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RESEARCH ARTICLE

Simple micropropagtion method of ginger (*Zingiber officinale* Rosc.)

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Abstract

Simple micropropagation method was established for *Zingiber officinale* Rosc using fresh rhizome sprouting bud in semisolid culture media. Fresh rhizome sprouting buds were surface sterilized by sodium hypochlorite (2, 2.5, or 3%) for 20 minutes. A concentration of 3% Clorox significantly reduced contamination of rhizome sprouted bud explants, where contamination-free culture was 90%, at the same time; the aseptic explants' survival rate was high. Plantlets cultured on Murashige and Skoog's (MS) medium were supplemented with different concentrations (2, 3, 4, or 5 mg/l) of BAP (6-benzyl- amino-purine) with or without 3% AC (activated charcoal), and combinations of BAP (2, or 3 mg/l) with NAA (α- Naphthalene acetic acid) (0.5, or 1 mg/l) for shoot and root induction. The maximum shoot and root number was obtained from MS medium containing 3 mg/l BAP with a mean of 5.4 shoot per explant, each shoot possesses in mean 10 leaves and 17.7 roots. Plantlets were acclimatization before transplanting, and high survival rate was recorded. Plants would be transferred to the field to produce rhizomes, the useful and economical part of ginger. The success of field culture will open new era of ginger production in Libya.

Key words: Ginger, micropropagation, plant growth regulators, activated charcoal, rhizome

Introduction

Ginger (*Zingiber officinale* Rosc) belongs to the family *Zingiberaceae*. This family is an herbaceous moderate sized of relatively advanced monocotyledonous plant of the order Zingiberales perennial grown as an annual crop, rhizomatous, and aromatic herbs often of large size, bearing flowers either terminally on aerial leaf shoots or from ground level. These are plants of tropical and subtropical regions. Several authors have mentioned different figures for the total number of genera and species, but it is reasonably appropriate to estimate the world record at least 51 genera and 1500 species (Newman, 2001). Ginger, the spicy and aromatic rhizome, is one of the most commonly used spices and herbal medicine in the world due to its health promoting properties (Nafi *et al.*, 2014). Ginger sexual propagation and breeding cannot be easily achieved due to poor flowering and seed set, therefore it is usually propagated vegetatively through rhizome (Kambaska and Santilata, 2009).

Improving seed propagation and breeding is highly desired, development of female parent of Sorghum for instance has been reported (Maiga *et al.*, 2021). A rhizome produces about 12 lateral buds in a season of 9 months. Moreover, vegetative propagation of ginger has a high risk of spreading infections. Slow propagation rate and the risk of disease transmission by sectioning of the rhizomes have deprived propagation by conventional means. Therefore, plant tissue culture is considered the best alternatives method that may supply a large number of planting materials (Hamirah *et al.,* 2010). The in vitro technique's success largely depends on the aseptic culture establishment, shoot regeneration capacity, rooting, and acclimatization. During plant tissue cultures, contamination normally occurs by different microorganisms that reduce productivity and can completely inhibit their cultivation. The contamination of explants present on the surface or stuck in the cracks and scales. The contamination of underground parts such as rhizomes is very high and the establishment of contamination frees cultures is difficult; therefore, successful tissue culture protocols start with effective explant sterilization (Mihaljevic *et al.*, 2013). There is no standard sterilization procedures available that apply to all plants; moreover, no study was able to standardize the sterilization protocol for micropropagation of *Zingiber officinale.* Therefore, one of our objectives in this study, was the sterilization for explants of *Zingiber officinale* Rosc for micropropagation, using different concentrations of sodium hypochlorite.

Shoot regeneration capacity depends on the formulation of culture media and plant growth regulators, mainly cytokinins and auxins. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) complemented with 6-benzylaminopurine (BAP) is more usually used for ginger shoot multiplication (Mehaboob *et al*., 2019). Other cytokinins such as kinetin and thidiazuron (TDZ) also showed different effect on shoot multiplication of different varieties of ginger (Ayenew *et al.*, 2012; Lincy and Sasikumar, 2010). Additionally, developing root system is the crucial step in plant tissue culture, which guarantees a high survival rate of micropropagated plantlets during the acclimatization stage (Miri, 2020). *In vitro,* root induction of ginger is mainly induced by auxins (Mehaboob *et al*., 2019). Different types and concentrations of auxin differently affected *in vitro* root induction in different Zinger species (Mehaboob *et al*., 2019; Jualang *et al*., 2015). Determining the appropriate type and concentration of auxin may improve the *in vitro* root induction of ginger, and consequently, enables acclimatization and successful establishment of the micropropagation-raised plantlets in the field conditions.

