Rapid and efficient leaf regeneration propagation system for *Euonymus bungeanus*

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Abstract

Rapid propagation of plants by tissue culture is of great significance for large-scale production, molecular genetics research, and breeding. Currently, a rapid and high-efficient tissue culture protocol for *Euonymus bungeanus* is needed. To develop a propagation system for this species, we established a new regeneration system from leaves. Callus formation was induced on Murashige and Skoog (MS) medium supplemented with 0.5 mg dm⁻³ 6-benzylaminopurine (6-BA) and 0.5 mg dm⁻³ α -naphthalene acetic acid (NAA) and the induction rate almost reached 100 % under red radiation within 21 d. The medium for proliferation of adventitious buds comprised of MS medium with 1.0 mg dm⁻³ 6-BA and 0.5 mg dm⁻³ NAA, and the induction rate within 20 d nearly reached 100 %. When, the adventitious buds were transferred to the rooting medium containing ½MS, 2.0 mg dm⁻³ indole-3-butyric acid (IBA), and 0.05 mg dm⁻³ NAA, adventitious root formation was achieved within 20 d. Collectively, the rapid and high-efficient regeneration system from *E. bungeanus* leaves was established, providing useful references for effective mass propagation and it could serve as an enabling technology for future genetic engineering.

Keywords: adventitious buds and roots, 6-benzylaminopurine, callus induction, indole-3-butyric acid, α -naphthalene acetic acid.

Introduction

Euonymus bungeanus is a small deciduous tree species native to northern China from the Heilongjiang province to Yangtze River and also is widely distributed in Ussuri region, southern Siberia, and Korean Peninsula (Liu *et al.* 2019). *E. bungeanus* has few pests and diseases and preferentially requires high irradiance but it is capable of surviving in harsh and extreme environmental conditions (Wang *et al.* 2016). The species unique growth characteristics (branch and crown shapes) increased its value for landscaping in southern China (Zhang *et al.* 2010). Moreover, the leaves of *E. bungeanus* are usually green in spring and summer, and in autumn the color of leaves turn red. Colored-leaf plants gain popularity in

landscape architecture (Gang *et al.* 2019).

E. bungeanus is generally reproduced sexually through seed sowing and vegetatively propagated using rooted cuttings (Song and Ding 2013). However, the species largescale sexual reproduction is restricted for two reasons: complicated seed pretreatment and undesirable progeny characteristics resulting from mitotic genetic segregation (Xu *et al.* 2004). Similarly, propagation capacity through cuttings is also limited after the identification of desirable varieties. Contrary to the traditional propagation using seed and cuttings, propagation through tissue culture has several advantages owing to the outstanding qualities such as rapid propagation, scale-up, low cost, space-saving, and season-free characters (Smith and Jernstedt 1989). Furthermore, tissue culture has an enabling technology role

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Abbreviations: 6-BA - 6-benzylaminopurine; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α -naphthalene acetic acid.

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in plant molecular breeding and biotechnology (Da Silva *et al.* 2019). However, it should be stated that the ability to rapidly propagate plants *in vitro* has its own challenges such as different genotypic characteristics, explant survival, phytohormones, culture medium composition, and culture conditions (Dobránszki and Da Silva 2010, Magyar-Tábori *et al.* 2010, Da Silva *et al.* 2019).

With *E. bungeanus* immature stems, Li and Shi (2009) have successfully generated auxiliary buds and obtained callus tissues. In 2010, the optimum culture medium for *E. bungeanus* propagation was discovered and the explants were mature embryos (Zhang *et al.* 2010). To date, information on regeneration system from *E. bungeanus* leaves remains scant. The objective of the present study was to develop a rapid and high-efficient *E. bungeanus* protocol of vegetative propagation through tissue culture. We expect that the resulting protocol would be of interest to the development of *E. bungeanus* genetically engineered plants.

