Research Article

Seied Mehdi Miri*

Micropropagation, Callus Induction and Regeneration of Ginger (*Zingiber officinale* Rosc.)

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Abstract: The present study describes a protocol for micropropagation, callus induction, and shoot regeneration of ginger (*Zingiber officinale*). The rhizomes were surface-sterilized with ethanol (70%) for 45 s, sodium hypochlorite (2.5%) for 10 min, and mercuric chloride (0.1%) for 10 min. Multiple shoots were induced from sprouting bud explants cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) combined with kinetin (Kin). The maximum shoot number was obtained from MS medium containing 10 mg/l BA with a mean of 20.6 shoots per explant. The leaf explants were cultured on MS medium supplemented with indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), Dicamba, or BA for callus culture. Green-red compact calli were induced using 2,4-D, Dicamba or BA. Also, BA successfully induced plant regeneration. The multiplied shoots that were transferred to the rooting medium (½MS supplemented with 0, 1 and 2 mg/l IAA, indole-3-butyric acid (IBA) or NAA) showed development of roots (100%). The rooted plantlets were transferred to pots containing a 1:1 mixture of cocopeat and perlite, and acclimatization was successful, resulting in 85% survival of the plantlets in the greenhouse.

Keywords: media, multiplication, plant growth regulators, tissue culture, Zingiberaceae

1 Introduction

Ginger (*Zingiber officinale*) from the family Zingiberaceae is a herbaceous perennial grown as an annual crop. It is native to southern Asia (China and India) and widely cultivated in the tropics and subtropics (Gang and Ma 2008; Ravindran and Nirmal Babu 2005; Ross 2005). Ginger is erect and has many fibrous roots, pseudostem with leaves, and an underground stem (rhizome). The rhizome is the tissue used for a lot of purposes, such as vegetative propagation and the storage of food materials (Gang and Ma 2008; Ravindran and Nirmal Babu 2005).

Ginger has been used as a popular spice and in traditional medicine for thousands of years (Al-Achi 2008; Gang and Ma 2008). The plant contains volatile oil composed of Shogaol, Gingerols, Zingiberene, and Bisabolene (Al-Achi 2008). Due to its anti-inflammatory properties, ginger is used as a medicinal plant to treat a wide array of illnesses and disorders, such as arthritis, inflammatory bowel disease, cancer, Alzheimer’s disease, the common cold (Gang and Ma 2008), and dyspepsia (Al-Achi 2008). It has potential antidiabetic (Al-Achi 2008), antiinflammatory, antiemetic, antihepatotoxic, anti-inflammatory, antiinvasive, antioxidant, antiparasitic, antiplatelet, antipyretic, antiseptic, antitussive, cardiovascular, digestive, and hypoglycemic activities (Duke et al. 2003). Due to its use as a traditional medicinal herb, ginger has drawn the attention of both the general public and medical communities. It is one of the top 20 selling herbal supplements in the United States (Gang and Ma 2008).

Ginger is reproductive sterile and is only propagated vegetatively (Ravindran and Nirmal Babu 2005). Micropropagation has become an important method of commercial propagation for many horticultural crops (Erfani et al. 2017; Fallahpour et al. 2015; Hasanloo et al. 2014; Miri and Roughani 2018b), especially those that are propagated vegetatively or the slow-growing monocots because conventional breeding programs are hampered by the lack of fertility and natural seed set (Gheisari and Mri 2017; Iliev et al. 2010; Mousavi et al. 2010b; Ravindran and Nirmal Babu 2005). On the other hand, rhizome rot caused by *Pythium* spp. and *Ralstonia solanacearum* are major diseases affecting ginger. These diseases primarily spread through infected rhizomes. Tissue culture techniques can be used to produce pathogen-free planting material of high-yielding varieties (Hasanloo et al. 2014; Mousavi et
al. 2010a; Ravindran and Nirmal Babu 2005). In addition, biotechnological approaches, specifically callus culture, play a vital role in the search for alternatives to the production of secondary metabolites from plants (Karuppusamy 2009). Also, adventitious shoot formation from callus cultures has been suggested as a reliable and potential technique for rapid clonal propagation of selected plant species (Bhojwani and Razdan 1996; Miri et al. 2016). Although several investigations have reported the tissue culture technique of ginger (Seran 2013), it is very important to develop a protocol because the efficiency of tissue culture is dependent on several factors, such as plant growth regulators, physiological state of the explants, etc. The present study was carried out to develop an effective protocol for in vitro propagation, callus induction, and plant regeneration of ginger.

