

Assessment of genetic uniformity in micro propagated plantlets of turmeric (*Curcuma longa* L.) through DNA markers

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ABSTRACT

Turmeric is herbaceous plant, characterized by long lance-shaped leaves sprouting from its rhizomatous underground stem. Notably, its rhizomes contain the bioactive compounds curcuminoids, renowned for its medicinal and culinary significance. However, meeting the surging demand for turmeric, particularly during off- seasons, presents a formidable challenge due to the sluggish vegetative propagation rate in *Curcuma longa*. Given the scarcity of sexual reproduction in turmeric, micropropagation emerges as the convenient method for obtaining disease-free seeds. In addressing the challenge of direct regeneration of the native Erode local cultivar of turmeric, this study endeavors to establish a protocol for *in vitro* plantlet production. Results indicate that multiple shoots were successfully induced, notably with $13.32 \mu\text{M}$ of 6-benzylaminopurine (BAP), yielding a response rate of $73.2 \pm 4.7\%$ and an average shoot count of 6.95 ± 0.81 per explant. Subsequently, a concentration of $17.76 \mu\text{M}$ of BAP demonstrated a response rate of $56.5\% \pm 4.7\%$, with an average shoot count of 5.94 ± 0.81 per explant. Additionally, a combination of $13.32 \mu\text{M}$ BAP and $2.68 \mu\text{M}$ Naphthaleneacetic acid (NAA) resulted in 8.65 ± 0.47 shoots per explant, with a response rate of $73.66 \pm 1.25\%$. Similarly, $13.32 \mu\text{M}$ BAP combined with $5.37 \mu\text{M}$ NAA yielded 7.32 ± 0.47 shoots per explant, with a response rate of $72.33 \pm 0.47\%$. The acclimatization of plantlets in a greenhouse exhibited a remarkable survival rate, ranging from 90% to 98%. Importantly, all regenerated plantlets closely resembled the mother plants morphologically. Genetic uniformity assessment, employing 10 ISSR and 4 DAMD markers, indicated more than 90% uniformity among one mother plant and regenerants. This indicates a significant genetic uniformity, ensuring consistency in desired traits across the regenerated plantlets.

KEYWORDS: Genetic uniformity, DNA markers, Micropropagation, Erode local

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INTRODUCTION

Turmeric (*Curcuma longa* L.) is a rhizomatous herbaceous plant belonging to the family Zingiberaceae, it grows in tropical and subtropical regions throughout the world and possesses high nutritional value. India is one of the main producers and exporters of turmeric in the world. It is extensively used in the food, dietary spice and dairy industries for its natural coloring (Downham & Collins, 2000; Nair, 2019; Pittampalli *et al.*, 2022). The rhizome of turmeric possesses anti-oxidant, germicidal, anti-tumor, and anthelmintic properties. The content of curcumin is a major component of the rhizome is an important factor in its exportation into the global market and the powder contains 2-8% curcumin, which is the main biologically active phytochemical compound (Singh *et al.*, 2018). *C. longa* has 63 chromosomes and is indigenous to Southeast Asia and India (Singh *et al.*, 2018) and it is a cross-pollinated triploid species,

flowers rarely and does not set seed (Ravindran *et al.*, 2007; Nair, 2019). Hence, it is challenging to develop new turmeric cultivars through conventional breeding methods which can be vegetatively propagated using underground rhizomes (He & Gang, 2014; Raju *et al.*, 2015). Since, hybridization is ineffective in most cases, genetic improvement is often limited to germplasm selection and mutation breeding (Ravindran *et al.*, 2007). A viable seed set can be obtained under certain conditions, which enables recombination breeding through hybridization and open-pollinated progeny selection (Sigrist *et al.*, 2011). Turmeric plants are only propagated by vegetative means, using subterranean rhizomes, as there is no seed production. In most cases, micropropagation aids in the multiplication of identical plantlets. It is the quick method of propagation of plants in a specified nutritional medium and with high light intensity in an *in vitro* environment. Rapid plant growth is performed through micropropagation using explants such as stem, and axillary

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buds (Bhojwani & Razdan, 1986). Several plant regeneration procedures have been reported in turmeric through direct shoot induction from various explants including axillary buds vegetative buds (Salvi *et al.*, 2002), sprouting buds (Gomathy *et al.*, 2014) shoot bud, half-shoot, and shoot slice explants (Prathantharug *et al.*, 2003; Pittampalli *et al.*, 2022), and terminal buds (Prathantharug *et al.*, 2003).

