Acta Scientific AGRICULTURE (ISSN: 2581-365X)

Volume 7 Issue 4 April 2023

Research Article

Effect of Ascorbic Acid on the Cryopreservation of Kenaf (*Hibiscus cannabinus* L.) Seeds

Olatunji M Olabisi* and Esther E Uchendu

Department of Agronomy, University of Ibadan, Nigeria

***Corresponding Author:** Olatunji M Olabisi, Department of Agronomy, University of Ibadan, Nigeria.

Received: June 01, 2022

Published: March 09, 2023

Abstract

Cryopreservation (storage in liquid nitrogen) is a proven method for long-term ex-situ conservation of germplasm. However, the success of cryopreservation depends on the management of oxidative stress imposed on the plant during the cryopreservation procedure. In this study, the role of ascorbic acid (AA) added at the regrowth stage as an antioxidant that enhances the recovery of cryopreserved Kenaf seeds (cv. Ifeken) was examined.

Kenaf seeds were frozen using Plant Vitrification Solutions (PVS) 3 and 4 as the cryoprotectants. The seeds were cultured in the regrowth media supplemented with different concentrations of AA (0.14, 0.28, 0.43, and 0.58 mM) following cryopreservation. The control had no AA in the regrowth medium. Each of the treatments had a sub-control of non-cryopreserved seeds. It was a 2 x 3 factorial experiment laid out in a completely randomized design with three replicates. Effects of AA on survival and germination were determined.

The results showed that there was no significant difference among concentrations of AA on survival and germination of seeds after cryopreservation. A less than 40% survival rate was observed for seeds vitrified using PVS 3. However, for PVS 4, survival increased to a maximum of 63.3% with 0.28 mM AA. Germination of seeds pretreated with PVS 4 generally increased with an increase in AA concentration for both frozen and unfrozen controls.

This is the first report on the cryopreservation of Kenaf seeds and the results showed that AA added at the regrowth stage fairly improved the survival of cryopreserved Kenaf seeds compared to non-AA treatments. Further studies to evaluate other stages of cryopreservation with a wider range of AA concentrations are needed before AA can be recommended for use in the cryopreservation of Kenaf seeds.

Keywords: Ascorbic Acid; Antioxidant; Kenaf Seed; Regrowth; Cryopreservation

Abbreviations

AA: Ascorbic Acid; ANOVA: Analysis of Variance; GENSTAT: General Statistics; HEPA: High-Efficiency Particulate Arrestance; IAR and T: Institute of Agricultural Research and Training; IITA: International Institute of Tropical Agriculture; JICA: Japan International Cooperation Agency; LN: Liquid Nitrogen; LSD: Least Significant Difference; MS: Murashige and Skoog; NLN: Not Subjected to Liquid Nitrogen; OS: Oxidative Stress; PSI: Pound per Square Inch; PVS: Plant Vitrification Solutions; ROS: Reactive Oxygen Species

Introduction

Kenaf (*Hibiscus cannabinus* L.), belongs to the family Malvaceae and has chromosome number 2n = 36. It is a short-day annual herbaceous plant with major economic uses [1]. Kenaf is the most widely cultivated fiber plant in Nigeria after cotton [2]. The plant grows to an average height of 16 feet in 7 months [3] under favourable conditions with a stalk made up of an outer fiber and an inner fiber. The outer fiber also known as bast is like a softwood tree fiber, while the inner fiber called the core is similar to a hardwood fiber [4].

A whole mature Kenaf when cultivated yields 15.33 to 17.78 ton/ha dry stock [5]. Different parts of the plant are processed into different products after harvest. The leaves and flowers are eaten as vegetables while the seeds serve as a good source of oil [2]. The stalk is used to make rope, twine, bag, packaging material, paper,

and fences. It is also used as animal litter due to its ability to absorb urine [4]. Kenaf plants can be used to improve the environment. For example, it can be used for soil remediation and erosion control [6]. Kenaf plants can also be used industrially in paper production. Kenaf paper mills are more environmentally friendly and require less energy, production costs, and water usage when compared to conventional mills. It produces less pollution and good quality paper that can be recycled [3,7].

