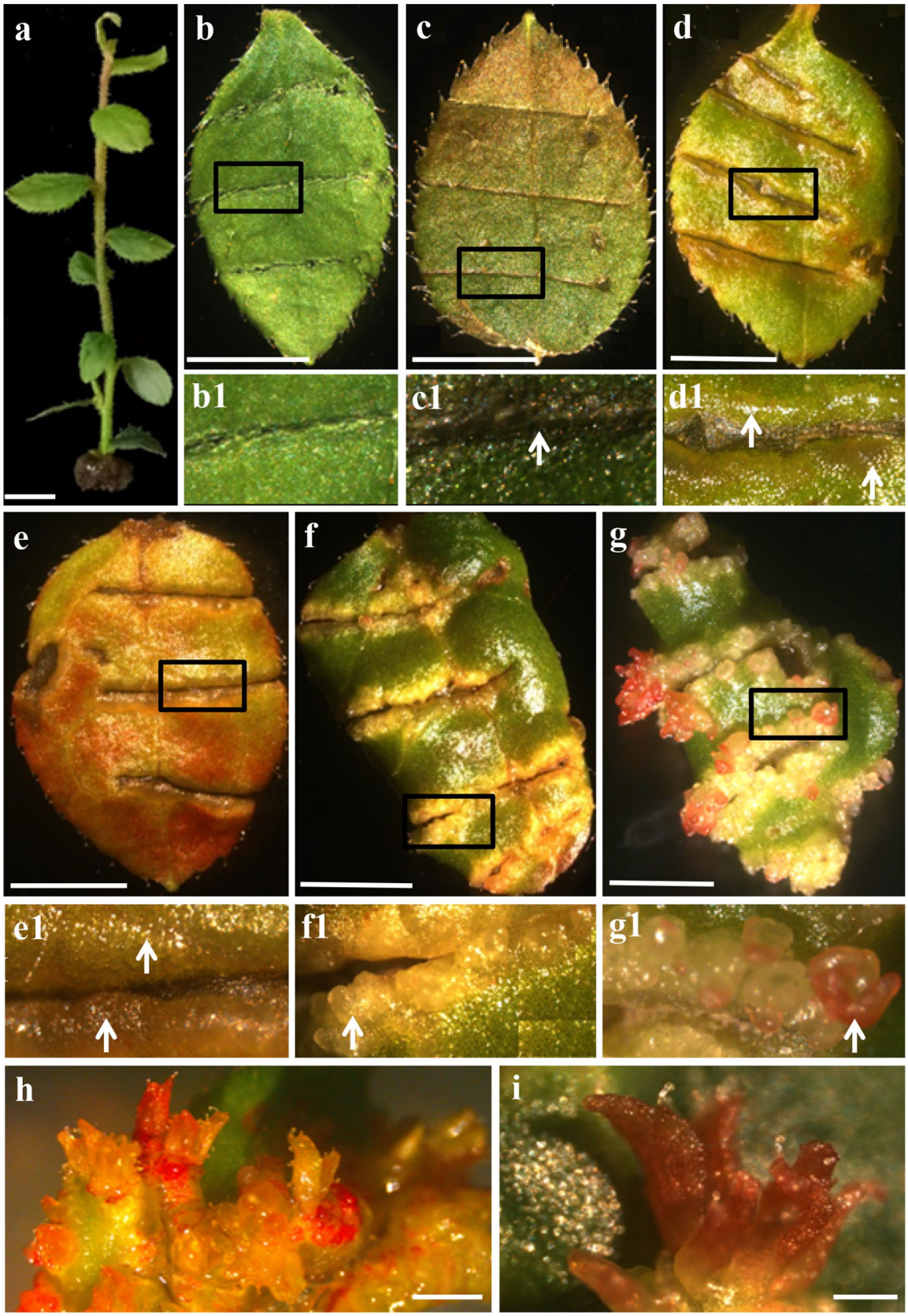
## Introduction

Highbush Blueberry (*Vaccinium corymbosum*), the most economically important cultivated blueberry among the *Vaccinium* species, is a perennial evergreen or deciduous shrub and native to North America (Strik and Yarborough 2005). Blueberries are a rich source of flavonoids, particularly anthocyanins, flavanols, and chlorogenic acid (Prior et al. 2001; Rodriguez-Mateos et al. 2012). These biochemical compounds have numerous health benefits such as increasing anticancer activity, reducing risk of heart disease, improving cognitive performance and preventing oxidative stress and inflammation (Sweeney et al. 2002; Kalt et al. 2007; Erlund et al. 2008; Williams et al. 2008;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◂**Fig. 1** Adventitious bud regeneration from leaf explants of highbush blueberry 'Misty'. Leaf explants were cultured on Woody Plant Medium supplemented with 20 µM zeatin and grown under the light condition. A 4-weeks old in vitro stock shoots from which leaf explants were excised and used for adventitious bud regeneration (**a**). A leaf explant used for adventitious bud regeneration at day 0 of culture (**b**). A close view in the square of **b** showing transverse cuts through the midrib (**b1**). A leaf explant at day 3 of culture (**c**). A close view in the square of **c** showing necrosis, as indicated by the arrow, along the cut (**c1**). A leaf explant at day 5 of culture (**d**). A close view in the square of **d** showing small protuberances, as indicated by the arrow, at both sides of the cuts (**d1**). A leaf explant at day 7 of culture (**e**). A close view in the square of **e** showing continuous lines of small protuberances, as indicated by the arrow, along the two sides of the cuts (**e1**). A leaf explant at day 15 of culture (**f**). A close view in the square of **f** showing young adventitious buds, as indicated by the arrow, along the two sides of the cuts (**f1**). A leaf explant at day 18 of culture (**g**). A close view in the square of **g** showing matured adventitious buds, with several leaf primordia, as indicated by the arrow, along the two sides of the cuts (**g1**). Elongated adventitious buds, as indicated by the arrows, regenerated in the leaf explant at day 21 of culture (**h, i**). Bars = 0.5 cm

Basu et al. 2010). Blueberries are also of characteristics of attractive colors, good taste and rich flavours, and therefore are widely grown in many parts of the world (Strik and Yarborough 2005; Prodorutti et al. 2007; Retamales and Hancock 2012).

