



## Assessment of genetic fidelity *in vitro* regenerated plantlets of *Zingiber zerumbet* (L.) Roscoe ex Sm

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### Abstract

In the given study, we established an efficient and reproducible *in vitro* plant regeneration protocol of *Zingiber zerumbet* Smith developed from rhizome explants. The shoot bud explant developed from rhizome produced the highest number of shoots (20 shoots/ explant) on medium amended with 1 mg<sup>-1</sup>L BAP, 2 mg<sup>-1</sup>L TDZ and 0.4 mg<sup>-1</sup>L NAA. Approximately 80% of shoots were induced simultaneously rooting on shoot induction media itself i.e., MS media fortified with 1 mg<sup>-1</sup>L BAP, 2 mg<sup>-1</sup>L TDZ and 0.4 mg<sup>-1</sup>L NAA. These plantlets were acclimatized under greenhouse conditions, and the survival rate was recorded as 90%. Micropropagated plantlets thus obtained were seemed to be morphologically similar to that of mother plants. In this study, Random Amplified Polymorphic DNA (RAPD) molecular markers were deployed to determine the genetic stability of plants obtained from the tissue culture of the *Z. zerumbet*. Our findings using molecular markers revealed the genetic uniformity of all *in vitro* regenerants were similar to their mother plant. Thus this *in vitro* regeneration procedure could be a helpful method for the genetic improvement of *Z. zerumbet*.

**Keywords:** Genetic fidelity, medicinal plant, shoot bud, shoot regeneration, rhizome, *Z. zerumbet*

### Introduction

*Zingiber zerumbet* (L.) Roscoe ex Sm is a perennial, aromatic rhizomatous plant species in the Zingiberaceae family, it is primarily found in Asia's tropical and subtropical regions (Anon, 1976) <sup>[1]</sup>. Traditional uses of the *Z. zerumbet* rhizomes include food and medicine (Yob *et al.* 011) <sup>[2]</sup>. Numerous pharmacological effects of plants have been documented, including anti-inflammatory, anti-tumor, anti-allergic, antioxidant, antibacterial, anti-viral, and treatments for sore throat, stomachaches, and worms (Rahman *et al.* 2014 <sup>[3]</sup>; Nhareet and Nur, 2003 <sup>[4]</sup>; Huang *et al.* 2005 <sup>[5]</sup>; Tewtrakul and Subhadhirasakul, 2007 <sup>[6]</sup>).

*Z. zerumbet* deserves special consideration and wider cultural dissemination due to its multiple health benefits, just like *Z. officinale*. Rhizomes are the conventional method of propagating *Zingiber zerumbet*. Since the rhizome is prone to fungal diseases that degrade the tuber quality, it cannot be kept for an extended period of time (Nalawade *et al.*, 2003 <sup>[7]</sup>). Modern cultivation and conservation measures are necessary to prevent overexploitation, which goes well beyond their natural potential for regeneration due to declining populations. For sustainable cultivation and conservation, high-frequency multiplication techniques in particular need to be developed (Sharmin *et al.* 2014 <sup>[8]</sup>). Through quick multiplication rates, *in vitro* propagation (also known as tissue culture) can address the problems and aid in sustainable breeding, cultivation, and preservation (Anis and Ahmad, 2016 <sup>[9]</sup>). True-to-type progenies and homogeneity in the products, such as the bioactive chemicals, are offered by micropropagation. Additionally, the preservation of germplasm *in vitro* (from healthy meristems) has benefits such as reducing the need for space and labor as well as preventing the buildup of infections and mutations (Alam *et al.* 2013 <sup>[10]</sup>). As a result, micropropagation can be

employed as a substitute technique to generate a large number of homogeneous, pathogen-free plants with high-quality tubers (Poosapaya and Kraisintu, 1993 <sup>[11]</sup>). According to Nalawade *et al.* (2003) <sup>[7]</sup>, the use of *in vitro* propagation technique resulted in the production of a significant number of pathogen-free uniform clones of elite, rare, and significant native medicinal plants so that they could be reintroduced in their natural habitats, promoting the safe exchange of germplasm across international borders.

