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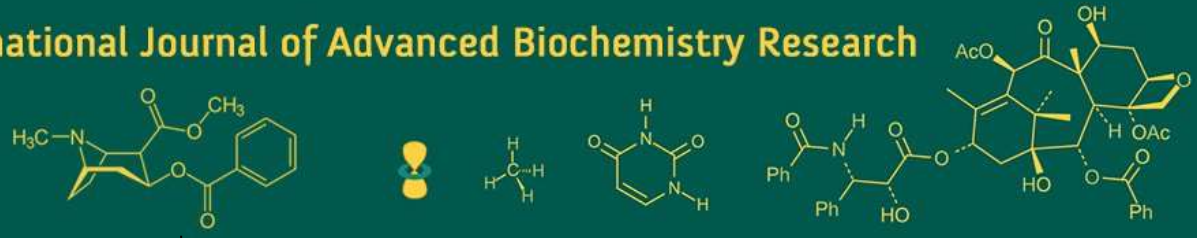

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Unravelling the influence of osmotic agents on *in vitro* conservation, regeneration and genetic fidelity in Banana cv. Nanjanagud Rasabale (AAB)

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Abstract
Nanjanagud Rasabale (AAB) is a well-known mostly farmed cultivar that has been given a GI (Geographical Indication) tag for its provenance, distinctive flavour, aroma, colour, fibrous texture and nutritional value. Due to its great susceptibility to the soil-borne panama wilt disease, which is brought on by *Fusarium oxysporum* f.sp. cubense, this leading cultivar is currently failing to maintain its GI designation. The present investigation was carried out on *in vitro* conservation by media manipulation using osmotic agents through slow growth culture technique. Shoot tip explants were cultured on MS basal medium supplemented with osmotic agents (Mannitol, Sorbitol, Sucrose and Maltose). Among different osmotic agents treated media effective storage (145.66 days) was recorded in sorbitol 35 g/L. Determination of genetic stability was performed by assessment of Inter Simple Sequence Repeat amplification (ISSR). All the 15 primers amplified unambiguous, readable and showed monomorphic bands. The cultures were conserved by extending the subculture period and prolonged storage period without any genetic variation by using osmotic agents.

Keywords: Banana, osmotic agents, *in vitro*, conservation

1. Introduction
Banana (*Musa* sp.) stands as one of the ancient fruits known to mankind, with its ancient roots tracing back to the garden of paradise, earning the nickname "Apple of Paradise." It has been a staple food in the diets of sages since time immemorial. In terms of trade, it holds the title of the world's most crucial fresh fruit commodity. It was the first plant cultivated, the inaugural food consumed by humans and the primary fruit introduced to infants. It has flourished due to its adaptability to diverse agro-climatic conditions and its resilience to climate changes.

From a botanical perspective, banana is a monocotyledonous, monocarpic giant herbaceous plant categorized under the section Eumusa in the family Musaceae, belonging to the order Zingiberales. It possesses chromosome numbers of 22, 33 and 44. The centre of origin is South-East Asian countries, particularly eastern Malaysia (Albany *et al*., 2005) [1]. Recognized as an ancient fruit crop, which is consumed in various forms, including fresh, cooked, steamed, roasted and brewed.

Nanjanagud Rasabale cultivar classified as AAB within the Silk sub-group, gained renown for its distinct characteristics, including its origin, unique taste, aroma, colour, fibrous texture and nutritional content. Recognizing these qualities, it received a Geographical Indication (GI) tag in 2005 under the Goods (Registration and Protection) Act, 1999. Originally a prominent cultivar in Mysore district, Karnataka, India it has struggled to regain its GI tag due to a significant drawback: high susceptibility to the soil-borne Panama wilt disease, attributed to *Fusarium oxysporum* f. sp. cubense. The symptoms manifest just before the flowering stage, marked by yellowing and withering leaves, stem base splitting and root rot, ultimately leading to the plant's demise. Consequently, there is an imperative need to conserve this cultivar to prevent its extinction (Babu, 2019) [2].