The traditional methods using controlled hybridization and deliberate selection have so far been most frequently used in blueberry breeding (Prodorutti et al. 2007; Retamales and Hancock 2012) and genetic transformation is also becoming a promising means (Prodorutti et al. 2007; Song and Hancock 2012). Availability of and easy access to diverse genetic resources are necessary of breeding programs in both the traditional and biotechnological strategies for plant genetic improvements.

Cryopreservation, i.e. storage of biological samples such as cell, shoot tips or seeds at ultra-low temperatures, usually in that of liquid nitrogen (LN, − 196 °C), has been considered an ideal means for long term conservation of plant genetic resources (Li et al. 2018; Wang et al. 2018). To date, several shoot tip cryopreservation protocols have been reported for blueberry, including two-step cooling (Reed 1989; Uchendu and Reed 2009), encapsulation-dehydration (Kami et al. 2009, 2010; Uchendu and Reed 2009), vitrification (Kami et al. 2009; Uchendu and Reed 2009), encapsulation-vitrification (Kami et al. 2009) and D cryo-plate technique (Dhungana et al. 2017). Results obtained in these studies provided a basic technical platform for setting-up cryo-bank of blueberry germplasm. Further development of high efficient and wide spectrum cryopreservation protocols that are applicable to a wide range of blueberry genotypes would certainly assist speeding-up establishment of blueberry cryo-banks. Recently, Wang et al. (2017) reported a droplet-vitrification cryopreservation. In their method, a mean shoot regrowth level of 66.5% was obtained across the five valuable blueberry genotypes, which represent a wide range of blueberry cultivars in China (Wang et al. 2017).

In vitro techniques provided important tools for blueberry genetic improvement programs. There have been a number of studies reporting adventitious shoot regeneration in blueberry (Rowland and Ogden 1992; Cao and Hammerschlag 2000, 2002; Debnath 2007, 2009, 2011; Meiners et al. 2007; Liu et al. 2010; Zhao et al. 2011), and transgenic plants have also been achieved in blueberry (Song and Hancock 2012; Gao et al. 2016). Leaf segments are the most frequently used explant in these studies.

The objective of the present study attempted, therefore, first to develop a highly efficient regeneration procedure of adventitious buds from leaf explants, and then to cryopreserve by droplet-vitrification small leaf squares (SLSs)-bearing adventitious buds, thus eliminating excision of shoot tips. Results reported here would largely simplify the cryopreservation procedure and improve the efficiency of shoot tip production.

## Materials and methods

### Plant materials

Highbush blueberries 'Misty', 'Northland' and 'Blueglod' (*V. corymbosum*) were used in the present study. 'Misty' was used for optimizing the procedures of adventitious bud regeneration and cryopreservation, and 'Northland' and 'Blueglod' were subsequently used to test the procedures established. In vitro stock shoots were maintained on a shoot maintenance medium (SMM) composed of Woody Plant Medium (WPM, Lloyd and McCown 1980) supplemented with 2 µM zeatin (ZT), 30 g $L^{-1}$ sucrose and 7 g $L^{-1}$ agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. The stock cultures were maintained at 22 ± 2 °C under a 16-h photoperiod with a light intensity of 50 µmol $s^{-1}$ $m^{-2}$ provided by cool-white fluorescent tubes. Subculturing was performed every 4 weeks.

### Adventitious bud regeneration

Three-four fully-opened leaves next to the shoot terminal bud of 'Misty' were excised from 4-weeks old stock shoots (Fig. 1a), and three transverse cuts were made through the midrib (Fig. 1b, b1). These prepared leaf explants were cultured for adventitious bud regeneration, with their adaxial surface down, in 90-mm sterilized Petri dishes containing 20 ml of adventitious bud regeneration medium (ABRM) composed of WPM supplemented with 20 µM ZT, 30 g $L^{-1}$ sucrose and 7 g $L^{-1}$ agar (pH, 5.8). The cultures were placed in the light condition, as used for stock cultures. Adventitious bud was defined as a structure containing an apical

dome (AD) and at least two leaf primordia (LPs). Number of adventitious buds per leaf explant, and size and LP number of adventitious buds were recorded at different time durations of culture. Histological studies were conducted on adventitious bud initiation and development, as described below.

## Droplet-vitrification cryopreservation

SLSs (2 × 3 mm in size), each bearing about 4–6 visible adventitious buds (Fig. 2a), were excised from leaf explants that had been cultured on ABRM for 21 days, and used for droplet-vitrification cryopreservation of 'Misty', as described by Wang et al. (2017). The SLSs were precultured on WPM containing 0.3 M sucrose for 24 h in the light conditions, as used for the in vitro stock shoots. The precultured SLSs were treated for 30 min with a loading solution (LS) composed of WPM containing 1.0 M sucrose and 2 M glycerol, followed by exposure to plant vitrification solution 2 (PVS2) (Sakai et al. 1990) at 0 °C for 40 min. PVS2 is composed of liquid WPM supplemented (w/v) with 30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose (pH 5.8, Sakai et al. 1990). Each of dehydrated SLS was then transferred onto 30-mm sterilized aluminum foils, each carrying 10 SLSs, with small holes and PVS2 was dropped until it covered the SLSs (Fig. 2b), followed by a direct immersion in liquid nitrogen (LN) contained in thermo jugs (EDISH, Beijing, China), with helps of forceps (Fig. 2c). The foils with the SLSs were kept in LN for cryostorage for at least 10 min. Frozen foils with the SLSs were removed out from LN and immediately placed into an unloading solution composed of liquid WPM containing 1.2 M sucrose at room temperature for 20 min. Cryopreserved, unloaded SLSs were then post-thaw cultured in 90-mm sterilized Petri dishes containing 20 mL SMM. The cultures were placed at 23 ± 2 °C in the dark for 1 day and then transferred to the light conditions for recovery.