Numerous studies have demonstrated that there are differences between micropropagated plants; these differences can be identified by comparing morphological features, cytogenetic analysis, biochemical markers, and molecular markers (Wani *et al.* 2012 <sup>[12]</sup>). Molecular markers are currently preferred by researchers over morphological traits, chemical markers, and cytological data because the later are affected by the environment and the stage of plant development. Molecular markers, specifically RAPD, were employed in the current investigation to assess genetic variability. Using a single primer with a known sequence, RAPDs amplify random genomic DNA segments and create numerous bands (Amin *et al.* 2018 <sup>[13]</sup>; Wani *et al.* 2016 <sup>[14]</sup>). Multiple loci may be evaluated very fast because fragments are frequently generated from various sections of the genome (Alam *et al.* 2013 <sup>[10]</sup>). Several DNA-based techniques have been effectively used in recent years to define medicinal plants and herbal remedies for quality control and standardization (Hamoud, 2019 <sup>[15]</sup>). Utilizing molecular markers based on polymerase chain reaction (PCR), such as AFLP, SSR, RAPD, and RFLP, it is possible to assess genetic variants within plant varieties (Galatali *et al.* 2021 <sup>[16]</sup>).

In the given study, we successfully established *in vitro* regeneration and assessed genetic fidelity of *in vitro*

regenerated plantlets of *Z. zerumbet* using RAPD markers. The present study aims to determine the species' molecular identification and create a successful micropropagation methodology backed by a DNA marker-based genetic fidelity. These would be helpful for the phytomedicine industry's precise identification of the medicinal plant as well as for cultivation and conservation techniques.

## Materials and methods

### Collection and preparation of explant

Rhizomes of *Zingiber zerumbet* were collected and maintained in a plot. The rhizomes at sprouting stage were collected and made free from soil traces by washing under running tap water followed by detergent wash. The bud parts of rhizomes were excised, trimmed and performed sterilization using bavistin (5 g/L), tween 20 (few drops) then explants were washed with sterile distilled water followed by mercuric chloride (0.1%) and sodium hypochlorite (15%) wash in aseptic conditions. The explants were washed with sterile distilled water for many times and dried on sterile tissue papers under flow then inoculated on medium.

### Culture medium for plantlet formation

The rhizome bud explants were inoculated on the medium with KN (0.2, 0.4, 0.6, 0.8 and 1.0 mg/L) and NAA (0.4 mg/L) and incubated for 4 weeks. BAP was introduced to the MS medium at various doses of 1, 2, 3, 4 and 5 mg/l in conjunction with NAA (0.4mg/L) and Kn (0.4mg/L) on to which the explants were subcultured. To increase shoot number, TDZ in different concentrations (1, 2, 3, 4 and 5 mg/L) was employed to the above medium. Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962 [17]) served as the foundation for all the media. It was supplemented with 30 g/L sucrose. After the medium was solidified with 8 g/L agar and autoclaved at 121°C, 15 psi for 20 min, the pH was adjusted to 5.8. All cultures were maintained at 25±2°C with a cool fluorescent lamp with photosynthetic flux of 150 mol m<sup>-2</sup> s<sup>-1</sup> and a 16-hour photoperiod. Plantlets with established roots were taken out of the medium, properly cleaned under running water to remove the adherent solid MS medium, transplanted into plastic pots with sterilized peat moss soil, and housed in a net house that provided 50% shade. In order to maintain a

high humidity and prevent plant dryness due to water loss, the plants were covered by transparent perforated polyethylene bags. The plants received regular watering to maintain a high degree of humidity. After 15 days, the plastic bags were taken off, and the plantlets were allowed to acclimatize for an additional 2 weeks. Plantlets were successfully moved to the *ex vitro* setting for maturation after 30 days of acclimatization. Different molecular traits were examined between plants that were grown in the field and those that were micropropagated.

### Collected of data

The following metrics were measured after eight weeks of culture: the number of shoots and roots; the length of the shoots and roots.

### Genomic DNA isolation

Using a modified Cetyl trimethyl ammonium bromide (CTAB) approach, the complete genomic DNA of *in vitro* propagated as well as the mother plant was separately isolated from fresh leaves. This procedure was described by Doyle and Doyle (1987) [18]. DNA was measured at 260 nm, and the purity was assessed using the 260/280 nm ratio. 25 ng of template DNA, 5 pM of each primer, 10X reaction buffer containing 15 mM MgCl<sub>2</sub>, 200 M of each dNTP, and 3U/μl of Taq polymerase were used in the PCR reactions, which were carried out in a final volume of 25μl. A thermal cycler was used for the amplification of genomic DNA and was programmed as follows: 5 min of 94 °C initial heating is followed by 40 cycles of 1 min at 94 °C denaturation, 1 min at 35°C annealing, 2 min at 72 °C extension, and 7 min at 72 °C final extension. Amplified reaction products were separated electrophoretically on a 1.5% agarose gel stained with 0.5 g/ml ethidium bromide, and were then seen under ultraviolet light using a gel documentation system (BioRad, CA, USA).

