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Clonal Propagation of Turmeric (*Curcuma longa*) and Confirmation of Genetic Fidelity of the Micropropagated Shoots by RAPD Markers

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Abstract

Turmeric (*Curcuma longa*) is used as a spice and has excellent medicinal value. However, the field multiplication rate of turmeric plants is not satisfactory. *In vitro* methods could be a feasible alternative to vegetative propagation in the field. This study was aimed at developing a suitable protocol for the clonal propagation of turmeric confirming the genetic fidelity of the *in vitro* regenerated plantlets. The explants of shoot bud (full, half, and quarter), root, rhizome, and leaf were cultivated on MS medium with BAP, NAA, and Kinetin. Cultured shoot buds showed a higher regeneration frequency and shoots per explant than those obtained from other explants. The highest shoot regeneration was 72.49% from shoot buds, followed by rhizome (68.76%) and the lowest was 53.93 % from ¼ shoot buds. There were significant effect**s** of growth regulator combinations and concentrations in shoot regeneration from shoot bud explant. The highest shoot regeneration per shoot bud explant was 4.42 with BAP at 20.0 mg/l and NAA at 0.5 mg/l at 24 weeks. The best rooting response with 9.75 roots per explant was reported with 1.0 mg/l BAP with 1.0 mg/l NAA. The rooted plants were successfully transferred to pots for their acclimatization. Both the micropropagated shoots and their mother plant produced monomorphic bands with the six RAPD markers that confirm the genetic fidelity of micropropagated clones. These results showed that *in vitro* raised plantlets of turmeric had no risk of somaclonal variations and were found to be true-to-type in nature over the culture period. The protocol developed through this investigation has the potential application for the rapid multiplication of turmeric plants.

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Introduction

Turmeric (*Curcuma longa*) is cultivated as an important spice in Bangladesh. Turmeric contains several different active substances that exhibit anti-inflammatory, hypocholesterolemic, choleretic, antibiotic, antidiabetic, anticancerous, antiviral, antivenomous, antirheumatic, aromatic, germicidal, carminative, antioxidant, antihelmintic, anti-tumor, antimicrobial, neuroprotective and cholesterol-lowering properties besides its use in Alzheimer's disease (Kikuzaki and Nakatani 1993, Cao et al. 2001, Sasaki et al. 2002, Rao and Mittal 2014). The presence of curcumin (1.8-5.4%) and essential oil (2.5-7.2%) in turmeric rhizome (Mohanta et al. 2015) has made it a valuable ingredient in the cosmetic, pharmaceutical, and food (Sumathi 2007, Goncalves et al. 2014, Shaw and Panda 2015).

Turmeric is propagated exclusively vegetatively using rhizomes at the expense of 20-25% of the annual production of the previous year. The field multiplication of turmeric takes a long time (8-10 months) to produce rhizomes with 10-15 lateral bulbs (Stanly et al. 2010) giving a very low rate of vegetative propagation. It requires much labor, attention, time, and space (Nasirujjaman et al. 2005). Moreover, soil-dowelling microorganisms including bacteria, fungi, and nematodes contaminate rhizomes that are used as propagules (Faridah et al. 2011) and cause considerable loss of the quantity as well as the quality of the turmeric vegetative propagules.

Propagation by tissue culture is the best alternative to the field multiplication of disease-free quality propagules in many sterile plant species overcoming the hurdles of low propagation rates and quality maintenance (Haque et al. 1997, Haque et al. 2007). Tissue culture has enormous potential for mass clonal multiplication with secondary metabolites production for medicinal, food, and industrial uses (Gupta and Sharon, 2012). This technique obtains pathogen-free true-to-type plants in large quantities and produces seedlings year-round (Ahmadian et al. 2013, Haque et al. 1997, Haque et al. 1998a). Micropropagation requires an appropriate species-specific tissue culture system (Sridhar and Aswath, 2014). Hence, improvement of the protocol is essential to enhance the efficiency of micropropagation. Different plant growth regulators affect shoot proliferation and somatic embryo formation (Haque et al. 1997, Haque et al. 1998b, Haque et al. 2000). Several studies on plant regeneration in *Curcuma sp.* via either direct or indirect organogenesis using explants like leaf sheath (Raju et al. 2013), leaf base (Raju et al. 2015), rhizome buds (Shanthala et al. 2020, Jena et al. 2020), leaf petiole (Jie et al. 2019), callus from apical and lateral bud (Gurav et al. 2020) explants have been reported. In addition, in vitro induction of micro-rhizomes was reported in *C. caesia* and *C. longa* using 5-9% sucrose in the regeneration medium (El-Hawaz et al. 2015, Cousins et al. 2008, Sarma et al. 2021). Unfortunately, none of the studies was carried out with local varieties of turmeric.

