An improved micropropagation via nodal segments of *Zingiber officinale*

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Summary

An improved micropropagation via nodal segments of *Zingiber officinale* was described in the present study. Nodal segments containing 1–3 nodes were obtained from 5-weeks old stock shoots and cultured on Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 1 mg L⁻¹ thidiazuron (TDZ) and 0.5 mg L⁻¹ naphthaleneacetic acid (NAA), to promote shoot elongation from axillary buds. With this protocol, more than 90% of nodal segments containing one and two nodes formed axillary shoots, with 2.2–2.3 shoots per explant produced after 2 weeks of culture. When the shoots were proliferated on MS supplemented with 3 mg L⁻¹ 6-benzylaminopurine (BAP) and 0.2 mg L⁻¹ NAA, 6.3 shoots per explant with well-developed roots, each shoot being 6.2 cm in length, were obtained after 5 weeks of proliferation. The plants were established in soil following acclimatization. Analysis of inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) did not detect any polymorphic bands in micropropagated plants. To the best of our knowledge, this is the first report on micropropagation using nodal segments of ginger native to China. Results reported here provide a technical support for micropropagation of *Zingiber* spp.

Keywords

ginger, nodal segments, axillary shoot formation, genetic stability

Significance of this study

What is already known on this subject?

- Most of the previous studies of ginger micropropagation focused on indirect shoot regeneration, while only a few described direct shoot regeneration. Nodal segments with one node were frequently used as explants.

What are the new findings?

- An improved micropropagation via nodal segments was described for *Zingiber officinale* native to China. Genetic stability was assessed in micropropagated plants using ISSR and RAPD.

What is the expected impact on horticulture?

- Results reported here provide a technical support for micropropagation of *Zingiber* spp.

Introduction

Ginger (*Zingiber officinale*), a perennial herbaceous plant, is mainly grown in Asia, including India, China, Thailand, Nepal, Japan, Indonesia, and Sri Lanka (Seran, 2013; Wilson et al., 2013). Currently, India and China are the major ginger producing countries of the world. Ginger is widely used as a spice in food (Seran, 2013), beverage (Seran, 2013) and a medicine for anti-inflammatory, cancer prevention, post-operative anti-emetic, and mental disorders (Grzanna et al., 2005; Chaiyakunapruk et al., 2006; Shukla and Singh, 2007; Yi et al., 2009; Seran, 2013; Wilson et al., 2013; Prasad and Tyagi, 2015; Ali et al., 2018).

Ginger is vegetatively propagated and the conventional propagation of ginger by matured rhizomes division resulted in low multiplication rates (Seran, 2013; Abbas et al., 2018), and virus accumulation in the progenies (Wang and Valkonen,

2009; Legg et al., 2015). *In vitro* culture technique provides an alternative method for efficient shoot regeneration and rapid micropropagation of virus-free plants of ginger (Sharma and Singh, 1997; Seran, 2013; Abbas et al., 2018). *In vitro* cultures can also be used for preserving ginger germplasm (Tyagi et al., 2006; Sundararaj et al., 2010) and production of virus-free ginger plants (Sharma and Singh, 1997; Manisha et al., 2018). *In vitro* shoot regeneration of ginger has been established using various explants such as shoot tips (Noguchi and Yamakawa, 1988; Malamug et al., 1991; Rout et al., 2001; Rajani and Patil, 2009; Ibrahim et al., 2015; Ali et al., 2018), immature inflorescence (Babu et al., 1992a), aerial stem buds (Sharma and Singh, 1997; Lincy et al., 2009; Rajani and Patil, 2009; Lincy and Sasikumar, 2010; Manisha et al., 2018), leaf segments (Babu et al., 1992b; Mehaboob et al., 2019), stem segments (Noguchi and Yamakawa, 1988), rhizome buds (Balachandran et al., 1990; Kambaska and Santilata, 2009; Abbas et al., 2011; Mohanty et al., 2011), and thin cell sections of microrhizomes (Thingbaijam and Huidrom, 2014).

