Original article

Micropropagation via multiple shoot induction in *Emmenopterys henryi* Oliv.

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Summary

Emmenopterys henryi Oliv., an endemic to China, is currently endangered. The aim of this research was to establish the procedure for rapid propagation of *E. henryi* via multiple shoot induction. Results showed that on media supplemented with 6-benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D), three types of calli developed from the leaf explant, such as green, granular and compact calli liable to form shoots; white and loose calli inclined to produce hair-like roots; yellowish-white watery and translucent calli with no regeneration ability. The increase of BA to 2,4-D ratio in culture medium helped to induce shoot-forming callus. The optimal hormone combination for the callus initiation of *E. henryi* was 2 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D. BA alone was more effective than combinations of BA and IBA in shoot differentiation. 2 mg L⁻¹ BA treatment gave the highest shoot differentiation frequency, 91%, however, resulted in the most serious callus browning simultaneously. Multiple shoot induction provided effective solutions for the problem. 2 mg L⁻¹ BA treatment proved to be most efficient for shoot proliferation and the proliferated shoots were morphologically normal. Multiple shoot initiation by 2 mg L⁻¹ BA provided an effective way for rapid propagation of *E. henryi*. In addition, the effects of indole-3-butyric acid (IBA) and activated charcoal (AC) on rooting of *E. henryi* were explored. The highest rooting rate, 96.2%, was obtained on medium with 1 mg L⁻¹ IBA and 0.3 mg L⁻¹ AC. About 94% of rooted plantlets survived and grew normally.

Keywords

acclimatization, browning, callus, differentiation, regeneration, rooting

Introduction

Emmenopterys henryi Oliv., the only species of the genus *Emmenopterys* belonging to the family *Rubiaceae*, is a monotypic species and endemic to China. It originated from the Cretaceous period of the Mesozoic era and is the relict plant of quaternary glaciers, which is one hundred million years old (Li and Jin, 2008). *E. henryi* mainly distributes in south-western China and the Yangtze River valley. Academically, it is highly valued for morphology evolution, phylogenetic analysis in the *Rubiaceae* and floristic region study of China (Peng et al., 2013; Zeng et al., 2014; Peng et al., 2017; Lu et al., 2018). As rare species, *E. henryi* was first introduced to England in 1907 by Ernest Wilson, who described *E. henryi*

Significance of this study

What is already known on this subject?

- *Emmenopterys henryi* Oliv., an endemic to China with high ornamental value, is currently endangered. Research on rapid propagation for *E. henryi* is in urgent need.

What are the new findings?

- This research shows that multiple shoot initiation provides effective solution for the problems resulted from callus browning in the plantlets regeneration of *E. henryi*.

What is the expected impact on horticulture?

- The objective of the present study was to develop an efficient protocol for multiplication and production of this species via de novo shoot organogenesis, which is important for the recovery and landscape application of the endangered species.

as one of the most strikingly beautiful trees in the Chinese forests. *E. henryi* was not only an excellent landscape tree for the beautiful shape and ecological function, but also a precious timber species for its fine wood (Wang, 2009; Peng et al., 2017). Its fine and flexible fibers are high-quality raw material for waxed paper and artificial cotton manufacturing. In addition, *E. henryi* is a valuable medicinal plant for its antitumor activity and therapy effect for tummy bug (Wu et al., 2013).

In recent years, the wild population of *E. henryi* has been found to decline rapidly. Besides habitat destruction and overexploitation, low nature regeneration rate plays a main role (Kang et al., 2007; Guo et al., 2017). In natural environment, *E. henryi* is until thirty years old when it matures and blooms every two or four years. Additionally, the seeds of *E. henryi* germinate less in natural habitat because of limitation of light and humidity (Li and Jin, 2008). So far, the species is being endangered (Qin et al., 2017). In view of this, *E. henryi* has been taken as the national second class protection plant of China (Fu, 1991). The aim of this study was to develop an efficient propagation system for *E. henryi*.

