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RESEARCH ARTICLE

Development of Cryoconservation Protocol for *In-vitro* Shoot Bases of *Allium ampeloprasum* L. for Long-term Conservation

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Abstract

Critical factors for the development of cryo conservation protocol for *Allium ampeloprasum* L. conserved in the *in-vitro* Genebank (IVGB) at ICAR-NBPGR of India were investigated. Shoot bases (1.0 x 1.5 mm) excised from 8-wk-old shoot cultures grown on growth media (MS + 0.1 mg/l NAA + 0.02 mg/l 2iP + 0.3 M sucrose) and pregrown at 22/ 5°C for 16/8 hours alternate temperature regime, precultured on MS medium supplemented with 0.3 M sucrose at standard culture conditions (SCC) for 16 hours followed by loading solution treatment for 60 minutes, PVS2 dehydration for 40 minutes were successfully cryoconserved using vitrification and droplet-vitrification technique. Droplet-vitrification technique improved post-thaw regrowth by ~58.33% as compared to vitrification. Thus, a standardized protocol was used for cryobanking of shoot bases of *A. ampeloprasum*.

Keywords: *Allium ampeloprasum*, Vitrification, Droplet-vitrification, Shoot bases, Cryobanking.

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Introduction

Genus *Allium* is one of the largest monocot genera comprising >1,100 taxa present globally, and India has a rich diversity of this genus (Pandey *et al.*, 2022). Since 1986, efforts have been made towards collecting and conservating cultivated and wild *Alliums* (Pandey *et al.*, 2022). In India, *Allium* genetic resources conservation is achieved through a systematic *trans-situ* approach and a total of 5,349 germplasm accessions of *Allium* genetic resources (~20 species) (both indigenous and exotic collections) are conserved in National Genebank at ICAR-NBPGR, New Delhi in its Seed genebank, *in-vitro* gene bank (IVGB), Cryogenebank, and Field genebank at NBPGR regional station, Bhowali (Anuradha *et al.*, 2023). The species *Allium ampeloprasum* L. (syn *A. lineare* Mill.) is a species with an onion-like flavor. The species originated in Middle Asia, with secondary centres of development and distribution in Western Asia and the Mediterranean countries (Swami and Veeregowda, 2006). The etymology of specific epithet is from Greek words '*ampelos*' connoting 'vineyard' and 'prason' meaning 'leek'. This species has multifarious uses, *viz.,* consumed as raw vegetable, as ingredient in many foods, also used in preparation of herbal medicines due to its immense medicinal properties like antioxidant activity, antimicrobial activity, hepatoprotective activity, anticancer activity, anti-osteoporotic activity, antidiabetic activity, anti-inflammatory activity, gastroprotective activity, anti-hypercholesterolemic/hypolipidemic activity, immunomodulatory activity, platelet anti-aggregation activity spasmolytic activity, insecticidal and antifungal properties (Lim, 2015). Although the

species produces seeds, due to its outbreeding nature, cryopreservation has been suggested as a viable method of long-term conservation of its germplasm (Volk *et al*., 2004).

A. ampeloprasum accession EC328492 was procured from the United States Department of Agriculture- Agricultural Research Service, USDA-ARS (Variety: PI-369525) and this species was conserved in field genebank, which is prone to biotic and abiotic stresses; germplasm conserved in the IVGB at ICAR (Indian Council of Agricultural Research) - National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India for over two decades requires periodic subculturing at 16-week intervals, which is labor intensive and time-consuming and only provides short- to medium-term storage (Pandey *et al*., 2022). Hence, there is a need for safe, cost-effective and long-term conservation of this species. So far, cryo conservation is the only available method for long-term conservation of plant genetic resources (PGR) (Agrawal *et al*., 2022a; Sharma *et al*., 2020). In our laboratory we had developed cryo conservation protocols for *in-vitro* conserved germplasm of several species (Agrawal *et al*., 2022b) *viz*., *Bacopa monnieri* (Sharma *et al*., 2011, 2017), Gowthami *et al*., 2023a), Dahlia (Gowthami *et al*., 2023b), *Dioscorea deltoidea* (Dixit-Sharma *et al*., 2005; Mandal and Dixit, 2000; Mandal and Dixit-Sharma, 2007; Sharma *et al*., 2022), *Fragaria* × *ananassa* (Gupta and Tewari, 2020), *Gentiana kurroo* (Sharma *et al*., 2021), *Musa* spp. (Agrawal *et al*., 2004, 2008, 2014), *Picrorhiza kurroa* (Sharma and Sharma, 2003) and *Rubus* spp. (Gupta and Reed, 2006). So far, no reports exist on the cryoconservation of *A. ampelprasum*. With this background, the present study was formulated with the objectives (a) to investigate the critical factors for cryoconservation of *in-vitro* shoot bases; (b) to develop an efficient cryoconservation protocol for cryobanking of *A. ampeloprasum*.

