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Full Length Research Paper

Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants

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A successful protocol was developed for mass propagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl., an important medicinal plant. Numerous shoots were induced from young shoot bud of *B. rotunda* mature rhizome on Murashige and Skoog (1962) medium supplemented with 30.0 g/l sucrose, 2.0 g/l gelrite, different concentrations of 6-benzylaminopurine (BAP) and α-naphtaleneacetic acid (NAA). Plant medium supplemented with different concentrations of BAP alone or with NAA produced varying degree of multiple shoots. A supplementation of 2.0 mg/l BAP and 0.5 mg/l NAA gave the best result. Ninety percent of the explants induced multiple shoots within 10 to 14 days of inoculation with five maximum numbers of shoots per explant. The numbers of multiple shoots was low during initial subculture but increased after third subculture and were slightly decreased after fourth subculture. Rooting was spontaneous in almost all the treatments after 10 to 14 days of culture. Micropropagated plantlets were successfully acclimatized.

Key words: *Boesenbergia rotunda*, micropropagation, medicinal plant, Zingiberaceae.

INTRODUCTION

Boesenbergia rotunda (L) Mansf. Kulturpfl. (Larsen, 1996), is a perennial herbaceous plant that belongs to the family Zingiberaceae and is believed to have originated from India and South-East Asia region. The pharmacological importance of this plant is mainly due to the presence of flavanoids, essential oil and chalcones (Jaipetch et al., 1982; Pandji et al., 1993; Trakoontivakorn et al., 2001). Moreover, rhizomes contain a potential antipyretic, analgesic, anti-mutagenic, anti-inflammatory, antioxidant enzymes and anti-human immunodeficiency virus 1 (anti-HIV-1) protease inhibition (Pathong et al., 1989; Murakami et al., 1993; Tuchnida et al., 2002; Fahey and Stephenson, 2002; Tewtrakul et al., 2003). In a recent study, 4-hydroxypanduratin A and panduratin A, isolated from the rhizome of *B. rotunda* were found to show high inhibitory activity towards dengue-2 virus protease at 120 ppm (Tan et al., 2006).

B. rotunda is traditionally propagated by vegetative techniques using a rhizome segment which is lengthy for large-scale multiplication. Moreover, many of the Zingiberaceae species are susceptible to rhizome and soft rot diseases, leaf spot and easily infected with pathogens such as *Coleotrichum* species (Balachandran et al., 1990; Chan and Thong, 2004). A rapid multiplication method is required to supply sufficient disease-free plant material for large scale cultivation using *in vitro* propagation. *In vitro* propagation of Zingiberaceae has already been reported, for example *Alpinia galanga* (Inden and Asahir, 1988) and *Zingiber officinale* (Hosoki and Sagawa, 1977; Pillai and Kumar, 1982; Inden and Asahira, 1988; Bhagyalakshmi and Singh, 1988; Ikeda and Tanabe

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Abbreviations: BAP, N6-Benzylaminopurine; **NAA,** α-naphthalene acetic acid; **MS,** Murashige and Skoog; **HIV,** human immunodeficiency virus.

1989). Several investigators reported micropropagation of ginger either from buds (Nadgauda et al., 1980) or shoot tips (Inden and Asahira, 1988), meristems (Bhagyalakshmi and Singh, 1988), or even from callus (Malamug et al., 1991; Nirmal et al., 1992). *In vitro* clonal multiplication of *Curcuma* species through rhizome buds also has been reported (Nadgauda et al., 1978; Balachandran et al., 1990; Barthakur and Bordoloi, 1992; Salvi et al., 2002; Loc et al., 2005, Yusuf et al., 2007; Naz et al., 2009). However, to date, there are no reports on the rapid micropropagation of *B. rotunda*, which will be discussed in this paper.

MATERIALS AND METHODS

Establishment of aseptic explants

Mature rhizomes of *B. rotunda* were purchased from herbal supplier at a local Market in Kuala Lumpur, Malaysia. The rhizomes were cleaned and rinsed. The cleaned rhizomes were then placed in an open container to allow shoots to sprout two to four cm in length. The sprouting buds were collected and washed with 20% (v/v) clorox for 15 min. Under aseptic conditions, shoots were surface sterilized with 0.5% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) solution for five minutes and followed by three rinses in sterile distilled water. The shoot buds had their external leaves removed and trimmed down until the size ranged from 0.5 to 0.8 mm and used as explants (Figure 1A to C). They were then inoculated into 350 ml glass jar containing MS (Murashige and Skoog, 1962) medium supplemented with 30.0 g/l sucrose and 2.0 g/l gelrite for 30 days. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C for 21 min. The cultures were maintained in a culture room with 16/8 h photoperiod (light/dark) at 25 ± 2°C. After 30 days, the aseptic buds of this species were transferred onto MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l NAA, the optimum medium was formulated for other Zingiberaceae species by Yusuf et al. (2007) for shoot multiplication for four (4) weeks (Figure 1D). The multiple shoots produced were separated and transferred into MS medium devoid of plant growth regulators for four (4) weeks and were used for all subsequent experiments.