### Statistical analysis

The Sigma Plot software, version 12.0, was used to analyse the collected data. To compare the means of various treatments, analysis of variance (ANOVA) with mean separation by Holms-Sidak (P<0.05) was utilized. Three times each experiment was conducted.

**Table 1:** Details of RAPD primers employed for genetic homogeneity assessment in *Zingiber zerumbet*.

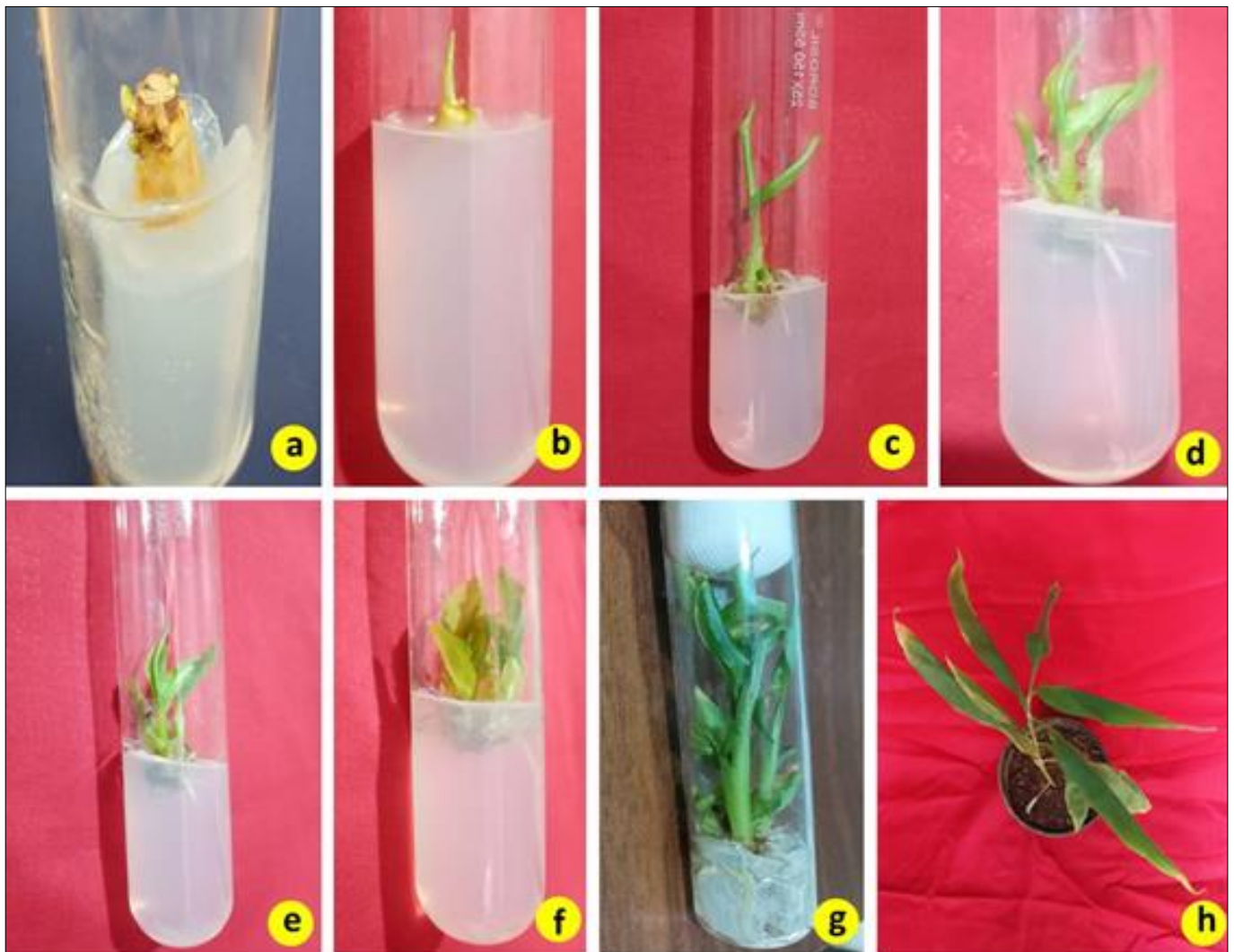
Primer	Primer sequence (5'-3')	Monomorphic bands	Polymorphic bands	Band range
AM11	CTCTCTCTCTCTCTG	8	0	350-2000bp
AM3	GCATGGCAAGCTGCA	7	0	500-2000bp
AM7	CTTCGGCAGCATCTTCAT	6	0	50-100bp
C12	GAGGCGTCGC	5	0	40-100bp
AM 2	GGATGGAATAGTCTC	7	0	1500-above
AM 4	GAGAGAGAGAGAGAC	5	0	500-1000bp
AM 5	ACACACACACACACC	6	0	300-1500bp
A6	TGGCCACCTG	1	0	1700 bp
AM8	CAGTGTGGAAGCCGATTATG	4	0	200-700bp
AM9	ATGTGTTGCTGGCTTGGTA	8	0	400-1000bp