Material and Methods

Plant Material

In-vitro shoot cultures of *A. ampeloprasum* accession EC328492 maintained in the i*n-vitro* Gene Bank (IVGB) of ICAR- NBPGR, New Delhi, India, were utilized as mother culture.

Establishment of *In-vitro* Cultures

Initially, the *in-vitro* grown plants were cultured on shoot multiplication medium (SM) comprising Murashige and Skoog medium (MS) supplemented with 0.1 mg/l naphthalene acetic acid (NAA), 0.02 mg/l 6-γ,γ-dimethylallylamino purine (2iP) (Sigma-Aldrich, St. Louis, MO), 3% (w/v) sucrose (HiMedia Laboratories, Mumbai, India) with pH adjusted to 5.8 prior to autoclaving and solidified using 0.8% (*w/v*) agar. Shoot bases of 1.0 to 1.5 cm were excised in a sterile laminar flow chamber and inoculated in each culture tube and cultures were maintained under standard culture room conditions (SCC) comprising 25 ± 2 °C, 16/8 hours photoperiod, and a light intensity of 40 μEm^{-2} s^{-1} using white fluorescent light (Philips, India). Subculture was performed every 4 to 6 weeks by transferring to fresh medium to generate sufficient explants for cryoconservation experiments.

Shoot base excision

In-vitro shoots were dissected under a stereo zoom microscope (Olympus India, Haryana, India). Initially, the top portion of the shoots were cut, followed by removing 3 or 4 layers of the shoot bases until the transparent shoot base was visible with 1.0 to 1.5 mm size (Figure 4A). Shoot bases were cryoconserved using vitrification (V) and droplet-vitrification (DV) techniques as described below. Initially, different parameters were standardized using the vitrification technique.

Vitrification

The excised shoot bases were precultured on MS medium supplemented with 0.3 M sucrose in 60 mm diameter petri dishes (Hi-Media®) (10 mL medium/Petridish) at SCC for 16 hours. Precultured shoot bases were placed carefully in 1.8 mL Nunc™ cryovials (Thermo Fisher Scientific®, Roskilde, Denmark) containing loading solution (LS) [liquid MS medium + 2.0 M glycerol (Sigma-Aldrich, St. Louis, USA) + 0.4 M sucrose] for 60 minutes at room temperature (25 ± 2°C) under sterile laminar flow chamber. Thereafter, LS was removed using a Pasteur pipette and 1.0 mL of pre-chilled plant vitrification solution 2 (henceforth referred as PVS2 solution) was added. PVS2 comprising 0.4 M sucrose, 30% (*w/v*) glycerol, 15% (*w/v*) ethylene glycol and 15% (*w/v*) dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA) in MS medium (pH 5.8) (Sakai *et al*., 1990) was incubated for 0, 10, 20, 30, 40, 50 and 60 minutes (depending on experiment) at room temperature (25 ± 2°C). After the desired period of PVS2 dehydration, cryovials with the shoot bases in PVS2 solution were placed in cryocanes, immediately plunged into liquid nitrogen (LN) in a Dewar flask, and retained for at least 1-hour. For thawing, cryovials were removed from LN and rewarmed rapidly by immediately plunging in warm water at 42°C for 2 minutes with constant stirring. After thawing, PVS2 solution was quickly removed using a Pasteur pipette and the shoot bases were rinsed 4 to 5 times with unloading solution (US; 1.2 M sucrose in liquid MS medium; pH 5.8) for 20 minutes at room temperature.