Kenaf is mostly propagated by seeds [8]. Seeds are probably the best plant tissue for *ex-situ* conservation of plant germplasm. It is cost-effective, can be distributed without difficulty, and holds the potential to develop into a genetically novel plant [9]. The length of time that the seeds remain viable may be significantly influenced by their environment. When stored under room temperature, seeds lose viability faster as well as when stored at higher relative humidity and lower temperatures [10,11]. Toole., *et al.* [10] were able to store Kenaf seeds effectively for 5.5 years at 8% relative humidity and -10°C temperature. There is a need for the best seed preservation technique that guarantees the biological characteristics of the seed are not significantly altered [12].

The only alternative for the long-term preservation of seed is cryopreservation. Cryopreservation is the process of preserving biological material at extremely low temperatures, often liquid nitrogen (-196°C), in a way that ensures the viability of the materials even after they have been warmed up [13]. Seeds of other crops such as *Bletilla formosana* [14] have been successfully cryopreserved. So far, there is no cryopreservation history for Kenaf, thus there is a need to develop a long-term preservation method using cryogenic techniques to minimize the loss of seeds due to loss of seed viability during storage.

In theory, if a plant material can be cryopreserved successfully, then it can stay protected for an endless number of years due to cessation in metabolic processes under LN temperature [13]. Explants are stored and protected against contamination in cryotubes. The approach has been found useful for secure and cheap long-term protection of genetic material of problematic species. [15]. Some explants, like dormant buds or orthodox seeds, exhibit natural dehydration processes and therefore can be cryopreserved without any pretreatment. Yet, one major difficulty with germplasms that have high quantities of cellular water and are thus not naturally resistant to freezing damage is explant impairment owing to oxidative stress. Oxidative stress is caused by ROS resulting from abiotic factors such as temperature, light, desiccation, etc. So, it is necessary to artificially dehydrate cells to keep them from the injury that results from intracellular water freezing [16].

There are two broadly classified artificial techniques for dehydrating cells which are freeze-induced dehydration and vitrification. Vitrification is the foundation for new dehydration procedures. It is the transformation of water directly from liquid to glass without the formation of ice [17]. The method entails the treatment of samples with cryoprotective agents, dehydration with highly concentrated vitrification solutions, fast cooling, and rewarming to remove cryoprotectants, and recovery [18]. The vitrification technique has been used for effective cryopreservation of seeds of other plants [14].

Experiments have also shown that antioxidants can be used to alleviate the damage caused by oxidative stress. The initiation of intracellular oxidative stress happens before cell injury, irrespective of the cause [19]. Therefore, plants have developed a defense system of antioxidants against oxidative stress [20]. An antioxidant is a molecule or compound that slows down, stops, or reverses cellular injury caused by oxidative stress [21]. There are two types of antioxidants which are enzymatic and non-enzymatic antioxidants. AA is a non-enzymatic antioxidant. It is the most important intracellular protection the plant uses against oxidative stress. It defends the plant by scavenging O_2, $O_2^{\square}$, ·OH, and regenerating α-tocopherol from the tocopherol radical [22]. AA has been shown to improve the regrowth of cryopreserved meristems by alleviating the damage of cells caused by ROS-induced oxidative stress [9]. However, to preserve the cell there must be a balance between intracellular oxidation and antioxidants. Cells can be damaged by both excessive oxidation and antioxidation (19).

The objective of this study, therefore, is to examine the effect of different concentrations of AA added at the regrowth stage of the cryopreservation process of seeds of Kenaf, determine the best AA concentration for the two common vitrification procedures (PVS 3 and PVS 4) on germination of cryopreserved Kenaf seeds, and generally compare the two different cryopreservation protocols for effective storage of Kenaf seeds.

Materials and Methods

Study location and source of explant

This study was carried out in the Biotechnology Laboratory, Department of Agronomy, University of Ibadan. Kenaf seeds (cv. *Ifeken*) were obtained from the Institute of Agricultural Research and Training (IAR and T), Ibadan, and kept in a dry paper bag and then transferred into a plastic container in the 3°C refrigerator till the time of use.

Growth room conditions

Cultured plants were kept in the growth room with an average temperature of 25°C ± 1, relative humidity of 85%, photoperiod of 16hr light/8hr darkness, and light intensity of 25µmols^{-1}m^{-1} provided by cool white fluorescent bulbs. These growth room conditions were maintained as the standard throughout the entire study.