Molecular marker technology is an efficient tool that facilitates plant genetic resource conservation and its management as well. Several molecular markers like inter simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), Directed amplification of minisatellite-region DNA (DAMD), and start codon targeted (SCoT) markers overcome the limitations of other biochemical and morphological markers by providing phenotypic variability and helping in detecting variations at the DNA level (Singh *et al.*, 2018). Under the long-term *in vitro* process, various factors such as media composition and plant growth regulators may result in variations in regenerated plants. Therefore, the genetic uniformity assessment of regenerated plants is of great importance. In recent years RAPD and ISSR have been successfully used for assessing the genetic fidelity of regenerated plantlets in many plant species (Bennici *et al.*, 2004; Ravindran *et al.*, 2007; Chavan *et al.*, 2015; Ahmed *et al.*, 2017; Cui *et al.*, 2019; Tikendra *et al.*, 2019). In the current study, we utilized shoot buds from the well-known *C. longa* variety Erode local rhizomes as explants. This was followed by a series of steps including regeneration, mass production of superior clonal seedlings, rooting, hardening, and acclimatization. The genetic uniformity was evaluated using ISSR and DAMD markers. Therefore, micropropagation techniques are essential for both the conservation and large-scale production of medicinally significant species within a limited timeframe and space.

MATERIALS AND METHODS

Plant Materials and Explant Preparation

The *Curcuma longa* var Erode local, was sourced from Salundi, Mysore District, Karnataka (11°43'26.4"N, 76°41'56.4"E). Upon collection, rhizomes washed thoroughly under running water and subsequent treatment with detergent and Bavistin. Additional rhizomes were segmented into small pieces, subjected to surface sterilization with mercuric chloride for 5-10 minutes, and then meticulously rinsed with double-distilled water under sterile conditions. Utilizing sprouted multiple shoot buds, explants were cut in 2-3 cm, ensuring surface sterilization of the buds and subsequent blotting dry under aseptic conditions. The shoot bud explants were carefully obtained by removing the outer leaf sheath (Raju *et al.*, 2015; Pittampalli *et al.*, 2022).

Culture Media and Growth Conditions

Morishige and Skoog (MS) medium were chosen for the regeneration of *C. longa*. The medium was composed of 30 g/L sucrose, solidified with 7 g/L agar, and adjusted to a pH of 5.8 before being autoclaved at 121 °C for 15 minutes. For shoot bud induction and multiplication, the medium consisted

of varying concentrations of BAP (4.44 µM to 17.76 µM per liter) and NAA (2.68 µM to 5.37 µM). After preparing the media, explants were carefully inoculated onto half-strength MS medium supplemented with different concentrations and combinations of plant hormones to induce regeneration. The cultures were then placed in the laboratory at 24±2 °C under a 16/8-hour (light/dark cycle) photoperiod, with cool white fluorescent light provided at 1500-3000 Lux. The inoculated culture bottles were monitored weekly and clear shoot initiation was observed within two weeks of inoculation (Murashige & Skoog, 1962; Zhang *et al.*, 2015).

Hardening and Rooting

After four to five weeks of growth, rooted plantlets were transferred to greenhouse conditions. The plantlets were carefully removed from the culture and rinsed to remove any adhered media. Subsequently, rooted plants were transplanted into plastic micro pots filled with a mixture of coco peat and soil in a ratio of 3:1. The survival rate was calculated.

Plant DNA Isolation

To check the genetic uniformity ten randomly selected plantlets were used. The genomic DNA was extracted as per CTAB method using fresh leaf tissues (Doyle & Doyle, 1990). After extraction quantitative measurement was carried out to check the DNA yield and to check the quality of genomic DNA 0.8% agarose gel stained with Goodview nucleic acid stain to visualize in the gel and DNA was stored at 4 °C for further use.