Effect of toxicity of PVS2 dehydration duration

For this experiment, shoot bases were excised from 4-week-old cultures pregrown on SM medium and precultured on MS + 0.3 M sucrose for 16 hours under SCC. Precultured shoot bases were subjected to LS treatment for 60 minutes, followed by different PVS2 dehydration durations (10, 20, 30, 40, 50 and 60 minutes) and US treatment for 20 minutes.

Effect of pregrowth duration, media and temperature

For this experiment, shoot cultures were pregrown for 4, 6 and 8 weeks on SM and MS medium supplemented with 0.1 mg/l NAA, 0.02 mg/l 2iP (Sigma-Aldrich, St. Louis, MO), 0.3 M (*w/v*) sucrose (HiMedia Laboratories, Mumbai, India) with pH adjusted to 5.8 prior to autoclaving and solidified using 0.8% (w/v) agar (hereafter called as SM10 medium) and incubated under 25 ± 2°C and 22/5°C. For pregrowth under 25 ± 2°C, cultures were maintained in a culture room with SCC and for pregrowth at 22/5°C, cultures were maintained in a biological oxygen demand (BOD) incubator (Ocean Life Science Corporation, India) for 16 hours of 22°C and 8 hours of 5°C, 16/8 hours of photoperiod using white fluorescent light.

Droplet-Vitrification

The excised shoot bases were precultured on MS medium supplemented with 0.3 M sucrose in 60 mm diameter Petri dishes at SCC for 16 hours and treated with LS (60 minutes) and PVS2 (40 minutes) as followed in the vitrification protocol. Before 2 minutes of the completion of PVS2 dehydration, 10 shoot bases were placed on a 5 µL droplet of PVS2 solution on a sterile aluminium foil strip (20 × 5 mm). Subsequently, the aluminium strip with shoot bases was plunged directly in LN for a few seconds and later placed into cryovial (1.8 mL) (filled with LN) in a polycarbonate cryo-box, held in a thermocol ice box, for at least an hour. For rewarming, aluminium strips were removed from LN, immediately placed into the US solution in a petri plate (35 mm), and incubated for 20 minutes at room temperature. In both techniques, shoot bases treated with all the solutions (LS and PVS2) but not frozen in LN and rinsed with US for 20 minutes, are treated as controls.

Regrowth and In-vitro Plantlet Development

After US treatment, shoot bases were placed on sterile dry filter papers placed in petri plates to remove the adhered solution. Later shoot bases were placed to 60 mm petridishes containing regrowth medium (SM medium) (10 mL medium/ petridish) and incubated in dark (covered with aluminum foil) at 25 ± 2°C for five days to avoid photo-oxidation. Thereafter, the petri plates with shoot bases were shifted to SCC. After 6 weeks, surviving shoot bases were transferred in culture tubes with SM medium (150 x 25 mm) and incubated under SCC. Regrowth was determined by dividing the shoot bases regrowing into normal shoots after four weeks of plating by the total number of shoot bases inoculated and expressed as a percentage.

Statistical Analysis

All the experiments were carried out in using completely randomized design (CRD) repeated thrice with 20 shoot bases in each replication. Analysis of variance (ANOVA) and duncan's multiple range test (DMRT) was used for comparison and for significant differences among means ($p \leq 0.05$) using SPSS statistics version 22.0 software package. Prior to analysis, the original percentage data were arcsin transformed ($y' = \arcsin y^{1/2}$) to stabilize the variance of data. Results are presented as mean (%) ± standard error (SE). Graphs were prepared by using Microsoft Excel 2010.