In vitro culture technique

Sterilization of plant materials, equipment, and glass wares

Seeds were sterilized following a modified version of the IITA standard operating procedure for *in vitro* genebank [24]. Explants were kept in 70% ethanol solution for 5 min, after which it was transferred to 35% sodium hypochlorite solution for 20 min. It was then rinsed in sterile water three times before moving into 35% sodium hypochlorite solution for 10 min. Explants were rinsed again in sterile water three times and kept in a sterile container. Seeds were transferred from one container to the other using sterilized forceps.

Seed culture was carried out in the laminar airflow cabinet under aseptic conditions. A horizontal laminar flow cabinet (JICA Technical Cooperation, Matsunga Manufacturing Company Limited, Tokyo, Japan) with High-Efficiency Particulate Arrestance or HEPA filter was used. Paper towels wet with 70% ethanol were used to sterilize the cabinet surface intermittently.

Surgical instruments, glass wares, and paper towels were autoclaved at 121°C and 15 psi for 15 min. The scalpels and forceps were frequently dipped into 70% ethanol and flamed to maintain sterility.

Culture media compositions

The media consisted of MS [25] inorganic salts (4.43g/L), agar (8g/L), myo-inositol (0.1g/L), sucrose (30g/L), and distilled water.

Preparation of the regrowth culture media

The regrowth culture media was prepared by mixing MS, sucrose, myo-inositol, and ascorbic acid (AA) in a beaker with distilled water. The water-soluble and acidic AA was added to the solution before mixing with the magnetic flea and adjusting the pH to 5.8. The agar was then added and media melted in the microwave oven for 30 mins after which it was dispensed into local or Bama bottles and autoclaved for 15 min at 121°C and 15 psi. The control experiment media was prepared following the same procedure but without AA.

Preparation of stock solutions.

Stock solutions of plant vitrification solution 3 (PVS 3), and plant vitrification solution 4 (PVS 4) as well as the rinse solution were prepared following the procedure described by [26] Sakai., *et al*. The solutions were then adjusted to pH 5.8 before autoclaving for 15 mins at 121°C and 15 psi. The ascorbate stock solution of 1mg/ml concentration was prepared by dissolving 0.3g of the salt in a few drops of water before making up to 300 ml with water. All stock solutions were kept in the refrigerator at 3°C till the time of use.

Preliminary experiment

Five samples of seeds were each kept in a PVS 3 solution for 10, 20, 30, 40, 50, and 60 min prior to culturing. The control was cultured without treatment with the cryoprotectant. The experiment had three replicates, and the optimal time for the seeds to be treated with cryoprotectant before exposure to LN was determined to be 30mins by counting the number of survivals after exposure to the cryoprotectant mixture. The culture medium was prepared following the procedure described above but without AA.

The main experiments

Cryopreservation of kenaf seeds using PVS 3 and PVS 4

The seeds were cryopreserved by modifying the vitrification procedure of [27] Sakai and Engelmann. Briefly, the sterilized seeds were placed in a 2-ml cryotube and PVS 3 solution was added. The cryovials with samples and PVS 3 were shaken and allowed to stand for 30 min. The cryovials were plunged into the LN for 1 min after which they were thawed by rapidly transferring into a water bath of 40°C. The cryotubes were stirred while in the water bath for 1.5 min after which the PVS 3 solution was drained from the cryotubes using a micropipette and immediately replaced with 2 ml basal culture medium supplemented with 1.2 M sucrose [28]. Explants were kept in this solution for 1 min after which they were transferred to the regrowth medium. The same procedure was repeated using PVS 4 solution.

The experimental layout

It was a 2 x 3 factorial experiment laid out in a completely randomized design with three replicates. The treatments consisted of different concentrations of AA: T_0 is 0 mM (control), T_1 is 0.14 mM, T_2 is 0.28 mM, T_3 is 0.43 mM, and T_4 is 0.58 mM. These concentrations were chosen based on the literature [23]. They were added to the regrowth media. Each treatment consisted of 20 seed samples (plunged into LN) and a sub-control that consisted of 20 seed samples (not plunged into LN).

Growth assessment

The survival based on the unchanged morphological appearance of the seeds after treatments was assessed two days after culture. Germination was determined based on the emergence of plumule and or radicle seven days after culture.