Two experiments were performed to optimize the parameters affecting survival and recovery of cryopreserved SLSs of 'Misty'. In the first experiment, leaf explants that had been cultured on ABRM for 21 days were cut into SLSs in 2 × 2 mm, 2 × 3 mm and 2 × 4 mm, respectively and used for droplet-vitrification cryopreservation, to select an optimal size of SLSs for cryopreservation. In

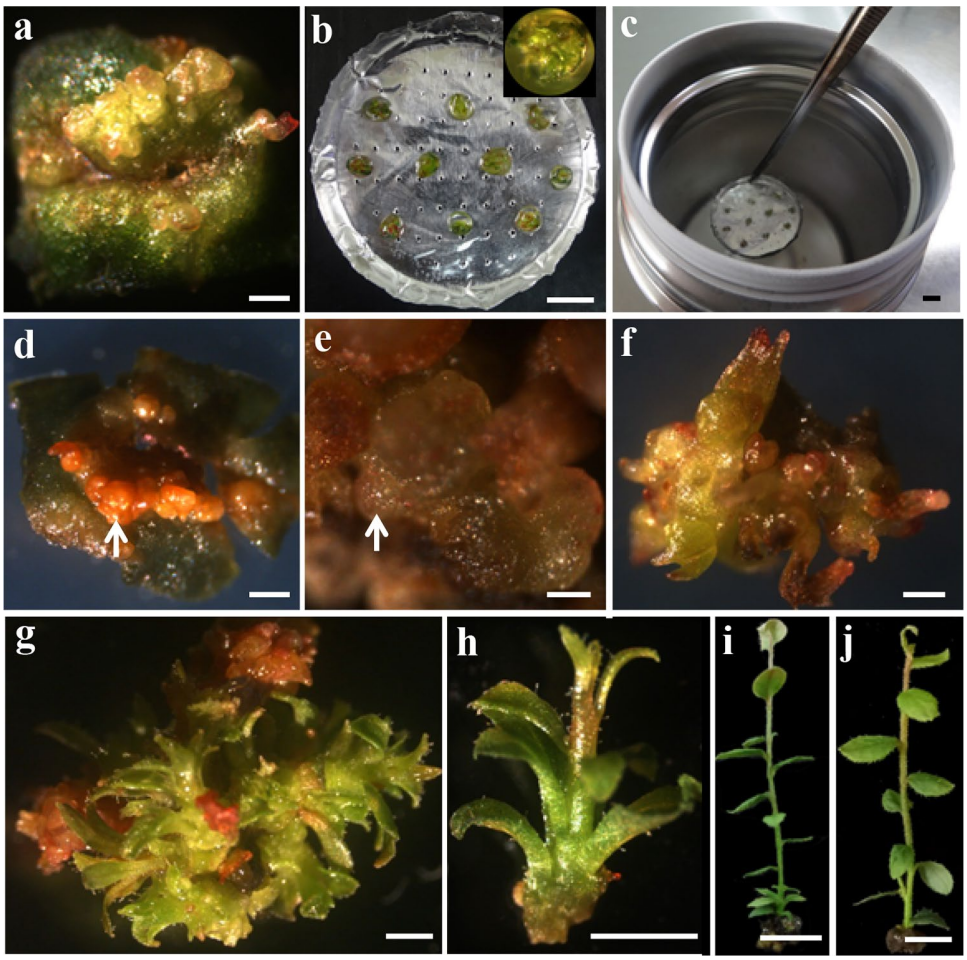


**Fig. 2** Droplet-vitrification cryopreservation of small leaf squares (SLSs)-bearing adventitious buds in highbush blueberry 'Misty'. A small leaf square used for cryopreservation (**a**). Droplets containing SLS-bearing adventitious buds covered with a few drops of PVS2 carried on aluminum foils (3 × 3 cm) with small holes (**b**). A close view of a droplet in **b** was placed at right upper part of **b**. Droplets immerged in liquid nitrogen contained in thermo jugs (**c**). Surviving (**d**) and dead (**e**) adventitious buds after 3 days of post-thaw culture following cryopreservation, as indicated by the arrow. Elongated adventitious buds after 10 days of post-thaw culture following cryopreservation (**f**). Multiple shoots regenerated from cryopreserved small leaf squares after 21 days of post-thaw culture (**g**). A close view of a regrown shoot in **g** (**h**). Plantlets regenerated from cryopreserved adventitious buds (**i**) and from in vitro-derived shoots (**j**). Bars in **a, d, e** and **f** = 1 mm; in **b**, c, **g** = 5 mm; in **h, I** and **J** = 1 cm

the second experiment, SLSs in 2 × 3 mm were excised from leaf explants that had been cultured in ABRM for 15, 18, 21, 24 days, respectively, and used for droplet-vitrification cryopreservation, to select an optimal age of adventitious buds for cryopreservation.

Survival was expressed as the percentage of SLSs containing any adventitious buds showing bright purple color of the total samples after 3 days of post-thaw culture. Shoot regrowth was defined as the percentage of SLSs containing any shoots (≥ 5 mm) of the total samples after 3 weeks of post-thaw culture. The number of shoots per SLS and length of shoots regenerated from cryopreservation was also recorded after 3 weeks of post-thaw culture.

After establishment of the procedures of adventitious bud regeneration and cryopreservation in 'Misty', 'Northland' and 'Blueglod' were used for testing these optimized procedures.

## Histological studies

Histological observations were conducted on adventitious bud initiation and development in leaf explants cultured on ABRM for different time durations in 'Misty', as described by Feng et al. (2013). In brief, samples were fixed with FAA [ethanol (50%): formalin: acetic acid = 18:1:1], dehydrated, and embedded in paraffin. Sections (5 μm) were cut with a microtome (Leica DM2000, Germany), mounted on glass slides, and stained with 0.01% toluidine blue (TB, Sakai 1973). The stained sections were observed using a light microscope (Leica DM 2235, Germany).

## Assessment of genetic stability

'Misty' was used for assessments of genetic stability by inter-simple sequence repeat markers (ISSR) and random amplified polymorphic DNA (RAPD). Shoots regenerated from leaf explants that had been cultured on ABRM for 21 days and plantlets recovered from cryopreserved shoot tips after 6 months of regeneration were used for assessment of genetic stability. In vitro stock shoots were used as control.

### DNA extraction

Genomic DNA was extracted from 150 mg fresh leaf tissue usinga Plant Genomic DNA Kit (Tiangen, Beijing, China), according to themanufacturer's instructions. Purified total DNA was quantified andits quality verified by ultraviolet spectrophotometry. Each sample was diluted to 50 ng $\mu L^{-1}$ in Tris-EDTA buffer and stored at − 20 °C until use.

