Genetic Stability of Micropropagated Ginger Derived from Axillary Bud through Cytophotometric and RAPD Analysis

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A protocol was developed for the *in vitro* propagation of ginger (*Zingiber officinale*) cv. Suprava using dormant axillary buds from unsprouted rhizomes. The dormant axillary buds embedded in the rhizome nodes were induced to sprout when cultured on MS medium supplemented with 6-benzyladenine (BA) alone (1–6 mg/l) or with a combination of BA (1–6 mg/l) and indole-3-acetic acid (IAA) (0.5, 1 mg/l). *In vitro* sprouted buds were transferred to the multiplication medium containing various combinations of auxins and cytokinins. MS basal medium supplemented with BA (1 mg/l), IAA (1 mg/l) and adenine sulfate (100 mg/l) was found optimum for the *in vitro* multiplication of shoots producing (8.2 ± 0.2) shoots from a single explant within 30 days of culture. The multiplication rate remained unchanged in subsequent subcultures. Rooting of shoots occurred in the same multiplication media. Upon transfer of the *in vitro* culture to *ex vitro* in pots, 96% of plants survived and established successfully under natural conditions. Tissue culture-raised plantlets of ginger could be conserved *in vitro* through subculturing at an interval of 4 months. The genetic stability of micropropagated clones was evaluated at regular intervals of 6 months up to 24 months in culture using cytophotometric estimation of 4C nuclear DNA content and random amplified polymorphic DNA (RAPD) analysis. Cytophotometric analysis revealed a unimodal distribution of the DNA content with a peak corresponding to the 4C value (23.1 pg), and RAPD analysis revealed monomorphic bands showing the absence of polymorphism in all fifty regenerants analyzed, thus confirming the genetic uniformity among *in vitro* grown somaclones of *Z. officinale*. This study is of commercial significance as axillary bud explants are available throughout the year for initiating a fresh culture of the elite ginger cv. Suprava to be used as a source of true-to-type disease-free planting material thereby minimizing the adverse effect of repeated subculturing from the same explant source.

Key words: Zingiber officinale, Micropropagation, Genetic Stability

Introduction

Ginger (*Zingiber officinale* Rosc.), a monocotyledonous perennial herb having a respected history as a spice crop, is known to human generation since time immemorial (Anonymous, 1950). It is used in medicine as a carminative and an aromatic stimulant to the gastrointestinal tract and externally as a rubefacient and counter irritant (Rout and Das, 2002). Besides, the use of ginger oil and oleoresin in various food and drink items has increased its economic importance in the global market. Conventionally ginger is propagated vegetatively by underground rhizomes with a slow multiplication rate. The rhizome pieces which are used as planting material contain apical buds which become active and sprout at the onset of monsoon, but latent buds embedded within scales of nodes remain dormant throughout the growing phase and occasionally sprout late in the season. Furthermore, the high susceptibility of this crop to soft rot and bacterial wilt is a major constraint in the production of ginger. A protocol for rapid multiplication is necessary to provide enough true-to-type disease-free planting material especially for the newly developed lines that are available in small quantities. Tissue culture technique is a powerful tool which can be employed as an alternative to the conventional method of vegetative propagation with the objective of rapid clonal multiplication of desired genotypes (Hussey, 1986; Murashige and Skoog, 1990). Periodic monitoring of the degree of genetic stability of *in vitro* con-

Abbreviations: BA, 6-benzyladenine; NAA, α-naphthalene acetic acid; IAA, indole-3-acetic acid; Ads, adenine sulfate.

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served plants is of utmost importance for commercial utilization of the technique for large scale production of true-to-type plants of the desired genotype. The assessment of the genetic integrity of *in vitro* grown regenerants of regular intervals can significantly reduce or eliminate the chance of occurrence of somaclonal variations (Larkins and Scowcroft, 1981) at the early or late phase of culture. Cytophotometric analysis is very useful for quick determination of the extent of the ploidy level of regenerated plants with a large number of cells (Nayak and Sen, 1995). Of several molecular markers used for the assessment of the genetic stability, random amplified polymorphic DNA (RAPD) analysis is the simplest, cheapest and quickest method for determining the genetic fidelity of *in vitro* grown plants as reported in many species (Williams *et al*., 1990; Rout *et al*., 1998; Martins *et al*., 2004; Venkatachalam *et al*., 2007). Until now *in vitro* propagation through multiple shoot induction has been reported in ginger using sprouting apical bud and meristem tips (Hosoki and Sagawa, 1977; Bhagyalakshmi and Singh, 1988; Balachandran *et al*., 1990; Sharma and Singh, 1997; Rout and Das, 2002; Tyagi *et al*., 2006). Genetic analysis has only been made from meristem culture-derived micropropagated ginger by Rout *et al.* (1998).