The conservation of germplasm can be conducted through in situ methods (within the natural habitat) or ex situ methods (outside the natural habitat). In situ conservation, poses significant challenges such as the need for extensive space, high operational costs, intricate

management and susceptibility to damage from both biotic and abiotic factors, especially in the face of changing climate conditions. Consequently, the risks associated with maintaining plants in the field have prompted the exploration of secure and efficient *in vitro* protocols for the effective conservation of plant germplasm (Pandey *et al*., 2015) [23]. Among, the various conservation methods available for banana germplasm, *in vitro* conservation continues to hold advantages over alternative approaches. *In vitro* preservation allows for the protection of genetic material within controlled conditions, facilitating sterile plant propagation, production of disease-free plantlets, secure and convenient global or local exchange of genetic resources and a comparatively lower cost of conservation. The process of *in vitro* conservation involves inducing slow growth conditions. The objective of slow or minimal growth is to reduce the frequency of sub-culturing to a level that does not negatively impact the stability of the genetic material or the regenerated plants. This conservation approach employs various techniques, one of which involves altering the composition of the medium through the use of osmotic agents. This modification leads to the inhibition of cell division, thereby restricting both callus formation and shoot development. These methods of slow growth enable the conservation of plants for several months to years under aseptic conditions (Engelmann, 2011) [11]. Throughout the process of *in vitro* conservation, plants are subjected to various stresses, encompassing physico-chemical and physiological factors, potentially causing a loss of genetic stability in the conserved plants.

The most precise methods for evaluating variability in plantlets involve the use of morphological and molecular markers. Early detection of somaclonal variants holds significant value for ensuring quality control in germplasm conservation efforts. A meticulous examination of the morphology of plantlets raised *in vitro* is crucial for distinguishing between normal plants and somaclonal or epigenetic variants (Sandoval *et al*., 1997) [26]. Consequently, the present study was conducted with the aim of establishing a standardized *in vitro* conservation protocol for the endangered banana cultivar, Nanjanagud Rasabale.

2. Materials and Methods

The current study was conducted at Centre for Horticultural Biotechnology Research, Department of Biotechnology and Crop Improvement, College of Horticulture, Bagalkot, Karnataka, India.

2.1 Plant material

The explant chosen for the experiment was shoot tips due to their ability to withstand genetic variability. Stem bases were gathered from healthy sword suckers sourced from the mother block of cv. Nanjanagud Rasabale cultivated in Nanjanagud Taluka of the Mysore district, Karnataka. The macro stem-cuttings underwent standard processing procedures to extract the meristem, as outlined below.

2.2 Establishment of aseptic culture

The explants were rinsed under flowing tap water and subsequently immersed in a detergent solution for 5 minutes. The stem disc was then precision-cut using a scalpel and placed in a pre-treatment solution containing 0.50% carbendazim + 0.05% streptocycline sulphate for 8 hours, followed by soaking in a 1% carbendazim solution for 30 minutes. This was succeeded by two washes with sterilized double-distilled water. The explants were then moved to a laminar air-flow environment and stirred in 0.05% cetrimide for 30 minutes, followed by 2-3 times double distil water wash.

The explants underwent surface sterilization using 0.10% mercuric chloride for 10 minutes. After removing the outer scales, they were thoroughly rinsed 5-6 times with sterilized double-distilled water and air-dried on a cushion of sterilized tissue paper. Shoot tip explants were cultivated for two weeks under standard culture conditions (25±1 ℃, 70% RH and a photoperiodic cycle of 16/8 hours light and dark). The greening and swelling observed in the explants indicated the successful establishment of the cultures [Fig. 1]. Subsequently, the discoloured tissues were trimmed and the explants were transferred to glass jars containing an *in vitro* conservation medium consisting of osmotic agents (Mannitol, Sorbitol, Sucrose, Maltose) based on the specified treatment details.