Materials and methods

Explants collection and sterilization: Two-year-old, wild-type *E. bungeanus* plants provided the material for this study. Plants were cultivated in a greenhouse and grown in pots (diameter 38 cm, height 40.5 cm) with 3:1 (v/v) peat soil (Klasmann-Deilmann 418#, Geeste, Germany) and Perlite (particle size specification: 3~6 mm, Altea, Lachine, Canada) and watered every three days without fertilizer. The greenhouse is located at the Jinling Institute of Technology's Horticultural Experimental Station (Nanjing, China). Young leaves (explants) were harvested from the upper branches, cleaned by 5 % (m/v) washing powder (Tide, Guangzhou, China) solution, rinsed with tap water for 2 h, and washed in 50 dm³ tubes on a shaker with distilled water for 10 min. After that, the leaves were soaked in 75 % (v/v) alcohol solution for 30 s and sterilized by 15 % (m/v) NaClO solution. The 15 % NaClO solution sterilization time was determined through a range of sterilization experiments (sterilization times ranging from 1 to 7 min, with 1 min increments, thus 7 treatment times). After immersion in the 15 % NaClO solution, leaves from each treatment time were rinsed by sterile water within 500 dm³ glass beaker for 2 min. Under the aseptic condition, the sterilized leaves were cut into small segments (5 × 5 mm) (Fig. 1 Suppl.) and then placed on (Murashige and Skoog 1962; MS) medium. Each treatment was done in three replications, yielding totally 630 leaf explants. To examine the effects of sterilization time, the pollution rate and survival rate percentages were calculated as follows:

Pollution rate = (No. of explants polluted/No. of explants inoculated) × 100 % [1]

Survival rate = (No. of explants survived/(No. of explants inoculated - No. of explants polluted)) × 100 % [2]

Callus formation: The sterilized leaf explants were cultivated on basal MS medium (Murashige and Skoog 1962) containing 6.5 g dm⁻³ agar and 30 g dm⁻³ sucrose and

adjusted to pH 5.8. In this medium, six combinations of different hormones (T1 - T6) were tested (Table 1). In these combinations, 6-benzylaminopurine (6-BA) concentration was fixed at 0.5 mg dm⁻³, while α -naphthalene acetic acid (NAA) concentration varied from 0.05 to 0.5 mg dm⁻³ (Table 1). Each of these hormonal combinations in medium was placed in 30 glass culture bottles (250 dm³) and this process was replicated three times. Each bottle contained five pieces of leaf explants and was cultivated in tissue culture room for 21 d for callus induction. The incubation was conducted under red radiation ($\approx$ 660 nm; an irradiance of 70 μ mol m⁻² s⁻¹; 16-h photoperiod), and a temperature of 24 $\pm$ 2 °C. The induction rate of callus was calculated as (No. of induced calluses/No. of inoculated leaf explants) × 100 % [3].

To evaluate callus growth state, the fresh and dry masses of the resulting calli were measured using 1/1000 electronic balance (Sartorius, Goettingen, Germany). To estimate the dry mass, calli were oven-dried at 105 °C for 30 min, then at 60 °C for 3~4 h to a constant mass (BOXUN, Shanghai, China).

Differentiation of adventitious buds: Well-induced callus was cut into small pieces in the size of 0.8 × 0.8 × 0.8 cm³ and then placed on the MS medium containing one of the hormonal combinations for 20 d (Table 1). Each hormonal medium combination was placed in 90 bottles (one replicate 30) with a total of three replicates, and five small callus pieces were placed in each bottle. For adventitious buds differentiation, we utilized white radiation $\approx$ 730 nm (an irradiance of 110 μ mol m⁻² s⁻¹; 16-h photoperiod) and a temperature 24 $\pm$ 2 °C. The growth of adventitious buds was monitored every three days and the induction rate of adventitious buds was calculated as (No. of adventitious buds/No. of inoculated calluses) × 100 % [4]. The length of adventitious buds was measured using Vernier caliper after pressing the samples flat.

Induction of adventitious roots: Adventitious buds in 1.5 - 1.8 cm length were selected and transferred into rooting medium for 20 d. Two types of rooting medium ($\frac{1}{2}$ MS and MS medium) were selected for the induction of adventitious roots. The $\frac{1}{2}$ MS and MS media were separately coupled with NAA (concentration from 0.05 to 0.5 mg dm⁻³) and indole-3-butyric acid (IBA; concentration from 0.05 to 2.0 mg dm⁻³) (Table 1). Each hormonal combination medium was placed in 90 bottles (one replicate 30) with three replicates, and five adventitious buds were placed in each bottle. For adventitious roots induction, we used the same conditions as for bud induction. The growth was monitored every three days and the induction rate of adventitious roots from adventitious buds was calculated as (No. of rooted plantlets/No. of inoculated adventitious buds) × 100 % [5]. A number of adventitious roots were counted after 20 d, and their length was measured using Vernier caliper after pressing the samples flat.