Results and Discussion

Cryoconservation is the only available method for safe and long-term conservation of plant genetic diversity. It is being used in many genebanks across the globe, as a complementary conservation strategy for backup to field and/or *in-vitro* collections to safeguard against loss of PGR (Agrawal *et al.*, 2022a; Sharma *et al.*, 2019, 2020). So far, in *Alliums*, cryopreservation (garlic and shallots) was attained using shoot bases, bulbils excised from field cloves which eliminates the establishment of *in-vitro* cultures following vitrification, droplet-vitrification and encapsulation-dehydration (Wang *et al.*, 2020 and references therein). However, contamination is the major encounter through this procedure, many other species do not produce bulbils and also obtaining sufficient explants is very difficult. Hence, in the present study we attempted cryoconservation using *in-vitro* derived shoot bases. The development of cryo conservation protocol involves a series of events and protocols are species/genotype-specific. Hence, in each species, standardization of different parameters *viz.,* explant size, age of mother plants, cold hardening, preculture media and duration, cryopreservation technique, regrowth media etc. are required (Zamecnik *et al.*, 2021). Accordingly, different critical parameters were tested in the present study to develop an efficient cryoconservation protocol for *A. ampeloprasum*.

In the first set of experiments, shoot bases were exposed to six different PVS2 dehydration durations to optimize cryoprotectant treatment duration. No shoot tips

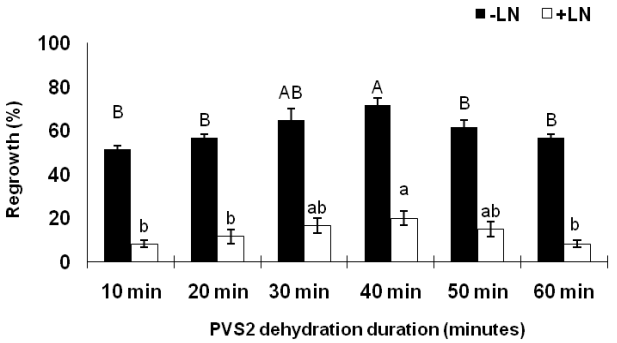

Figure 1: Effect of PVS2 dehydration duration on regrowth of non-cryoconserved (-LN) and cryoconserved (+LN) shoot bases. Data are presented in vertical bars represent mean ± SE. Significant differences ($P \leq 0.05$) in regrowth among non-cryoconserved shoot bases (upper case) and among cryoconserved shoot bases (lower case) are presented by different alphabets analyzed by Duncan's Multiple Range Test

survived cryoconservation without PVS2 treatment, and PVS2 dehydration duration significantly influenced the regrowth of both cryoconserved and non-cryoconserved shoot bases (Figure 1). Gradual increase in regrowth was observed when shoot bases treated with PVS2 solution for 10 to 40 minutes *i.e.,* 51.7 to 71.7% (-LN) and 8.33 to 20% (+LN). However, further increase in PVS2 duration to 50 and 60 minutes resulted in significant decrease in regrowth to 61.7 and 56.7% of non-cryoconserved and 15 and 8.33% of cryoconserved shoot bases. A similar toxic effect of lower and higher PVS2 treatment duration on post-thaw regrowth was reported by Sharma *et al.* (2021) in *Gentiana kurroo*. Hence, for further experiments to improve regrowth, PVS2 dehydration for 40 minutes was followed. Optimization of treatment duration of explants with cryoprotectants is one of the major critical steps to enhance the tolerance to LN exposure and among several cryoprotectants, PVS2 used in many species (Zamecnik *et al.*, 2021). A decrease in the optimum treatment time has been shown to damage explants after LN exposure due to intra- and inter-cellular ice crystal formation and extended period treatment damages explants due to chemical toxicity (Zamecnik *et al.*, 2021).