2 Materials and Methods

2.1 Sterilization and establishment

Healthy rhizomes of ginger (*Zingiber officinale*) were planted in sterilized sand to initiate sprouting. The sprouted rhizomes were, then, removed and washed thoroughly by tap water for 30 min. These rhizomes were chopped to a size of 4-5 cm containing sprouting rhizome buds and surface-sterilized in 70% (v/v) ethanol for 45 s and 2.5% (v/v) sodium hypochlorite for 10 min followed by 0.1% (w/v) mercuric chloride for 10 min. After rinsing three times with sterile distilled water, the shoots were cut apart from the rhizomes and trimmed to a final size of 1 cm. The explants were cultured in Murashige and Skoog (1962) free-hormone medium containing 20 g/l sucrose and 7 g/l agar. The pH of the medium was adjusted to 5.5-5.7 prior to autoclaving at 121ºC and 1.05 kg.cm⁻² for 20 min. The cultures were kept in a growth chamber at a temperature of 25±1ºC with a 16-h photoperiod under an irradiance of 45 μmol/m²/s provided by cool white fluorescent light.

2.2 Multiplication

When the growth started, the shoots were inoculated with a multiplication medium supplemented with 30 g/l sucrose and different concentrations of 6-benzyladenine (BA) (0, 1, 5 and 10 mg/l) combined with kinetin (Kin) (0 and 1 mg/l). After four weeks of culture, the number and length of the shoots, as well as their fresh and dry weights, were recorded. The shoot dry weight was recorded after oven-drying at 70ºC for 48 h.

2.3 Callus induction and shoot regeneration

For callus induction, in vitro leaf segments (1×1 cm) were placed on MS medium containing 0, 0.5, 1 and 2 mg/l indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), Dicamba, or BA. The calli were collected after four weeks and weighed using fresh and dry weight. The leaves derived from the calli were then placed in a basal medium with 0, 0.5, 1 and 2 mg/l BA or Kin for shoot induction. The percentage of explants forming shoots, the number of regenerated shoots per callus, and shoot length were recorded after four weeks.

2.4 Rooting

The multiplied shoots (2-3 cm) were separated after elongation and were transferred to ½MS enriched with 20 g/l sucrose, 6 g/l agar, and 0, 1 or 2 mg/l of IAA, indole-3-butyric acid (IBA), or NAA for rooting.

2.5 Acclimatization

After four weeks, the well-rooted plantlets were carefully taken out of the jars and the roots were washed thoroughly under running tap water. They were, then, placed in small pots containing a mixture of sterilized cocopeat and perlite (1:1). These pots were covered with polythene bags and sprayed with water at 2-day intervals to maintain humidity. They were kept in a greenhouse for six weeks to acclimatize. Mean daily greenhouse temperature and relative humidity were set at 20±2ºC and 75%, respectively. The polythene bags were, gradually, removed to expose the plantlets to the outer normal environment.

2.6 Data analysis

In all experiments, each treatment consisted of 5 replicates (4 explants each replicate). The mean values of the parameters were compared by analysis of variance using the SPSS (Ver. 21) software package. The significant differences among treatments were compared using a least significance difference (LSD) test at a 5% probability level.
3 Results

The described sterilization method resulted in 65% aseptic cultures whereas 35% of explants were browned or infected.

Table 1 shows the formation of axillary shoots from buds grown in the MS medium supplemented with various concentrations of two cytokinins. In this study, the type and concentration of cytokinin incorporated into the media influenced shoot proliferation. No multiplication occurred when shoot tips were cultured in the cytokinin-free media. Callus formation was observed at the base of the explants. Furthermore, rooting occurred in the media containing up to 1 mg/l BA. The multiplication response of ginger to BA and Kin cytokinins is shown in Figure 1. An increase in the BA concentration from 1 to 10 mg/l resulted in an increase in the induction of axillary shoots. The maximum shoot proliferation was achieved in the MS medium containing 10 mg/l BA, which was followed by 10 mg/l BA and 1 mg/l Kin with a mean of 20.6 and 17.2 shoots per explant, respectively. When 1 mg/l Kin was combined with different levels of BA (except 10 mg/l), it had no significant effect on the shoot number. As the concentration of BA was increased from 5 to 10 mg/l in combination with 1 mg/l Kin, the shoot number diminished.

On the other hand, the shoot number was negatively correlated with the shoot length and shoot fresh and dry weights, so that these parameters were decreased with the increase in the number of shoots (Table 2). The occurrence of hyperhydrycity, which is typical for high concentrations of BA, was not recorded. The results showed that with an increase in the concentration of BA in the medium, the shoot length was decreased significantly so that the mean shoot length was decreased from 6.2 cm to 1.0 cm when BA concentration was increased from 0 to 10 mg/l. The highest shoot fresh and dry weights were obtained from the control (hormone-free media).