Induction of multiple shoots

Isolated shoot buds were inoculated onto MS (Murashige and Skoog, 1962) medium containing 30.0 g/l sucrose and 2.0 g/l gelrite as solidifying agent supplemented with cytokinins (6-benzylaminopurine (BAP)) ranging from 0.0, 0.5, 1.0, 2.0, 3.0 and 5.0 mg/l and auxin (α-naphthaleneacetic acid (NAA)) ranging from 0.0, 0.5, 1.0 and 2.0 mg/l, for shoot multiplication. A single shoot was cultured into each 350 ml glass jar and 10 experimental units were used for each medium combination.

Scoring of data

All cultures were examined periodically, and the morphological changes were recorded on the basis of visual observations and were made after one to four weeks of culture for four consecutive subcultures. There were 10 cultures per treatment for shoot multiplication and subculturing was carried out at an interval of four weeks. The effect of different treatments and subcultures were quantified on the basis of percentage of cultures showing response for multiple shoots formation.

Acclimatization

In vitro rooted plantlets of *B. rotunda* (Figure 1E) were removed from the solid MS medium and the roots were washed under running tap water to remove residual agar. Each plantlet was then planted into pots containing mixture of organic soil and sand (1:1) without direct sunlight. The percentage of surviving plantlets was recorded after four weeks of transfer.

RESULTS AND DISCUSSION

Initiation of shoot buds

Establishment of contamination free cultures was a major task due to the fact that the explants originated from underground rhizomes (Hosoki and Sagawa, 1977). In this study, rhizomes were initially sprouted on soil free condition until shoot buds appearred. These shoot buds were excised from rhizomes and were used as explants. The buds from the rhizomes of *B. rotunda* that were surface sterilized with mercury chloride solution and commercial bleach (Clorox®) solution for 15 min could establish more than 70% aseptic and surviving explants and remained free of contamination after four (4) weeks in MS medium. Chan and Thong (2004) also reported that the use of $HgCl_2$ with two-stage surface sterilization using Clorox® solution was extremely efficient for establishing aseptic buds of other Zingiberaceae species. Similar sterilization process was also used to establish the aseptic buds of *Cymbopogon nardus* (Chan et al., 2005). Once the contamination free cultures of the shoot buds were established, they were easily maintained by sub culturing on fresh medium.

Shoots growth and multiplication

The aseptic shoots of *B. rotunda* cultured on MS medium supplemented with different concentrations of BAP alone (0.0 to 5.0 mg/l) or in combinations with NAA (0.0 to 2.0 mg/l) resumed their growth, produced shoots and roots, simultaneously (Table 1). The simultaneous production of shoots and roots were also reported in other Zingiberaceae species (Balachandran et al., 1990; Chan and Thong, 2004; Bharalee et al., 2005). The growth of the shoots and subsequent multiplication could not be achieved in medium without growth regulators. After 4 to 6 weeks, about 90% of the shoots of *B. rotunda* that were cultured on MS medium supplemented with 0.5 to 3 mg/l of BAP and 0.5 mg/l NAA showed different levels of development with high micropropagation frequency (induced 4 to 5 multiple shoots, Table 1) and variable number of leaves with 10 to 15 roots hairs including secondary roots per explants (data not shown). The maximum number of multiple shoots (5) was obtained in the medium containing 2.0 mg/l of BAP and 0.5 mg/l NAA four (4) weeks after culture initiation (Figure 2). However, the buds cultured on MS medium containing higher