Activated charcoal (AC) is normally used in micropropagation to improve cell growth and development (Pan and van Staden, 1998). The addition of AC to the media is a recognized practice, and its effect on growth and development could be attributed to the adsorption of inhibitory substances in the culture medium (Theander and Nelson, 1988), severe reduction in the phenolic oxidation (Teixeria *et al*., 1994), alteration of medium pH to an ideal level (Owen *et al*., 1991) and creation of a darkened environment in medium, and therefore simulate its conditions (Dumas and Monteuuis, 1995).

Zingiber officinale Rosc micropropagation has not been practiced in Libya. Therefore, this study was carried out to establish a successful protocol for direct *in vitro* regeneration of *Zingiber officinale* Rosc. This study's objectives were to determine Clorox's effective concentration for explant surface sterilization, and to assess the effect of different concentrations of BAP with or without AC, and combinations of BAP with NAA, on plantlets growth and development.

Materials and methods

Explants and nutrient medium: The experiment was carried out at the National Research Center of Biotechnology, Tripoli, Libya in 2020. Rhizomes of *Zingiber officinale* Rosc were imported from China by Mecca Trade Company (Cairo-Egypt). Healthy rhizomes were kept in the dark 15 days for sprouting. The sprouted rhizome buds (fig. 1, A) were collected in beaker and kept under running tap water for 10 minutes prior to sterilization in the laminar airflow cabinet, then were chopped to fragments and surface-sterilized in 70% (v/v) ethanol for 2 minutes. For the explant sterilization experiment, (2, 2.5, and 3%) of Sodium hypochlorite (NaOCl) were used for 20 minutes of exposure. After rinsing three times with sterile distilled water, the shoots were cut apart from the rhizomes and trimmed to a final size of 1 to 2 cm. The explants were cultured in MS free-hormone medium containing 30 g/l sucrose and 7 g/l agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121ºC and 100 kPa 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.

Effects of plant growth regulators and activated charcoal on explants development

In the first experiment, the addition of BAP at 2, 3, 4, and 5 mg/l into MS medium with or without 30 g/l AC was established.

In the second experiment, the medium was supplemented as follows; 2.0, or 3.0 mg/l BAP and 0.5, or 1 mg/l NAA in combinations, each explant was cultured in an individual jar.

After four weeks, plantlets were subjected to two weeks of in vitro acclimatization, then the rooted plantlets were carefully taken out of the jars and the roots were washed under running tap water, data related to number of shoots per explant, shoot length (cm), leaves number, root length and number per shoot were recorded. Plantlets were then placed in small pots containing beat-moss for ex vitro acclimatization. These pots were covered with polythene transparent bags, and sprayed with water 2 to 3 times a day to ensure high humidity around plantlets. The plastic bags were punctured and gradually removed to expose the plantlets to the outside environment; greenhouse temperature and relative humidity were about 25±2°C and 70%, respectively. Plantlets were kept in the greenhouse four weeks then the survival rate was determined.

According to the experiment, the design used was either a completely randomized design or completely randomized block design. Eleven replicates per treatment were used. Data were analyzed statistically, mean separation was analyzed by Duncan´s multiple range test at 5% level of significance.

Results and discussion

Establishment of contamination-free culture with high survival rate

Our study's approach was to establish a micropropagation method to *Zingiber officinale* Rosc. First, we examined the effect of Clorox concentration (2, 2.5, and 3%) to establish contamination-free culture with high survival rate. The concentrations used in this experiment is the normal range used in sterilizing of most plants in tissue culture, especially tubers and rhizomes, which ranges between 1.5 to 5.5%. A concentration of 3% Clorox significantly improved contamination-free culture of ginger rhizome sprouted bud explants to 90%, the aseptic explants' survival rate was high, and no damage effect on explants due to Clorox was noticed. While 2 or 2.5% of Clorox concentration showed less contamination-free culture of 76% and 62% respectively, with no difference in the aseptic explants' survival rate among all treatments.