In earlier studies of micropropagation with many crops, shoot proliferation from even organized buds did not always show genetic purity (Devarumath et al. 2002). Checking the genetic fidelity of the regenerants is, therefore, important for the production of progeny that are genetically uniform and either significantly reduce or eliminate the somaclonal variations (Larkins and Scowcroft, 1981) and is considered an essential step in micropropagation. Previous studies of the turmeric micro propagated reported the absence of (Salvi et al. 2001) and a high degree of genetic purity (Salvi et al. 2002). Molecular markers are popular for analyzing the genetic integrity of tissue culture-derived plants due to their consistency, reproducibility, independence of environmental effect, and quickness (Rani and Raina, 2003, Agarwal et al. 2008). Assessment of genetic purity of in vitro propagated turmeric shoots and plants using cytophotometry and RAPD analyses is scanty (Tyagi et al. 2007, Panda et al. 2007). The present study evaluates the plantlet regeneration capacity of various explants of turmeric and the genetic integrity of the regenerated shoots employing RAPD markers for the first time in Bangladesh for the multiplication of true-to-type clones of turmeric.

Materials and Methods

Contamination-free turmeric rhizomes were collected from the local farmers. Rhizomes, sprouted immature shoot buds (1-2 cm long), ½ shoot buds, ¼ shoot buds, roots, and leaves were used to culture on MS (Murashige and Skoog, 1962) tissue culture medium. Explants were prepared, and washed with water and 1% Savlon. All the explants were then surface sterilized by rinsing with 70% ethanol and dipping in 0.1% $HgCl_2$ solution for 15 min.

Rhizome bud explants (1-2 cm long) were cultured on various combinations and concentrations of BAP, Kn, and NAA for shoot induction. In each vial, 3-5 rhizome buds were placed. Sprouted shoot buds were cut into 1-2 cm pieces using sterilized surgical blades. In each vial, 3-4 pieces of shoot buds were inoculated for shoot induction. *In vitro,* shoot tips were cut into ½ and ¼ shoot buds and cultured. Root tips collected from germinated seedlings cut into 1 cm in length using sterilized surgical blades were cultured. The root tips were arranged horizontally on a medium containing BAP, Kn, and NAA at various concentrations. Healthy and disease-free leaves from field-grown plants were collected and dissected into 1-2 cm long and then cultured on the medium. The explants were incubated in the room at 25 ± 2°C, 1500 lux light intensity, and 16 hrs photoperiod. The vials were checked routinely to record the response and collection of appropriate data.

After 4 weeks, the shoots that developed on the culture were removed and placed again on a fresh medium. The regenerated micro-shoots were excised several times and subcultured on the same medium. Subculture was carried out at 4 weeks intervals. There was no additional treatment for rooting. Rooting was spontaneous in almost all media used for shoot induction and multiplication.

The plantlets with adequate roots were transplanted into pots filled with the potting mixture of soil and cow dung. The pots, after transplantation, were covered with polythene bags to protect the planlets from desiccation. The pots were placed in a growth room for an initial 7-15 days in a controlled environment. After 15-20 days, the plantlets were transferred to the field. Data were collected on shoot regeneration percentage, number of shoots and leaves per plantlet, root length, and number. The experiments were arranged in a Completely Randomized Design with 12 treatments; each replicated 3 times. The statistical analysis was done by the MSTAT computer program, ANOVA was calculated and the means were compared following DMRT.

Young leaves were used to extract genomic DNA following the procedure described in Haque et al. (2017). Twenty (20) primers were screened and six primers with clear, and distinct bands were selected and used for evaluation of diversity across 15 turmeric genotypes including 14 regenerants and one mother plant (Table 1). PCR was performed with DNA samples of 25 µl mixer composed of 12.5 µl PCR master mix (2X), 3µl of 10 pmol/µl respective primer, 2µl of turmeric genomic DNA, and 7.5 µl of nuclease-free water. Then the PCR reactions were run for 36 cycles with initial denaturation at 95°C for 2 mins, denaturation at 95°C for 1 min; Annealing at 32°C for 1 min, Primer elongation or extension at 72°C for 2 min, and final elongation at 72°C for 10 min. After completion, the PCR-amplified DNAs were separated by electrophoresis using 1% agarose gel and documented by a gel documentation system.