### ISSR

Forty ISSR primers were screened to select suitable primers for assessment of genetic stability in the samples. Ten ISSR primer pairs selected were used for providing PCR products. Procedures of PCR and electrophoresis of PCR products were conducted as described in detail by Wang et al. (2017).

### RAPD

Thirty-six RAPD primers were screened to select suitable primers for assessment of genetic stability in the samples. Ten ISSR primer pairs selected were used for providing PCR products. Procedures of PCR and electrophoresis of PCR products were conducted as described in detail by Wang et al. (2017).

## Experimental design and data analysis

Thirty samples were included in experiments of adventitious bud regeneration and the whole experiment was repeated twice. Ten samples were collected from each of the two experiments and used for histological studies. In cryopreservation experiments, ten samples were included in every treatment of two replicates and the whole experiments were repeated three times. Data of percentages obtained in cryopreservation experiments were converted to ASIN values and then subject to statistical analysis. Data were analyzed using one way ANOVA. The significant differences were calculated at $P < 0.05$ by Turkey's test. Thirty samples from leaf-explant-derived adventitious buds and recovered from cryopreservation were randomly selected from a population of 150 regenerants, and 200 in vitro stock shoots and subjected to assessments of genetic stability.

# Results

## Adventitious bud regeneration

Leaf explants of 'Misty' turned slightly brown–purple color and tissues at the cut edges became necrosis at day 3 of culture (Fig. 1c, c1). Small protuberances were visible beside the cut edges at day 7 of culture (Fig. 1d, d1). These small protuberances formed two continuous lines along both sides of the cut edges at day 9 of culture (Fig. 1e, e1), and continued growth and developed into young adventitious buds at day 12 of culture (Fig. 1f, f1). These young adventitious buds further matured at day 15 of culture (Fig. 1g, g1) and elongated into shoots at day 21 of culture (Fig. 1h, i). About 28–35 adventitious buds were produced in each leaf explant at day 21–27 of culture (Fig. 3A). Number of adventitious buds per leaf explant (Fig. 3A), and size (Fig. 3B)

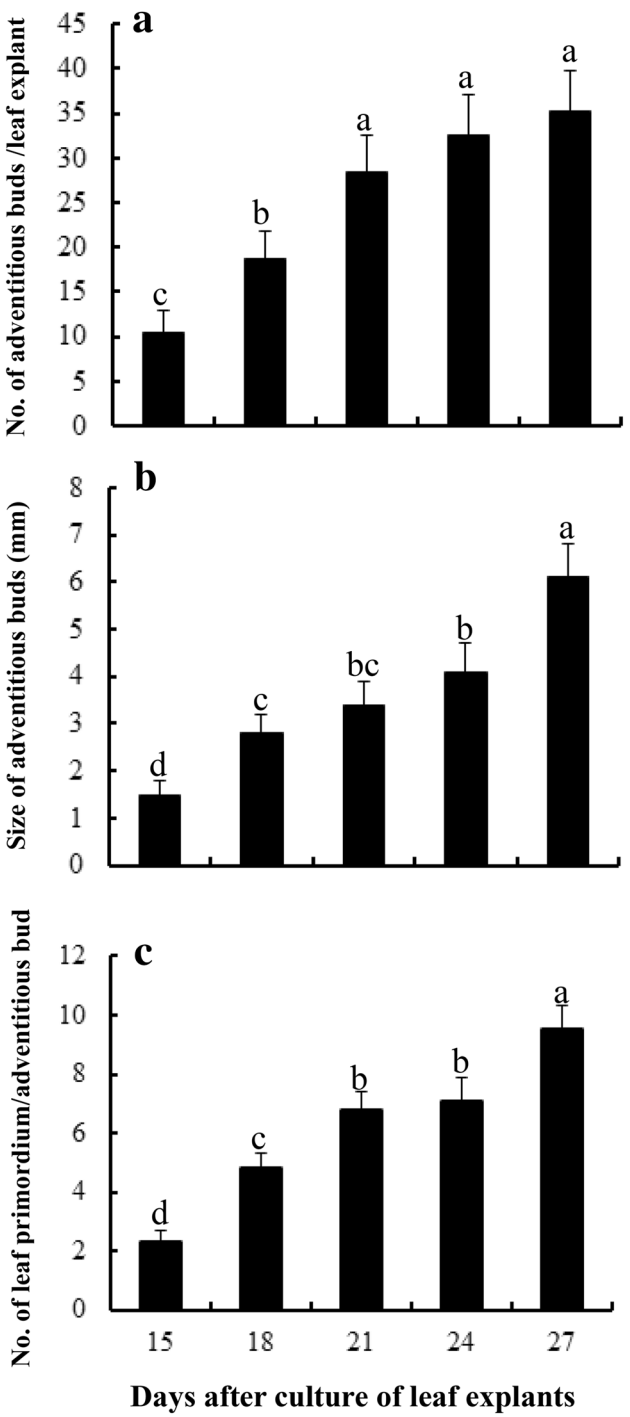


**Fig. 3** Number (**A**), size (**B**) of adventitious buds and number of leaf primordium (**C**) of adventitious buds in leaf explants cultured on adventitious bud regeneration medium for different time durations in highbush blueberry 'Misty'. Data are presented as means ± SE and with different letters indicate significant differences at $P < 0.05$ by Tukey's test

and LP number (Fig. 3C) of adventitious buds significantly ($P < 0.05$) increased as culture time durations increased from 15 to 27 days (Table 1).

## Histological observations

The pattern of adventitious bud formation in transverse sections of leaf explants of 'Misty' revealed changes in leaf structure from day 0 (Fig. 4a) to 5 (Fig. 4b), when subepidermal cells outside of the vascular bundle dedifferentiated and started the first cell division. These cells contained dense cytoplasm and conspicuous nucleus. These dividing cells formed two continuous lines at the surface along the both sides of the cut edge at day 9 of culture (Fig. 4c). Primary adventitious buds containing apical dome developed in the two continuous lines at day 12 of culture (Fig. 4d). These primary adventitious buds further developed into matured buds containing apical dome (AD) and the youngest leaf primordia (LPs) at day 15 of culture (Fig. 4e), and elongated into shoots at day 21 of culture (Fig. 4f).