Normally, the rhizome sprouted buds were responsive explant, but contamination-free culture establishment was a challenging task. Low concentration of Clorox has been reported not to be effective in controlling contamination especially those caused by bacteria (Nisar et al., 2021). Using high concentration of Clorox (5.25%) significantly reduced the contamination level, which resulted in about 75% contamination-free explants. Even though 5.25% Clorox significantly reduced contamination of ginger rhizome sprouted bud explants, the aseptic explants' survival rate decreased in the culture due to the damaging effect of a high Clorox concentration (.Azhar *et al.,* 2018; Nisar *et al.,* 2021).

Effects of growth regulators and activated charcoal on shoots multiplication

Our results in table. 1 clearly exhibited that the number of produced multiple shoots/explant was increased in most treatments, with the exception of those grown on MS medium supplemented with 5 mg/l BAP accompanied with 3% AC, where a slight decrease but not significant was recorded. MS medium supplemented with 3 mg/l BAP recorded the maximum numbers of shoots multiplication (5.4 shoots/explant), with 10 leaves per shoot (fig. 1, D). Conversely, the lowest number of shoots multiplication (1.8, and 1.5 shoot/explant) was recorded with control (MS free growth regulators) and MS supplemented with 5mg/l BAP combined with AC. The positive effect of MS medium supplemented with BAP on increasing of shoots number was also documented (Abbas *et al.,* 2011; Balachandran *et al.,* 1990), who reported that, concentration of 4.5 and 3.0 mg/l BAP was found to be the optimum for in vitro ginger multiplication. In agreement to our results, Panda *et al.,* (2007) showed that in vitro propagation of Curcuma longa was successfully established and gave the maximum number of shoots per explant when MS medium was supplemented with BAP alone. Adetiloye *et al.,* (2020) reported that the optimum result for regeneration and proliferation of one Banana cultivar was observed at 4.5mg/L in both BAP and Kinetin. It was previously mentioned that the efficiency of BAP as an obvious growth regulator on shoot formation compared to other cytokinins could be attributed to its high stability in vitro cultures (Buah *et al.,* 2010). However, Kambaska and Santilata (2009) indicated that, MS supplemented with high concentration of BAP combined with NAA gave high shoots multiplication rate of *Z. officinale*, which was to some extend in agreement with our results since the medium supplemented with 3 mg/l BAP combined with NAA showed increase in shoot number compared to control (table 1). Number of leaves/shoot as it is presented in Table 1, showed differences among the different treatments. Furthermore, similar to number of shoots/plant, MS medium supplemented with 3 mg/l BAP with or without AC recorded the maximum numbers of leaves/explant (12.4, and 10 leaves/explant), these results are in agreement with those obtained by Shukla *et al.,* (2007), who found that MS medium supplemented with 3.0 mg/l BAP produced the optimum number of leaves of *Curcuma angustifolia*. Combinations of BAP and NAA in MS medium did not seem to improve leaves number/shoot with the exception of those grown on MS medium supplemented with 3 mg/ BAP and 0.5 mg/l NAA, which showed increase but not significant of about 26% in leaves number/shoot, compared to control, which was in agreement with Jagadev *et al.,* (2008) , who stated that MS medium supplemented with 3.0 mg/l BAP and 0.4 mg/l NAA was ideal for maximum number of leaves formation in *Z. officinale* Rosc among all treatments used of BAP and NAA combinations. The addition of 3% AC on medium containing BAP showed a positive effect on leaves number/shoot with the exception of those supplemented with 5 mg/l BAP, which showed a reduction in leaves number compared to control.

Our data revealed that shoot length was not affected in medium supplemented with 3% AC, while all other treatment showed slight reduction in shoot length compared to control.(table 1). These results disagree with that obtained by Kambaska and santilata (2009), who reported that, in *Z. officinale* cultures, the combination of MS with BAP (2.0 mg/l) and NAA(0.5 mg/l) provide optimal response in shoot length.

Effects of growth regulators and activated charcoal on roots multiplication

Rooting responses include number of roots/shoot and mean root length are presented in Table 1. It is well known that profuse rooting in vitro is important for successful establishment of regenerated plants in soil, therefore, beside shoot multiplication, we focused on root multiplication parameters. In this experiment, the maximum number of roots 17.7 MS was recorded on medium supplemented with 3 mg/l BAP, while other BAP concentrations used showed slight but not significant reductions of root number/shoot. Beside BAP' s high stability in vitro cultures (Buah *et al.,* 2010), Cheng *et al.,* (2020) illustrated that the positive effects of BAP on roots and shoots of micropropagated potato attributed to up-regulation of 22 proteins, which are involved in metabolism and bioenergy, storage, redox homeostasis, cell defense and rescue, transcription and translation, signaling and transport.