Table 1. Primers used in the present study with names and sequences

Primer name	Base Sequence (5' -3')	GC Content (%)
OPP04	GTG TCT CAG G	70
OEP07	AGA TGC AGC C	70
OPD20	ACC CGG TCA C	60
OPE20	AAC GGTGAC C	70
OPC01	TTC GAG CCA G	70
OPA10	GTG ATC GCA G	70

Data analysis: All data were analysed statistically, and means were calculated and differentiated by Duncan's Multiple Range Test (DMRT). A 1 kb DNA ladder was used to estimate the band size using Alpha Ease FC (version 4.0).

Results and Discussion

The establishment of a contamination-free culture was challenging in using underground rhizome bud explants (Fig. 1A). Initially, the explants were cultivated *in vitro* on a

medium with various growth regulators in combinations. After the plantlets formation, *in vitro*-derived leaves, roots, and the intersection of shoot buds were further cultured. There was a gradual increment in the shoot regeneration frequency and the shoots regenerated in each explant over time in all the explants. Shoot buds had a higher frequency of shoot regeneration and number of regenerated shoots per explant than rhizome bud, ½ shoot bud, and ¼ shoot bud explants. The highest shoot regeneration (72.49%) was recorded at 24 weeks from shoot bud explant, followed by rhizome (68.76%) explant (Table 1). The lowest rate of shoot regeneration was 53.93 % at 24 weeks from ¼ shoot bud explant.

Table 1. Effects of turmeric explants on *in vitro* regeneration of shoots

Explants	Shoot regeneration (%)			Shoots per explant		
	4 Week	12 Week	24 Week	4 Week	12 Week	24 Week
Rhizome	37.53 b	54.78 b	68.76 a	1.71 b	2.53 b	3.18 b
Shoot bud	47.65 a	61.55 a	72.49 a	2.41 a	3.12 a	3.71 a
½ shoot bud	32.41 c	48.12 c	58.59 b	1.35 c	1.94 c	2.88 c
¼ shoot bud	27.66 d	43.57 d	53.93 c	1.11 d	1.74 d	2.53 d
LSD(0.05)	4.29	4.29	4.27	0.185	0.18	0.19
CV (%)	30.13	21.05	17.15	28.62	19.97	15.84

Figures followed by the same letter(s) are not statistically significantly different by DMRT.

The type of explants was the significant factor that affected the shoot regeneration. *In vitro* propagation through micro rhizome induction from rhizome bud and shoot bud explants has been described in many countries by other groups (Ferrari et al. 2016, Tan 2016, Wahengbam et al. 2015, Jala 2012, Rahayu et al. 2012, Behara et al. 2010). Meristematic tissue is always considered the best explant for regeneration in many other crops because these tissues have high cell division rates. Pattnaik et al. (1996) found shoot buds to be the best available explant for *in vitro* propagation, because of the pre-existing meristem that develop quickly into shoots. Identical results were observed in this study.

There was a notable variation in shoots per explant and the highest number of shoots (3.71) was achieved from shoot bud explant at 24 weeks, followed by rhizome explant. The lowest number of shoots per explant was 2.53 at 24 weeks from ¼ shoot bud explant (Table 1).

Significant effect**s** of various combinations and concentrations of growth regulators were observed in shoot regeneration. The highest shoot regeneration per shoot bud explant was 4.42 in 20.0 mg/l BAP and 0.5 mg/l NAA at 24 weeks. The highest percentage (84.17%) of plantlet regeneration was recorded in the same treatment combination at 24

weeks (Table 2). Tyagi et al. (2007) reported 11.2 or 22.2 µM BAP for efficient shoot regeneration in *Curcuma*. On the other hand, Roy and Raychaudhuri (2004) using a kinetin (5.0 mg/l) and NAA (4.0 mg/l) combination obtained the best regeneration of shoots (~4 shoots) using nodal segments as explants. However, Preethi et al. (2010) recommended BAP (2.22 µM) and NAA (2.69 µM) as the best combination for turmeric. The highest shoot length (8.43 cm) was found in 20.0 mg/l BAP and 0.5 NAA mg/l at 24 weeks. Although BAP at the higher level (8.0 mg/l) had the highest shoot length, multiple shooting was observed in lower concentrations (2.0 and 4.0 mg/l) of BAP (Behara et al. 2010). This type of variation in tissue culture response is common in many species showing differential PGR responsiveness and shoot regeneration probably for genotypes.