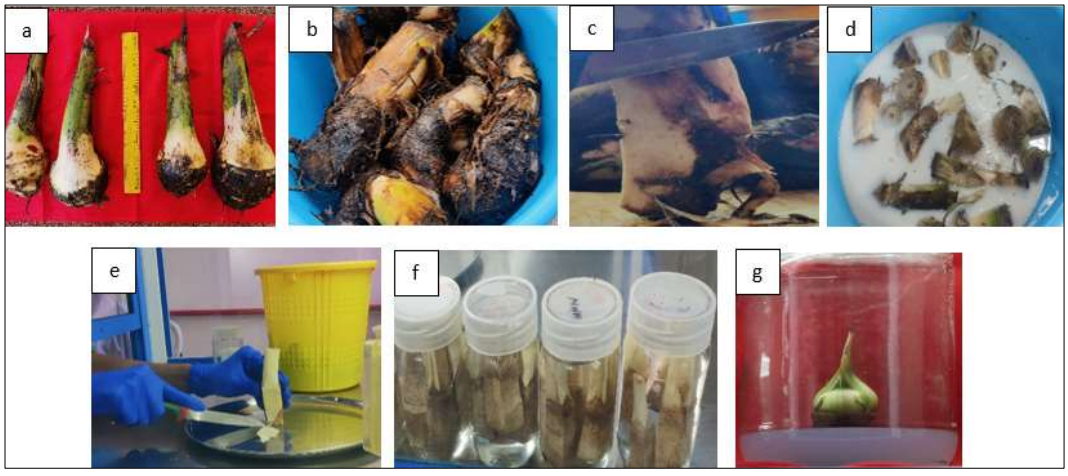

Fig 1: Establishment of aseptic culture in banana cv. Nanjanagud Rasabale (AAB): a) Sword sucker. b) Washing with water and tween 20. c) Removal of roots and outer leaf sheaths. d) Treatment with 0.5% carbendazim and 0.05% streptocyclin. e) Reducing explant size in laminar air flow. f) Treatment with 0.10% mercuric chloride. g) Established cultures.

2.3 Regeneration of the conserved cultures

Aseptic shoot tip cultures preserved in a sterile environment were regenerated following the protocol established by Guranna *et al*. (2017) [15]. Subsequently, the cultures were placed in a growth chamber with a temperature maintained

at 25±1 ℃, RH of 70±2% and a photoperiodic cycle of 16/8 hours of light and dark.

2.4 Genetic fidelity test of regenerated plantlets

The plants that underwent regeneration were evaluated for genetic stability through the utilization of morphological characteristics and ISSR markers. A total of 15 ISSR primers were chosen for screening and the reliability of PCR amplification was examined by employing selected primers with diverse DNA samples. The DNA samples were isolated using the Cetyl Trimethyl Ammonium Bromide (CTAB) method as outlined by Gawel and Jarret in 1991 [13].

2.5 Statistical analysis

The experimental statistical analysis employed a Completely Randomized Design (CRD) and was conducted using Wasp 2 statistical software (ICAR-Research Complex, Goa). Prior to subjecting the percentage data to ANOVA, an Arc Sin transformation was applied. Decimal data underwent transformation using the square root method (Gomez and Gomez 1984) [14].

Results and Discussion

When osmotic agents like sorbitol, mannitol, sucrose and maltose are used for *in vitro* conservation, the water potential of the medium is decreased, reducing the amount of water availability and as a result, the cell's ability to absorb nutrients and carry out metabolic activities is restricted (Huang *et al*., 2014) [17]. An ideal osmotic agent would be non-toxic, non-penetrating yet reducing the osmotic potential of the medium (Bundig *et al*., 2016) [5]. There are various benefits to this conservation technique, including longer intervals between subcultures, lower expenses to maintain the germplasm bank and a lesser risk of somaclonal variation (Chauhan *et al*., 2016) [7].

Among different osmotic agents used for *in vitro* conservation of banana cv. Nanjanagud Rasabale, sorbitol (35 g/L) treated media showed the highest storage period [Table 1, Fig. 2]. Bud sprouting was not observed, but no mortality and no abnormalities (senescence or browning) with reduction in growth rate. At the end of the storage period tip browning (145.66 days) was observed in the cultures. It helped in extending storage period approximately for 20 weeks. Media exhaustion is directly proportional to storage period. Hence, days taken for media exhaustion was highest (126.33 days) in sorbitol supplemented conservation media. These observations are in conformity with the findings of Edrisinghe *et al*., 2017 in banana cv. Ambar, Rath Kesel and Pulathisi. Increase in acyclic polyols such as sorbitol have been observed in response to stress in many plants. These compounds can act as osmoregulators as well as oxygen radical scavengers and reduce the growth of the plants. The authors hypothesized that sorbitol function might go beyond those of a carbon supply and as an osmotic regulator. It was proposed that sorbitol might affect molecular and physiological processes through chemical signaling (Feng *et al*., 2011) [12].