Acclimatization of plantlets: After incubating the adventitious buds in the rooting medium (T1 combination) for 20 d, 150 aseptic plantlets belonging to three replicates

Table 1. Hormonal combinations of *E. bungeanus* tissue culture medium used in different growth stages.

Growth stages	Combinations	Hormone concentration [mg dm ⁻³]			Medium types
		6-BA	NAA	IBA	
Callus formation	T1	0.5	0.05	0	MS
	T2		0.1		MS
	T3		0.2		MS
	T4		0.3		MS
	T5		0.4		MS
	T6		0.5		MS
Adventitious bud differentiation	T1	1	0.05	0	MS
	T2		0.1		MS
	T3		0.3		MS
	T4		0.5		MS
	T5		0.7		MS
	T6		0.9		MS
Adventitious root induction	T1	0	0.05	2	½MS
	T2		0.2	0.6	½MS
	T3		0.5	0.05	½MS
	T4		0.05	2	MS
	T5		0.2	0.6	MS
	T6		0.5	0.05	MS

were successfully rooted. To acclimate these plantlets to natural environment, the glass bottles lids were removed in the tissue culture room for three days. Then the bottles with plantlets were placed in a greenhouse for three days. Subsequently, the roots of aseptic plantlets were rinsed by sterile water before planting in the plastic pots (diameter 10 cm, height 9 cm). A total of 150 plantlets (one replicate with 50) were cultivated in the peat soil mixed with *Perlite* in the ratio of 3:1 (v/v), and then the plantlets were incubated in the greenhouse. After 14 d, their survival rate was recorded.

Statistical analysis: Data were analyzed using *IBM SPSS v. 21.0* statistic software. One-way analysis of variance (*ANOVA*) was used to determine the significant differences between treatments' means by Duncan's multiple comparison test ($P < 0.05$).

Results

To optimize the sterilization time of leaf explants, young leaves were divided into seven groups and were submerged in 15 % NaClO solution for different periods ranging from 1 to 7 min with 1-min increments. The pollution rates of leaf explants sterilized for the first 4 sterilization treatments (1 to 4 min) were 100, 91.3, 50.1, and 33.5 %, respectively, while their survival rates were 0, 8.7, 49.9, and 66.5 %, respectively. On the other hand, the pollution rates for the 6- and 7-min sterilization treatments were 0 %, while their survival rates were 40.3 and 32.4 %, respectively. Interestingly, the pollution rates and survival

rates of the 5-min sterilization treatment were 0 and 100 %, respectively, suggesting that the 5-min sterilization time is the best (Fig. 1A).

Callus induction was evaluated using constant 6-BA concentration (0.5 mg dm⁻³) across 6 different NAA concentrations ranging from 0.05 to 0.5 mg dm⁻³ (Table 1). After 21 d of culture, a significant difference ($P < 0.05$) was observed towards increased NAA concentrations (Fig. 1B). The best rate of callus formation was observed at NAA (0.5 mg dm⁻³) and 6-BA (0.5 mg dm⁻³) hormonal combination reaching 100 % (Fig. 1B), with callus fresh and dry masses of 0.174 ± 0.041 g and 0.027 ± 0.011 g, respectively, representing 338.9 and 186.3 % increase from T2 to T5 (Table 2). In view of the growth status of the calli (Fig. 1C-E), we determined that T6 combination represents the optimal medium for *E. bungeanus* calli induction from leaf explants (that is MS + 0.5 mg dm⁻³ 6-BA + 0.5 mg dm⁻³ NAA).