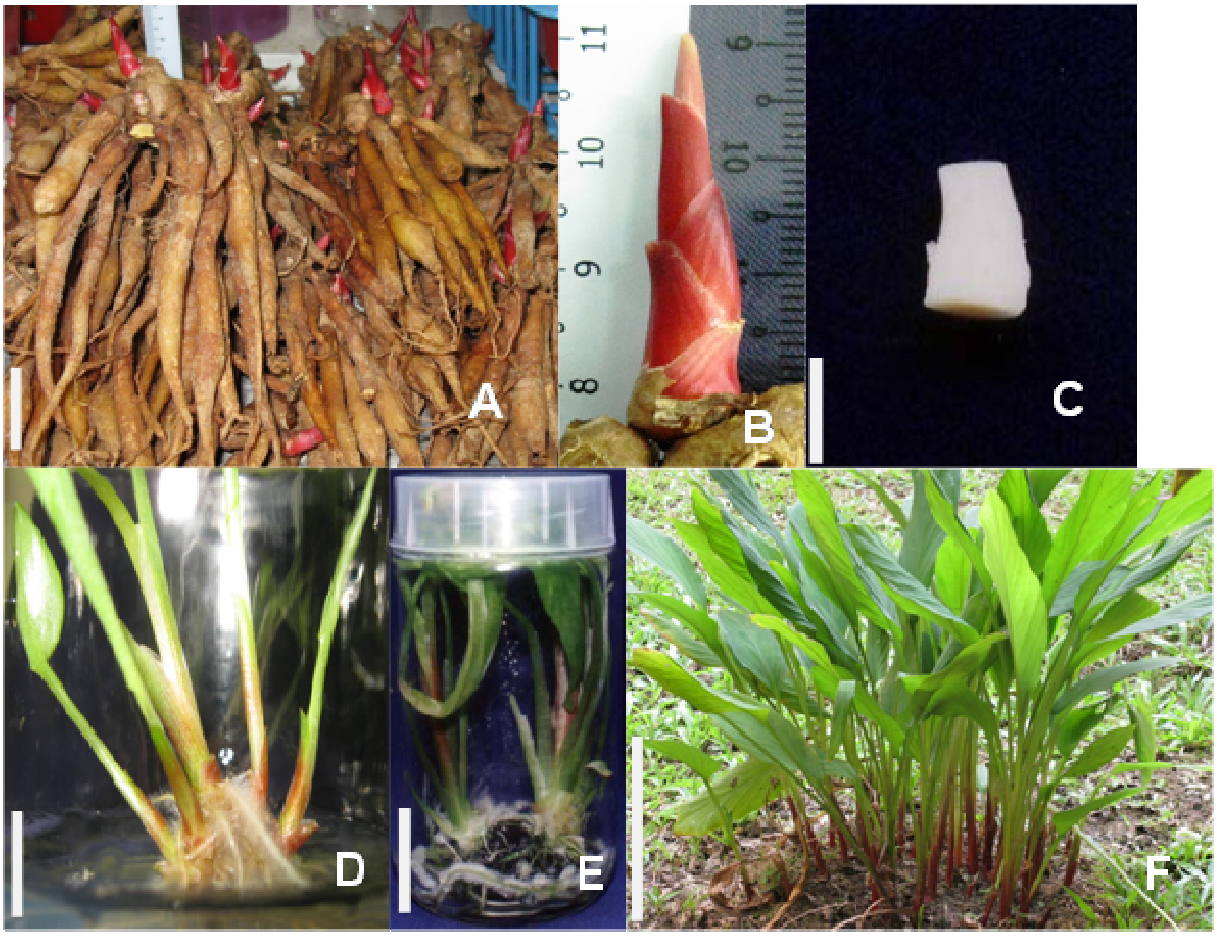

Figure 1. *In vitro* regeneration and plant establishment of *B. rotunda*. (A) Shoots sprouted from mature rhizomes of *B. rotunda*. Bar represents 25 mm. (B) Sprouted buds (30 to 50 mm) collected as explants. (C) Shoots after been surface sterilized under aseptic conditions with external leaves removed and trimmed down (5 to 8 mm) as explants. Bar represents 5 mm. (D) Induction of multiple shoots of *B. rotunda* on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA after four (4) weeks of culture. Bar represents 15 mm. (E) Formation of roots on acclimatized semi-solid MS medium devoid of plant growth regulators with 2.0% (w/v) activated charcoal after four (4) weeks of culture. Bar represents 20 mm. (F) The *in vitro*-derived plants germinated in soil with normal morphology. Bar represents 100 mm.

concentration of BAP (5.0 mg/l) and NAA (2.0 mg/l) showed low level of development and low micropropagation frequencies (1 to 2 shoots per explant) and also showed abnormalities; even though it produced multiple shoots, the plantlets were stunted and the leaves became yellowish within four (4) weeks of culture. These results indicated that there was an optimal concentration of plant growth regulators required for normal shoot multiplication of *in vitro* cultures of *B. rotunda* species. Balachandran et al. (1990) also reported that higher concentration of kinetin was not suitable for *Zingiber officinale*. Aseptic cultures in MS supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA induced more roots than shoots. Micropropagation was quantified by the number of shoot. Well-formed shoots was BAP-dependent and was inhibited by NAA. The medium having NAA alone had no effect on shoot multiplication or growth. The increase of NAA concentration higher than 1.0 mg/l sup-pressed multiplication rate but was BAP-dependent. Multiplication frequency was increased until BAP concentration reached 3.0 mg/l.