All combinations of BAP with NAA used exhibited a slight but not significant increase in number of roots/shoot with the exception of those grown on MS medium supplemented with 3 mg/ BAP and 0.5 mg/l NAA, which showed a significant increase in root number (13.4 root per shoot). The current finding was in line with Kambaska and Santilata (2009), who illustrated that the application of low NAA concentration was suitable for in vitro root induction of Zingiber officinale Rosc. cv Suprava and Suruchi. According to Abdelmageed *et al.,* (2011), auxin promoted the growth of intact roots and excised root sections but only at a relatively low concentration range. If high concentration of auxin is applied, it will suppress morphogenesis in cultured plants (Smith, 2013). Rout *et al.,* (2001) confirmed the role of low auxin concentration on the induction of rooting ability of some *Z. officinale* cvs, and revealed the increased level of peroxidase activity at different intervals during the process of rhizogenesis. Various studies on other plant species (Asparagus, Populus and Prunus) have shown the fundamental role played by peroxidase activity during root initiation and expression (Hausman, 1993). Root length was either not effected or slightly diminished in all treatments used (table 1). We expected not to perceive any improvement in root length since MS complete medium is rich medium and normally does not encourage shoot length.

The addition of 3% AC on medium containing different concentrations of BAP showed a positive effect on roots number/shoot with the exception of those grown on medium supplemented with 5 mg/l BAP and 3% AC which showed a significant reduction in number of roots/shoot compared to control. AC is often used in tissue culture to improve cell growth and development. The stimulated effects of AC on morphogenesis may be mainly because of its irreversible adsorption of inhibitory compounds in the culture medium and considerably diminishing the toxic metabolites, phenolic exudation, brown exudate accumulation and alteration and darkening of culture media. Despite of these supportive roles, the negative effect of AC in micropropagation was also reported such as adsorption of some ions, vitamins and growth regulators. In cashew (*Anacardium occidentale*), AC suppressed bud sprouting from shoot nodes but shoot elongation was improved and more vigorous than those without AC (Boggetti *et al.,* 1999).

Table 1: Effects of different concentrations of BAP and combinations of BAP either with AC or with NAA on shoot multiplication and root induction of rhizome bud of *Zingiber officinale* Rosc after 6 weeks of culture

Treatment BAP (mg/l)	AC (g/l)	Number of shoots per explant (mean ± SE)	Number of leaves per shoot (mean ± SE)	Shoot length in cm (mean ± SE)	Number of root per shoot (mean ± SE)	Root length in cm (mean ± SE)
0.0	0.0	1.8 ± 0.24 c	5.0 ± 0.62 bc	5.5 ± 0.30 ab	7.5 ± 1.09 de	4.7 ± 0.40a
2.0	0.0	3.7± 0.74 ab	5.2 ± 1.31 bc	3.5 ± 0.27 bcd	6.8 ± 1.25 de	4.8 ± 0.38a
3.0	0.0	5.4± 1.14 a	10.0 ± 2.35 a	4.5 ± 0.42 abcd	17.7 ± 3.24 a	3.9 ± 0.41ab
4.0	0.0	3.3 ± 0.27 b	4.4 ± 0.40 bc	3.4 ± 0.35 cd	6.8 ± 0.88 de	4.4 ± 0.37a
5.0	0.0	2.6 ± 0.44 bc	4.0 ± 0.56 bc	4.8 ± 0.40 abcd	5.8 ± 0.81 ef	3.3 ± 0.37ab
2.0	30	2.7 ± 0.47 bc	7.2 ± 1.23 ab	5.1 ± 0.22 ab	9.8 ± 1.18 cd	4.0 ± 0.27ab
3.0	30	3.9 ± 0.63 ab	12.4 ± 1.46 a	5.6 ± 0.22 ab	10.8 ± 1.06 bc	4.1 ± 0.41ab
4.0	30	2.3 ± 0.32 bc	5.8 ± 0.62 bc	5.8 ± 0.31 a	8.1 ± 0.87 cde	3.5 ± 0.32ab
5.0	30	1.5 ± 0.17 c	3.3 ± 0.62 c	4.9 ± 0.52 ab	3.2 ± 0.32 f	3.5 ± 0.46ab
BAP(mg/l)	NAA(mg/l)					
2.0	0.5	2.2 ± 0.40 bc	4.4 ± 0.79 bc	4.0 ± 0.32 bcd	8.2 ± 1.37 cde	2.8 ± 0.45b
2.0	1.0	2.8 ± 0.46 bc	4.7 ± 0.93 bc	4.0 ± 0.44 bcd	9.7 ± 0.71 cd	3.4 ± 0.46ab
3.0	0.5	3.1 ± 0.27 b	6.3 ± 0.75 bc	4.9 ± 0.41 ab	13.4 ± 1.58 b	3.4 ± 0.21ab
3.0	1.0	3.0 ± 0.46 b	3.3 ± 0.88 c	4.1 ± 0.55 bcd	9.2 ± 1.40 cd	3.0 ± 0.30ab