Adventitious bud induction was evaluated using a constant 6-BA concentration of 1.0 mg dm⁻³ across 6 different NAA concentrations ranging from 0.05 and 0.9 mg dm⁻³ (Table 1, Fig. 2A). Among these the NAA concentration of 0.5 mg dm⁻³ produced the highest, adventitious bud induction rate reaching approximately 100 % (Fig. 2B), with an average of 2.8 cm adventitious bud length as compared to other combinations. Thus, we concluded that the hormonal medium containing 1.0 mg dm⁻³ 6-BA and 0.5 mg dm⁻³ NAA was the best for regenerating plants with green leaves and strong stems (that is MS + 1.0 mg dm⁻³ 6-BA + 0.5 mg dm⁻³ NAA) (Fig. 2C).

Fig. 1. Effect of different sterilization time and callus formation under different hormone combinations. *A* - Pollution and survival rates of *Euonymus bungeanus* leaf explants with different sterilization time; *B* - Callus induction rate under different hormonal combinations; *C, D, E* - Callus differentiation at 7, 14, and 21 d on MS medium containing 0.5 mg dm^{-3} 6-BA and 0.5 mg dm^{-3} NAA (bar 5 mm).

Table 2. Fresh and dry masses of *E. bungeanus* calli cultured under different hormonal combinations. Means $\pm$ SDs, $n = 3$; means followed by the same letter are not significantly different from each other's at $P < 0.05$.

Combinations	Hormone concentration [mg dm^{-3}]		Fresh mass [g]	Dry mass [g]
	6-BA	NAA		
T1	0.5	0.05	---	---
T2	0.5	0.1	0.019 ± 0.002 cd	0.005 ± 0.001 cd
T3	0.5	0.2	0.045 ± 0.008 cd	0.010 ± 0.003 bcd
T4	0.5	0.3	0.063 ± 0.017 bc	0.014 ± 0.004 bc
T5	0.5	0.4	0.099 ± 0.039 b	0.019 ± 0.007 ab
T6	0.5	0.5	0.174 ± 0.041 a	0.027 ± 0.011 a

To determine the optimum medium, sturdy adventitious buds were cut into small pieces and cultured in $\frac{1}{2}$ MS and MS coupled with different hormonal combinations (Table 1). Our results revealed that the rooting rate, number of adventitious roots, and length of adventitious roots showed significant difference when they were grown under NAA (0.05, 0.2, 0.5 mg dm^{-3}) and IBA (0.05, 0.6, 2.0 mg dm^{-3}) in $\frac{1}{2}$ MS and MS medium ($P < 0.05$) (Fig. 3A,B,C). When the hormone combinations of NAA and IBA were the same, rooting rate, root number, and root length of the adventitious roots grown on $\frac{1}{2}$ MS medium (T1, T2, T3) produced better results than those grown on MS medium (T4, T5, T6). For instance, the rooting rates of the adventitious buds in T1, T2, and T3 were 99.7, 41.5, and 28.2 %, respectively, while the rooting rates of the adventitious buds in T4, T5, and T6 were 36.0, 12.6, and 0 %, respectively (Fig. 3A). These results indicated that $\frac{1}{2}$ MS medium containing different concentrations of NAA

and IBA effectively contributed to the adventitious root growth. Moreover, when NAA and IBA concentrations were 0.05 and 2.0 mg dm^{-3} (namely T1), the rooting rate was approximately 100 %, and an average number of adventitious roots of 15 was the greatest (Fig. 3B), and the length of adventitious roots was 7.5 cm, representing the longest of all treatments (Fig. 3C). Additionally, the adventitious roots had a strong main root and abundant lateral roots (Fig. 3D,E). These results allowed us to conclude that T1 (that is $\frac{1}{2}$ MS + 0.05 mg dm^{-3} NAA + 2.0 mg dm^{-3} IBA) is the most suitable for adventitious roots growth.

To investigate the adaptability of the rooted plantlets to natural environmental condition, a total of 150 aseptic rooted plantlets grown in T1 ($\frac{1}{2}$ MS + 0.05 mg dm^{-3} NAA + 2.0 mg dm^{-3} IBA) for 20 d were transplanted into plastic pots in a greenhouse. Surprisingly, after 14 d of plantlets grown in pots, all survived the greenhouse environment,

Fig. 2. Induction rate (A) and length (B) of *Euonymus bungeanus* adventitious buds under different hormonal combinations. C - Growth of adventitious buds in T4 medium after 20 d (bar 5 mm).

indicating that these plantlets had excellent growth status (Fig. 2 Suppl.).