Since there was no increase in the number of shoots formed and as it would also be economically feasible, low concentration of BAP (2.0 mg/L) and NAA (0.5 mg/L) supplemented into MS medium formulation was chosen as the shoot multiplication medium for *B. rotunda*. Other researchers reported higher concentration of plant growth regulators for the induction of multiple shoots formation for some of the Zingiberaceae species. Loc et al. (2005) reported that MS medium supplemented with 20% (v/v) coconut water, 3 mg/l BA and 0.5 mg/l IBA, could induce the formation of an average of 5.6 shoots per explant for *Curcuma zedoaria*. Bharalee et al. (2005) found that MS medium supplemented with 4 mg/l BAP and 1.5 mg/L NAA was the best medium for shoot multiplication of *C. caesia* (average 3.5 shoots per explant) and MS plus 1.0

Table 1. Effects of MS medium supplemented with BAP (0, 0.5, 1.0, 2.0, 3.0 and 5 mg/l) and NAA (0, 0.5, 1.0 and 2.0 mg/l) on multiple shoots formations of *B. rotunda* over a period of four (4) weeks of culture.

BAP (mg/l)	NAA (mg/l)	Mean no. of multiple shoots
0.0	0.0	1.0 ± 0.0
0.5	0.0	2.9 ± 0.2
1.0	0.0	3.3 ± 0.2
2.0	0.0	4.1 ± 0.3
3.0	0.0	3.9 ± 0.3
5.0	0.0	2.7 ± 0.2
0.0	0.5	1.0 ± 0.0
0.5	0.5	3.8 ± 0.1
1.0	0.5	4.2 ± 0.1
2.0	0.5	4.9 ± 0.1
3.0	0.5	4.0 ± 0.3
5.0	0.5	2.8 ± 0.2
0.0	1.0	1.0 ± 0.0
0.5	1.0	2.7 ± 0.3
1.0	1.0	2.9 ± 0.3
2.0	1.0	3.6 ± 0.4
3.0	1.0	3.2 ± 0.4
5.0	1.0	2.4 ± 0.3
0.0	2.0	1.0 ± 0.0
0.5	2.0	2.4 ± 0.3
1.0	2.0	2.5 ± 0.2
2.0	2.0	2.8 ± 0.2
3.0	2.0	2.6 ± 0.3
5.0	2.0	1.8 ± 0.2

Values represent means ±SE for 10 cultures per treatment.

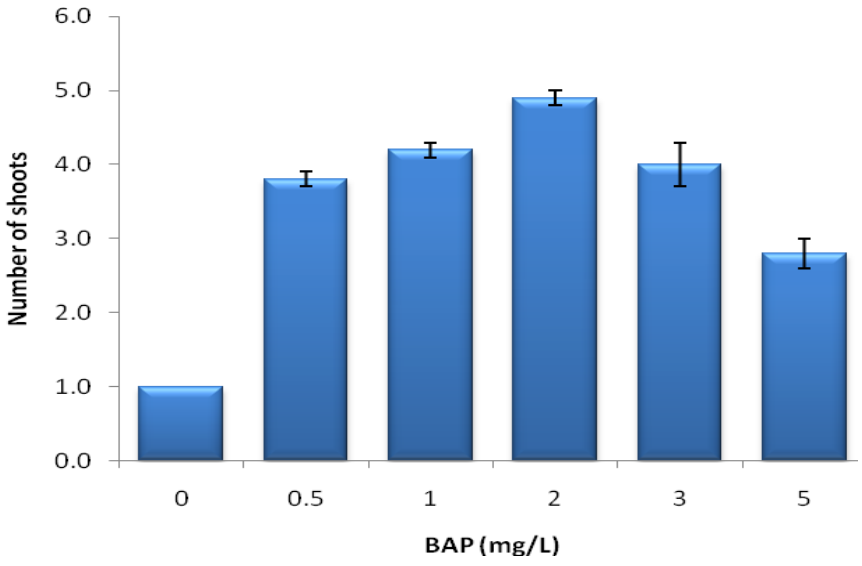

Figure 2. Effects of MS medium supplemented with BAP (0, 0.5, 1.0, 2.0, 3.0 and 5 mg/l) and low concentration of NAA (0.5, mg/l) on multiple shoots formations of *B. rotunda* over a period of four (4) weeks of culture. Values are mean ± standard deviation for 10 cultures per treatment.