Table 1: Effect of osmotic agents on culture storage period in banana *cv.* Nanjanagud Rasabale (AAB).

Treatment	Culture storage period (days)
T_1 : MS B* (Control)	39.70
T_2: MS B + Mannitol 20 g/L	89.30
T_3: MS B + Mannitol 25 g/L	96.30
T_4: MS B + Mannitol 30 g/L	124.33
T_5: MS B + Sorbitol 25 g/L	107.33
T_6: MS B + Sorbitol 30 g/L	113.66
T_7: MS B + Sorbitol 35 g/L	145.66
T_8: MS B + Sucrose 50 g/L	76.26
T_9: MS B + Sucrose 55 g/L	81.33
T_{10}: MS B + Sucrose 60 g/L	93.30
T_{11}: MS B + Maltose 30 g/L	61.33
T_{12}: MS B + Maltose 45 g/L	68.66
T_{13}: MS B + Maltose 60 g/L	74.50
S. Em ±	1.23
CD at 1%	4.82

MS B*- Murashiage and Skoog basal medium

Fig 2: Effect of osmotic agents on growth parameters of *in vitro* conserved banana cv. Nanjanagud Rasabale. a)MS B* (Control); b) MS B + Mannitol 20 g/l; c) MS B + Mannitol 25 g/l; d) MS B + Mannitol 30 g/l; e) MS B + Sorbitol 25 g/l; f) MS B + Sorbitol 30 g/l; g) MS B + Sorbitol 35 g/l; h)MS B + Sucrose 50 g/l; i) MS B + Sucrose 55 g/l; j) MS B + Sucrose 60 g/l; k)MS B + Maltose 30 g/l; l) MS B + Maltose 45 g/l; m) MS B + Maltose 60 g/l

Media browning was found highest as the concentration of osmotic agent was increased especially in conservation media supplemented with sucrose (55 g/L and 60 g/L) and maltose (45 g/L and 60 g/L) [Table 3]. Media browning occurs through the action of copper containing oxidase enzymes, often called polyphenol oxidases, phenolase and tyrosinase (Lerch, 1981) [20] which are released or synthesized with suitable substrates and oxidative conditions when tissues are wounded or under stress condition. Higher concentration of sucrose and maltose in conservation media drastically reduced hydric potential and water availability was restricted. Hence, this might be possible reason for higher stress condition in cultures which had directly increased phenol exudation and increased media browning. Similar findings were reported by Rukundo *et al.* (2012) [24] in *in vitro* growth of banana plantlets.

Vitrification symptoms were not observed in any of the treatments [Table 2]. As stated earlier factors influencing vitrification are providing less than optimal growth condition (temperature, light, relative humidity), liquid media (Davis *et al*., 1997) [9], nutrient composition (symptoms occurs on high nutrient concentrated media than that of low concentration media), lack of oxygen, pH of the medium (Buds of begonia became glassy when pH of medium was adjusted to 4 but the symptoms did not occur when the medium was adjusted to 5) (Berghoef and Bruinsma, 1979) [3]. In case of osmotic agent increase in concentration in a medium solidified with 1% agar had same effect as increasing agar concentration. Similar findings were reported by Langford and Wainwrigth (1987) [18] that glassiness was rare in rose shoot tip cultures grown in MS medium with 40 g/L sucrose, but increased as the concentration of sucrose decreased.