Fig. 1. Regeneration of rhizome bud explant on MS medium with 20 mg/l BAP+ 0.5 mg/l NAA (A), Shoot regeneration failed on roots (B), a rooted plantlet (C), elongated shoot on MS medium with 5.0 mg/l BAP and 0.5 mg/l NAA (D), 10 mg/l BAP and 0.5 mg/l NAA (E), 20 mg/l BAP + 0.5 mg/l NAA (F).

The use of the lower levels of auxins with cytokinins was reported to increase the numbers of shoots in different species (Phulwaria et al. 2012, Loc et al. 2005, Jeong et al. 2001). Several plants have been successfully multiplied on a medium with BAP and Kn. BAP alone and in combinations with NAA and Kn in combinations with NAA were tested for assessing the appropriate levels of the cytokinins to maximize shoot number. BAP while combined with NAA was more effective than BAP without NAA on shoot multiplication of *C. longa*.

The efficient use of BAP and NAA in the formation of multiple shoots in turmeric was reported earlier (Ghosh et al. 2013). Several reports are also available where a medium with BAP and NAA at various combinations was successfully used for the regeneration of multiple shoots from different explants of turmeric and ginger (Wahengbam et al. 2015, Ikeda and Tanabe 1989, Bhagyalakshmi and Sing 1988). The results revealed that NAA at a lower concentration with BAP gave the best shoot multiplication in *C. zedoaria* (Shahinozzaman et al. 2013) and *C. amada* (Prakash et al. 2004, Ferdous et al. 2012). The superiority of BAP over Kn for producing *in vitro* shoots has also been established in other plants like *Psidium guajava* (Liu and Yang 2011), *Citrus clementina* (Lombardo et al. 2011), garlic (Haque et al. 1997, 1998a) and *Citrus limon* (Goswami et al. 2013). The present findings are in line with the above information.

Table 2. Effects of growth regulators on shoot regeneration, rooting, and plantlet formation

Treatments		% shoot regeneration	Shoots/ explant	Roots/ plantlet	Root wt./ plantlet (mg)	Root length (cm)	Leaves/ plantlet	Shoot length (cm)
NAA (mg/l)	BAP (mg/l)							
0	0	38.08 e	1.92 f	3.84 h	0.5 e–g	1.92 f	1.92 f	8.04 d
0	1.0	49.92 d	2.42 e	6.84d	0.7 ab	1.93 f	1.92 f	7.87 f
	5.0	56.67 cd	2.92 d	6.67 d	0.7 bc	2.75 b	1.93 f	8.09 cd
	10.0	53.25 d	2.25 ef	2.75 i	0.6 c–e	1.0 i	2.75 b	7.57 g
	20.0	66.58 bc	3.17 cd	2.75 i	0.5 e–g	1.50 h	1.00 i	7.45 h
0.5	1.0	70.00 b	3.17 cd	5.75 e	0.6 cd	1.75 g	1.50 h	7.93 ef
	5.0	58.17 cd	2.42 e	4.92 f	0.5 d–f	2.00 e	1.75 g	7.54 g
	10.0	71.67 b	3.42 bc	4.58 fg	0.5 fg	3.84 a	2.00 e	8.23 b
	20.0	84.17 a	4.41 a	2.92 i	0.4 g–i	1.75 g	3.84 a	8.43 a
1.0	1.0	64.92 bc	3.33 cd	9.75 a	0.8 a	2.00 e	1.75 g	7.55 g
	5.0	63.33 bc	2.92 d	9.00 b	0.7 ab	2.59 c	2.00 e	7.45 h
	10.0	73.33 b	3.17 cd	7.59 c	0.7 bc	2.58 c	2.59 c	7.61 g
	20.0	63.33 bc	3.00 cd	4.92 f	0.6 cd	5.09 de	2.58 c	7.04 k
LSD(0.05)		8.784	0.393	0.6031	0.081	0.0989	0.0625	0.0847
CV (%)		17.15	15.84	15.23	4.34	2.70	3.51	1.39

Figures followed by the same letter(s) are not statistically significantly different by DMRT.

In this experiment, root regeneration was spontaneous on the shoot regeneration medium (Fig. 1E). Therefore, no additional treatment for rooting was necessary. This study is supported by Nasirujjaman et al. (2005).