PCR Amplification

The PCR amplification was carried out in a volume of 10 µL containing 50 ng/µL template DNA, 1 µL of 0.5 µM concentration of primers, 5 µL of the reaction mixture (TSS, Takara) and 3 µL of Nucleus free water. A total of 12 ISSR and 4 DAMD markers were used to estimate the molecular variability of accessions in the present study (Table 1). Amplification was performed in a thermal cycler (Applied Biosystems, Life Technologies, USA). The PCR amplification was performed as follows: initial denaturation (at 94 °C for 5:00 min) followed by 30 cycles of denaturation (at 94 °C for 1:00 min), annealing temperature (48-60 °C) for 0:45 min, extension (at 72 °C for 2.00 min) and a final extension (at 72 °C for 7:00 min) followed by cooling at 4 °C. Amplified products were separated on agarose gel (2% w/v) electrophoresis in 1X TAE buffer, along with 2-Log DNA ladder (New England Bio-Labs). The gels were photographed using a gel documentation system (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

In vitro Regeneration and Hardening of Plants

The present study was to establish a reliable and efficient method for regenerating plants from the Erode local variety of

C. longa. Shoot buds were predominantly utilized as explants, as depicted in the Figure 1. Various plant growth regulators (PGRs), primarily BAP and NAA were used (Salvi *et al.*, 2002; Pittampalli *et al.*, 2022). Initiation and induction of multiple shoots varied depending on the concentrations and combinations of PGRs. In this study, BAP concentrations ranged from 2.22 μ M to 22 μ M, while NAA concentrations were limited to 2.68 μ M and 5.37 μ M. Notably, the combination of BAP (13.32 μ M) and NAA (2.68 μ M) yielded the most promising results, with an average of 8.65 ± 0.47 shoots per shoot bud explant and a response rate

of $73.66 \pm 1.25\%$. Similarly, the combination of BAP (13.32 μ M) and NAA (5.37 μ M) produced 7.32 ± 0.47 shoots per explant, with a response rate of $72.33 \pm 0.47\%$. Furthermore, BAP alone demonstrated significant efficacy, particularly at a concentration of 13.32 μ M, resulting in an average of 6.95 ± 0.81 shoots per explant and a response rate of $73.2 \pm 4.7\%$. Researchers have investigated the efficacy of different PGRs such as BAP and TDZ (thiazine) in inducing shoot proliferation in turmeric and related species like *C. longa*. Several plant regeneration protocols have been developed and reported from different

Table 1: Details of the Inter simple sequence repeat (ISSR 1-10) and DAMD (11-14) primers used for genetic uniformity assessment in turmeric (*C. longa*), including the average number of scorable bands and the approximate range of band size

S. No.	Primer name	Primer Sequence (5'-3')	Average No of scorable bands	Approximate range of amplification
1	UBC 808	AGAGAGAGAGAGAGAGC	2	400-1500
2	UBC 809	AGAGAGAGAGAGAGAGG	7	350-1500
3	UBC 812	GAGAGAGAGAGAGAGAA	2	400-700
4	UBC 818	CACACACACACACACAG	3	450-1000
5	UBC 827	ACACACACACACACACG	3	400-1300
6	UBC 835	AGAGAGAGAGAGAGAGYA	6	150-1500
7	UBC 850	GTGTGTGTGTGTGTGTYC	2	500-700
8	UBC 855	ACACACACACACACACYT	8	300-1400
9	UBC 856	ACACACACACACACACYA	4	300-1000
10	UBC 857	ACACACACACACACACYG	2	400-1000
11	HBV	GGTGTGTGTGTGTCAT	5	300-1500
12	HBV3	GGTGAAGCACAGGTG	6	400-900
13	HVA	AGGATGGAAAGGAGGC	5	300-800
14	33.6	AGGGCTGGAGG	3	200-1000