Among the different treatments the highest per cent of contamination (11%) was registered in treatment media maltose (30 g/L) [Table 2]. As stated, earlier tissue culture plants may be contaminated by fungi including yeasts, bacteria and bacteria-like organisms (Herman, 1990) [16] (Cassells, 2000) [6]. Most of the problems caused by contamination of cultures are due to the use of inefficient methods such as explants (high microbial load) which have been taken from: (1) plant tissues exposed to or near to soil; (2) plants grown in tropical climates in the field; or (3) overhead irrigated stock plants. Inappropriate surface sterilization protocol, aseptic handling of cultures and the laboratory environment (especially the cleanliness of floors, work surface inside the laminar flow cabinets and the laboratory air) has also been shown to affect the rate of contamination (Leifert, 1990) [19]. Inappropriate sterilization and preparation of media along with some heat resistant Bacillus species have been reported to survive even after autoclaving (121 ℃ 20 min) the growth media. Higher concentration of carbohydrates (osmotic agents) in media also increases contamination mainly fungal growth is observed (Odutayo *et al*., 2007) [22].

Table 2: Effect of osmotic agents on microbial contamination, media exhaustion, vitrification and media browning in banana cv. Nanjanagud Rasabale (AAB)

Treatment	Per cent contamination	Days taken for media exhaustion	Vitrification	Media browning
T_1 : MS B* (Control)	5.33 (13.34) *	28.83	-	2
T_2: MS B + Mannitol 20 g/L	0.00 (1.17)	71.40	-	1
T_3: MS B + Mannitol 25 g/L	0.00 (1.17)	79.00	-	1
T_4: MS B + Mannitol 30 g/L	0.00 (1.17)	96.30	-	1
T_5: MS B + Sorbitol 25 g/L	0.00 (1.17)	88.26	-	1
T_6: MS B + Sorbitol 30 g/L	5.33 (13.34)	90.90	-	1
T_7: MS B + Sorbitol 35 g/L	0.00 (1.17)	126.33	-	1
T_8: MS B + Sucrose 50 g/L	5.33 (13.34)	61.40	-	1
T_9: MS B + Sucrose 55 g/L	5.33 (13.34)	68.93	-	2
T_{10}: MS B + Sucrose 60 g/L	0.00 (1.17)	82.33	-	2
T_{11}: MS B + Maltose 30 g/L	11.00 (19.35)	49.90	-	1
T_{12}: MS B + Maltose 45 g/L	5.33 (13.34)	50.20	-	2
T_{13}: MS B + Maltose 60 g/L	0.00 (1.17)	69.16	-	2
S. Em ±	0.29	0.64		
CD at 1%	1.17	2.52		

Note: MS B*- Murashiage and Skoog basal medium

Visual scoring (Vitrification): (+) Presence, (-) Absence

Visual scoring (Media browning)- 0: No browning, 1: Browning only at explant base, 2: Browning extended beyond explant base, 3: Complete browning of the medium

Analysis of genetic stability of regenerated plantlets using morphological and molecular markers

Genetic variation poses a significant concern in micropropagation due to the elevated risk of variability. The term "somaclonal variation" denotes stable and genetically transmissible variation in plant growth and development (Sandoval *et al*., 1997) [26]. Despite the potential of biotechnology for widespread banana propagation, the challenge lies in the early detection of somaclonal variation before transplanting to the field. To distinguish between regular plants and somaclonal or epigenetic variants, a thorough examination of the morphology of *in vitro* plants is essential.

Healthy and actively multiplying shoots were recovered from slow-growth cultures [Table 3 Fig. 3]. To stimulate growth, these cultures were cultivated in standard regeneration media, as outlined by Guranna *et al*. (2017) [15]. The results indicated successful growth with the observation of a normal morphology. However, there were variations in phenotypic characteristics in the regeneration media. This divergence might be attributed to the lingering influence of osmotic agents from the conservation media. Despite these variations, Orchid demonstrated successful growth with a normal morphology and gradual progression after *in vitro* conservation utilizing osmotic agents (Lopez-Puc, 2013) [21].

