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Preservation of a GI-tagged banana cultivar: Insights from tissue culture and DNA barcoding of Nanjangud Rasabale

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ABSTRACT

Nanjanagudu Rasabale (NRB) is a Geographical Indication (GI)-tagged banana cultivar from Karnataka, known for its unique aroma and taste. The cultivar faced a serious threat of extinction due to a major outbreak of Panama disease. This study aimed to conserve and verify the genetic identity of NRB using tissue culture and DNA barcoding techniques. Shoot tip culture was used to produce disease-free saplings, and DNA barcoding was performed using the ITS2 region as a marker. The tissue culture protocol showed a high rate of micropropagation, enabling large-scale production of healthy plantlets suitable for field planting. However, DNA barcoding alone was not sufficient to distinguish NRB from closely related cultivars, highlighting the need for morpho-molecular analysis and whole genome sequencing for accurate identification. To support conservation, the disease-free saplings were distributed to farmers across different districts of Karnataka, and their contact information has been recorded for follow-up.

Key words: Genome sequencing, DNA barcoding, tissue culture, ITS2, biodiversity conservation.

INTRODUCTION

Nanjanagudu Rasabale (NRB), a unique banana cultivar (*Musa* spp.), is primarily grown in the Mysuru and Chamarajanagar districts of Karnataka, India. It is well known for its distinct aroma and flavor, attributed to the region's black, clayey, saline alluvial soil. Due to these unique qualities, NRB has received Geographical Indication (GI) status from the Department of Horticulture, Government of Karnataka (Application No. 35) (Raghavendra *et al*., 11). Historical records shows its cultivation back to the Mysore Wadiyar dynasty, where it was favoured by the royal family and promoted among local farmers (Attia *et al*., 2). In addition to its cultural significance, NRB is valued for its nutritional properties, including dietary fibre and antioxidants (Ahmad *et al*., 1). Between 2016 and 2017, the cultivar suffered a major decline due to a widespread outbreak of Panama wilt disease, caused by *Fusarium oxysporum f. sp. cubense*. A survey reported disease incidence as high as 60.57% in NRB, which severely affected its cultivation (Chand *et al*., 5). As a result, many farmers shifted to other banana varieties, leading to a sharp drop in NRB cultivation and raising concerns about its possible extinction. Conventional propagation of NRB is limited by the low number of suckers and the continued threat of soil-borne diseases. To address this, tissue culture-based micropropagation provides an effective method for large-scale production of disease-free plantlets. Shoot tip culture is a commonly used technique for banana propagation (Suman 13), involving the use of phytohormones like cytokinins and auxins to promote multiplication, along with ascorbic acid to reduce browning caused by polyphenol oxidation.

Due to high demand and limited availability, there have been instances where other banana varieties are falsely marketed as NRB. Therefore, reliable methods to confirm the identity of the cultivar is required. DNA barcoding is a molecular tool used for species identification and biodiversity assessment (Li *et al*., 10).Based on the previous research, among the different barcode the internal transcribed spacer 2 (ITS2) of nuclear ribosomal DNA has shown good potential in plant barcoding (Yao *et al*.,15). In this study, we employed ITS2 to distinguish NRB from other cultivars and confirm its genetic identity. Additionally, we established a standardized tissue culture protocol for NRB and demonstrated the utility of ITS2-based DNA barcoding for its precise genetic verification

MATERIALS AND METHODS

To verify the authenticity of the NRB cultivar, leaf samples were collected from different sources across the Mysuru district. A total of seven samples were obtained from both farmer fields and tissue culture laboratories. The details of sample sources are listed in Table 1.

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Table 1: Source details of plant samples collected for DNA barcoding.

Sl No.	Source	Region
1	Farmer 1	Madralli, Taluk – Nanjangudu, Mysuru.
2	Farmer 2	Madralli, Taluk – Nanjangudu, Mysuru.
3	Farmer 3	Devarasanalli, Nanjangudu, Mysuru.
4	Farmer 4	Nanjangudu, Mysuru.
5	Farmer 5	Hemmaragala, Taluk – Nanjangudu, Mysuru.
6	Tissue culture Lab 1	Mysuru
7	Tissue culture Lab 2	Mysuru

Genomic DNA was extracted from freshly collected leaf tissues using the DNeasy Plant DNA Isolation Kit (Qiagen), following the manufacturer's protocol. The extracted DNA samples were stored at -20°C until further use. Prior to PCR amplification, the quality and integrity of the DNA were assessed. The ITS2 region was amplified using specific primers as described by Bhat *et al.* (4). PCR amplification was performed using EmeraldAmp® GT PCR Mix (Takara Bio) under the following conditions: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 5 minutes. PCR products were resolved on 1% agarose gel pre-stained with ethidium bromide and visualized under a UV transilluminator.