In the present communication, we report on the rapid *in vitro* multiplication of a promising ginger cultivar (Suprava) using dormant axillary buds of rhizome segments as explants and *in vitro* detection of the genetic stability of micropropagated plants through cytophotometric estimation of the 4C nuclear DNA content and RAPD analysis of somaclones at regular intervals. 'Suprava' is a high yielding genotype released from High Altitude Research Station, Pottangi, Orissa, India in 1988, and its cultivation is confined to limited areas. Considerable genetic differences found among different ginger cultivars (Nayak *et al.*, 2005) necessitate the standardization of the protocol for large scale production of a disease-free stock of planting material of elite genotypes through tissue culture. The use of different genotypes and different explants of same genotype proved to have a marked effect on the induction of the somaclonal variation (Nayak and Sen, 1991, 1998). So in the present study latent axillary bud-derived micropropagated ginger somaclones conserved *in vitro* were periodically assessed for genetic integrity.

Material and Methods

Preparation of plant material

Rhizomes of a high yielding variety of ginger (cv. Suprava) were collected at the High Altitude Research Station (HARS), Pottangi, Orissa, India and were grown in the medicinal plant garden of Siksha 'O' Anusandhan University, Bhubaneswar, Orissa, India. Rhizomes containing latent axillary buds were used as explants. The explants were thoroughly washed with water, and the scale leaves were carefully cleaned so that impression of embedded buds could be marked without being damaged. Those explants were kept under running water for 10–15 min and then dipped in liquid detergent (Extran, Merck) for 3–5 min. They were thoroughly washed with distilled water to remove the last drop of detergent. Surface-sterilization was done by 0.1% mercuric chloride solution for 8–10 min. After sterilization the explants containing embedded buds were washed several times with sterile distilled water under aseptic conditions prior to inoculation.

In vitro sprouting and multiplication

Explants were inoculated on basal medium of Murashige and Skoog (MS) (1962) with varying combinations of 6-benzyladenine (BA) (1–6 mg/l), indole-3-acetic acid (IAA) (0.5, 1 mg/l) and adenine sulfate (Ads) (70, 100 mg/l) as mentioned in Table I. 15 replicas were used for each treatment. Prior to inoculation, MS media containing hormones were autoclaved at 121 °C and 1.05 kg/cm^2 of pressure for 20 min. pH value of the media was adjusted to 5.7. Culture tubes containing the inoculated explants were kept under white fluorescent light with 55 μmol/(m^2 s^2) light intensity. Percentage of response of the explants to different hormonal combinations was observed from time to time. To induce multiplication of *in vitro* sprouted axillary buds and plant regeneration, inoculants were transferred to a fresh medium after 4–6 weeks with varying hormonal combinations (Table II). The culture conditions were similar to those used at the time of initial explant inoculation.

Establishment of plant in soil

In vitro grown plantlets having well developed shoots and roots were transferred to pots containing sterilized soil. These were then kept in a greenhouse for acclimatization. After one month they

were transferred to normal field conditions and grown to maturity.

Cytophotometric analysis

For analysis of the 4C nuclear DNA content through cytophotometry, root tips of *in vitro* grown regenerants were collected aseptically from culture tubes and were fixed over night in propionic acid/ethanol (1:3). This treatment was followed by hydrolysis in 1 M HCl (v/v) at 60 °C for 12 min. The root tips were washed in distilled water, stained in Schiff's reagent for 2 h at 14 °C and squashed with 45% acetic acid. The DNA content of nuclei was measured with a Leitz Wetzlar microspectrophotometer using the single wavelength (550 nm) method (Sharma and Sharma, 1980). *In situ* DNA values were obtained on the basis of optical densities which were converted to picograms (pg) by using Van't Hof's 4C nuclear DNA value of *Allium cepa* (67.1 pg) as standard (Van't Hof, 1965).