Table 3: Analysis of growth parameters and morphology of plantlets regenerated from culture conserved by using osmotic agents

Treatment	Number of shoots per explant	Length of the shoot (cm)	Number of leaves per shoot	Number of roots per explant	Length of the longest roots (cm)	Appearance of plant
T_1 (Control)	1.70	7.12	5.30	0.00	0.00	+
T_2: MS B + Mannitol 20 g/L	1.40	6.90	5.00	4.10	3.05	+
T_3: MS B + Mannitol 25 g/L	1.40	6.58	4.40	4.00	3.00	+
T_4: MS B + Mannitol 30 g/L	1.20	5.92	4.10	4.00	2.94	+
T_5: MS B + Sorbitol 25 g/L	1.40	5.72	4.00	4.10	2.65	+
T_6: MS B + Sorbitol 30 g/L	1.20	5.45	4.10	3.90	2.71	+
T_7: MS B + Sorbitol 35 g/L	1.20	5.36	4.00	3.20	2.32	+
T_8: MS B + Sucrose 50 g/L	1.00	5.22	4.00	3.20	2.22	+
T_9: MS B + Sucrose 55 g/L	1.00	4.90	3.80	3.10	2.19	+
T_{10}: MS B + Sucrose 60 g/L	1.00	4.75	3.70	3.00	2.17	+
T_{11}: MS B + Maltose 30 g/L	1.00	4.82	3.90	3.50	2.18	+
T_{12}: MS B + Maltose 45 g/L	1.00	3.25	3.10	3.20	2.10	++
T_{13}: MS B + Maltose 60 g/L	1.00	3.14	3.00	2.20	2.02	++
S. Em ±	0.13	0.06	0.17	0.17	0.13	
CD at 1%	0.52	0.27	0.70	0.69	0.51	

Note: + = Normal, ++ = Subnormal, +++ = Abnormal

Fig 3: Morphology of plantlets regenerated from cultures conserved using osmotic agents a)MS B* (Control); b) MS B + Mannitol 20 g/l; c) MS B + Mannitol 25 g/l; d) MS B + Mannitol 30 g/l; e) MS B + Sorbitol 25 g/l; f) MS B + Sorbitol 30 g/l; g) MS B + Sorbitol 35 g/l; h)MS B + Sucrose 50 g/l; i) MS B + Sucrose 55 g/l; j) MS B + Sucrose 60 g/l; k)MS B + Maltose 30 g/l; l) MS B + Maltose 45 g/l; m) MS B + Maltose 60 g/l

During *in vitro* conservation, genetic changes may occur leading to a variation that is not identical to the mother plant. The absence of an assessment of morphological polymorphism in plants does not eliminate the possibility of genetic variations; instead, it necessitates a thorough analysis. Accurate identification is particularly crucial in clonally propagated crops like bananas. Various techniques can be employed to monitor *in vitro* raised and conserved plants, with molecular marker techniques being the most reliable. Early detection of potential somaclonal variations is considered vital for quality control in plant tissue culture. Among the numerous molecular markers available, inter simple sequence repeat (ISSR) markers stand out for their cost efficiency and the lower amounts of DNA they require (Zietkiewicz *et al*., 1994) [28]. ISSR markers have been widely utilized to assess the genetic stability of various micropropagated plants, especially in horticultural crops like bananas.

15 ISSR primers were used to assess the genetic fidelity of *in vitro* conserved plants. All 15 primers successfully amplified genomic fragments, generating reproducible bands and produced monomorphic bands across all regenerated plantlets from the conserved cultures [Table 4 Fig. 4]. Consequently, the regenerated plantlets maintained genetic stability even after prolonged conservation on the same media without subculturing. In case of bananas, the level of genetic stability appears to be influenced by the interplay between genotype and tissue culture conditions (Vuylsteke *et al.*, 1991) [27]. These findings align closely with observations in *Populus alba*, as reported by Salim *et al.* (2019) [25].

Table 4: Analysis of genetic stability of *in vitro* regenerated plants of banana cv. Nanjanagud Rasabale using ISSR markers

Primer No.	Nucleotide sequence (5' to 3')	No. of monomorphic bands	No. of polymorphic bands	Per cent Monomorphism	Per cent Polymorphism
UBC 829	ACACACACACACACACG	14	0	100	0
UBC 850	CTCTCTCTCTCTCTCTRC	13	0	100	0
UBC 840	TGTGTGTGTGTGTGTGG	14	0	100	0
UBC 852	GTGTGTGTGTGTGTGTYA	12	0	100	0
UBC 808	AGAGAGAGAGAGAGAGC	14	0	100	0
UBC 864	TCTCTCTCTCTCTCTCRT	13	0	100	0
UBC 815	CTCTCTCTCTCTCTCTG	12	0	100	0
UBC 836	TGTGTGTGTGTGTGTGC	13	0	100	0
UBC 855	ACACACACACACACACCTT	12	0	100	0
UBC 813	CTCTCTCTCTCTCTCTT	13	0	100	0
UBC 821	CACACACACACACACAG	14	0	100	0
UBC 841	GAGAGAGAGAGAGAGAY	13	0	100	0
UBC 899	GAGCAACAACAACAACAA	13	0	100	0
UBC 901	GAGCAACAACAACAACAA	11	0	100	0
UBC 862	AGCAGCAGCAGCAGCAGC	12	0	100	0
Total		193	0	1500	0
Average		12.8	0	100	0