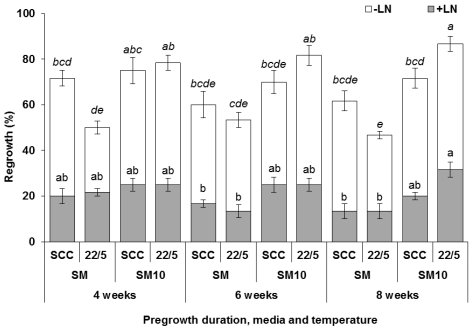

Figure 2: Effect of pregrowth duration, media and temperature on regrowth (%) of non-cryoconserved (-LN) and cryoconserved (+LN) shoot bases. Data are presented in vertical bars represent mean ± SE. Significant differences ($P \leq 0.05$) in regrowth among non-cryoconserved shoot bases (lower case italicized) and among cryoconserved shoot bases (lower case non-italicized) are presented by different alphabets analyzed by Duncan's Multiple Range Test

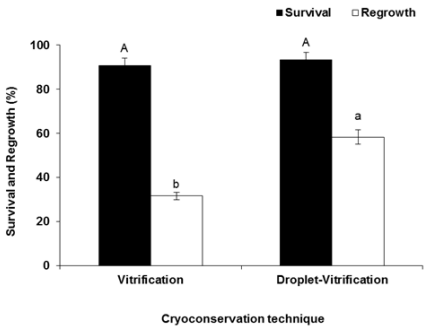

Figure 3. Comparison of two cryoconservation techniques (vitrifcation and droplet-vitrification) on post-thaw regrowth of shoot bases. V- Vitrification, DV- Droplet-Vitrification. Data are presented in vertical bars represent mean ± SE. Significant differences ($P \leq 0.05$) in survival (upper case) and regrowth (lower case) are presented by different alphabets analyzed by Duncan's Multiple Range Test

Induction of dehydration tolerance of explants before before exposure to LN is an essential step in cryoconservation, which is generally achieved by pregrowth of mother plants and preculture of explants, either at low temperature or different temperature regimes, using high sucrose media (commonly 0.3 M sucrose) and growth regulators (Suzuki *et al.*, 2005; Reinhoud *et al.*, 2000; Bettoni *et al.*, 2021). Hence in the present work, to increase the post-thaw regrowth, experiments were conducted to identify the optimum pregrowth duration, pregrowth media and pregrowth temperature to enhance the tolerance to LN freezing and results are presented in Figure 2. Explants survived (46.67–86.67%) and exhibited normal growth in non-frozen controls. These three factors have a significant effect on post-thaw regrowth. It was observed that when shoot bases were excised from 4-week-old cultures pregrown on multiplication medium had 20% regrowth, which increased to 25% when pregrown on shoot multiplication medium with 0.3 M sucrose (SM10) maintained at SCC. Shoot bases excised from 6-week-old cultures pregrown on multiplication medium, resulted in reduction of post-thaw regrowth (16.67%), which further decreased to 13.33% when maintained at 25 ± 2°C. However, similar regrowth of 25% was obtained, when pregrown on SM10 medium maintained at SCC. Similarly, a further increase in pregrowth duration to eight weeks had positive direction improvement when pregrown on SM10 medium at 25/5°C resulting in increase in post-thaw regrowth to 31.67% in comparison to 20% (pregrown on SM10 maintained at SCC). In contrast, negative direction regrowth was observed when pregrown on SM medium and cultures maintained at SCC. From this study it is evident that, pregrowth duration from 8-week-old cultures in comparison to 4 weeks and 6 weeks, pregowth of cultures on shoot multiplication medium with 0.3 M sucrose instead of shoot multiplication medium with 3% sucrose and pregrowth temperature of 25/5°C instead of SCC significantly increased post-thaw regrowth (10%). Thus, the experiment clearly indicated that excision of shoot bases from 8-week-old cultures pregrown on multiplication media supplemented with 0.3 M sucrose and pregrown at two alternate temperature regimes *i.e,* 25/5°C for 16/8 hours has increased the osmotolerance further to enhance the tolerance to LN exposure. Positive impact of pregrowth at low temperatures on post-thaw recovery has been reported in apple, pear, *Rubus, Prunus*, kiwifruit, birch and mulberry (Suzuki *et al.*, 2005). In conformity with previous reports, pregrowth at low temperature of 5°C for 8 hours, alternatively with 22°C for 16 hours enhanced the post-thaw regrowth. Yoon *et al.* (2006) found that cold hardening, the addition of osmotic agents, culture conditions such as high light intensity, ventilation of culture vessels, and low planting density led to increased post-thaw survival in potato.