Materials and methods

Plant material and explant preparation

Leaves of *E. henryi* were collected during May–July from trees (3 years old) of natural habitats growing at ravines of State-owned Miaoshou Forest Farm, Jingde, Anhui, China (latitude 30°12'N, longitude 118°20'W). The leaves were

rinsed in running water for 1 h and then surface-sterilized in 75% ethanol for 15 seconds, followed by surface sterilization in 0.2% HgCl₂ (w/v) for 10 min. This was followed by rinsing five times with sterile water. The sterilized leaves were cut into pieces about 2 mm² and used for callus induction.

Callus induction

The sterilized explants were cultured on ½ WPM medium (Phytotech) supplemented with different concentrations of 6-benzyladenine (Sigma) (BA, 0.5 or 2 mg L⁻¹) and 2,4-dichlorophenoxyacetic acid (Sigma) (2,4-D, 0.5 or 2 mg L⁻¹). All media contained 2% (w/v) sucrose and 6.0 g L⁻¹ agar. The pH of all media was adjusted to 5.8 before autoclaving at a pressure of 105 kPa for 20 min at 121°C. All cultured materials were incubated under 16 h/8 h light/dark cycles at 24 ± 1°C with relative humidity of 55–60%. Each treatment was repeated three times. Each replicate consisted of at least 100 pieces of explants. Callus formation frequency (number of explants with callus/total number of explants cultured) was recorded after 4 weeks of culture.

Multiple shoot induction

For shoot differentiation, calli were transferred to ½ WPM medium containing different combinations of growth regulators, including BA (1 or 2 mg L⁻¹), kinetin (Sigma) (KT, 1 or 2 mg L⁻¹) and indole-3-butyric acid (Sigma) (IBA, 0 or 1 mg L⁻¹). For multiple shoot induction, the shoot tip (2–3 cm in length) were excised and cultured on ½ WPM medium containing different concentrations of BA (0, 1, 2 and 3 mg L⁻¹). The culture conditions were similar to those used for callus induction. Each treatment was repeated three times and each replicate consisted of at least 100 pieces of calli or shoot tips. Shoot differentiation frequency (number of calli with shoots/total number of calli cultured) was recorded after 4 weeks of culture. Proliferation coefficient (total number of shoots/number of mother shoots) was recorded after 4 weeks of culture.

Rooting and acclimatization

For rooting, shoots were cultured on ½ WPM medium supplemented with 0, 0.5, 1, or 2 mg L⁻¹ of IBA in combina-

tion with different concentrations of activated charcoal (Sigma) (AC, 0, 0.3 or 0.5 mg L⁻¹). The culture conditions were similar to those used for callus induction. Each treatment was repeated three times and each replicate consisted of at least 100 shoots, and rooting frequency (%) were recorded after 4 weeks. The rooted plantlets were transplanted into the substrate (peat soil:perlite = 2:1), maintained in seedling boxes with breathable lids in greenhouse at 25 ± 2°C. A week later, the lids were removed and the plantlets were kept in the greenhouse for one more week. Then the plantlets might be transferred to the open field.

Statistical analysis

Each treatment was repeated three times. Each replicate consisted of at least 100 pieces of explants or calli or shoots. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 19. The means were compared using Duncan's multiple range test at the 5% level.