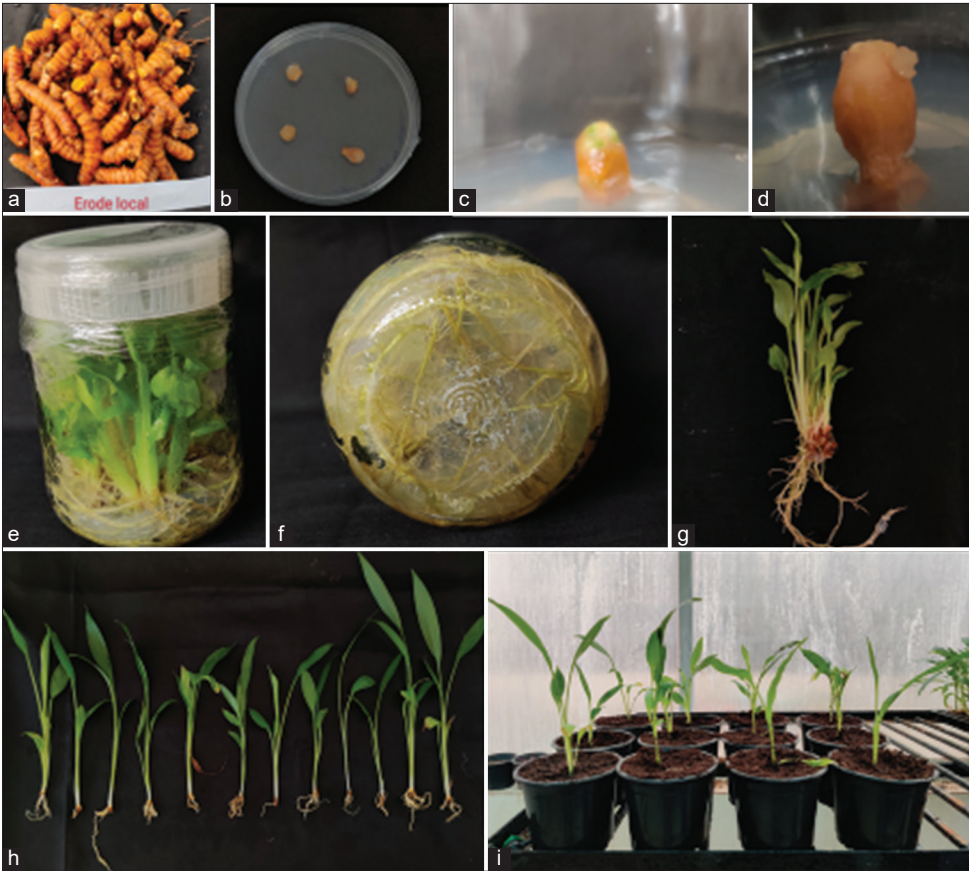


Figure 1: *In vitro* propagation of *C. longa* using mature bud explants. (a) Matured rhizomes, (b) Rhizome explants on half MS medium, (c, d) Callus started from rhizome explant, (e) Regenerated plantlets, (f) Well-established root system, (g, h) Rooted plantlets and (i) Acclimatized plants

types of turmeric explants from different genotypes and cultivars using a different combination of PCR in turmeric, Salem, Duggirala Red, Prathibha, and PCT-13 cultivars used

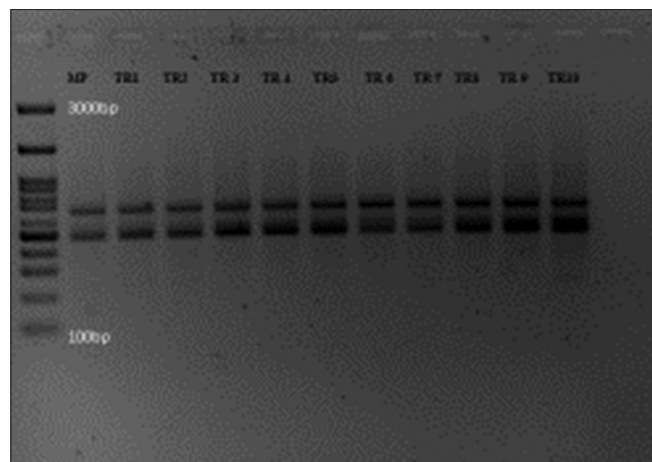


Figure 2: ISSR Profiles generated by PCR amplification with primer UBC 850, MP- Mother plant and TR1-TR10 (Turmeric regenerants 1-10)