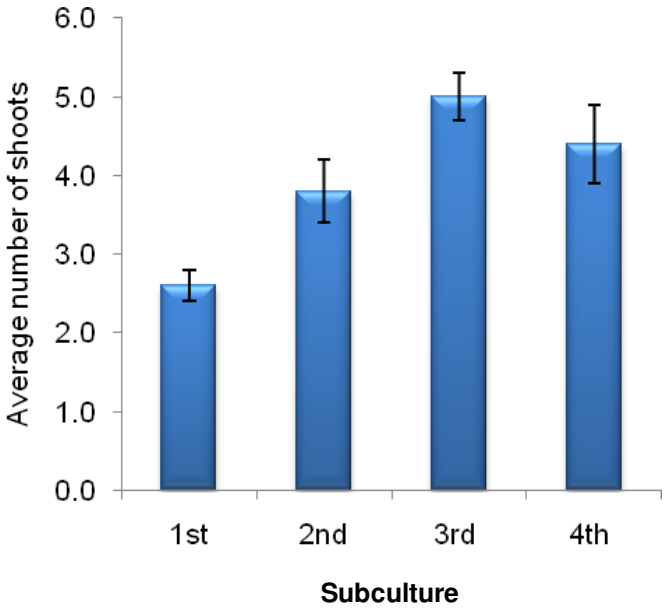

Figure 3. Effects of subculture on multiple shoots formations of *B. rotunda* over a period of four (4) subcultures in MS medium supplemented with 2.0 mg/l BAP and 0.5, mg/l NAA. Values are mean ±standard deviation for 10 cultures per treatment.

mg/L BAP and 0.5 mg/L NAA for *C. zedoaria* (average 4.5 shoots per explant). Balachandran et al. (1990) reported that *C. domestica C. caesia* and *C. aeruginosa* could produced an average of 3.4, 2.8 and 2.7 shoots per explant respectively using MS medium supplemented with 3 mg/L BAP. Nayak (2000) reported MS medium supplemented with 5 mg/L BAP was most effective for shoot multiplication of *C. aromatic* producing an average 3.3 shoots per explants. Similar results indicating cytokinin and auxin effects on shoot multiplication had been reported earlier in ginger (Hu & Wang, 1983; Palai et al., 1997). Hosoki and Sagawa (1977) reported that the requirement of a cytokinin for high-frequency shoot multiplication of ginger was as high as 5.0 mg/L IBA. In addition, Palai et al. (1997) indicated that the combination of two cytokinins along with auxin increased the rate of shoot multplication (16-fold) within four (4) weeks of culture. The present findings suggested that a high frequency of shoot production from a single shoot meristem could be achieved by manipulating the plant growth regulators.

The number of shoots generated from *B. rotunda* shoot explants cultured on the proliferation medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA remained unchanged after either four (4) or eight (8) weeks of subculturing. Thus, the *in vitro* plantlets could be subcultured every eight (8) weeks in terms of culture preservation. In addition, the *in vitro* plantlets could be maintained on solid basic MS medium for two (2) months without any subculturing been required and the plants remain healthy. Hence MS medium without the addition of plant growth regulator(s) could be potentially used for the *in vitro* germplasm conservation of *B. rotunda*.

The effect of subculture on shoot multiplication was tested in MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA for 16 weeks. The results indicated that the number of multiple shoots were low during initial subcultures, and increased to 5.0 ± 0.3 at 3rd subculture and then decreased slightly to 4.4 ± 0.5 at 4th subculture (Figure 3). *In vitro* plantlets of *B. rotunda* with well developed roots were successfully acclimatized with 85% survival of the plantlets when transplanted to the field environment. *In vitro* plantlets were morphologically similar to their respective mother plants (Figure 1F).

Conclusion

A supplementation of 2.0 mg/l BAP and 0.5 mg/l NAA gave the best result to stimulate a successful production of multiple shoots of *B. rortunda* with 90% of the inoculated explants inducing multiple shoots. Clonal propagation and germplasm conservation of *B. rotunda* was attractive because the propagated material was shown to possess high genetic stability (Rout et al., 1998). *In vitro* plantlets can be an important source of disease-free planting material, ideally suited for germplasm exchange, transportation and conservation.

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