Results and discussion

Callus induction

As shown in Table 1, on media supplemented with varying concentrations of BA and 2,4-D, the callus induction frequency was 100%, respectively. Three types of callus developed from the leaf explants. Type 1: Green callus with granular and compact structure (Figure 1b). When transferred to differentiation medium, this type of callus was inclined to form shoots. Type 2: White callus with loose structure (Figure 1a). When transferred to differentiation medium, this type of callus usually produced hair-like roots. Type 3: Yellowish-white watery and translucent callus with loose and soft structure, which was non-regenerable on differentiation medium (Figure 1a). In medium supplemented with 0.5 mg L⁻¹ BA and 2 mg L⁻¹ 2,4-D, higher percentage of type 2 and type 3 calli but only a small percentage of type 1 calli were induced. Comparatively, type 1 calli occurred more while type 2 and 3 calli developed less in medium supplemented with 0.5 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D. However, in medium supplemented with 2 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D, a higher percentage of type 1 callus and a lower percentage of type 2 and 3 callus were found, suggesting that the increase of BA to 2,4-D ratio in culture medium helped to induce shoot-forming callus (Table 1). This was in agreement with investigations of *Miscanthus × ogiformis* Honda 'Giganteus' (Holme and Petersen, 1996; Petersen, 1997), sorghum (Bhaskaran and Smith, 1989), mango ginger (Sundar Raju et al., 2013), and bermudagrass (Chaudhury and Qu, 2000). In *Miscanthus × ogiformis* Honda 'Giganteus', only 2.1% embryogenic and no shoot-forming callus was induced in medium without BA whereas the inclusion of BA dramatically decreased the percentage of root-forming callus and, simultaneously, increased the percentage of embryogenic and shoot-forming callus (Petersen, 1997). Similarly, in bermudagrass, addition of BA in the callus induction medium promoted the formation of regenerable callus, as reported in other grass species (Griffin and Dibble, 1995; Van der Valk et al., 1995; Chaudhury and Qu, 2000). Likewise, in sorghum, a high auxin to cytokinin ratio advanced primarily root differentiation while a high cytokinin to auxin ratio helped multiple bud development. Therefore, the optimal hormone combination for the callus initiation of *E. henryi* was 2 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D.

TABLE 1. Effects of 2,4-D and BA on callus induction from leaves of *E. henryi* in vitro.

Treatment		Callus induction frequency (%)	Type of callus
2,4-D (mg L ⁻¹)	6-BA (mg L ⁻¹)		
2	0.5	100 a	Most type 2 and type 3 Fewer type 1
0.5	0.5	100 a	Decreasing type 2 and type 3 Increasing type 1
0.5	2	100 a	Most type 1 Fewer type 2 and type 3

2,4-D = 2,4-dichlorophenoxyacetic acid; BA = 6-benzyladenine; Type 1 = green callus with granular and compact structure; Type 2 = white callus with loose structure; Type 3 = yellowish-white watery and translucent callus with loose and soft structure.

Each treatment was repeated three times. Each replicate consisted of at least 100 pieces of explants. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 19. Different letters mean significant difference at the 0.05 level according to Duncan's multiple range test.

FIGURE 1. Callus development, shoot regeneration, multiple shoot induction on media with different plant growth regulators. (a) White and loose callus (type 2 callus, white arrow) and yellowish-white, watery, translucent and loose callus (type 3 callus, red arrow) formation. (b) Green, granular and compact callus formation (type 1 callus, red arrow). (c) Regeneration of shoots on type 1 callus (red arrow). (d) Multiple shoots induced on medium with 2 mg L⁻¹ BA.

Multiple shoot induction

For shoot differentiation, type 1 calli were selected and subcultured in differentiation medium supplemented with different combinations of BA and IBA. Small shoots were observed to emerge from the appearance of green and granular calli (type 1 calli) and gradually develop into small plantlets (Figure 1c). As shown in Table 2, the highest shoot differentiation frequency obtained in medium supplemented with 2 mg L⁻¹ BA alone was 91% while that in medium supple-

mented with 1 mg L⁻¹ BA alone, 63.6%, was next best. But in media supplemented with BA or KT in combination with IBA, the shoot differentiation frequency were significantly lower than those obtained in media with BA alone, which agrees with previous results (Lin and Griffin, 1992; Nin et al., 1996; Panizza et al., 1997; Khanam, 2000), suggesting that BA alone was effective for shoot differentiation of *E. henryi*. In addition, the shoot differentiation frequencies on media containing only KT were remarkably lower than on those with

TABLE 2. Effects of BA, KT and IBA on shoot differentiation in *E. henryi* in vitro.