Isolation of DNA

Fresh young leaves collected for DNA isolation were washed thoroughly with cold autoclaved distilled water and then blotted to dryness. About 2 g of leaves were taken for extraction. The genomic DNA was isolated following the protocol of Doyle and Doyle (1990) with a little modification. Insoluble polyvinylpolypyrrolidone was added to the leaf sample prior to grinding. The crude DNA was purified with RNase A (@ 60 μg/ml of crude DNA solution) followed by washing thrice with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and subsequently with chloroform/isoamyl alcohol (24:1 v/v). Quantification of DNA was done by 0.8% agarose gel electrophoresis of the samples along with a known amount of uncut lambda DNA (Bangalore Genei Pvt. Ltd, Bangalore, India) as standard. DNA samples were diluted to 25 ng/μl for RAPD-PCR analysis.

RAPD analysis

A total of 20 random primers was used for RAPD analysis out of which 8 random decamer primers (Operon Tech, Alameda, USA) from A, C, D, and N series (OPA02, OPA03, OPC02, OPC05, OPD08, OPD18, OPD20, OPN06) were selected on the basis of the clarity of banding pattern. The RAPD analysis was performed as described by Williams *et al.* (1990). PCR was performed in a volume of 25 μl containing 25 ng of template DNA, 2.5 ml of 10X assay buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 0.1% gelatine), 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 15 ng of primer (Bangalore Genei), 0.5 U Taq DNA polymerase (Bangalore Genei Ltd). DNA amplification was performed in a thermal cycler (Model Gene Amplification PCR System 9700, Applied Biosystems, CA, USA). The first step consisted of holding the samples at 94 °C for 5 min for complete denaturation of the template DNA. The second step consisted of 42 cycles having three ranges of temperature, *i.e.* at 92 °C for 1 min for denaturation of template DNA, at 37 °C for 1 min for primer annealing and at 72 °C for 2 min for primer extention followed by running the samples at 72 °C for 7 min for complete polymerization. The PCR products were electrophoresed in 1.5% agarose gel containing ethidium bromide [@ 0.5 μg/ml of gel solution in TAE buffer (40 mM Tris, 20 mM acetic acid, 20 mM EDTA to pH 8)] for 3 h at 60 V. The size of the amplicons was determined using size standards GeneRuler 100 bp DNA Ladder Plus (MBI Fermentas, Garcenovalis, Lithuania). DNA fingerprints were visualized under UV light and photographed using a gel documenting system (Bio-Rad, CA, USA). RAPD analysis using each primer was repeated at least twice to establish the reproducibility of banding pattern of different DNA samples of ginger.

Results and Discussion

In vitro sprouting

The present paper reports on *in vitro* sprouting of dormant axillary buds of rhizomes and subsequent induction of multiple shoots unlike all other reports dealing with sprouted buds for *in vitro* shoot multiplication (Rout and Das, 2002). Axillary buds of rhizome used as explants in the present study are available throughout the year whereas sprouted buds used as explants are available only at the onset of monsoon. The effect of various plant growth regulators on sprouting of these latent axillary buds of rhizome segment was observed. Explants were inoculated on MS media containing BA (1–6 mg/l) alone and in combination with IAA (0.5, 1 mg/l) (Table I). Although sprouting of buds occurred on media with BA alone, percentage of response was higher on media with a combination of BA and IAA. Media with kinetin had negligible effects towards *in vitro*

Table I. Effect of different growth regulators on *in vitro* sprouting of latent axillary buds of *Z. officinale* (cv. Suprava).

Sl. No.	MS medium + growth regulators [mg/l]	% of sprouting of explants (mean ± SE)[a]
1	BA (1.0)	26.6 ± 0.8
2	BA (2.0)	32.8 ± 0.9
3	BA (3.0)	33.3 ± 1.01
4	BA (4.0)	40.4 ± 1.1
5	BA (6.0)	25.96 ± 1.0
6	BA (1.0) + IAA (0.5)	50.36 ± 0.8
7	BA (1.0) + IAA (1.0)	51.5 ± 0.8
8	BA (2.0) + IAA (0.5)	61.9 ± 0.6
9	BA (3.0) + IAA (0.5)	86.5 ± 0.8
10	BA (3.0) + IAA (1.0)	80.6 ± 1.03
11	BA (4.0) + IAA (1.0)	96.23 ± 1.19
12	BA (6.0) + IAA (1.0)	73.4 ± 0.8
F value		671.5
P value		< 0.001

The differences in mean values among the treatments are statistically significant ($p < 0.001$).
[a] Data represent the mean of 15 replicates for each treatment.

sprouting (data not shown). Of the various combinations tried, media with 4 mg/l BA and 1 mg/l IAA showed maximum response (96.23 ± 1.19) towards *in vitro* sprouting, which was significantly higher than with other hormonal combinations (Table I). About 96.2% of the explants sprouted on this medium within 7 days of inoculation. Sprouted buds elongated further attaining about 2 cm height within 30 days of explantation. With increasing concentration of BA beyond 4 mg/l, percentage of sprouting was reduced (Table I). Sprouting of buds did not occur in the presence of either IAA or kinetin alone.