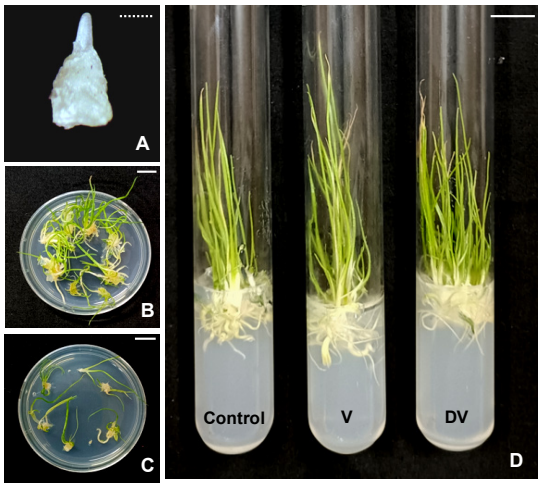

Figure 4: Cryoconservation of *in-vitro* shoot bases of *Allium ampeloprasum* L. using vitrification and droplet-vitrification techniques. (A) Shoot bases for cryoconservation (1.5 mm x 1.0 mm), (B) Control (+PVS2, -LN of DV protocol), (C) Post-thaw regrowth of shoot bases after droplet-vitrification, (D) *In-vitro* plantlet development Control: PVS2, -LN, V: post-thaw regenerated shoot bases of vitrification protocol, DV: post-thaw regenerated shoot bases of droplet-vitrification protocol. Scale bar: dotted line (0.5 mm), solid line (1.0 cm)

Figure 3 presents the feasibility of DV technique using parameters standardized for vitrification technique. In the present study, irrespective of technique, the non-frozen controls (but PVS2 treated) exhibited 100% survival (data not presented) (Figure 4). As reflected in Figure 3. comparison of vitrification and droplet-vitrification on post-thaw regrowth showed significant differences based on DMRT ($P \leq 0.05$). DV technique resulted in significant improvement in post-thaw regrowth (58.33%) (Figure 4C), which was almost twice as compared to vitrification (31.67%). The increased post-thaw survival or regrowth using DV technique in comparison to vitrification has been reported in many species (Agrawal *et al.*, 2004; Panis *et al.*, 2005; Park and Kin, 2015; Benelli *et al.*, 2021; Sharma *et al.*, 2021). The cryoconserved shoot bases produced shoots without transitory callus formation, similar to non-treated controls. All the survived shoot bases developed into plantlets *in-vitro* (Figure 4D).

Conclusion

To the best of our knowledge, this is the first report on cryoconservation protocol development for *Allium ampeloprasum* shoot bases. Droplet-vitrification technique improved post-thaw regrowth by ~58.33% as compared to vitrification. Thus, a standardized protocol was successfully used for cryobanking of *A. ampeloprasum* accession EC328492.

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References

Agrawal, A., Gowthami, R., Srivastava, V., & Chander, S. (2023). *Trans situ* conservation of PGR. In: RK Gautam et al., (Eds.) *Plant Genetic Resources Management: Theory and Practices. The Director, ICAR-NBPGR, New Delhi, India*, pp 44-52.

Agrawal, A., Swennen, R., & Panis, B. (2004). A comparision of four methods for cryopreservation of meristems in banana (*Musa* spp.). *CryoLetters*, **25(2)**: 101-110.

Agrawal, A., Tyagi, R. K., & Goswami, R. (2008). Cryopreservation of subgroup Monthan (ABB) of Indian cooking banana (*Musa* spp.) germplasm. *Curr. Sci.*, **94(9)**: 1125-1128.