Amplicon purification was carried out using the using the NucleoSpin® Gel and PCR Clean-Up Kit (Takara Bio) and sequenced commercially (Eurofins India, Bengaluru). The raw sequence chromatograms were manually checked, and low-quality bases were trimmed. Multiple sequence alignment of ITS2 regions was carried out using BioEdit v5.0.6 to assess sequence similarity among the samples. The basal medium consisted of Murashige and Skoog (MS) medium supplemented with 1% agar. The pH of the medium was adjusted to 5.8 before autoclaving. After sterilization, medium was supplemented with 6-benzylaminopurine (6-BAP) – 2mg/L, naphthaleneacetic acid (NAA) - 0.5 mg/L, and 100 mg/L ascorbic acid. The medium was poured into sterile tissue culture bottles for inoculation. Three to four month old sword suckers from one year old mother plants were collected, thoroughly washed under running water, and trimmed into 2–3 cm segments. Shoot tips were excised and surface sterilized using 0.15% mercuric chloride ($HgCl_2$) for 15 minutes with continuous shaking, followed by multiple washes with sterile distilled water to remove residual $HgCl_2$. Sterilized shoot tips were aseptically transferred to the prepared medium. Cultures were incubated in complete darkness at 25°C for 30 days. Subsequently, Cultures were maintained under a photoperiod regime consisting of 16 hours of light followed by 8 hours of darkness. Subculturing was performed every 40–45 days. *In vitro* plantlets were acclimatized in a polyhouse for 15 days with loosened bottle caps to facilitate air exchange. Primary hardening involved washing the plantlets to remove the medium and soaking them in a nutrient solution containing 1% NPK, 0.05% micronutrients, and 0.1% fungicide (Bavistin) for 3 hours. The plantlets were transferred to seedling trays containing a soil-to-cocopeat mixture (1:2) with a 1% mycorrhizae mixture and maintained under high humidity until new leaves emerged. Secondary hardening was performed by transferring the plantlets to nursery bags filled with a soil to cocopeat mixture (1:1) supplemented with NPK (2 g/kg).

RESULTS AND DISCUSSION

The conservation and authentication of Nanjangud Rasabale (NRB), a Geographical Indication (GI)-tagged banana cultivar, are essential for preserving its unique characteristics and supporting local biodiversity. Known for its distinctive aroma, taste, and nutritional properties, NRB has faced a significant decline in cultivation due to disease outbreaks and reduced commercial interest. In this study, shoot tip explants were successfully used to develop a reliable *in vitro* micropropagation protocol for NRB. The use of Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (6-BAP) - 2 mg/L , naphthaleneacetic acid (NAA) - 0.5 mg/L, and 100 mg/L ascorbic acid promoted shoot proliferation and multiplication. Our results demonstrated a high rate of multiplication, producing healthy and disease-free plantlets suitable for field-level distribution (Fig. 1 & 2).

Unlike conventional protocols that use different media for initiation, multiplication, and rooting stages (Babu 3; Guranna *et al*., 6)and the cultivated edible types are mainly triploid in nature with basic chromosome number 11 (Salaria, 2004, our method employed a single standardized medium throughout

Fig. 1. Initiation of shoot tip culture (A). Collection of 3-4 month old banana suckers (B). Explant preparation (C). Inoculation of shoot tip in the nutrient medium.

Fig. 2. Shoot formation and multiplication after 6 months of inoculation and subculture.

the tissue culture process. This simplification not only reduced the cost and time involved but also enhanced reproducibility and scalability. After successful acclimatization and hardening in greenhouse conditions (Fig. 3), the plantlets were distributed across various districts in Karnataka to aid in on-field conservation (Fig. 4).

Fig. 3. Primary and secondary hardening stage of tissue cultured banana saplings in greenhouse(A): Primary hardening (B): Secondary hardening.

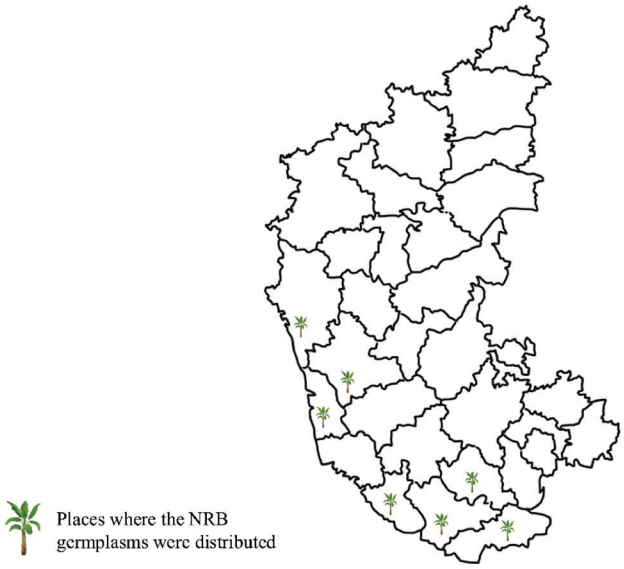

Fig. 4. Distribution details of tissue cultured Nanjangudu Rasabale Saplings. Total number of plants 51, Locations (Dist.) Mysore, Mandya, Chamarajanagara, Tumkur, Kodagu, Shivamogga, Uttarakannada, Udupi.