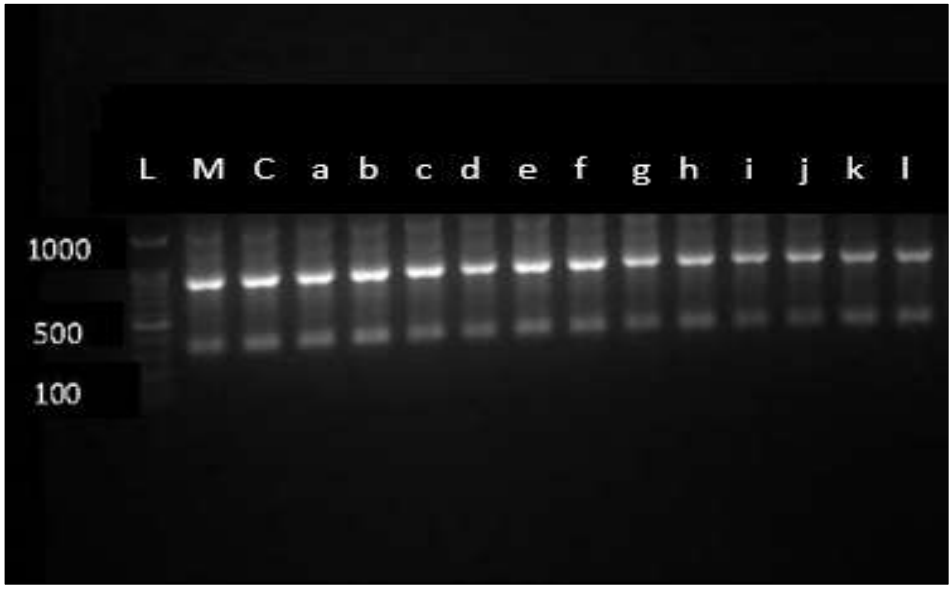

Fig 4: Genetic fidelity test of *in vitro* conserved banana *cv.* Nanjanagud Rasabale (AAB) showing ISSR based DNA amplification pattern on agrose gel electrophoresis for UBC 829. L Ladder. M Mother plant. C Control. a) MS B + Mannitol 20 g/L; b) MS B + Mannitol 25 g/L; c) MS B + Mannitol 30 g/L; d) MS B + Sorbitol 25 g/L; e) MS B + Sorbitol 30 g/L; f) MS B + Sorbitol 35 g/L; g)MS B + Sucrose 50 g/L; h) MS B + Sucrose 55 g/L; i) MS B + Sucrose 60 g/L; j)MS B + Maltose 30 g/L; k) MS B + Maltose 45 g/L; l) MS B + Maltose 60 g/L

Conclusion

To enhance the subculture duration and prolong the storage period in the *in vitro* conservation of banana, the manipulation of media with osmotic agents is suggested. Sorbitol-treated cultures exhibited successful conservation for an extended period of 145.66 days without the need for subculturing. Furthermore, regeneration occurred with normal morphology, establishing sorbitol as the optimal osmotic agent for the *in vitro* conservation of the Nanjanagud Rasabale banana cultivar. Additionally, ISSR markers proved to be valuable for assessing the genetic fidelity of the conserved cultures.

Author contribution

All authors contributed to the study conception and design. The first draft of the manuscript was written by A and all authors commented on previous versions of the manuscript. All authors read and agreed to the published version of manuscript.

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Conflict of Interest Statement

The authors declare that they have no competing interests

Data availability statement

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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