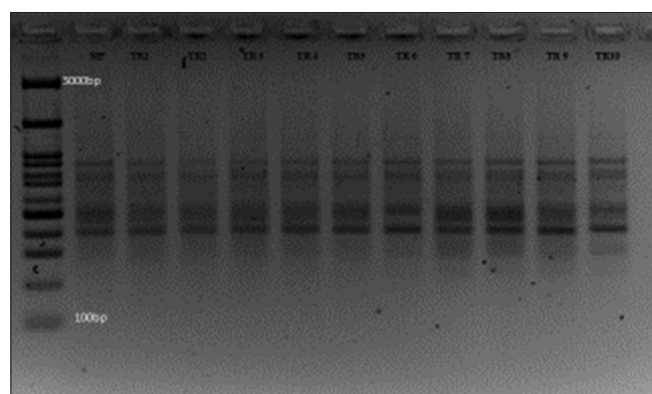


Figure 3: DAMD Profiles generated by PCR amplification with primer HVA, MP- Mother plant and TR1-TR10 (Turmeric regenerants 1-10)

for direct regeneration with a combination of PCR (Pittampalli *et al.*, 2022). Different concentrations of BAP were observed as the most suitable PCR to induce the maximum number of shoots of *C. longa* (Sarma *et al.*, 2011; Thingbaijam *et al.*, 2012; Gomathy *et al.*, 2014), and several species of *Curcuma* (Tyagi *et al.*, 2004; Zuraida, 2013). Many studies were suggested that TDZ was an efficient plant growth regulator to induce more shoots than BAP in *C. longa* (Prathantharug *et al.*, 2003) and *C. vama* (Bejoy *et al.*, 2012).

Genetic Uniformity and Phylogenetic relationship

A subset of regenerants from the field underwent genetic fidelity testing to verify their similarity to the donor plant. Analysis using ten ISSR and four DAMD primers consistently produced clear, scorable, and reproducible monomorphic bands. This outcome indicates a high level of uniformity among the *in vitro* plants and confirms their close genetic resemblance to the mother plant. Further phylogenetic analysis using ISSR and DAMD markers reinforced the genetic fidelity of the *in vitro*-raised plants. The analysis revealed two distinct clusters, with DAMD markers showing 98-99% similarity between them. Additionally, ISSR markers uncovered three sub-clusters with 90-95% similarity (Figures 2-5). These results affirm that the regenerated plants faithfully retained the genetic characteristics of the original plant material. Similar findings regarding genetic uniformity have been documented across various plant species, including *C. longa* (Salvi *et al.*, 2002; Pittampalli *et al.*, 2022), *Magnolia sirindhorniae* (Cui *et al.*, 2019), *Helicteres isora* (Muthukumar *et al.*, 2016), *Rauwolfia tetraphylla* (Rohela *et al.*, 2019), *Pittosporum eriocarpum* (Thakur *et al.*, 2016), *Cicer arietinum* (Sadhu *et al.*, 2020), *Morus alba* (Rohela *et al.*, 2020), *Scaevola taccada* (Manokari *et al.*, 2020), and *Crinum malabaricum* (Priyadharshini *et al.*, 2020). Ensuring and understanding the genetic fidelity of plant species is crucial for preserving and propagating desirable traits, thereby enhancing their utility in agriculture, horticulture, and medicine. Moreover, the application of molecular marker techniques in molecular