Further improvements of *in vitro* shoot regeneration would facilitate wider applications of biotechnology in ginger. It is well-known that genotype-specific is very common *in vitro* shoot regeneration and a regeneration system suitable for some genotypes may not be suitable for other genotypes in a given species (George et al., 2008). Studies on *in vitro* shoot regeneration of ginger native to China were quite limited. Therefore, developments of *in vitro* shoot regeneration protocols of China's ginger genotypes are of significance.

Genetic stability is a major concern in *in vitro* regener-

ants, and was investigated in a number of plants including perennial species like apple (Li et al., 2014) and grapevine (Bi et al., 2018), and annual ones like lily (Yin et al., 2013) and potato (Wang et al., 2014). However, such studies have been quite limited in *Zingiber* (Mohanty et al., 2011).

The objective of the present study was, therefore, to develop an efficient micropropagation using nodal segments of *Z. officinale* native to China. Genetic stability was assessed in the regenerants using inter-simple sequence repeat markers (ISSR) and random amplified polymorphic DNA (RAPD).

Materials and methods

Plant materials, culture media and conditions

Z. officinale 'Qianwei', a cultivar widely grown in south-west China (Zhang et al., 2012), was used in the present study. Matured rhizomes collected in 2018 from commercial ginger field in Leshan City, China, were surface-disinfected with 0.1% mercuric chloride (HgCl_2) for 20 min, followed by

rinsing three times with sterile distilled water. Buds were isolated from the surface-disinfected rhizomes and cultured on a basic medium (BM) composed of Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g L⁻¹ sucrose and 7 g L⁻¹ agar (Sigma Chemical Co., St. Louis, MO, USA). The pH of the medium was adjusted to 5.8 with 1 M NaOH, prior to autoclaving at 121°C for 20 min. The cultures were grown at 25 ± 2°C under a 16-h photoperiod of 45 μmol m⁻² s⁻¹ provided by cool-white fluorescent tubes. After 7 days of culture, the cultures were transferred on a shoot maintenance medium (SMM) and cultured in the same conditions for production of *in vitro* stock shoots. SMM was composed of BM supplemented with 1 mg L⁻¹ α-naphthaleneacetic acid (NAA). *In vitro* stock shoots were subcultured every 5 weeks.

Five-week-old stock shoots (Figure 1a) were used for obtaining nodal segments. After removal of leaves from basal parts of the stock shoots (Figure 1b), nodal segments containing one node (2–3 mm in length, Figure 1c), two nodes

FIGURE 1. Preparation of nodal segments, shoot formation and proliferation, and re-establishment of micropropagated plants in soil of *Zingiber officinale* 'Qianwei'. a. An *in vitro* stock shoot used for excision of nodal segments for shoot formation; b. Preparation of a nodal segment, as indicated by a red rectangle; Segments with one node (2–3 mm in length, c); two nodes (5–6 mm in length, d); three nodes (8–10 mm in length, e); f. An axillary bud on a node, as indicated by a white arrow; g. A small shoot elongating from an axillary bud on the surface of a nodal segment at day 3 of culture, as indicated by a red circle; h. Shoots (0.5–1.0 cm in length) formed in a nodal segment at day 12 of culture; i. A shoot with 3 fully-opened leaves after 2 weeks of culture on SFM5; j. Proliferating shoots with well-developed roots on SPM8 after 5 weeks of proliferation; k. Micropropagated plants re-established in soil in open field for three months.

TABLE 1. Effect of size of nodal segments and combination of TDZ and NAA on shoot formation of *Zingiber officinale* 'Qianwei'.