In vitro shoot multiplication and growth of plantlets

Sprouted axillary buds which attained a height of about 2 cm in induction media were transferred to multiplication media. As we were using unsprouted buds, this step was necessary to promote a rapid growth of the sprouted shoots to multiply. MS media containing BA (1–6 mg/l), IAA (0.5, 1 mg/l) and Ads (70, 100 mg/l) were used for the multiplication of sprouted buds (Table II). Though multiplication of shoot buds occurred in all media mentioned, MS basal medium with 1 mg/l BA, 1 mg/l IAA and 100 mg/l Ads was optimum for shoot multiplication. On this medium, percentage of response was (86.2 ± 0.3) and the number of shoots formed was (8.2 ± 0.2). Simultaneously root development occurred in this medium. Percentage of multiplication was reduced with increased concentration of BA beyond 1 mg/l (Table II). MS medium containing kinetin and α-naphthalene acetic acid (NAA) showed no significant results. The effectiveness of BA for shoot multiplication using apical bud explants has been reported in other zingiberaceous species (Hosoki and Sagawa, 1977; Bhagyalakshmi and Singh, 1988; Balachandran *et al.*, 1990; Ikeda and Tambe, 1989; Smith and Hamil, 1996; Inden *et al.*, 1988). In all these cases, formation of 4–7 shoots per explant has been reported. The multiplication rate ob-

Table II. Effect of various growth regulators on *in vitro* shoot multiplication of *Z. officinale* (cv. Suprava).

Sl. No.	MS medium + growth regulators [mg/l]	% of shoot multiplication (mean ± SE)[a]	No. of shoots per explant (mean ± SE)[a]
1	No growth regulator	13.6 ± 0.5	1.4 ± 0.16
2	BA (1.0)	16.0 ± 0.3	1.6 ± 0.16
3	BA (3.0)	33.8 ± 0.3	3.3 ± 0.16
4	BA (5.0)	30.0 ± 0.3	3.1 ± 0.1
5	BA (6.0)	17.0 ± 0.3	1.7 ± 0.15
6	BA (1.0) + IAA (0.5)	34.4 ± 0.2	3.5 ± 0.16
7	BA (2.0) + IAA (1.0)	35.2 ± 0.3	3.5 ± 0.16
8	BA (1.0) + IAA (0.5) + Ads (70)	44.0 ± 0.3	4.4 ± 0.16
9	BA (1.0) + IAA (0.5) + Ads (100)	44.6 ± 0.2	4.5 ± 0.16
10	BA (1.0) + IAA (1.0) + Ads (100)	86.2 ± 0.3	8.2 ± 0.2
11	BA (3.0) + IAA (0.5) + Ads (100)	53.6 ± 0.2	5.2 ± 0.2
12	BA (3.0) + IAA (1.0) + Ads (100)	56.8 ± 0.3	5.7 ± 0.15
13	BA (4.0) + IAA (1.0) + Ads (100)	56.2 ± 0.3	5.3 ± 0.26
14	BA (6.0) + IAA (1.0) + Ads (100)	27.0 ± 0.3	2.6 ± 0.16
F value		475.9	116.9
P value		< 0.001	< 0.001

The differences in mean values among the treatments are statistically significant ($p < 0.001$).
[a] Data represent the mean of 15 replicates for each treatment.

served in our experiment remained the same in subsequent subcultures. MS media containing kinetin and NAA when tried for *in vitro* shoot multiplication showed no significant result as reported earlier for *Z. officinale* (Sharma and Singh, 1997). This difference could be due to the use of different genotypes for culture establishment. Both solid and liquid media were tried to compare the rate of multiplication. Though the multiplication rate remained the same in both media, plants grown in liquid media became weak and etiolated with stunted growth. This report is in close agreement with that of Bhagyalakshmi and Singh (1988), who reported solid media as superior to liquid media for shoot multiplication for *Z. officinale*. For enhancement of growth, *in vitro* grown plantlets were transferred to different media (Fig. 1). MS medium containing 1 mg/l BA was the most effective for shoot growth and development. The length of shoots in this medium was (9.8 ± 0.17) cm after 15 days of transfer. This medium was also best effective for root development. With increasing concentration of BA beyond 1 mg/l the growth rate was retarded. Our report showing an enhanced growth rate using solid MS medium is in close agreement to the report of Bhagyalakshmi and Singh (1988), but in contrast to Ikeda and Tambe (1989). In *Z. officinale* liquid media revealed as superior over the solid ones. This difference in reports by various authors might be due to a genotypic effect of *Z. officinale* as different cultivars were used.