In this study, green-red compact and friable calli were observed in the leaf explants. As shown in Figure 2A, there was no callus formation from the leaf explants that were cultured in the medium lacking growth regulators or containing lower concentrations of IAA and NAA (0.5 and 1 mg/l). However, the frequency of callus production was increased with the increase in the concentration of 2,4-D and Dicamba (1 mg/l) from 33% and 55% to 60%, respectively and then, it was decreased at higher concentrations. Similarly, highly proliferating calli were observed in the MS medium containing up to 1 mg/l BA, and at higher concentrations, a decline was observed in the percentage of cultures forming calli. Therefore, 0.5 and 1 mg/l BA were proven to be the best for the maximum callus induction frequency (100%).

Table 1: Effect of BA and Kin on shoot multiplication of ginger.

| Cytokinin (mg/l) | Mean no. of shoots/explant | Mean shoot length (cm) | Mean shoot fresh weight (mg) | Mean shoot dry weight (mg) |
|-----------------|---------------------------|------------------------|-----------------------------|---------------------------|
| BA              | Kin                       |                         |                             |                           |
| 0               | 0                         | 1.1 e                   | 6.2 a                       | 1030.0 a                  | 79.2 a                    |
| 0               | 1                         | 2.8 de                  | 5.6 b                       | 780.0 b                   | 60.0 b                    |
| 1               | 0                         | 4.0 d                   | 1.7 cd                      | 366.6 c                   | 33.3 c                    |
| 1               | 1                         | 4.2 d                   | 1.4 cd                      | 275.3 de                  | 22.0 bc                   |
| 5               | 0                         | 12.4 c                  | 1.4 cd                      | 217.5 de                  | 15.7 f                    |
| 5               | 1                         | 10.9 c                  | 1.6 cd                      | 267.7 de                  | 26.0 d                    |
| 10              | 0                         | 20.6 a                  | 1.0 d                       | 283.5 de                  | 21.0 e                    |
| 10              | 1                         | 17.2 b                  | 0.9 d                       | 176.5 e                   | 17.3 ef                   |

Means with shared letter(s) in each column for each factor showing insignificant differences (LSD, α≤ 0.05).
Table 2: Correlation coefficient between shoot multiplication characteristics.

| Characteristic          | Shoot No. | Shoot length | Shoot fresh weight |
|------------------------|-----------|--------------|--------------------|
| Shoot length           | -0.672**  |              |                    |
| Shoot fresh weight     | -0.651**  | 0.947**      |                    |
| Shoot dry weight       | -0.669**  | 0.930**      | 0.993**            |

**: α ≤ 0.01

Table 3: Effect of auxins on in vitro rooting of ginger.

| Auxin (mg/l) | Rooting (%) | Mean no. of roots/explant | Mean roots length (cm) |
|--------------|-------------|----------------------------|------------------------|
| IAA          | IBA         | NAA                        |                        |
| 0            | 0           | 0                          | 100                    | 3.1 c   | 2.6 b   |
| 1            | 0           | 0                          | 100                    | 3.3 c   | 2.3 b   |
| 2            | 0           | 0                          | 100                    | 6.6 b   | 1.8 b   |
| 0            | 1           | 0                          | 100                    | 4.2 bc  | 3.9 ab  |
| 0            | 2           | 0                          | 100                    | 8.9 a   | 4.6 a   |
| 0            | 0           | 1                          | 100                    | 4.4 bc  | 2.5 b   |
| 0            | 0           | 2                          | 100                    | 3.8 c   | 3.4 ab  |

Means with shared letter(s) in each column for each factor showing insignificant differences (LSD, α ≤ 0.05).

Figure 1: In vitro shoot multiplication of ginger four weeks after cultivation on MS medium supplemented with 1 mg/l Kin (A) and 10 mg/l BA (B).
Fresh and dry weights of calli are shown in Figure 2B. The highest fresh and dry weights were achieved in 1 mg/l 2,4-D (Figure 4A), all tested concentrations of Dicamba, 0.5 mg/l BA, and 1 and 2 mg/l BA.

The friable calli were transferred to the shoot induction media containing different concentrations of BA or Kin. Increasing BA and Kin from 0 to 2 mg/l resulted in an increase in the shoot regeneration ability (Figure 3A). Between the two studied cytokinins, BA was found to be superior to Kin as it induced 6.0 and 5.5 shoots per callus at 0.5 (Figure 4B) and 1 mg/l respectively, while Kin at the same concentrations gave 0.4 and 1.2 shoots per callus after 4 weeks of incubation (Figure 3B).