* Means followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

When *Gymnema sylvestre* was micropropagated on media containing AC to overcome phenolic exudation, shoots number was reduced most likely due to the adsorption of essential factors required for tissue growth (Komalavalli and Rao, 2000). High frequency shoot regeneration from cotyledon was achieved in Vigna radiate on MS medium supplemented with different plant growth regulators. However, the addition of 0.5 g/l AC to the medium completely inhibited the shoot initiation (Tivarekar and Eapen, 2001).

Percentage of survival plantlets

After 2 weeks of in vitro acclimatization, the derived plants (fig. 1, E) were acclimatized for two weeks under ex vitro conditions in the greenhouse, where plantlets were transferred to pots containing peat-moss taking special care not to harm the roots and moistened uniformly at periodic intervals. In general, about 90% of the regenerated plantlets (fig. 1, F) tolerated and survived under ex vitro environment in greenhouse conditions. Few number of plantlets were lost due to less adaptive abilities for photoautotrophic and/or lowing of relative humidity during acclimatization in ex vitro conditions. Many similar observations have been described, for instance, Preece and Sutter (1991) reported that plantlets growing in vitro exhibit low photosynthetic capacity, and during acclimatization, there is a need for rapid transition from the heterotrophic to the photoautotrophic state for survival. Pospisilova *et al.*, (1999) demonstrated that photosynthetic parameters are very important for the growth of in vitro plantlets during transition stage to greenhouse. Hence, transfer of plantlets from in vitro to ex vitro condition should be carefully handled specially in term of photosynthetic and humidity considerations. Therefore, most researchers focused on these factors to reach high survival rate.

For example, Cha-um *et al.*, (2005) found that in vitro acclimatization of ginger plantlets under high relative humidity with CO_2 enrichment, to enhance photoautotrophic, produced plantlets possess vigorous shoot and root systems with high survival rate.

We can conclude that this study demonstrated a simple and economical in vitro propagation of *Zingiber officinale* Rosc. Surface sterilization of rhizome sprouted bud explants was achieved by the soaking of the explants in 3% Clorox for 20 minutes. The contamination-free culture was determined to be 90%, and at the same time; the aseptic explants' survival rate was high. Shoot multiplication and root induction from rhizome bud was significant on MS medium supplemented with 3 mg/l BAP. This protocol is useful for rapid clonal propagation of healthy *Zingiber officinale* Rosc plants for commercial production. Furthermore, the combined application of cytokinins and auxins positively influenced the shoot and root multiplication of many *Zingiberaceae* plants (Kambaska and Santilata, 2009; Ayenew *et al.*, 2012). Hence, further studies need to be taken in account to obtain the optimal cytokinin or auxin type and their combination for higher shoot and root multiplication rate of ginger. Finally, it is recommended to assess other *Zingiber officinale* cvs to establish simple and economical protocols for their micropropagation.

Figure 1. Micropropagation of *Zingiber officinale* Rosc. (A) Rhizome sprouted buds of *Zingiber officinale* Rosc used as explant for culture initiation; (B) Aseptically established culture when surface sterilized with 3% Clorox after one week of culture on MS medium supplemented with 3 mg/l BAP; (C) I*n vitro*-raised shoot used as explant for shoot multiplication (after two weeks of incubation on MS medium supplemented with 3 mg/l BAP; (D) Multiple shoots produced in MS medium supplemented with 3 mg/l BAP after four weeks of culture; (E) *In vitro*-rooted plantlets derived from MS medium supplemented with 3 mg/l BAP after four weeks of culture followed by two weeks of *in vitro* acclimatization; (F) *Zingiber officinale* Rosc plants after two weeks of *in vitro* followed by two weeks of *ex vitro* acclimatization.

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