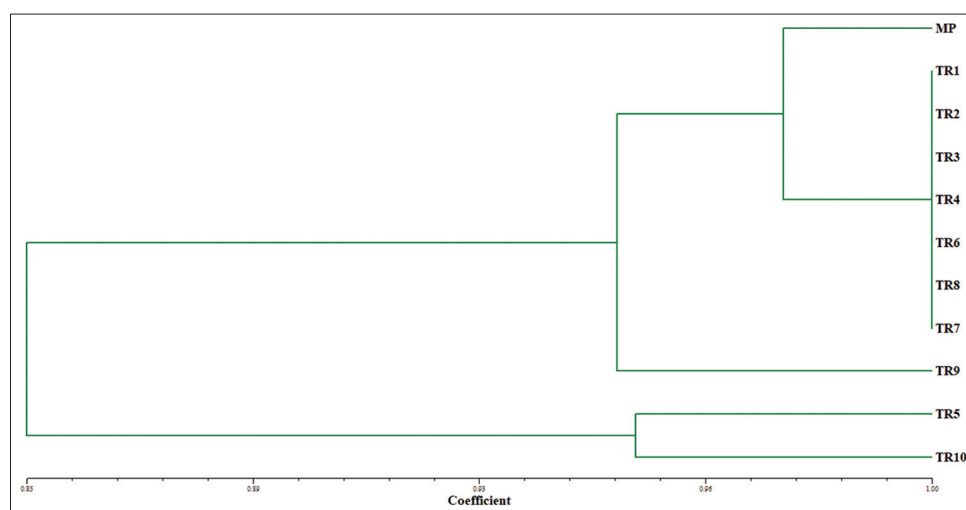


Figure 4: Dendrogram (UPGMA, NTSYS) representing phylogenetic relationship in ten regenerants of *C. longa* based on ISSR marker genetic uniformity matrix data

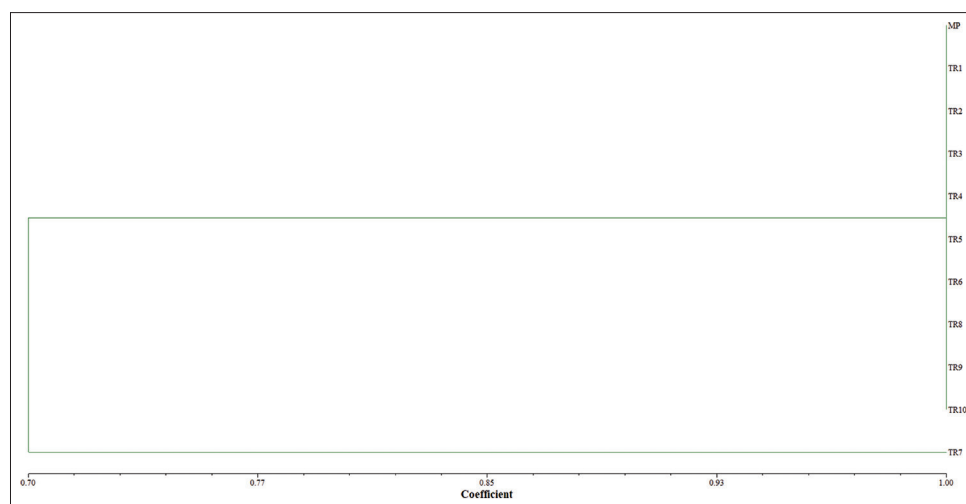


Figure 5: Dendrogram (UPGMA, NTSYS) representing phylogenetic relationship in ten regenerants of *C. longa* based on DAMD marker genetic uniformity matrix data

breeding and genetic resource management strategies plays a vital role in optimizing plant cultivation practices and ensuring long-term sustainability and productivity.

CONCLUSION

The study presents a successful direct regeneration protocol for the *C. longa* variety Erode local, utilizing shoot bud explants treated with various combinations of BAP and NAA. The findings demonstrate the efficacy of this method in generating multiple shoots, thus providing a valuable approach for *in vitro* propagation of turmeric plants. Moreover, the genetic homogeneity observed among plants derived from these shoot bud explants indicates the reliability and reproducibility of the regeneration process. Genetic uniformity was confirmed through DNA-based ISSR and DAMD markers, highlighting the stability of the regenerated plants. The significance of maintaining genetic stability cannot be overstated, particularly in germplasm conservation efforts. This study's success in achieving high genetic uniformity contributes to the preservation and propagation of desirable traits within the *C. longa* variety Erode local. Overall, the established regeneration protocol offers a promising avenue for further genetic improvement of turmeric through micropropagation. By providing a reliable method for mass propagation of plants with consistent genetic characteristics, this study lays a foundation for advancing research and applications in turmeric cultivation and utilization.

AUTHOR CONTRIBUTIONS

SMR and SR: Tissue culture, Molecular work and other experiment done by SMR. The manuscript drafted by SR.

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