Treatment			Shoot differentiation frequency (%)	Callus browning frequency (%)
BA (mg L ⁻¹)	KT	IBA (mg L ⁻¹)		
1		0	63.6 b	26.1 c
1		1	5.1 de	24.9 c
2		0	91 a	65.8 a
2		1	10 c	50.3 b
	1	0	3.8 e	10.3 e
	1	1	5.6 de	12.5 de
	2	0	7.4 d	14.7 d
	2	1	7.1 d	12.8 de

BA = 6-benzyladenine; KT = kinetin; IBA = indole-3-butyric acid. Each treatment was repeated three times. Each replicate consisted of at least 100 pieces of calli. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 19. Different letters mean significant difference at the 0.05 level according to Duncan's multiple range test.

TABLE 3. Effect of BA on multiple shoots induction in *E. henryi* in vitro.

Concentration (mg L ⁻¹)	Proliferation coefficient
BA	
0	0 d
1	3.7 b
2	7.4 a
3	2.6 c

BA = 6-benzyladenine.

Each treatment was repeated three times. Each replicate consisted of at least 100 pieces of shoot tips. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 19. Different letters mean significant difference at the 0.05 level according to Duncan's multiple range test.

only BA (Table 2). BA has been considered to be more effective than kinetin for shoot differentiation (Tan et al., 2011; Grzegorzczak-Karolak et al., 2015; Wei et al., 2015; Zaheer and Giri, 2015), which was also consistent with our work.

What is more, while BA alone was in favor of shoot differentiation, it was found that the calli cultured on differentiation medium gradually turned brown and eventually died after the first subculture. The most serious callus browning was observed on the media supplemented with 2 mg L⁻¹ BA only, which was especially best for shoot differentiation (Table 2). Callus browning caused by BA treatment was also reported in bermudagrass, *Piper chaba* Hunt. and bamboo (Chaudhury and Qu, 2000; Rani and Dantu, 2016; Huang et al., 1989). Polyphenol oxidase (PPO), a copper-containing metalloprotein, catalyzes the oxidation of phenolics to quinones, which make brown pigments in tissues. Previous work indicated that browning callus had significantly higher PPO activity and total phenolic content than control callus (Tang and Newton, 2004; Kaewubon et al., 2015), implying that polyphenol oxidase (PPO) played a role in enzymatic callus browning (He et al., 2009; Sandhu et al., 2018). Antisense analysis on apple callus indicated that the browning potential of the transgenic apple callus, in which antisense

PPO gene was introduced and the expression of PPO was repressed, was only half the browning potential of non-transgenic callus, further proving the involvement of PPO in callus browning (Murata et al., 2001). BA had been reported to increase PPO activity (Dhruve and Vakharia, 2008; Dhruve et al., 2009), implicating that callus browning of *E. henryi* might be resulted from the promotion of BA on PPO activity. However, there is also evidence that the browning rate of callus was negatively correlated with the activity of PPO in *Corydalis saxicola* callus (Cen et al., 2016). What is more, in oilseed rape, it was found that application of BA had no influence on PPO activity (Wan et al., 2012). So, more research is necessary to elucidate the regulation mechanism of BA on callus browning of *E. henryi*.

In order to avoid the influence of callus browning on rapid propagation, the shoot tips were excised and transferred to multiple shoot induction media supplemented with varying concentrations of BA. As shown in Table 3, there was a significant difference in shoot formation between treatments lacking and containing BA. On medium with no BA, no multiple shoots formed. When BA was included in the medium, new shoots were observed to appear sequentially from both the leaf axis and the base of the stem (Figure 1d). A single shoot could multiply several times during a 4-week period. The highest shoot proliferation coefficient, 7.4, was obtained in medium supplemented with 2 mg L⁻¹ BA (Table 3). Higher and lower concentrations of BA yielded fewer shoots per explant. Previous works indicated that BA was effective for multiple bud formation and higher concentrations of BA were less effective (Chand and Singh, 2004; Husain et al., 2008; Zaheer and Giri, 2015; Aziz et al., 2018), which is in agreement with our results. Research on *Piper chaba* Hunt. showed that although shoot proliferation was optimal in the medium with BA, the shoots and medium turned brown and did not last beyond the second subculture (Rani and Dantu, 2016). What is different, in our work, the proliferated shoots were morphologically normal after four subculture (Figure 1d). Therefore, 2 mg L⁻¹ BA applies to shoot regeneration and multiple shoot initiation, which provides an efficient pathway for rapid propagation of *E. henryi*.