## Recovery from cryopreservation

Surviving adventitious buds of 'Misty' showed bright purple color (Fig. 2d), while dead ones turned brown (Fig. 2e) after 3 days of post-thaw culture following cryopreservation. Surviving buds started to elongate (Fig. 2f) and developed into shoots (Fig. 2g, h) after 10 and 21 days, respectively, of post-thaw culture following cryopreservation. The morphology of plantlets recovered from cryopreservation was identical to that of the in vitro derived shoots (Fig. 2i, j).

Although size of SLSs did not affect survival and shoot regrowth levels, it significantly ($P < 0.05$) influenced the number of shoots per SLS in the treated control (− LN) and cryopreserved (+ LN) SLSs (Table 1). The number of shoots per SLS in the treated control (− LN) significantly ($P < 0.05$) increased from 5.4 to 10.8 when the size of SLS increased from 2 × 2 mm to 2 × 4 mm. Similar patterns were also obtained in the cryopreserved (+ LN) SLSs. Although the largest SLSs (2 × 4 mm) produced the greatest number of shoots (9.5) following cryopreservation (Table 1), they were difficult to adhere to aluminum foils than smaller ones (2 × 2 mm and 2 × 3 mm) and easily dropped from aluminum foils to LN, causing difficulties in handling samples. Due to this problem, SLSs of 2 × 3 mm were used in the following experiments. Age of adventitious buds had significant effects on cryopreservation. For the treated control (− LN), about 75 and 65% of SLSs survived and developed into shoots in 15 days old adventitious buds (Table 2). All SLSs survived and were able to regrow shoots in 18–27 days old adventitious buds (Table 2). However, the number of shoots per SLS significantly differed with the age of adventitious buds. The number of shoots per leaf segment was about 3.1–5.1 in 15–18 days old adventitious buds, which were much fewer ($P < 0.05$) than those (7.8–8.8) in 21–27 days old ones (Table 2). For cryopreserved (+ LN) SLSs, survival and shoot regrowth rates were 55 and 35% in 15 and 18 days

**Table 1** Effects of size of small leaf squares (SLSs)-bearing adventitious buds excised from leaf explants cultured on adventitious bud regeneration medium for 21 days in highbush blueberry 'Misty'

| Size of SLSs (mm × mm) | Survival (%) | | Recovery | | | |
|---|---|---|---|---|---|---|
| | | | Shoot regrowth (%) | | Number of shoots per SLS | |
| | −LN | +LN | −LN | +LN | −LN | +LN |
| 2×2 | 100a | 100a | 100a | 100a | 5.4 ± 0.4c | 4.8 ± 0.4c |
| 2×3 | 100a | 100a | 100a | 100a | 8.1 ± 0.5b | 7.8 ± 0.5b |
| 2×4 | 100a | 100a | 100a | 100a | 10.8 ± 0.6a | 9.5 ± 0.6a |

Survival and recovery was recorded after 3 and 21 days of post-thaw culture, respectively. Data are presented as means ± SE and with different letters indicate significant differences at $P < 0.05$ by Turkey's test

old adventitious buds and significantly ($P < 0.05$) increased to 100% in 21–24 days ones (Table 2). The number of shoots per SLS was about 2.1 in 15 days old adventitious buds and significantly ($P < 0.05$) increased to 4.7 in 18 days old ones (Table 2). The greatest number (7.5–7.9) of shoots per SLS was obtained in 21–24 days old ones. Age of adventitious buds was also found to significantly affect length of regrown shoots in the treated control (−LN) and cryopreserved (+LN) adventitious buds (Table 2). For the treated control (−LN), shoot length was about 1.5 cm in 15 days old adventitious buds, which was much lower than that (about 2.3 cm) in 18 days old ones. The longest shoot length (3.4–3.7 cm) was obtained in 21–24 days old ones. Similar patterns of shoot length were also found in cryopreserved (+LN) adventitious buds, with the longest shoot length (2.6–2.8 cm) produced in 21–24 days samples (Table 2).

### Adventitious bud regeneration and cryopreservation in 'Northland' and 'Blueglod'

With the procedures of adventitious bud regeneration and cryopreservation developed for 'Misty', 23.8 and 24.2 adventitious buds per leaf explant were produced in 'Northland' and 'Blueglod' (Table 3). All SLSs survived and resumed shoot regrowth in these two cultivars (Table 4). Each cryopreserved SLS produced 6.5 and 6.3 shoots (> 2.5 cm) in 'Northland' and 'Blueglod' (Table 3).

### Assessment of genetic stability

For ISSR, the ten primers tested produced strong, clear, reproducible bands and yielded 46 scored bands in each of samples in 'Misty' (Table 4; Fig. 5a, b), with 1380 bands in total produced across the 30 samples analyzed. No polymorphic bands were observed in the samples regenerated from leaf explants, cryopreserved shoot tips and in vitro stock shoots (Table 4). For RAPD, the ten primers produced strong, clear, reproducible bands and yielded 56 scored bands in each of the samples (Table 4; Fig. 5c, d), with 1680 bands in total produced across the 30 samples analyzed. No polymorphic bands were observed in the samples regenerated from leaf explants, cryopreserved shoot tips and in vitro stock shoots (Table 4).

## Discussion

In this study, leaf explants were cultured on ABRM containing 20 μM ZT to regenerate adventitious buds. About 28 adventitious buds per leaf explant were produced after 21 days of culture, which were similar to the results obtained by Rowland and Ogden (1992) and Meiners et al. (2007). These data confirm ZT is efficient to induce adventitious buds in blueberry (Rowland and Ogden 1992; Cao and Hammerschlag 2000, 2002; Liu et al. 2010; Meiners et al. 2007). Our histological studies showed that meristemoids initiated from sub-epidermal cells outside of the vascular bundles and eventually developed into adventitious buds, without callus formation. The time course of adventitious bud regeneration observed here is consistent with that reported by Pizzolato et al. (2014) using leaf explants of *V. corymbosum* 'Aurora' cultured on the medium containing thidiazuron. But in the study of Pizzolato et al. (2014), multiple origins of the adventitious buds were observed, including parenchyma cells from the midrib, palisade mesophyll, and epidermis adjacent to the xylem of the original veins. Blueberry genotypes and plant growth regulators used in the present study and the study of Pizzolato et al. (2014) are most likely responsible for these differences. Direct shoot regeneration is desired in organogenesis, because somatic variation may occur in shoots that regenerate from callus (George and Davies 2008).