**Table 2:** Effect of KN and BAP and in combination with NAA on multiple shoot induction from shoot bud explants in *Zingiber zerumbet*.

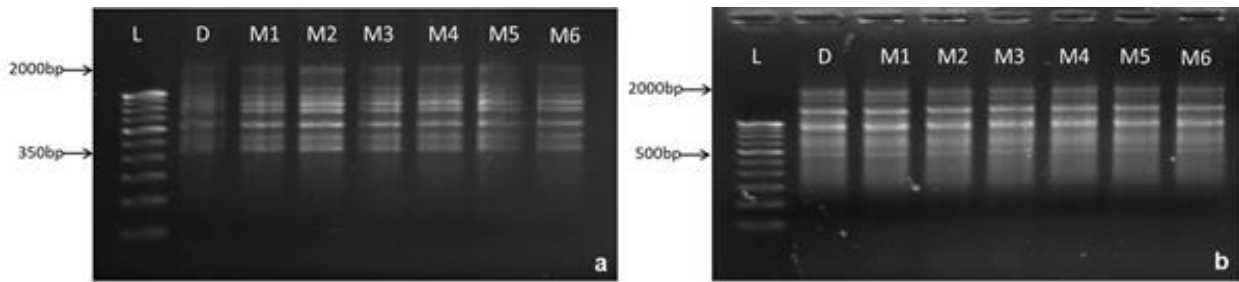
Hormones (mg/L)	Morphogenic Response	No. of shoots Mean ± SE	No. of roots Mean ± SE	Length of shoots Mean ± SE	Length of roots Mean ± SE
<b>KN+NAA</b>					
0.2+0.4	S+R	1.33±0.33	10.66±0.33 <sup>a</sup>	7.66±0.33	1.33±0.33
0.4+0.4	S+R	<b>2.33±0.33</b>	<b>11.66±0.33<sup>b</sup></b>	<b>9.33±0.33</b>	<b>2.96±0.33</b>