Nodal segments containing	Code of shoot formation medium (SFM)	Plant growth regulators		Shoot formation (%)	Number of shoots/nodal segment
		TDZ (mg L ⁻¹)	NAA (mg L ⁻¹)		
One node	SFM 1	0.5	0	86.7±6.7ab	1.7±0.1v
	SFM 2	0.5	0.5	76.7±3.3abc	1.3±0.1y
	SFM 3	0.5	1.0	76.7±3.3abc	1.7±0.2v
	SFM 4	1.0	0	83.3±3.3ab	1.5±0.2vw
	SFM 5	1.0	0.5	90.0±5.8a	2.3±0.1tu
	SFM 6	1.0	1.0	86.7±3.3ab	1.7±0.1v
Average				83.4	1.7
Two nodes	SFM 1	0.5	0	76.7±5.8abc	1.5±0.1 vwxy
	SFM 2	0.5	0.5	63.3±3.3cde	1.4±0.1wxy
	SFM 3	0.5	1.0	80.0±5.8abc	1.7±0.1v
	SFM 4	1.0	0	70.0±3.3bcd	1.5±0.1 vwxy
	SFM 5	1.0	0.5	93.3±3.3a	2.2±0.1u
	SFM 6	1.0	1.0	76.7±3.3abc	1.6±0.1vw
Average				76.7	1.7
Three nodes	SFM 1	0.5	0	46.7±3.3ef	1.6±0.1vw
	SFM 2	0.5	0.5	53.3±3.3def	1.3±0.2xy
	SFM 3	0.5	1.0	36.7±3.3f	1.5±0.1vwxy
	SFM 4	1.0	0	53.3±3.3def	1.1±0.1z
	SFM 5	1.0	0.5	53.3±3.3def	2.4±0.1t
	SFM 6	1.0	1.0	36.7±6.7f	1.6±0.1vw
Average				46.7	1.6

One node (2–3 mm in length), two nodes (5–6 mm in length) and three nodes (8–10 mm in length) were cultured on SFM for shoot formation. Results are presented as means ± SE, and with different letters in the same column indicate significant differences at $P < 0.05$ analyzed by Duncan's multiple range test.

TABLE 2. Effects of combinations of BAP and NAA in shoot proliferation medium on shoot proliferation of *Zingiber officinale* 'Qianwei'.

Code of shoot proliferation medium	Plant growth regulators		Number of proliferating shoots/explant	Shoot length (cm)
	BAP (mg L ⁻¹)	NAA (mg L ⁻¹)		
SPM 1	1.0	0.1	3.2±0.1e	3.7±0.2yz
SPM 2	1.0	0.2	2.6±0.1g	3.0±0.3z
SPM 3	1.0	0.5	4.2±0.1d	4.5±0.3wxy
SPM 4	2.0	0.1	3.1±0.1ef	4.4±0.2xy
SPM 5	2.0	0.2	4.7±0.2c	5.1±0.4vw
SPM 6	2.0	0.5	2.7±0.1fg	4.7±0.3vw
SPM 7	3.0	0.1	5.3±0.2b	5.6±0.3uv
SPM 8	3.0	0.2	6.3±0.1a	6.2±0.2u
SPM 9	3.0	0.5	5.5±0.2b	5.4±0.1uvw

Shoots were cultured on 9 SPM for shoot proliferation. SPM was composed of BM supplemented with different combination of BAP and NAA. Data were presented as means ± SE, and with different letters in the same column indicate significant differences at $P < 0.05$ analyzed by Duncan's multiple range test.

(5–6 mm in length, Figure 1d) or three nodes (8–10 mm in length, Figure 1e) were obtained. The nodal segments were cultured on six shoot elongation media (SEM) (Table 1), to promote shoot elongation from axillary buds. The culture condition was the same as used for production of *in vitro* stock shoots. Shoot formation percentages and number of shoots per nodal segment were recorded after 14 days of culture.

Shoot proliferation

Shoots (>2.0 cm in length) were excised from the nodal segments after 4 weeks of culture and transferred on 9 different shoot proliferation media (SPM), to select an optimal SPM for shoot multiplication (Table 2). The culture condition was the same as used for production of *in vitro* stock shoots. Number of proliferating shoots per shoot and shoot length were recorded after five weeks of culture.

TABLE 3. ISSR primer names, sequences, and number of amplified bands in micropropagated plants of *Zingiber officinale* 'Qianwei'.