Agrawal, A., Sanayaima, R., Singh, R., Tandon, R., Verma, S., & Tyagi, R. K. (2014). Phenotypic and molecular studies for genetic stability assessment of cryopreserved banana meristems derived from field and *in vitro* explant sources. *In In Vitro Cell. Dev. Biol. Plant.*, **50**: 345-356.

Agrawal, A., Gowthami, R., Chander, S., & Srivastava, V. (2022a). Sustainability of *In Vitro* Genebanks and Cryogenebanks. *Indian J. Plant Genet. Resources*, **35(3)**: 180-184.

Agrawal, A., Sharma, N., Gupta, S., Bansal, S., Srivastava, V., Malhotra, E. V., & Singh, K. (2022b). Biotechnological applications for plant germplasm conservation at ICAR-National Bureau of Plant Genetic Resources, India- recent achievements. *Acta Hortic.*, **1339**: 29-42.

Benelli, C., Carvalho, L. S., El Merzougui, S., & Petruccelli, R. (2021). Two advanced cryogenic procedures for improving *Stevia rebaudiana* (Bertoni) cryopreservation. *Plants*, **10(2)**: 277.

Bettoni, J. C., Bonnart, R., & Volk, G. M. (2021). Challenges in implementing plant shoot tip cryopreservation technologies. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **144(1)**: 21-34.

Dixit-Sharma, S., Ahuja-Ghosh, S., Mandal, B. B., & Srivastava, P. S. (2005). Metabolic stability of plants regenerated from cryopreserved shoot tips of *Dioscorea deltoidea*–an endangered medicinal plant. *Sci. Hortic.*, **105(4)**: 513-517.

Gowthami, R., Sharma, N., Chandra, R., Kurian, J. S., Malhotra, E. V. & Agrawal, A. (2023a). Development of an improved and simple shoot tip cryoconservation protocol for cryobanking of *Bacopa monnieri* (L.) Wettst. germplasm. *In Vitro Cell. Dev. Biol. Plant*, 1-13.

Gowthami, R., Chander, S., Pandey, R., Shankar, M., & Agrawal, A. (2023b). Development of efficient and sustainable droplet-vitrification cryoconservation protocol for shoot tips for long-term conservation of *Dahlia* germplasm. *Sci. Hortic.*, **321**: 112329.

Gupta, S., & Reed, B. M. (2006). Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. *CryoLetters*, **27(1)**: 29-42.

Gupta, S., & Tewari, P. (2020). Cryopreservation of Fragaria × ananassa using different techniques. *Acta Horticulturae*, **1298**: 161-166.

Lim, T. K., & Lim, T. K. (2015). *Allium ampeloprasum*. *Edible Medicinal and Non-Medicinal Plants: Volume 9, Modified Stems, Roots, Bulbs*, 103-123.

Mandal, B. B., & Dixit, S. (2000). Cryopreservation of shoot-tips of *Dioscorea deltoidea* Wall. -an endangered medicinal plant. *IPGRI Newsletter for Asia, the Pacific and Oceania*, w, 23.

Mandal, B. B., & Dixit-Sharma, S. (2007). Cryopreservation of in vitro shoot tips of *Dioscorea deltoidea* Wall., an endangered

medicinal plant: effect of cryogenic procedure and storage duration. *CryoLetters*, **28(6)**: 461-470.

Pandey, A., Malav, P. K., Semwal, D. P., Chander, S., Gowthami, R., & Rai, K. M. (2022). Repository of *Allium* Genetic Resources at ICAR-NBPGR: Prospects and Challenges for Collection and Conservation. *Indian J. Plant Genet. Resources*, **35(03)**: 185-190.

Panis, B., Piette, B. M. A. G., & Swennen, R. (2005). Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. *Plant Sci.*, **168(1)**: 45-55.

Park, S. U., & Kim, H. H. (2015). Cryopreservation of sweet potato shoot tips using a droplet-vitrification procedure. *CryoLetters*, **36(5)**: 344-352.