In the present study, size of SLSs significantly affected the number of the shoots per SLS, but not survival and shoot regrowth following cryopreservation. Small SLSs contained fewer adventitious buds than large ones before cryopreservation. Therefore, the number of shoots per SLS is logically fewer in the former than in the latter following cryopreservation. The age of adventitious buds was also found to affect cryopreservation in the present study. Similar results were also reported by Li et al. (2014), who found shoot regrowth levels in cryopreserved buds significantly increased as the age of adventitious buds increased from 8 to 11 weeks in

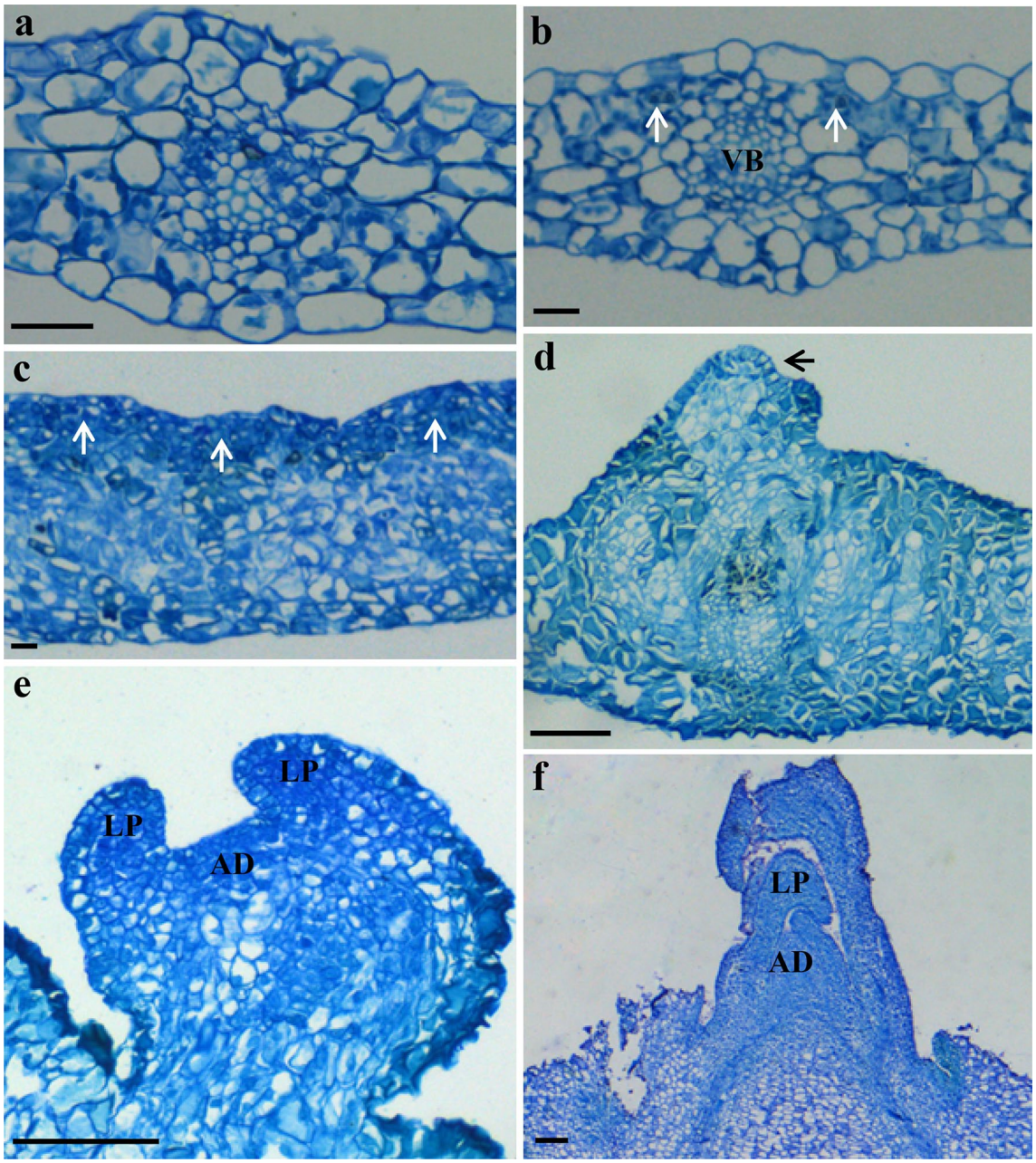


**Fig. 4** Histological observations in transverse sections of leaf explants of highbush blueberry 'Misty' during adventitious bud regeneration. **a** A leaf explant at day 0 of culture. **b** First cell divisions were observed in sub-epidermal cells outside of the vascular bundles (VB) of leaf explants on day 5 of culture. These cells contained dense cytoplasm and conspicuous nucleus, as indicated by arrows. **c** A continuous line, as indicated by arrows, was observed at the surface along the cut edge of the leaf explant at day 9 of culture. This line was constituted of dividing cells that contained dense cytoplasm and conspicuous nucleus. **d** Primary adventitious buds containing apical dome were easily seen, as indicated by arrows, in leaf explant at day 12 of culture. **e** A typical adventitious bud containing apical dome (AD) and the youngest leaf primordia (LPs) formed in leaf explant at day 15 of culture. **f** A matured adventitious bud that had already started shoot elongation in leaf explant at day 21 of culture. Bars = 100 µm

apple 'Gala' (*Malus* × *domestica*). The results obtained in the present study also showed the age of adventitious buds was positively related with size and leaf primordium number of the adventitious buds. Increased shoot regrowth levels in larger shoot tips following cryopreservation were found in other plants such as chrysanthemum (Wang et al. 2014a), apple (Li et al. 2016) and potato (Li et al. 2017).