Number	Primers	Sequence	Reference	Number of amplified bands	Number of polymorphic bands
1	SPS03	(GACA) ₄	Mohanty et al., 2011	4	0
2	SPS05	(GA) ₉ T		5	0
3	SPS06	T(GA) ₉ T		3	0
4	IS1	(CT) ₇ TG	Yamuna et al., 2007	5	0
5	ISSR02	(AGTG) ₃	Kizhakkayil and	4	0
6	ISSR05	(CT) ₇ TG	Sasikumar, 2010	6	0
7	ISSR06	(GA) ₈ C		7	0
8	ISSR07	(GA) ₈ G		5	0
9	ISSR08	(GA) ₈ T		5	0
10	ISSR09	(CT) ₈ G		3	0
11	ISSR12	(CAC) ₃ GC		7	0
12	ISSR14	(AGC) ₄ GT		6	0
13	ISSR16	(GAGAGA) ₂ GAGAT		5	0
Total				65	0

Establishment of plants in soil

Proliferated shoots (5–6 cm in length) with 3–4 fully opened leaves and well-developed roots were transferred into plastic pots (12 cm in diameter) containing a mixture of sterile soil: sand: coir dust: cow dung (3:1:1:1) and grown in greenhouse conditions. The pots were covered with plastic bags to maintain high humidity and gradually opened to reduce the humidity in a week. After two weeks of growth in greenhouse, the plants were re-established in soil in field conditions.

Assessment of genetic stability

Genetic stability was assessed in micropropagated plants after three months of growth in soil in the open field, using inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD).

DNA extraction

Genomic DNA was isolated from 0.5 g fresh leaf tissue of the samples, according to Doyle and Doyle (1990). Purified total DNA was quantified and its quality verified by ultraviolet spectrophotometry. Each sample was diluted to 50 ng μL^{-1} in Tris-EDTA buffer and stored at -20°C until use.

ISSR

Twenty-two primer pairs were screened to select suitable primers for amplifying PCR products, according to Mohanty et al. (2011), Yamuna et al. (2007), and Kizhakkayil and Sasikumar (2010) (Table 3). Procedures of PCR were conducted according to Yamuna et al. (2007). After amplification, the PCR products were separated by electrophoresis and revealed by silver staining according to the method of Bassam et al. (1991).

RAPD

Fifteen RAPD primer pairs were screened to select suitable primers for amplifying PCR products, according to Mohanty et al. (2011) (Table 4). Procedures of PCR were conducted according to Yamuna et al. (2007) and polyacrylamide gel electrophoresis of PCR products performed, as described by Bassam et al. (1991).

Experimental design and statistical analysis of data

In experiments of shoot formation and proliferation, 10 explants were included in each of three replicates. The whole experiment was performed twice. Results are presented as means $\pm$ SE. Data were analyzed using one-way ANOVA and significant differences were calculated at $P < 0.05$ by Duncan's

TABLE 4. RAPD primer names, sequences, and number of amplified bands in micropropagated plants of *Zingiber officinale* 'Qianwei'.

Number	Primers	Sequence	Reference	Number of amplified bands	Number of polymorphic bands
1	OPA07	GAAACGGGTG	Mohanty et al., 2011	9	0
2	OPA09	GGGTAACGCC		5	0
3	OPC02	GTGAGGCGTC		6	0
4	OPC05	GATGACCGCC		3	0
5	OPD03	GTCGCCGTCA		3	0
6	OPD07	TTGGCACGGG		4	0
7	OPD20	ACCCGGTCAC		5	0
8	OPN04	GACCGACCCA		2	0
Total				37	0

multiple range test. Thirty samples were randomly selected from each of micropropagated plants and *in vitro* stock shoots (control) and used for assessment of genetic stability. Bands were scored as present (1) or absent (0). Bands of equal molecular weight and mobility generated by the same primer were considered to represent the same locus. Both distinct monomorphic bands and polymorphic bands were scored. The experiments were repeated twice to confirm their repeatability, and only reliable and repeatable bands were included in the data analysis.