The best rooting response with 9.75 roots per explant was reported with 1 mg/l BAP in and 1 mg/l NAA. The highest root weight was 0.079 g and the longest root (6.375 cm) was on the same medium. In turmeric, rooting was on half-strength MS basal salts in response to NAA (Dipti et al. 2005). The maximum root number was found in the plantlets generated in response to 5.0 mg/l BAP and 1.0 mg/l NAA. Arimura et al. (2000) reported the highest number of longer roots in the same medium. The rooted plants (Fig. 1C) were transferred to pots containing soil.

Twenty (20) RAPD loci were initially screened to analyze the genetic integrity of the plantlets. Six primers namely OPP04, OEP07, OPE20, OPC01, OPD20, and OPA10 that produced distinct and clear bands of better quality and efficiency in detecting polymorphism were selected to detect if there were any variations of the 14 randomly selected turmeric regenerated shoots and mother plants at the molecular level. Six primers detected 15 loci, 2-3 loci per primer, with sizes ranging from 100bp to 1000bp. All the primers showed monomorphic banding in the 14 regenerated shoots and the mother plant. The banding patterns of three representative primers are shown (Figs 2-4). The DNA amplification obtained by six RAPD primers used in this study clearly showed that both micro-propagated shoots and their mother plant exhibited a monomorphism in the banding pattern. This pattern confirms the genetic fidelity of the micropropagated clones. These results also showed that the *in vitro* raised plantlets of turmeric remained free from the risk of somaclonal variations and true-to-type in nature over the culture period.

Table 3. RAPD primers used in this study with band score

Primer code	Sequences (5' - 3')	Total band number	Monomorphic bands	Polymorphic bands
OPP-04	GTG TCT CAG G	3	3	0
OEP-07	AGA TGC AGCC	3	3	0
OPD-20	ACC CGG TCA C	2	2	0
OPE-20	AAC GGTGAC C	3	3	0
OPC-01	TTC GAG CCA G	2	2	0
OPA-10	GTG ATC GCA G	2	2	0
Overall		15	15	0

The occurrence of somaclonal variations in vitro culture depends on the explant source and regeneration pathway (Goto et al. 1998). In general, plants regenerated from well-differentiated organs like adventitious buds or well-developed meristems yielded

fewer variations (Rout et al. 1998, Joshi and Dhawan 2007). On the contrary, regenerants obtained via a callus phase showed higher variations (Al-Zahim et al. 1999, Parvin et al. 2008). According to Potter and Jones (1991), plants that regenerate from preexisting meristems are genetically stable, and somaclonal differences are frequently linked to regeneration from undifferentiated tissues. These results confirm that micropropagation systems based on meristems are genetically far more stable than regeneration through a long callusing phase. Regeneration via callus is more sensitive to variations than direct regeneration without an intermediate callus phase.

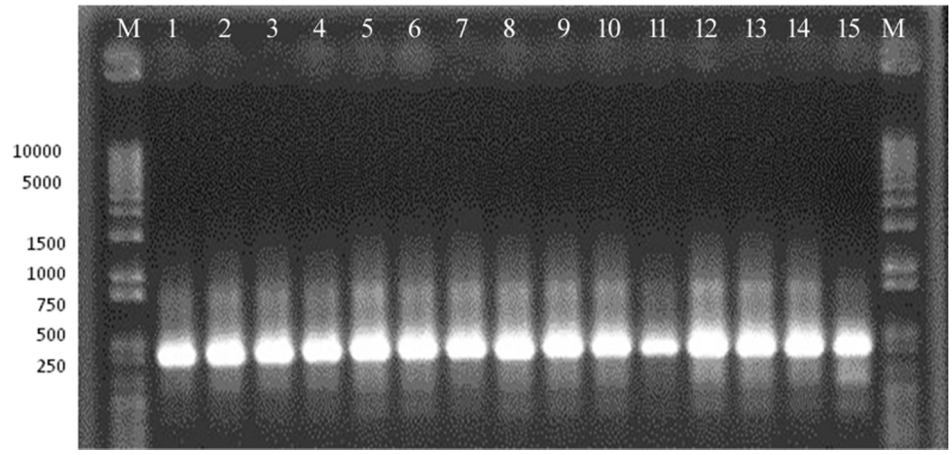

Fig. 2. RAPD profile of 14 *C. longa* Somaclones and mother plant using primer OPE20. Bands of lanes 1-14 represent 14 clones and lane 15 is the DNA band of the mother plant. M: Molecular Weight Marker (1kb DNA Ladder).