For preservation of genetic resources, shoot tips are preferred over other tissues or organs because the former are genetic more stable than the latter (Li et al. 2018; Wang

**Table 2** Survival and recovery of the treated control (−LN) and cryopreserved (+LN) small leaf squares (SLSs)-bearing adventitious buds excised from leaf explants cultured on adventitious bud regeneration medium (ABRM) for different time durations in highbush blueberry 'Misty'

| Days after culture | Survival (%) | | Recovery | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Shoot regrowth (%) | | Number of shoots per SLS | | Length of regrown shoots (cm) | |
| | −LN | +LN | −LN | +LN | −LN | +LN | −LN | +LN |
| 15 | 75 ± 5b | 55 ± 5b | 60 ± 6b | 35 ± 4b | 3.1 ± 0.3c | 2.1 ± 0.3c | 1.5 ± 0.3c | 0.8 ± 0.2c |
| 18 | 100a | 100a | 100a | 100a | 5.1 ± 0.6b | 4.7 ± 0.5b | 2.3 ± 0.4b | 1.6 ± 0.2b |
| 21 | 100a | 100a | 100a | 100a | 7.8 ± 0.7a | 7.7 ± 0.7a | 3.5 ± 0.4a | 2.6 ± 0.3a |
| 24 | 100a | 100a | 100a | 100a | 8.1 ± 0.7a | 7.9 ± 0.8a | 3.7 ± 0.4a | 2.8 ± 0.4a |
| 27 | 100a | 100a | 100a | 100a | 8.8 ± 0.8a | 4.8 ± 0.8b | 3.4 ± 0.5a | 2.3 ± 0.5a |

Survival and recovery was recorded after 3 and 21 days of post-thaw culture, respectively. Data are presented as means ± SE and with different letters indicate significant differences at $P < 0.05$ by Turkey's test

**Table 3** Adventitious bud regeneration, and survival and recovery of cryopreserved small leaf squares (SLSs)-bearing adventitious buds in highbush blueberry 'Northland' and 'Blueglod'

| Cultivars | No. of adventitious buds per leaf explant | Survival (%) | Recovery | | |
|---|---|---|---|---|---|
| | | | Shoot regrowth (%) | Number of shoots per SLS | Length of regrown shoots (cm) |
| Northland | 23.8 ± 2.0 | 100 | 100 | 6.5 ± 0.6 | 2.7 ± 0.4 |
| Blueglod | 24.2 ± 2.8 | 100 | 100 | 6.3 ± 0.5 | 2.5 ± 0.3 |

Number of adventitious buds was recorded after 21 days of culture on adventitious bud regeneration medium (ABRM). PVS2 droplets of SLSs (2 × 3 mm)-bearing adventitious buds were cryopreserved. Survival and recovery was recorded after 3 and 21 days of post-thaw culture, respectively. Data are presented as means ± SE from two independent experiments

**Table 4** Names and sequences of primers, and number of amplified bands analyzed by ISSR and RAPD in plantlets regenerated from cryopreserved shoot tips of highbush blueberry 'Misty'

| Molecular marker | Name of primer | Primer sequence (5′–3′) | Number of amplified bands | Number of ploymorphic bands |
|---|---|---|---|---|
| ISSR | UBC811 | (GA)8C | 4 | 0 |
| | UBC817 | (AC)8T | 4 | 0 |
| | UBC825 | (CA)8T | 5 | 0 |
| | UBC827 | (AC)8G | 4 | 0 |
| | UBC835 | (AG)8CTC | 6 | 0 |
| | UBC860 | (TG)8AGA | 4 | 0 |
| | UBC867 | (GGC)6 | 5 | 0 |
| | UBC873 | (GACA)4 | 4 | 0 |
| | UBC880 | (GGAGA)3 | 4 | 0 |
| | UBC881 | (GGGTG)3 | 6 | 0 |
| Total | | | 46 | 0 |
| RAPD | OPA-05 | AGGGGTCTTG | 5 | 0 |
| | OPA-07 | GAAACGGGTG | 7 | 0 |
| | OPA-16 | AGCCAGCGAA | 6 | 0 |
| | OPA-18 | AGGTGACCGT | 5 | 0 |
| | OPA-20 | GTTGCGATCC | 5 | 0 |
| | OPM-05 | GGGAACGTGT | 8 | 0 |
| | OPM-14 | AGGGTCGTTC | 5 | 0 |
| | OPP-16 | CCAAGCTGCC | 4 | 0 |
| | OPY-09 | AGCAGCGGAC | 4 | 0 |
| | OPY-15 | AGTCGCCCTT | 7 | 0 |
| Total | | | 56 | 0 |