0.6+0.4	S+R	1.66±0.33	9.33±0.33 <sup>a</sup>	6.00±0.57	1.33±0.33
0.8+0.4	S+R	1.66±0.66	8.66±0.33 <sup>a</sup>	5.66±0.33	1.66±0.33
1.0+0.4	S+R	1.00±0.00	11.00±0.57 <sup>a</sup>	6.66±0.33	2.00±0.57
<b>BAP+KN +NAA</b>					
1.0+0.4+0.4	S+R+C	<b>3.90±0.00<sup>a</sup></b>	<b>1.86±0.33<sup>a</sup></b>	2.00±0.57	<b>3.80±0.00</b>
2.0+0.4+0.4	S+R+C	2.66±0.33 <sup>ab</sup>	1.33±0.33 <sup>abc</sup>	<b>2.46±0.33</b>	2.33±0.33
3.0+0.4+0.4	S+R+C	3.66±0.33 <sup>ab</sup>	1.00±0.00 <sup>abc</sup>	2.33±0.33	2.00±0.00
4.0+0.4+0.4	S+R+C	3.33±0.33 <sup>ab</sup>	1.33±0.33 <sup>bc</sup>	1.66±0.33	1.33±0.33
5.0+0.4+0.4	S+R+C	2.33±0.33 <sup>b</sup>	1.00±0.00 <sup>bc</sup>	2.00±0.00	1.66±0.33
<b>BAP+TDZ +NAA</b>					
1.0+1.0+0.4	S+R+C	17.66±0.88 <sup>a</sup>	22.66±0.88 <sup>a</sup>	11.66±0.3330 <sup>a</sup>	1.33±0.33
1.0+2.0+0.4	S+R+C	20.00±0.57 <sup>b</sup>	31.00±0.57 <sup>b</sup>	13.66±0.6670 <sup>ac</sup>	2.73±0.33
1.0+3.0+0.4	S+R+C	10.66±0.88 <sup>cd</sup>	30.66±0.88 <sup>cd</sup>	11.00±1.0000 <sup>a</sup>	2.00±0.57
1.0+4.0+0.4	S+R+C	13.33±1.45 <sup>cd</sup>	27.33±1.45 <sup>cd</sup>	9.33±0.3330 <sup>ad</sup>	2.33±0.33
1.0+5.0+0.4	S+R+C	19.00±0.57 <sup>a</sup>	19.00±0.57 <sup>a</sup>	8.66±0.3330 <sup>b</sup>	1.00±0.00

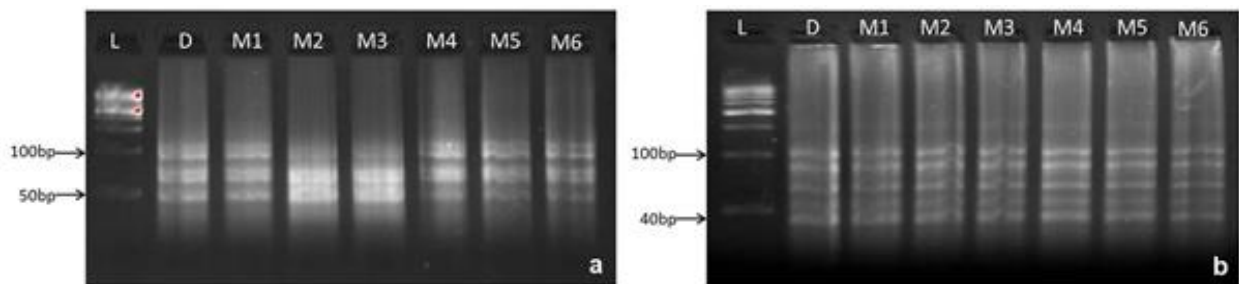
BAP, 6-benzylaminopurine; KN, Kinetin; NAA, 1-naphthaleneacetic acid. Each experiment had 20 replicates and was repeated three times. Values represent the mean ± standard error. According to Duncan’s new multiple range test at 5% level, mean values with the same *letter* within *columns* are not significantly different.



**Fig 1:** Different stages of the *in vitro* regeneration from rhizome explants of *Z. zerumbet*. (a) Inoculation of rhizome bud explants on MS medium containing different concentrations of Kn (0.2-1.0 mg/L) + NAA (0.4 mg/L); (b) Sprouted explant; (c) Sprout elongation; (d) Subculture on to the medium containing BAP (1-5 mg/L) + Kn (0.6 mg/L) +NAA (0.4 mg/L); (e) Responding with 3-4 shoots per explant; (f) Sub-culture on to the medium containing TDZ (1-5 mg/L) + BAP (1 mg/L) + NAA (0.4 mg/L); (g) Elongation of the sub-cultured shoot buds; (h) Maximum number of 20 shoots per explant resulted on BAP (1 mg/L) + TDZ (2 mg/L) + NAA (0.4 mg/L) containing medium and elongation of shoots and roots; (i) Hardening of *in vitro* raised plantlet of *Z. zerumbet*.



**Fig 2:** RAPD banding pattern with primers (a) AM1 and (b) AM3 in both micropropagated and field grown mother plant of *Z. zerumbet* (lane 1: ladder, lane 2: donor plant and lane 3-8: micropropagated plants).



**Fig 3:** RAPD banding pattern with primers (a) AM7 and (b) AM12 in both micropropagated and field grown mother plant of *Z. zerumbet* (lane 1: ladder, lane 2: donor plant and lane 3-8: micropropagated plants).