Discussion

Plant tissue culture is based on cell totipotency and has developed into a useful protocol for complete plant regeneration with great large-scale production potential (Shahzad *et al.* 2017, Lee and Pijut 2017). Successful plant tissue culture is dependent on the regeneration ability of different species, and also on variables, such as tissues or organs to develop into cloning, physiological age, growth stage, and position of explants directly or indirectly affecting the establishment of tissue culture (Ćosić *et al.* 2015, Raomai *et al.* 2015).

The selection of suitable explants, culture mediums, and hormonal treatments are the key factors in the success of plant tissue culture (Rout and Sahoo 2007). In woody plants, the leaves and petioles as explants, have a higher ability to undergo callus formation, as opposed to other explant types (Lee and Pijut 2017, Wei *et al.* 2017, Rusea *et al.* 2018, Hesami *et al.* 2019). In this study, the leaves of *E. bungeanus* were selected as explants and were sterilized by NaClO for a short period of time. Li *et al.* (2009) obtained auxiliary buds from *E. bungeanus* immature stems, which successfully grew. They further used the produced leaves as explants to induce the formation of callus, differentiation of adventitious buds, and the formation of adventitious roots (the process lasted 80 d).

These authors achieved 70.9, 50.0, and 82.5 % success in callus induction, adventitious bud differentiation, and rooting, respectively. By comparison, our *E. bungeanus* tissue culture system of callus induction by using leaves directly was more simple and efficient. Additionally, the entire timeframe needed for the whole culture cycle was reduced to about 20 d. Therefore, it can be said that we have optimized the process and successfully produced the new, simple and rapid tissue culture propagation system for *E. bungeanus*.

A major challenge in plant tissue culture is to sterilize explants while maintaining their viability (Duan *et al.* 2016). To date, a wide range of surface disinfectants has been generally used for sterilizing explants, such as ethanol, mercuric chloride aqueous solution, NaClO solution (commercial bleach), calcium hypochlorite, hydrogen peroxide, and benzalkonium bromide solution (Ahmad *et al.* 2016, Mahmoud and Al-Ani 2016, Gochhayat *et al.* 2017, Nisa *et al.* 2019). NaClO solution has been the most widely used due to its high efficacy against bacteria and fungi (Yildiz and Er 2002). The most effective leaf sterilization treatment in our study was 15 % NaClO for 5 min. This treatment yielded pollution and survival rates of 0 and 100 %, respectively.

In the traditional plant tissue culture process using leaf explants, the formation of callus is followed by adventitious buds and adventitious roots (Pérez-Tornero *et al.* 2000, Agarwal 2015, Liu *et al.* 2016, Guan *et al.* 2018). The ability to initiate formation of callus is controlled by the hormonal composition of culture medium, nutrition and irradiance

Fig. 3. *Euonymus bungeanus* adventitious roots induction and growth. A - Adventitious roots induction rate under different hormonal combinations; B - Number of adventitious roots under different hormonal combinations; C - Length of adventitious roots under different hormonal combinations; D,E - Growth of adventitious roots and plantlets in T1 medium after 20 d (bar 2.0 cm).

(Domir *et al.* 1992). Plant callus cultures play significant roles in agriculture and horticulture (Efferth 2019). Callus further differentiates into whole plantlet using appropriate culture media. In this study, the combination of 6-BA and NAA was used to induce callus formation. The callus induction rate approximately reached 100 % when the concentration of these two hormones was fixed at 0.5 mg dm^{-3} . The visible external protuberances were observed and enlarged on the cuts between 7 and 21 d, which might be caused by the high nutrient and hormone absorption efficiency at the cut edges (Sarwar and Skirvin 1997).