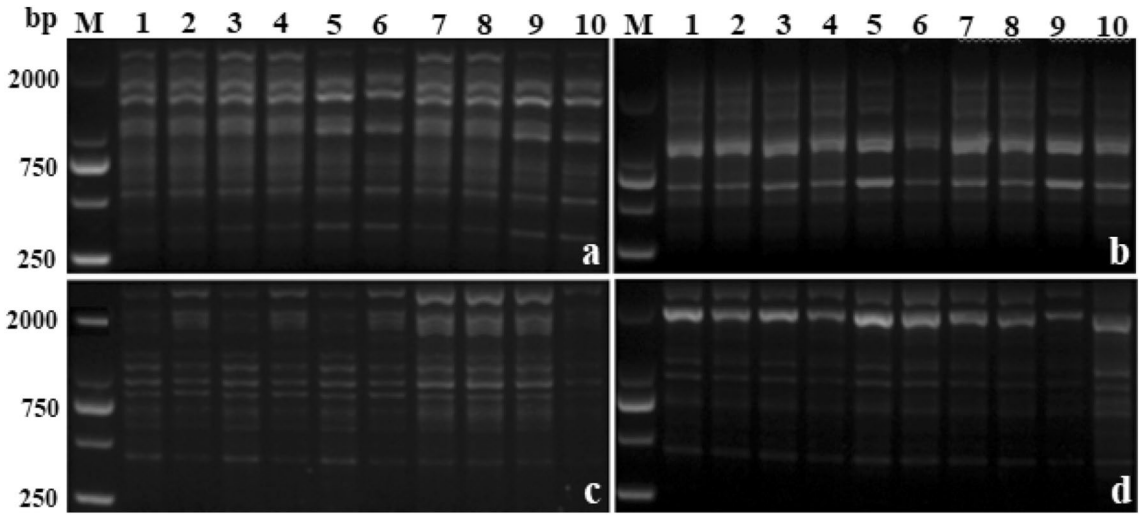


**Fig. 5** ISSR and RAPD analysis in stock shoots, and shoots regenerated from leaf explants and plantlets recovered from cryopreserved shoot tips of highbush blueberry 'Misty'. ISSR banding patterns produced by the primers UBC835 (**a**) and UBC881 (**b**). RAPD banding patterns produced by the primers OPA-07 (**c**) and OPA-16 (**d**). M = marker; lanes 1–2 = stock plantlets; lanes 3–6 = plantlets regenerated from leaf explants; lanes 7–10 = plantlets regenerated from cryopreserved shoot tips

et al. 2014b, 2018). In the traditional shoot tip cryopreservation, terminal buds are frequently used and axillary buds are sometimes used (Li et al. 2018; Wang et al. 2018). Only one or a few shoot tips can be produced from each in vitro stock shoot and the shoot tip production efficiency is low in the traditional method. Burritt (2008), Li et al. (2014) and Yin et al. (2014) reported successful cryopreservation of adventitious buds induced from leaf segments in *Begonia*×*erythrophylla* (Burritt 2008), *Lilium* (Yin et al. 2014) and *Malus* (Li et al. 2014). These studies considerably increased the efficiency of shoot tip production to at least eight times (Li et al. 2014) and about 20 times (Yin et al. 2014), compared with the corresponding traditional methods (Feng et al. 2013; Li et al. 2016; Chen et al. 2011).

In almost all shoot tip cryopreservation protocols available now, excision of shoot tips is still a necessary step. This step requires skilled staff and is the most time-consuming and labour-intensive in the whole procedure (Harvengt et al. 2004). In addition, surgical excision of shoot tips from stock cultures may cause physical damage to and induce browning of explants. More recently, Pan et al. (2018) reported cryopreservation of SLSs-bearing adventitious buds in *Lilium*. The study of Pan et al. (2018) used the same method of Yin et al. (2014) to regenerate adventitious buds from *Lilium* leaf segments. SLSs (3×4 mm), each bearing at least one adventitious bud, were cut from the leaf segments and used for cryopreservation. The greatest advantage in the study of Pan et al. (2018) is elimination of shoot tip excision, in addition to high efficiency of shoot tip production. The cryopreservation procedure established in the present study also uses SLSs and therefore has the same advantage of elimination of shoot tip excision, as reported by Pan et al. (2018). Additional three improvements are reported in the present study. First, terminal shoot tips were used in all studies of shoot tip cryopreservation in blueberry. Thus, only one shoot tip was obtained from each 4-week-old stock shoot. In the present study, three-four fully-opened leaves were taken from each 4-week-old stock shoot and cultured for adventitious bud regeneration. About 28 adventitious buds were produced in 3 weeks of culture, and thus, each stock shoot can produce at least 84 (28×3) adventitious buds in about 3 weeks. This productive ability is at least 84 times of the traditional cryopreservation studies in blueberry (Reed 1989; Uchendu and Reed 2009; Kami et al. 2009, 2010; Wang et al. 2017). Second, similarly high recovery results were produced in cryopreserved SLSs with their ages ranging from 21 to 24 days in the present study, indicating this cryopreservation protocol is much more flexible than the previously reported cryopreservation protocols using adventitious buds in *Begonia*×*erythrophylla* (Burritt 2008), *Lilium* (Yin et al. 2014; Pan et al. 2018) and *Malus* (Li et al. 2014). Third, at least seven shoots regenerated following cryopreservation in the present study, while only one shoot regenerated in all studies using adventitious buds (Burritt 2008; Li et al. 2014; Yin et al. 2014; Pan et al. 2018).

Genetic stability in the regenerants recovered from cryopreserved plants is a major concern (Harding 2004; Benson 2008; Wang et al. 2014b). In this study, adventitious buds regenerated from leaf explants were used for cryopreservation. Types and concentrations of plant growth regulators (PGRs) are key factors responsible for genetic variations in adventitious buds regenerated from in vitro cultures

(George and Davies 2008). The present study used 20 µM ZT in ABRM, and the same PGR at the same concentration was also repeatedly used in previous studies of shoot regeneration from leaf segments in blueberry (Rowland and Ogden 1992; Cao and Hammerschlag 2000, 2002; Liu et al. 2010; Meiners et al. 2007). Genetic stability in the blueberry plants regenerated from in vitro culture has been assessed by random amplified polymorphic DNA (RAPD) markers (Gajdošová et al. 2006), expressed sequence tagpolymerase chain reaction (Debnath 2011) and flow cytometry (Gajdošová et al. 2006), and all results were quite promising. In this study, genetic stability was assessed by ISSR and RAPD in adventitious shoots regenerated from leaf explants and no polymorphic bands were detected, compared with in vitro stock shoots. In our previous study using the same blueberry 'Misty' and droplet-vitrification cryopreservation, no polymorphic bands were detected in the regenerants following cryopreservation by ISSR and RAPD (Wang et al. 2017). The same results were obtained in the present study. These results indicated no obvious alternations in genetic integrity were caused by the cryoprocedure used. In this study, the morphology of plantlets regenerated from cryopreserved SLSs was identical to that of the in vitro-derived ones.

In conclusion, a droplet-vitrification method was described for cryopreservation of SLSs-bearing adventitious buds in blueberry. With this protocol, 100% of survival and shoot regrowth rates, and more than six shoots per SLS were produced following cryopreservation in the three highbush blueberry cultivars tested. Thus, the cryopreservation method reported here can be considered an efficient cryopreservation of plant shoot tips, and has potential applications to other plant species.

**Author contributions** H-YC and JL: performance of experiments, collection and analysis of data and preparation of manuscript; CP: preparation of plant materials and assistance to experiments; J-WY: assistance to data collection and analysis; Q-CW: chief scientist, financial supports, experimental design and preparation of manuscript.

## Compliance with ethical standards

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

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