Results

Overall process of shoot formation, proliferation and plant establishment in soil

Axillary buds were observed in nodes of the stem under a microscope (Figure 1f). Axillary buds started to elongate, with visible shoots (0.5–1.0 mm) formed on surface of nodal segments at day 3 of culture (Figure 1g). These small shoots continued to grow and formed shoots (0.5–1.0 cm) in nodal segments containing one node or two nodes at day 12 of culture (Figure 1h). Shoots (≥ 2.0 cm in length) with 3–4 open leaves developed after 4 weeks of culture on SFM (Figure 1i). Proliferating shoots with well-developed roots (Figure 1j) were produced after five weeks of culture on SPM. Micropropagated plants were successfully re-established and grew normally in soil in the open field (Figure 1k).

Effects of the size of nodal segments and combination of TDZ and NAA on shoot formation

Although three sizes of nodal segments formed shoots when cultured on SFM containing different combinations of TDZ and NAA concentrations, size of nodal segments and combination of TDZ and NAA concentration in SFM significantly influenced shoot formation (Table 1). Nodal segments containing one, two and three nodes produced average shoot formation of 83.4%, 76.7% and 46.7%, respectively, regardless of the combinations of TDZ and NAA concentrations (Table 1). When cultured on SFM5, nodal segments containing one and two nodes produced 90.0% and 93.3% shoot formation, and 2.3 and 2.2 shoots per nodal segment (Table 1). These were the optimal results of shoot formation obtained among the 18 media tested in the present study. Therefore, SFM5 containing 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA was consistently used for shoot formation in nodal segments in the present study.

Effect of combination of BAP and NAA on shoot proliferation

Although shoots could be proliferated on the nine SPM tested, combination of BAP ($1.0\text{--}3.0 \text{ mg L}^{-1}$) and NAA ($0.1\text{--}0.5 \text{ mg L}^{-1}$) considerably affected shoot proliferation (Table 2). SPM8 containing 3.0 mg L^{-1} BAP and 0.2 mg L^{-1} NAA produced the best shoot proliferation (Figure 1j; Table 2): 6.3 shoots per explant and 6.2 cm shoot length. Therefore, SPM8 was consistently used for shoot proliferation in the present study.

FIGURE 2. ISSR (a, primer ISSR06 and b, primer ISSR16) and RAPD (c, primer OPD 03 and d, OPD 07) banding patterns in micropropagated plants of *Zingiber officinale* ‘Qianwei’ after 3 months of re-establishment in soil. M marker = DL2000, lanes 1–2 = *in vitro* stock shoots, lanes 3–10 = micropropagated plants.

Assessment of genetic stability

1. ISSR. Thirteen primers selected from 22 primers produced clear and reproducible bands and yielded 65 scored bands in each samples (Table 3; Figures 2a, b), with total 1,950 bands produced across the 30 samples analyzed. The number of bands for each primer varied from 3 to 7 (Table 3), with an average of 5 bands per primer. No polymorphic bands were detected in the micropropagated plants tested (Table 3; Figures 2a, b).

2. RAPD. Eight primers selected from 15 primers produced clear and reproducible bands and yielded 37 scored bands in each sample (Table 4; Figures 2c, d), with a total of 1,110 bands produced across the 30 samples analyzed. The number of bands for each primer varied from 2 to 9 (Table 4), with an average of 4.6 bands per primer. No polymorphic bands were tested in the micropropagated plants (Table 4; Figures 2c, d).