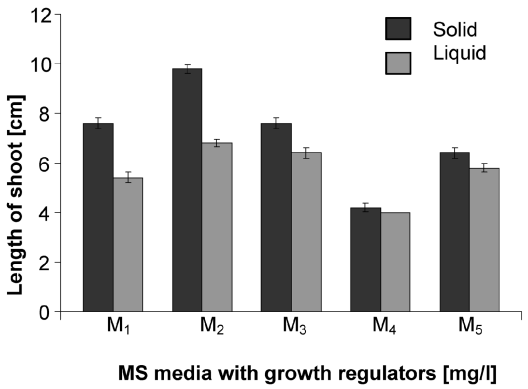

Fig. 1. Effect of solid and liquid media with different growth regulators on *in vitro* growth of *Z. officinale* (cv. Suprava). M_1, MS + no growth regulators; M_2, MS + BA (1 mg/l); M_3, MS + BA (3 mg/l); M_4, MS + BA (4 mg/l); M_5, MS + BA (1 mg/l) + IAA (1 mg/l) + Ads (100 mg/l).

In vitro grown plants could be conserved on modified MS medium supplemented with 1 mg/l BA, 1 mg/l IAA, 100 mg/l Ads and enhanced content of sucrose (6%) by subculturing at a 4-month-interval for more than 24 months. The role of an enhanced concentration of sucrose for prolonging the subculture interval for short term conservation has also been reported for turmeric (Nayak, 2002).

Establishment of plantlets in soil

In vitro grown plantlets were transferred to pots containing soil, cow dung and sand at a 1:1:1 ratio after 90 days of culture. These were then kept in a greenhouse for 30 days to acclimatize. About 96% of plants survived successfully with normal growth after being transferred to field conditions.

Cytophotometric analysis

The cytophotometric analysis of the 4C nuclear DNA content of root tips from a total of 50 *in vitro* grown regenerants carried out at 6-month-intervals up to 24 months in culture revealed diploidy in all with a unimodal distribution with one distinct peak corresponding to a 4C value (23.1 pg) same as obtained in the source plant. Thus the possible occurrence of a somaclonal variation was ruled out in the *in vitro* conserved plants of *Z. officinale* derived from axillary buds of rhizomes. Cytophotometric analysis has also been used for the assessment of the genetic stability of *in vitro* grown plantlets in other plants like *Ornithogalum thyrsoides* and *Ornithogalum umbellatum* (Nayak and Sen, 1991, 1995). The plantlets of *Z. officinale* used for the cytophotometric study were labeled properly to be used for the molecular analysis with RAPD markers.

RAPD analysis

In order to confirm the genetic integrity of micropropagated ginger maintained in culture over a period of 2 years, RAPD analysis was carried out at 6-month-intervals starting from one-month-old plantlets. A total of 50 plantlets over a period of 24 months were analyzed taking a minimum of 10 plants randomly at each culture period. 8 selected RAPD primers utilized in this study gave rise to a total of 40 scorable bands, ranging from 250 bp to $>$ 3000 bp in size (Table III). The number of bands for each primer varied from 3 to 8, with an average of 5 bands per RAPD primer. A total of 2000 bands [(number of plantlets analyzed) (number of

Table III. RAPD banding pattern of micropropagated and field-grown mother plants of *Z. officinale*.