The best results for shoot length were obtained from BA (2.1-2.5 cm) followed by Kin (1.0-1.8 cm) and MS medium without PGR (control) with a shoot length of 0.5 cm.

The isolated shoots were transferred to the ½MS medium supplemented with IAA, IBA or NAA at different rates. Auxins used in the experiment had a positive effect on in vitro root formation of ginger shoots. However, among all treatments (even the control), 100% of shoots formed roots (Table 3). The highest number of roots and mean root length were observed in the media supplemented with 2 mg/l IBA (Figure 5A) (8.9 roots per shoot and 4.6 cm, respectively). Good results were also obtained from 2 mg/l IAA (6.6 roots per shoot). The least roots formed were in the media without growth regulators and in the media containing 1 mg/l IAA or 2 mg/l NAA (3.1, 3.3 and 3.8 roots per shoot, respectively).

The rooted plantlets were hardened inside the greenhouse and the hardened plantlets were successfully acclimatized to the natural environment (Figure 5B). The percentage of healthy plantlets with well-developed roots adapted to the greenhouse conditions was 85%.
4 Discussion

In general, the absence of contamination on in vitro explants is a criterion for the successful establishment and subsequent shoot multiplication (Gheisari and Miri 2017; Seran 2013). As rhizome explants are underground part of ginger plants, the in vitro establishment of clean culture is not easy due to the presence of pathogens in these living materials (Seran 2013). So, in this study some disinfectants (including ethanol, sodium hypochlorite, and mercuric chloride) were used to sterilize rhizome buds to control the contamination. Similarly, Gheisari and Miri (2017) used the fungicide Captan, ethanol, and sodium hypochlorite to disinfect hyacinth bulbs. They stated that because the underground parts of the plants were highly infected, a combination of several sterilizing agents showed better synergistic effects on the control of the fungal and bacterial contamination.

Micropropagation has become a reliable approach for large-scale rapid multiplication of a selected or elite variety. The shoot tips cultured in cytokinin-free MS medium did not show any proliferative response. Generally, a cytokinin is required for shoot multiplication. Cytokinins induce cell division, overcome the apical dominance, and stimulate the initiation and activity of axillary meristems resulting in shoot formation (Ahmed and Anis 2014; Erfani et al. 2017; Fallahpour et al. 2015; Miri and Roughani 2018a; Miri et al. 2016; Seran 2013). However, its type and optimal concentration varies with species (Ahmed and Anis 2014). The stimulatory effect of BA on multiple shoot formation has been reported earlier in ginger by Rout et al. (2001), Hiremath (2006), Kambska and Santilata (2009), Kavyashree (2009), Abbas et al. (2011), and Ayenew et al. (2012) who have suggested that BA shows a strong effect on the multiplication of shoots. This may be due to its ability to induce the metab-

Figure 3: Effect of BA and Kin on the percentage of shoot regeneration (A) and the mean number of shoots/callus and shoot length of ginger (B). Means with shared letter(s) in each column for each factor showing insignificant differences (LSD, α≤ 0.05).
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Figure 4: Callus induction on MS medium supplemented with 1 mg/l 2,4-D (A) and shoot regeneration on MS medium containing 0.5 mg/l BA (B) of ginger four weeks after cultivation.

Figure 5: Rooting response of ginger cultured on MS medium containing 2 mg/l IBA four weeks after cultivation (A). Acclimatization of micropropagated ginger plantlet four weeks after transferring (B).

olizing or producing the natural endogenous hormones for morphogenesis induction (Ahmed and Anis 2014). The cytokinin Kin mainly influenced shoot and leaf growth of ginger whereas it made little impact on proliferation. Parzynies and Dąbski (2012) found that shoot tips of Clematis integrifolia were more proliferative in BA-containing media, but the quality of the plants was better with Kin or 2iP. Similarly, Abbas et al. (2011) reported that the best medium for shoot length of Z. officinale was obtained from the MS medium containing 3 mg/l Kin + 1 mg/l NAA. The application of higher concentrations of BA resulted in a significantly higher number of shoots. This result agrees with several studies (Abbas et al. 2011; Hiremath, 2006; Kavyashree 2009) in which it has been reported that BA
at higher concentrations is more stimulatory to shoot growth than the lowest concentrations in the culture medium. On the other hand, BA has also an effect on the reduction of multiplied shoot length and shoot fresh and dry weight. The negative correlation between the number and length of shoots can be due to the limited capacity of food-making of the in-vitro shoots so that the amount of food absorbed by each shoot was decreased at higher concentrations of BA (Erfani et al. 2017; Mousavi et al. 2010b). In several plant species, cytokinin combinations increase shoot multiplication (Baskaran and Narayanasamy, 2008). However, in the present study, the combination of BA and Kin was found to be not significantly different from BA alone in terms of the number of shoots. It was inconsistent with the results of Ayenew et al. (2012) who reported that shoot tip explants supplied with 2 mg/l BA and 1 mg/l kinetin gave the highest average shoot number. Baskaran and Narayanasamy (2008) also demonstrated that solid L2 medium containing BA and TDZ enhanced shoot proliferation in Psoralea corylifolia.