Discussion

Compared with the previous reports of shoot regeneration using nodal segments (Noguchi and Yumakawa, 1988) and aerial stem segments of ginger (Lincy and Sasikumar, 2010), two significant improvements were made in the present study. First, nodal segments with one node (Noguchi and Yumakawa, 1988) and aerial stem segments (1.0–1.5 cm) (Lincy and Sasikumar, 2010) were used as initial explants. In the present study, nodal segments with 1–3 nodes were compared for shoot formation, and identified the size of the nodal segments exerted significant effects of shoot formation in ginger. Second, more than 3 months and 140–148 days were required to complete the micropropagation cycle using stem segments (Noguchi and Yumakawa, 1988) and aerial stem segment (Lincy and Sasikumar, 2010) as initial explants. In the present study, about 9 weeks were required to complete the micropropagation cycle and 28–58 plantlets/initial explant can be produced using nodal segments as the initial explants. These two improvements largely simplified the procedure and shortened the time required in micropropagation of ginger. Therefore, the protocol described in the present study can be considered an improved protocol for efficient micropropagation of ginger.

Plant growth regulators (PGRs) play a critical role in shoot regeneration and proliferation of ginger (Seran, 2013). TDZ and BAP alone or in combination with auxin was frequently used to induce adventitious shoot formation (Seran, 2013). Working on *Z. officinale* 'Kintoki', Noguchi and Yumakawa (1988) reported that 1.0 mg L⁻¹ BAP produced the highest number (6.3) of shoots from shoot tips, while a combination of 3.0 mg L⁻¹ BAP and 5.0 mg L⁻¹ NAA was optimal for shoot regeneration from leaf segments. The best results of shoot regeneration were obtained in transverse thin cell layer sections (tTCLSs) of 'Nadia' microrhizome when cultured with a combination of 2.0–6.0 mg L⁻¹ BAP with 0.2 mg L⁻¹ 2,4-D (Thingbaijam and Huidrom, 2014). Among the different combination of PGRs tested, a combination of 1.0 mg L⁻¹ TDZ with 1.0 mg L⁻¹ IBA produced maximum shoot regeneration in stem segments of 'Jamaica' and 'Varada' (Lincy and Sasikumar, 2010). The present study found a combination of 1.0 mg L⁻¹ TDZ with 0.5 mg L⁻¹ NAA producing the best results of shoot formation from nodal segments of ginger 'Qianwei'.

There have been a number of studies on shoot proliferation in ginger. Although Mehaboob et al. (2019) reported the highest number of proliferating shoots was obtained on shoot proliferation medium containing 0.5 mg L⁻¹ BAP alone, most of the studies used a combination of BAP with IBA or

NAA for shoot proliferation. Kambaska and Santilata (2009) reported that 2.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ NAA gave an average of 7.5 shoots in ginger 'Suprava' and 'Suruchi', and a higher concentration of BAP (2.5–3.0 mg L⁻¹) induced callus formation and produced fewer number of shoots. Zuraída et al. (2016) reported that the highest number of proliferating shoots of 'Rubrum' were obtained when cultured in the medium containing 3.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ NAA. The present study found that shoot proliferation medium containing 3.0 mg L⁻¹ BAP and 0.2 mg L⁻¹ NAA was optimal for shoot proliferation of ginger 'Qianwei'. The differences in combinations of PGRs for shoot proliferation are most likely due to types of ginger genotypes.

The genetic stability is a major concern in *in vitro*-derived plants and therefore, assessment of genetic stability in regenerants is necessary (Yin et al., 2013). Molecular markers such as ISSR and RAPD were among the most widely used techniques for this purpose. Use of more than one marker system would produce more reliable results of genetic integrity analyses (Wang et al., 2014; Anandan et al., 2018). Information on assessment of genetic stability in the regenerants is lacking in *Z. officinale*. Mohanty et al. (2011) applied RAPD and ISSR to assess the genetic stability in the regenerants through the rhizome buds of *Z. rubens* and did not detect any polymorphic bands. The results of the present study are in line with those of Mohanty et al. (2011), indicating that the regenerants are genetically stable.

Conclusions

An improved micropropagation via nodal segments as initial explants was described for *Z. officinale*. To the best of our knowledge, this is the first report on *in vitro* micropropagation from nodal segments of ginger native to China. Results reported here provide technical support for *in vitro* micropropagation of *Zingiber* spp.

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