Data collection and statistical analysis

Data collected were subjected to analysis of variance (ANOVA) using GENSTAT statistical package Discovery Edition 4 while the least significant difference was used to separate treatment means at $P \leq 0.05$. The PVS 3 experiments were analyzed separately from the PVS 4 experiment as they were two different protocols before combination for comparison purposes.

Results

Effects of ascorbic acid on the survival and germination of cryopreserved seeds.

Survival of kenaf seeds following cryopreservation.

There was no significant difference among AA concentrations regarding the survival of cryopreserved seeds (Table 1). The percentage survival of seeds increased with an increase in the concentration of AA up to an optimal of 0.43 mM (38.3%) when pretreated with PVS 3 and then declined. The unfrozen PVS 3 controls all recovered above 50%. An increasing trend was also observed with PVS 4 treatment. Survival increased from 36.7% to 63.3% with an AA treatment of 0.28 mM.

Germination of Kenaf seeds following cryopreservation.

Data on germination of cryopreserved Kenaf seeds pretreated with PVS 3 was less than 40% survival and all died soon afterward (data not shown). Generally, for the seeds pretreated with PVS 4, the addition of AA to the regrowth media had little or no significant effect on the germination of Kenaf seeds after cryopreservation (Table 2). Exogenous AA (0.58 mM) increased the percentage of non-cryopreserved Kenaf seeds germination by about 10% compared to the control (Table 2). Among the AA treatments, the 0.58 mM AA increased germination the most by 10-13% for cryopreserved seeds (Figure 1) and up to 19% for non-cryopreserved seeds.

Direct comparison of the effectiveness of PVS 3 and PVS 4 as plant vitrification solutions for cryopreservation of Kenaf seeds.

The percentage survival of cryopreserved Kenaf seeds pretreated with PVS 4 (55.3%) was more than the survival of cryopreserved Kenaf seeds pretreated with PVS 3 (31%) as shown in table 3. Based on these broad comparisons, it appears that PVS 4 is more suitable for the cryopreservation of Kenaf seeds than PVS 3.

Ascorbic acid (mM)	PVS 3 Survival (%)	PVS 4 Survival (%)
0 (Control)	23.3 ± 3.33	36.7 ± 8.82
0.14	25.0 ± 2.89	56.7 ± 8.82
0.28	33.3 ± 8.82	63.3 ± 3.33
0.43	38.3 ± 1.67	56.7 ± 12.02
0.58	35.0 ± 5.77	63.3 ± 14.53

Table 1: Mean percentage survival in cryopreserved seeds pretreated with PVS 3 and PVS 4 and subjected to different concentrations of AA at the Regrowth Stage.

PVS 3: LSD of AA = 0.2654; LSD of LN = 0.1679; CV = 190.4%.

PVS 4: LSD of AA = 0.3606; LSD of LN = 0.2281; CV = 146.5%.

Ascorbic acid (mM)	Germination (%)	
	Liquid Nitrogen	
	+	-
0 (Control)	83.3 ± 16.67	88.9 ± 5.56
0.14	77.4 ± 12.43	81 ± 13.23
0.28	75.4 ± 16.97	83.3 ± 9.62
0.43	74.2 ± 12.94	95.8 ± 4.17
0.58	87 ± 6.68	100 ± 0.00

Table 2: Mean percentage germination in cryopreserved and non-cryopreserved seeds pretreated with PVS4 and subjected to different concentrations of AA at the regrowth stage.

Survival: LSD of AA = 0.3606; LSD of LN = 0.2281; CV = 146.5%; +, subjected to LN; -, not subjected to LN.

PVS Treatments	Survival (%)
0 (Control)	36.7
PVS 3	31.0 ± 2.50b
PVS 4	55.3 ± 4.67a

Table 3: Direct comparison of mean survival after PVS 3 and PVS 4 treatments on cryopreserved Kenaf seeds.

LSD of PVS = 0.2265; CV = 204.2%.