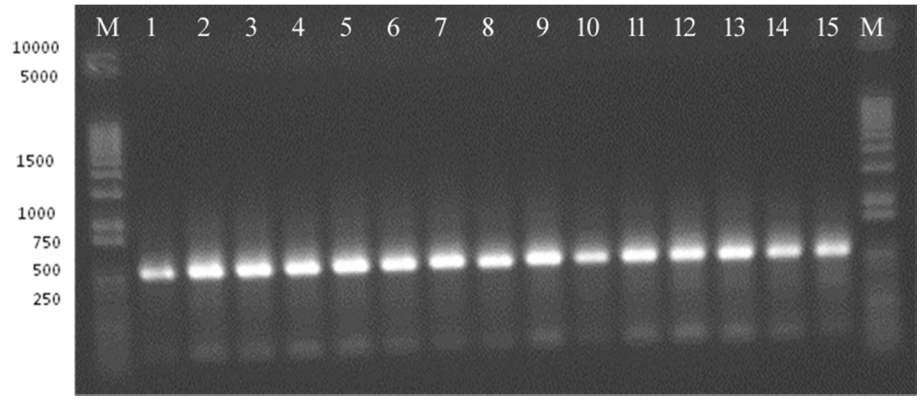

Fig. 3. RAPD profile of 14 *C. longa* clones and mother plant using primer OPC01. Bands of lanes 1-14 represent 14 shoots and lane 15 indicates the DNA band of the mother plant. M: Molecular Weight Marker (1kb DNA Ladder).

The type and concentrations of the growth regulators play a key role in inducing variations among the somaclones. The extraordinarily low or high levels of growth regulators, more especially the synthetic ones, are more likely to induce variations in tissue culture-derived plants (Martin and Pachathundkandi 2006). Even prolonged culture using growth regulators at optimum levels often may lead to epigenetic or somaclonal variations changing the fidelity of the clonal nature of the regenerated plants. In this study, a total of 15 distinct bands were recorded in regenerated clones and the mother plant all of which are monomorphic.

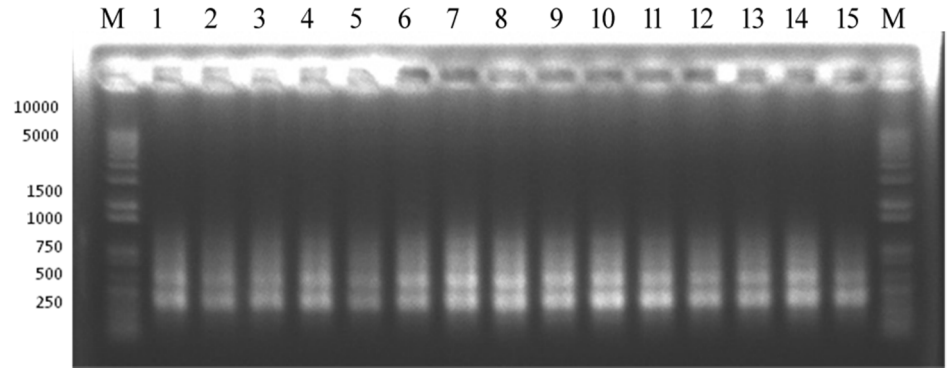

Fig. 4. RAPD profile of 14 *C. longa* Somaclones and mother plant using primer OPA10. Bands of lanes 1-14 represent 14 Somaclones and lane 15 indicates the DNA band of the mother plant. M: Molecular Weight Marker (1kb DNA Ladder).

According to Bhatia et al. (2010), cormel's shoot tip explants derived clones were true-to-type, while one clone from the leaf exhibited variations. A monomorphic banding profile in Gladiolus obtained using RAPD markers, as of ours, was indicative of no genetic variation among the plants produced by tissue culture in relation to the mother plant they were derived from (Kumar et al. 2018). All primers in our study showed monomorphism in banding. The total bands produced per primer ranged from 2-3 giving 2.5 bands on average. Primers OEP-07, OPP-04, and OPE-20 produced three bands each. The other three primers produced two bands. As expected, the primers produced 15 distinct bands ranging from 100 to 1000bp in each clone along with the mother. It can be confirmed that the shoots show genetic identity with the mother plant from which they were regenerated.

A protocol of *in vitro* clonal propagation turmeric was developed in this study. The results of the RAPD analysis showed that *in vitro*-raised plantlets of turmeric had no risk of somaclonal variations and were genetically identical in nature over the culture period. The protocol has potential applications for the rapid multiplication of turmeric plants maintaining genetic integrity.

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