To verify the genetic identity of NRB, DNA barcoding was performed using the ITS2 region as a molecular marker. The amplified ITS2 sequences from six different sources (including farmer fields and tissue culture labs) were aligned and compared with sequences of *Elakki Bale* and *Robusta* cultivars (Fig. 5). The analysis revealed that ITS2 sequences were highly conserved across NRB and closely related cultivars, showing minimal nucleotide variation.

This lack of resolution suggests that ITS2 alone may not be sufficient to distinguish NRB from morphologically similar banana cultivars. The challenge is particularly notable within the *Musaceae* family, where ITS regions often display incomplete concerted evolution, especially in interspecific hybrids (Hřibová *et al*., 7). Similar issues have been observed in other plant groups such as *Warburgia* and *Pinaceae*, where ITS-based barcoding failed to resolve varietal and sub-species level distinctions (Lamb and Naidoo 8; Sokołowska *et al*., 12). In addition, other commonly used chloroplast barcode markers—*rbcL*, *matK*, and *psbA* (Li *et al.,* 9; Vasconcelos *et al.,* 14) were evaluated *in silico* using sequences from the NCBI database. Multiple sequence alignments showed that these plastid markers also lacked discriminatory power to differentiate NRB from other banana cultivars (Fig. 6A-C).

These results highlight the limitations of currently available barcode regions for cultivar-level identification in banana. Therefore, a combination

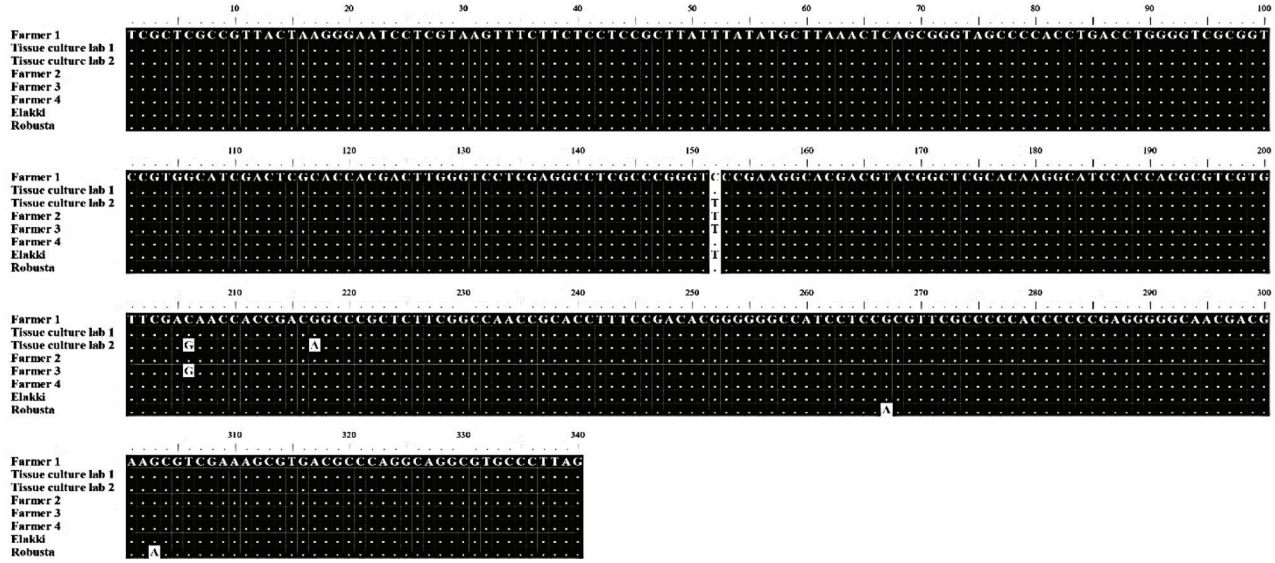

Fig. 5. Multiple alignment of ITS2 sequences of NRB samples from different source compared with Elakki and Robusta banana verities.

Fig. 6A. Multiple alignment of matK sequences of multiple banana samples.

Fig. 6B. Multiple alignment of rbcL sequences of multiple banana samples.

Fig. 6C. Multiple alignment of psbA sequences of multiple banana samples.

of morpho-molecular markers or whole genome sequencing (WGS) approaches may be necessary for the precise and reliable identification of NRB. Our study successfully developed a simplified and effective tissue culture protocol for large-scale propagation of *Nanjangud Rasabale*, contributing significantly to its conservation. Although ITS2-based DNA barcoding provided limited resolution, it underscored the need for advanced genomic tools for accurate cultivar authentication. The distribution

of disease-free NRB saplings to multiple regions and maintenance of grower contact details ensures traceability and long-term monitoring.

AUTHORS' CONTRIBUTION

Conceptualization of research work and designing of experiment (ARB, TCM, CR); Execution of field/ lab experiments and data collection (ARB); Analysis of data and interpretation (ARB, SRH, TCM, CR); Preparation of manuscript (ARB, SRH, TCM, CR)

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DECLARATION

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be as a potential conflict of interest

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