Discussion

Cryopreservation induces the formation of ROS whose activities impose stress on the plant. This oxidative stress (OS) is the principal cause of poor growth or death in cryopreserved plants processed for germplasm conservation [29]. The addition of an antioxidant to the regrowth media following cryostorage can help greatly reduce the damage resulting from oxidative stress [30]. This study provided the first report on the cryopreservation of Kenaf seeds

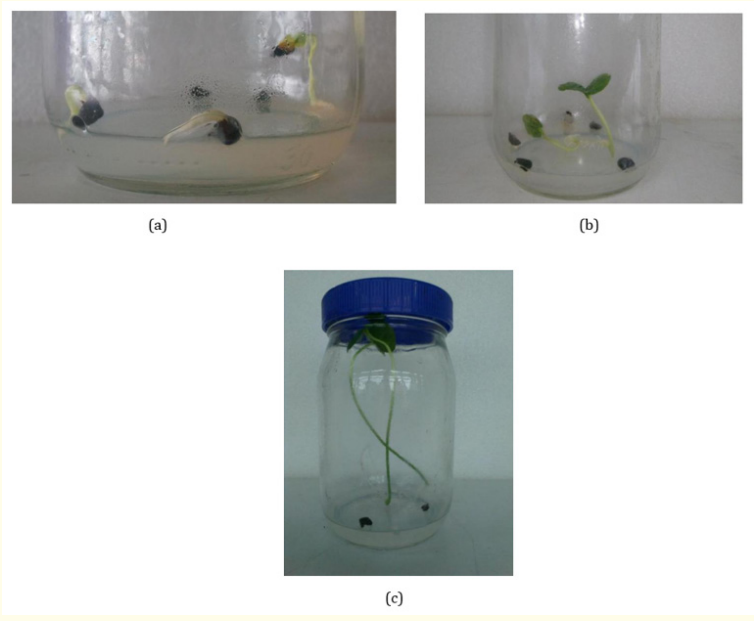

Figure 1: (a) Cryopreserved seed with emerging radicle at day 2 after liquid nitrogen. (b) Cryopreserved seeds at Day 7 after liquid nitrogen. (c) Typical Kenaf plant at day 10 after cryopreservation.

(Figure 1c). The results of this study indicate that the optimal concentrations of AA required for the cryopreservation of Kenaf seeds are 0.28 or 0.43 mM depending on the cryoprotectant used. These results suggest that AA had a beneficial effect on the cryopreservation of Kenaf (Figure 1a) using both cryopreservation protocols (PVS 3 and PVS 4). However, the addition of AA to the regrowth media generally did not significantly increase survival compared to the control. It also did not significantly improve the germination percentages following cryopreservation (Figure 1b).

The cryopreservation process involves a pretreatment phase (preventive stage) and a post-thaw recovery phase (Curative stage). Identifying the critical phase where the most stress or damage occurs is critical to solving the problems of low survival or germination of germplasm following cryopreservation, especially in the case of Kenaf which appears to lose viability easily. Chen., *et al.* [31] reported that AA is a major antioxidant that scavenges OH• and H_2O_2. This was also established by Polle [32] who observed that a decrease in AA resulted in an increase in H_2O_2, thus, AA can be suitably used to increase the recovery percentages following low temperature-induced stress or damage. AA also stops radical chain reactions by disproportionation to non-toxic or non-radical products. It regenerates lipophilic, membrane-associated α-tocopherol (vitamin E) which also helps to alleviate oxidative stress [33]. Uchendu., *et al*. [34] showed that AA (0.28 mM) significantly increased the recovery of cryopreserved blackberry species. Chua S. P. and Normah M. N. [35] also reported that AA when added at 0.28 mM to the loading solution improved the survival of *Nephelium ramboutan-ake* after cryopreservation. Fabian., *et al*. [36] found that the addition of AA to the vitrification solution increased the viability of wheat egg cells after cryopreservation. Further studies are recommended to determine the optimum concentration of AA required for increased survival and germination of Kenaf seeds. Also, the addition of AA at all the critical stages of cryopreservation other than the regrowth media should be examined.

The vitrification technique is generally used for the cryopreservation of tropical plant species because they do not have a natural tolerance to cold. It involves dehydrating the explants in a plant vitrification solution to optimum moisture content such that the remaining moisture in the cytosol is vitrified when the explant is immersed into LN [37,38]. This study showed that PVS 4 was more suitable as a cryoprotectant for the cryopreservation of Kenaf seeds compared to PVS 3 (Table 2). PVS 3 has a higher concentration of glycerol (5.43M) than PVS 4 (3.8M). Glycerol is highly viscous and may be toxic to plant tissues depending on the concentration and plant tolerance.