In this study, callus formation was achieved on the ginger explants derived from the in vitro-grown leaves after cultivation in the MS medium with various plant growth regulators. The high frequency of callus indicated the potential of ginger for secondary metabolite production in the pharmaceutical industry. A wide range of auxins and cytokinins have been used to induce callus in gingers (Ali et al. 2016; Hossain et al. 2010; Ibrahim et al. 2015; Sultana et al. 2009). Generally, 2,4-D and BA alone or in combination were found to be the best plant growth regulators for callus induction. In comparison with different auxin types, Dicamba was superior to 2,4-D in callus induction of Z. officinale (Sultana et al. 2009) and Paspalum vaginatum (Neibaur et al. 2008). However, Hossain et al. (2010) and Ibrahim et al. (2015) obtained a greater callus formation in the medium containing BA+IAA and BA+2,4-D in ginger. In the current research, BA followed by Dicamba and 2,4-D were as effective at inducing a callus formation rate from leaf disks of ginger as NAA and IAA. However, BA had no significant effect on callus induction in Paspalum vaginatum (Neibaur et al. 2008), and the effect of plant growth regulators was found to be species-specific.

Calli derived from leaf explants were used for adventitious shoot regeneration. An efficient callus induction and shoot regeneration system enable the genetic transformation of this important medicinal and spice species. The addition of BA induces shoot regeneration satisfactorily when compared to Kin. Li et al. (2013) also demonstrated that Lysionotus serratus plants are sensitive to BA to induce shoot regeneration, but not Kin. Indirect shoot regeneration has been reported in Helicteres isora (Shriram et al. 2008) and Z. officinale (Sultana et al. 2009) by using BA + Kin, except that Sultana et al. (2009) reported 3.8 shoots per callus whereas in our experiment, a higher regeneration (6.0 shoots per callus) was obtained. The results showed that BA at lower concentrations (0.5 and 1 mg/l) is suitable for indirect shoot regeneration of ginger. Similar results were reported by Shriram et al. (2008) for Helicteres isora, which was related to an endogenous level of auxins that stimulated organogenesis in the presence of an external source of cytokinin.

The key step of a vegetative plant’s propagation is a well-developed root system. The improvement of in vitro rooting techniques allows for easier and faster adaptation and helps in better survival of plantlets under ex vitro conditions (Krupa-Malkiewicz and Mgłosiek 2016). The application of auxins to in vitro cultures accelerates root formation and enhances rooting rate (Martins et al. 2013; Miri and Roughani 2018a). In the present study, the type and concentration of exogenous auxins influenced the average number of roots produced per explant as well as the mean length of the roots. It is well known that the rooting of excised shoots was easy. In our experiment, we even observed rooting in the multiplication phase with lower BA concentrations. This is probably due to the endogenous concentration of IAA in the shoots of ginger that moves to lower parts and its concentration increases at the basal end of the shoots where it stimulates root formation or provides an appropriate hormonal balance along with other exogenous auxins to improve rooting (Martins et al. 2013). The highest number and length of roots were observed in the media supplemented with IBA at higher concentrations (2 mg/l). The formation of the adventitious roots from in vitro shoots of the gingers promoted by auxins has been reported by various researchers (Abbas et al. 2011; Hiremath 2006; Kambaska and Santilata 2009). But, unlike those who reported that NAA was a suitable auxin for the rooting of gingers, in our study the maximum number and length of roots were obtained with IBA.

5 Conclusion

In this research, we established suitable media with different compositions of plant growth regulators for micropropagation, callus induction, and multiple shoot regeneration of gingers. The highest shoot proliferation was achieved by using the MS medium supplemented with 10 mg/l BA. The highest callus initiation and indirect shoot regeneration were obtained from BA. Also, the results
indicated that auxins are essential for in vitro rooting. The protocol described here provides an efficient tissue culture system for the massive multiplication and somaclonal variation via callogenesis in gingers, which can be further used in breeding and transformation studies.

Conflict of interest: Authors declare no conflict of interest.

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