He *et al.* (2019) applied various concentrations of

6-BA in combination with NAA to establish an efficient protocol of callus proliferation in Chinese kale (*Brassica oleracea* var. *alboglabra*). He found that the best callus proliferation is in the MS medium supplemented with 2.5 mg dm^{-3} 6-BA and 0.4 mg dm^{-3} NAA after cultivation for 30 d. In fact, the dosage and proportion of hormones is a critical factor influencing callus formation and subsequent differentiation. Mainly cytokinins and auxins added to various cultures effect the organogenesis (Yin *et al.* 2008). The ability of callus to differentiate into plantlets depends on the hormone concentration but different plants respond differently to the same hormone. He *et al.* (2019) used five

times higher concentration of 6-BA than our study. This differences may be due to using different plant material. Wei *et al.* (2015) also found that 6-BA has a dominant role in promoting bud induction and proliferation in *Bambusa ventricosa*. Our result was similar to that of He *et al.* (2019), who reported that the combination of 6-BA and NAA had optimum callus proliferation of Chinese kale. In addition, with respect to plant callus induction, it is pointed out that the use of polychromatic radiation from light-emitting diode does not sufficiently effect the callus induction as the use of monochromatic radiation (Budiarto 2010). It was found that monochromatic red radiation could promote callus formation more effectively in *Anthurium andraeanum* leaves (Budiarto 2010). Hamada *et al.* (2009) also found that the induction rate of protocorm callus of *Cymbidium finlaysonianum* was the highest under red radiation. Liu *et al.* (2011) also reported that red radiation was beneficial to the induction and proliferation of protocorm callus of *Oncidium hybridum*. Our results showed that the callus induction from leaves under red radiation could reach 100 % in *E. bungeanus*. Generally, the results varied in different plant species (Lin and Hsu 2004).

For the differentiation of adventitious buds from callus, our results showed that the concentrations of 1.0 mg dm⁻³ 6-BA and 0.5 mg dm⁻³ NAA was the most efficient treatment. Adventitious bud induction was nearly 100 %. A previous study showed that high concentrations of 6-BA could efficiently induce the formation of adventitious buds in Cavendish banana (*Musa* spp.) (Subramaniam *et al.* 2008). Another study showed that optimum hormone combination for differentiation of adventitious buds of *Orostachys fimbriata* was the MS medium supplemented with 1.5 mg dm⁻³ 6-BA and 0.2 mg dm⁻³ NAA (Liu *et al.* 2016). Similarly, the effect of 6-BA and NAA on adventitious bud formation in *Lilium longiflorum* cv. White fox was discovered, and the results demonstrated that the optimal medium for inducing adventitious buds is MS medium supplemented with 2.0 mg dm⁻³ 6-BA and 0.1 mg dm⁻³ NAA. Under these conditions, the highest differentiation frequency reached 95.6 % (Xi *et al.* 2012). All of the above results suggest that the combination of 6-BA and NAA contributes to the adventitious bud differentiation on MS medium.

NAA was also known as an effective phytohormone in inducing rooting from proliferated buds (Wei *et al.* 2015). A previous study indicated that 0.1 mg dm⁻³ NAA could efficiently promote the rooting process in *Orostachys fimbriata* tissue culture system (Liu *et al.* 2016). The combination of IBA + NAA, are found to be the most extensive hormone combination for rooting in tissue culture (Ullah *et al.* 2013). Our results showed that ½MS medium supplemented with 0.05 mg dm⁻³ NAA and 2.0 mg dm⁻³ IBA was an excellent combination for rooting, and the induction rate of adventitious roots was close to 100 %. A propagation system for Garnem rootstock (almond × peach) was conducted and the highest root number was achieved by using ½MS medium with 2.0 mg dm⁻³ IBA (Erfani *et al.* 2017). In addition, the vigorous roots are also generated in ½MS medium supplemented with 0.5 mg dm⁻³

IBA (Zang *et al.* 2017). However, high induction rate of adventitious roots could be achieved by using MS media supplemented with 2.0 mg dm⁻³ IBA in an efficient *in vitro* tissue culture system for *Crocus sativus* (Zeybek *et al.* 2012). Therefore, different types of medium (MS or ½MS) played distinct roles in plant rooting. Nevertheless, these findings repeatedly confirmed that IBA and NAA were required for the induction of adventitious roots.

Conclusions

We developed an efficient protocol for complete plant regeneration system through the organogenesis of adventitious buds from calluses using *in vitro*-derived leaf explants from the wild-type of *E. bungeanus*. This protocol will establish a basis for the realization of *E. bungeanus* large-scale production, as well as will serve as the foundation for the future genetic engineering.

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