Vitrification results in the formation of metastable glasses rather than ice crystals which can cause damage to the cell resulting in the death of the cell. The glasses are amorphous, non-crystalline, and lack organized structure, and as such, cause minimal in-

jury to the cell [38]. A critical factor that determines the success of cryopreservation by vitrification is the use of the most appropriate vitrification solution [39]. Also, a cryopreservation protocol is considered suitable for germplasm if it ensures a minimum of 40% survival after cryopreservation [39]. The PVS 3 gave an average survival of less than 40% for all cryopreserved seeds (Table 4.1) and the germination rate was also less than 40% with all the seeds dead soon afterward due to contamination (data not shown), making it unsuitable for cryopreservation of Kenaf seeds. However, PVS 4 gave an average survival of 55.3% which makes it more suitable than PVS 3.

Conclusion

This study provided the first report on the cryopreservation of Kenaf seeds which makes it easy to manage the problems of loss of viability of the seeds during storage. The results of this study indicated that the survival of Kenaf seeds increased following the addition of AA into the regrowth media and that PVS 4 is more effective for the cryopreservation of Kenaf seeds than PVS 3. Hence, PVS 4 is recommended for the cryopreservation of Kenaf seeds. Further investigations need to be carried out at the other critical stages of cryopreservation, including preculture, loading, unloading, etc., and a wider range of AA using the PVS 4 protocol to explore increasing survival and germination percentages of Kenaf seeds.

Acknowledgements

I appreciate my parents, Mr. and Mrs. Olabisi, for the financial support, and my siblings 'Tope and Tomi for the sacrifices, love, and support that brooked no denial.

I appreciate my colleague Akeem Nofiu's for his assistance with data analysis and Adenike Dada for the review.

Bibliography

1. Ogunniyan DJ. "Assessment of genetic divergence in kenaf (*Hibiscus cannabinus* L.) Genotypes using agro-botanical characteristics and multivariate analysis". *Sabrao Journal of Breeding and Genetics* 48.1 (2016): 61-71.

2. Alexopoulou E., *et al.* "Kenaf booklet". Prepared in the framework of the BIOKENAF project QLK5 CT2001 01729 (2007).

3. Fried R. "Kafus environmental industries: Producing forestry products without trees". *Corporate Environmental Strategy* 6.4 (1999): 378-386.

4. Lee C. "Kenaf". *Cooperative Extension Service* (2014): 1-2.

5. Liu Y and Labuschagne MT. "The influence of environment and season on stalk yield in kenaf". *Industrial Crops and Products* 29.2-3 (2009): 377-380.

6. Bada BS and Raji KA. "Phytoremediation potential of kenaf (*Hibiscus cannabinus* L.) Grown in different soil textures and cadmium concentrations". *African Journal of Environmental Science and Technology*, 4.5 (2010): 250-255.

7. Kaldor AF., *et al*. "Kenaf - a fast-growing fiber source for papermaking". *Tappi Journal* 73.11 (1990): 205-209.

8. Duke JA. "*Hibiscus cannabinus* L. Malvaceae Kenaf, Bimli, Bimlipatum, Jute, Deccan hemp". In: Handbook of Energy Crops (1983).

9. Pritchard HW. "Cryopreservation of Seeds". *Methods in Molecular Biology* 38 (1995): 133-144.

10. Toole EH., *et al.* "Preservation of Hemp and Kenaf Seed". *United States Department of Agriculture Techinal Bulletin*, Issue 1215. Washington, DC (1960).

11. Webber CL., *et al.* "Kenaf harvesting and processing". *Trends in New Crops and New Uses*. ASHS Press, Alexandria, VA (2002): 340-347.

12. Balešević-Tubić S., *et al*. "Seed viability of oil crops depending on storage conditions". *Helia* 52 (2014): 153-159.

13. Engelmann F. "Plant cryopreservation: Progress and prospects". *In Vitro Cellular and Developmental Biology - Plant* 40.5 (2004): 427-433.

14. Hu WH., *et al.* "Cryopreservation of the seeds of a Taiwanese terrestrial orchid, *Bletilla formosana* (Hayata) Schltr. by vitrification". *Botanical Studies* 54.1 (2013): 1-7.

15. Engelmann F. "Use of Biotechnologies for the Conservation of Plant Biodiversity". *In Vitro Cellular and Developmental Biology-Plant* 47 (2011): 5-16.