Reinhoud, P. J., Versteege, I., Kars, I., Iren, F. V., & Kijne, J. W. (2000). Physiological and molecular changes in tobacco suspension cells during development of tolerance to cryopreservation by vitrification. In *Cryopreservation of tropical plant germplasm: current research progress and application. Proceedings of an international workshop, Tsukuba, Japan, October, 1998* (pp. 57-66). International Plant Genetic Resources Institute (IPGRI).

Sakai, A., Kobayashi, S., & Oiyama, I. (1990). Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports*, *9*(1), 30-33.

Sharma, N., & Sharma, B. (2003). Cryopreservation of shoot tips of *Picrorhiza kurroa* Royle ex Benth., an indigenous endangered medicinal plant, through vitrification. *CryoLetters*, **24(3)**: 181-190.

Sharma, N., Satsangi, R., & Pandey, R. (2011). Cryopreservation of shoot tips of *Bacopa monnieri* (L.) Wettst by vitrification technique. *Acta Hortic.*, **908**: 283-288.

Sharma, N., Singh, R., Pandey, R., & Kaushik, N. (2017). Genetic and biochemical stability assessment of plants regenerated from cryopreserved shoot tips of a commercially valuable medicinal herb *Bacopa monnieri* (L.) Wettst. *In Vitro Cell. Dev. Biol. Plant*, **53***:* 346-351.

Sharma, N., Pandey, R., & Gowthwami, R. (2020). *In vitro* conservation and cryopreservation of threatened medicinal plants of India. In: Rajasekharan PE, Wani SH (Eds.) *Conservation and utilization of threatened medicinal plants*. Springer, India, pp 181–228.

Sharma, N., Gowthami, R., & Pandey, R. (2019). Synthetic seeds: a valuable adjunct for conservation of medicinal plants. In: Faisal M, Alatar AA (Eds) *Synthetic seeds: germplasm regeneration, preservation and prospects*. Springer International Publishing, Cham, pp. 181–216.

Sharma, N., Gowthami, R., Devi, S. V., Malhotra, E. V., Pandey, R., & Agrawal, A. (2021). Cryopreservation of shoot tips of *Gentiana kurroo* Royle–a critically endangered medicinal plant of India. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **144**: 67-72.

Sharma, N., Malhotra, E. V., Chandra, R., Gowthami, R., Sultan, S. M., Bansal, S., & Agrawal, A. (2022). Cryopreservation and genetic stability assessment of regenerants of the critically endangered medicinal plant *Dioscorea deltoidea* Wall. ex Griseb. for cryobanking of germplasm. *In Vitro Cell. Dev. Biol. Plant*, **58(4)**: 521-529.

Swamy, K. R. M., & Gowda, R. V. (2006). Leek and shallot. In Peter KV (Ed) *Handbook of Herbs and Spices*. Woodhead Publishing: Cembridge, UK, **3**: 365–389.

Volk, G. M., Maness, N., & Rotindo, K. (2004). Cryopreservation of garlic (*Allium sativum* L.) using plant vitrification solution 2. *CryoLetters*, **25(3)**: 219-226.

Wang, M. R., Zhang, Z., Zámečník, J., Bilavčík, A., Blystad, D. R., Haugslien, S., & Wang, Q. C. (2020). Droplet-vitrification for shoot tip cryopreservation of shallot (*Allium cepa* var. *aggregatum*): Effects of PVS3 and PVS2 on shoot regrowth. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **140**: 185-195.

Yoon, J. W., Kim, H. H., Ko, H. C., Hwang, H. S., Hong, E. S., Cho, E. G., & Engelmann, F. (2006). Cryopreservation of cultivated and wild potato varieties by droplet vitrification: effect of subculture of mother-plants and of preculture of shoot tips. *CryoLetters*, **27(4)**: 211-222.

Zamecnik, J., Faltus, M., & Bilavcik, A. (2021). Vitrification solutions for plant cryopreservation: Modification and properties. *Plants*, **10(12)**: 2623.