Primer	Sequence	Range of amplicons [bp]	Total no. of bands
OPA02	5′TGCCGAGCTG3′	250 – 2100	4
OPA03	5′AGTCAGCCAC3′	500 – 900	3
OPC02	5′GTGAGGCGTC3′	500 – 1950	8
OPC05	5′GATGACCGCC3′	1200 –> 3000	4
OPD08	5′GTGTGCCCCA3′	600 – 1700	4
OPD18	5′GAGAGCCAAC3′	300 – 1100	4
OPD20	5′ACCCGGTCAC3′	450 – 2200	7
OPN06	5′GAGACGCACA3′	500 – 1800	6
Total			40

bands with all primers)] was generated by the RAPD techniques, giving rise to monomorphic patterns across all 50 plantlets analyzed. The number of monomorphic bands was highest, *i. e.* 8, in case of primer OPC02 (ranging from 500–1950 bp in size) and lowest, *i. e.* 3, in case of primer OPA03 (ranging from 500–900 bp in size). The RAPD banding pattern showing monomorphic bands obtained among 15 regenerants from a 24-month-old culture is shown in Fig. 2 for RAPD markers. No RAPD polymorphism was observed in the micropropagated plants (Fig. 2).

RAPD analysis of micropropagated plants of ginger (*Zingiber officinale* cv. Suprava) showed a profile similar to that of the control indicating that no genetic variation had occurred *in vitro*. RAPD-based assessment of the genetic stability of *in vitro* grown micropropagated plants has been reported for many other plant species (Rani *et al.*, 1995; Rout and Das, 2002; Martins *et al*., 2004; Venkatachalam *et al*., 2007). We have used axillary buds as explants for micropropagation of *Z. officinale* through direct organogenesis because they lower the risk of genetic instability. Shenoy and Vasil (1992) reported that micropropagation through explants containing organized meristem is generally associated with a low risk of genetic instability. The culture conditions used to achieve plant regeneration from tissue where meristems are already present are less aggressive than those usually needed to induce shoots from differentiated cells via disorganized calli (Martins *et al.*, 2004). The present study provides the first information on the genetic stability of micropropagated ginger obtained from unsprouted axillary bud explants of rhizomes using RAPD analysis. RAPD-based genetic stability of ginger has been reported by Rout *et al*. (1998) among 15 micropropagated plants obtained from the sprouted apical buds of cv. V_3S_{18}. Vendrame *et al*. (1999) concluded that the genetic variation in a culture line could be affected more by a genotype than by the period in culture. Hammerschlag *et al.* (1987) suggested that the genotype and the nature of explants could influence the phenotypic stability of the plants obtained in his studies performed with micropropagated peach plantlets. Nayak and Sen (1998) reported that the degree of genetic instability in cultured tissue of *Ornithogalum* sp. was influenced by different genotypes used. Some authors, however, have re-

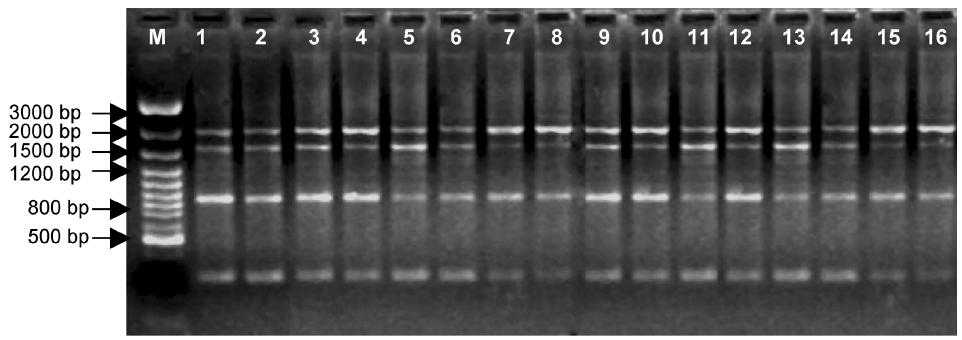

Fig. 2. RAPD banding pattern with primer OPA02 in both micropropagated and field-grown mother plants of *Z. officinale* (cv. Suprava). Lane 1, mother plant; lanes 2–16, micropropagated plants.

ported that the time of *in vitro* culture could promote somaclonal variation (Orton, 1985; Hartmann *et al*., 1989; Nayak and Sen, 1991). In our study the length of the culture period (for more than two years) with regular subculturing did not seem to affect the genetic integrity of *Z. officinale*. Similar results were also reported by Nayak and Sen (1991) for *Ornithogalum* sp., Salvi *et al.* (2002) for turmeric, and Martins *et al.* (2004) for almond plantlets.

The protocol developed in the present study gives scope for year round initiation of a fresh culture of true-to-type elite ginger cv. Suprava using dormant axillary buds. This method could be of significance for large scale commercial cultivation of ginger thereby minimizing the chances of loss of vigor of micropropagated plants in field obtained through repeated subculturing from the same stock *in vitro*.

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