16. Mazur P. "Freezing of living cells: mechanisms and implications". *The American Journal of physiology* 247 3 Pt 1 (1984): C125-42.

17. Fahy GM., *et al*. "Vitrification as an approach to cryopreservation". *Cryobiology* 21 (1984): 407-426.

18. Sakai A. and Engelmann F. "Vitrification, Encapsulation-Vitrification and Droplet-Vitrification: A Review". *CryoLetters* 28 (2007): 151-172.

19. Dalian D., *et al*. "Antioxidative stress-induced damage in cochlear explants". *Journal of Otology* 15.1 (2020): 36-40.

20. Gill SS and Tuteja N. "Reactive Oxygen Species and Antioxidant Machinery in Abiotic Stress Tolerance in Crop Plants". *Plant Physiology and Biochemistry* 48 (2010): 909-930.

21. Halliwell B. "Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life". *Plant Physiology* 141. 2 (2006): 312-322.

22. Şen A. "Oxidative Stress Studies in Plant Tissue Culture". *IntechOpen* (2012): 10.

23. Uchendu EE. "Cryopreservation of shoot tips: Antioxidant investigations with rubus and protocols for mentha and vaccinium". Ph.D. Thesis, Dept. of Hort. Oregon State Univ. USA (2009).

24. Badara G., *et al.* "Standard Operation Procedures (SOP) for IITA *in vitro* genebank". *International Institute of Tropical Agriculture*, Nigeria (2012): 1-59.

25. Murashige T and Skoog F. "A revised medium for rapid growth and bioassays with tobacco tissue cultures". *Plant Physiology* 15 (1962): 473-497.

26. Sakai A., *et al*. "Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis tanaka*) by vitrification". *Plant Cell Reports* 9 (1990): 31-38.

27. Sakai A and Engelmann F. "Vitrification, Encapsulation-Vitrification and Droplet-Vitrification: A Review". *CryoLetters* 28 (2007): 151-172.

28. Sakai A. "Conservation of plant genetic resources *in vitro*. General Aspects". *Science Publishers*, MKR Ed. 1 (1997): 53-66.

29. Benson EE. "Free radical damage in stored plant germplasm". *International Board for Plant Genetic Resources*, Rome (1990).

30. Reed BM. *et al.* "Are antioxidants effective for reducing oxidative stress during cryopreservation?" First International Symposium for In Vitro Conservation and Cryopreservation, Mexico (2012).

31. Chen G., *et al.* "Cryopreservation affects ROS-induced oxidative stress and antioxidant response in *Arabidopsis* seedlings". *Cryobiology* 70.1 (2015): 38-47.

32. Polle A. "Dissecting the superoxide dismutase-ascorbate-glutathione-pathway in chloroplasts by metabolic modeling. Computer simulations as a step towards flux analysis". *Plant Physiology* 126 (2001): 445-462.

33. Valpuesta V and Botella MA. "Biosynthesis of L-ascorbic acid in plants: New pathways for an old antioxidant". *Trends in Plant Science* 9.12 (2004): 573-577.

34. Uchendu E. E., *et al.* "Antioxidant and anti-stress compounds improve regrowth of cryopreserved *Rubus* shoot tips". *In Vitro Cellular and Developmental Biology - Plant* 46.4 (2010): 386-393.

35. Chua SP and Normah MN. "Effect of preculture, PVS 2 and vitamin C on survival of recalcitrant *Nephelium ramboutan-ake* shoot tips after cryopreservation by vitrification". *Cryoletters* 32.6 (2011): 506-515.

36. Fabian A., *et al*. "Cryopreservation of wheat (*Triticum aestivum* L.) Egg cells by vitrification". *Acta Physiologiae Plantarum* 30.5 (2008): 737-744.

37. Benson EE., *et al.* "Physical and engineering perspectives of *in vitro* plant cryopreservation". *Plant Tissue Culture Engineering*. Dutta Gupta S, Ibaraki Y (Eds.). Springer, Netherlands (2008): 441-473.

38. Day JG., *et al*. "Cryopreservation: Conservation of bioresources at ultra-low temperatures". *Molecular Biomethods Handbook.* Walker, J. M., and Rapley, R. (Eds.). Humana press, New Jersey (2008): 917-947.

39. Dussert S., *et al.* "Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections". *CryoLetters* 24 (2003): 149-160.