### Result and discussion

As fungal and bacterial contamination is the major challenge to overcome in tissue culture, we performed sterilization using different disinfectants. It was quite familiar from several studies that mercuric chloride was successfully employed as disinfectant during surface sterilization process. In the given study also, rhizome buds were sterilized using 0.1% HgCl<sub>2</sub> for 2 min and washed thrice with sterile distilled water. Further, the explants of *Z. zerumbet* grown on MS media supplemented with varying concentrations of KN (0.2, 0.4, 0.6, 0.8, and 1.0 mg/L) and NAA (0.4 mg/L), on which both shoots and roots concurrently generated, with shoot induction being the lowest (2.00). Explants were sub-cultured on MS media fortified with BAP at various doses of 1, 2, 3, 4, and 5 mg/l in conjunction with NAA (0.4 mg/L) and Kn (0.4 mg/L), which resulted in increased shoot quantity and length (Table. 2) (Fig. 1).

Our findings were in corroboration with the findings of Stanly and Keng (2007)<sup>[19]</sup> with similar simultaneous shoot and root formation in *Z. zerumbet* and several other studies suggested that using a mixed medium reduced the time required for plant regeneration (Chan and Thong, 2004<sup>[20]</sup>; Bharalee et al. 2005<sup>[21]</sup>; Yusuf et al. 2011<sup>[22]</sup>). The maximum number of shoots (13.90) was observed in MS media supplemented with 1.0 mg/L BAP, 0.4 mg/L Kn and 0.4 mg/L NAA. The quantity and length of shoots per explant increased at all BAP concentrations. As a result, it indicates that BAP has a beneficial influence on *Z. zerumbet* shoot multiplication in vitro. The proliferating shoots appeared green and robust. Other Zingiberaceae species have reported the role of BAP in shoot proliferation (Panda et al., 2007<sup>[23]</sup>; Mohanty et al., 2011<sup>[24]</sup>). In the given study also, the inclusion of 1 mg/L BAP, 2 mg/L TDZ and 0.4 mg/L NAA in the medium increased the number of proliferating shoots (20.00) significantly (Table. 2). Besides increasing the number of shoots, TDZ also increased the mean root number (31.00) and length (13.66) significantly

in 1.0 mg/L BAP, 2.0 mg/L TDZ and 0.4 mg/L NAA. Auxins are important in the induction of shoot buds from a variety of Zingiberaceae plants, including *Alpinia galanga* (Sahoo et al. 2020<sup>[25]</sup>); *K. angustifolia* (Haque and Ghosh, 2018<sup>[26]</sup>); and *Z. zerumbet*, and *Z. moran* (Das et al. 2013<sup>[27]</sup>)

Somaclonal variation, which includes a variety of genetic and epigenetic variants, is expected to be a regular phenomenon in *in vitro*-raised plants (Peredo et al. 2006<sup>[28]</sup>). RAPD markers were chosen for their ease of application and low cost, as well as their usefulness in detecting variability in DNA sequences across *in vitro* preserved plantlets (Mohanty et al. 2008<sup>[25]</sup>). In the given study, a total of 10 RAPD primers (AM1, AM2, AM3, AM4, AM5, A6, AM7, AM8, AM9, C12) were used and resulted scorable and reproducible amplifications to analyze *in vitro* grown plantlets (Table. 1). Amplification products found were 57 altogether with an average of 5.7 bands per primer. There found no polymorphic banding between mother and *in vitro* raised plantlets of *Z. zerumbet*. Highest number of 8 bands was produced by AM1 and AM9 primers in contrast (Fig. 2 & 3), the least number of 1 band was observed with A6 primers. The band range was from 40bp to 2000 bp for all primers together.

This study detected no variations among the micropropagated *Z. zerumbet* plantlets which serve as an evidence that the plant's *in-vitro* propagation method is reliable. Plantlets for the study were taken from rhizomatous buds; it confirms the substantially higher genetic stability of rhizome-based micropropagation systems compared to callus-based regeneration systems.

### Conclusion

This work outlines the morphological and molecular identities of *Z. zerumbet* and also provided details on an effective micropropagation method and PCR-based DNA marker techniques that revealed no genetic variation in micropropagated plants. This high-fidelity technique can be

used to commercially multiply the desired *Z. zerumbet* germplasm and preserve it *in vitro*.

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