FIGURE 2. Rooting and acclimation of *Emmenopterys henryi* Oliv. plantlets. (a) Rooting on medium supplemented with 1 mg L⁻¹ IBA in combination with 0.3 mg L⁻¹ AC. (b) The rooted plantlets were transplanted into the substrate (peat soil:perlite = 2:1) and acclimated in greenhouse.

TABLE 4. Effect of IBA and AC on rooting of regenerated shoots in *E. henryi*.

IBA (mg L ⁻¹)	AC (mg L ⁻¹)	Rooting frequency (%)
0	0	50.3 f
0.5	0	60.3 e
1	0	88.3 b
2	0	61.8 e
0.5	0.3	78 c
1	0.3	96.2 a
2	0.3	72.9 d
0.5	0.5	39.3 g
1	0.5	48.6 f
2	0.5	31.1 h

IBA = indole-3-butyric acid; AC = activated charcoal.

Each treatment was repeated three times. Each replicate consisted of at least 100 pieces of shoots. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS 19. Different letters mean significant difference at the 0.05 level according to Duncan's multiple range test.

Rooting and acclimatization

The effects of different concentrations of IBA and activated charcoal on rooting of *E. henryi* were investigated. On medium with no plant growth regulators, the rooting frequency was 50.3 %. Addition of IBA promoted the rooting of *E. henryi* and the highest rooting frequency, 88.3%, was obtained on medium with 1 mg L⁻¹ IBA. Inclusion of 0.3 mg L⁻¹ AC further improved the rooting of *E. henryi* and made the roots thicker and stronger (Figure 2a), which agrees with the results of *Lycium chinense* (Kim and Park, 2017). AC might function by delaying the disintegration of the auxin in the medium, adsorbing toxic compounds like polyphenols released by cultured tissues (Thomas, 2008) or establishing polarity by darkening the medium (Sanputawong et al., 2015). However, addition of 0.5 mg L⁻¹ AC resulted in the decrease of rooting frequency and the roots were thinner than the control (data not shown). More seriously, the plantlets grew slowly and leaves gradually turned brown, implying that higher concentration of AC was detrimental. Similarly, in Bitter Almond, addition of activated charcoal to the rooting medium caused a sharp decrease in the rooting percentage (Kassim et al., 2012), which was also in line with previous findings that activated charcoal inhibited the rooting of peach and deciduous fruit (Antoelli and Chiariotti, 1988; Kassim et al., 2012). Actually, AC not only absorbed toxic compounds in medium but also plant growth regulators and other organic compounds, such as thiamine hydrochloride and nicotinic acid, which were essential for root growth and development (Constantin et al., 1977; Weatherhead et al., 1978). Therefore, AC might be beneficial or harmful in root induction, depending upon the medium, the tissue cultured, and the concentration of AC. In our work, 0.3 mg L⁻¹ AC was the best for root induction of *E. henryi*. In the subsequent acclimation experiments, about 94% of rooted plantlets survived and grew to maturity normally (Table 4, Figure 2b).

Conclusion

In conclusion, an efficient propagation protocol for *E. henryi* was established. The increase of BA to 2,4-D ratio proved to be beneficial to shoot-forming callus induction. ½ WPM medium with 2 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D was the most effective for regenerable callus initiation. BA alone, especially, 2 mg L⁻¹ BA, was demonstrated to be optimal not

only for shoot differentiation but also multiple shoot induction, which helped to avoid the influence of callus browning problem resulted from higher BA concentration during subculture on propagation of *E. henryi*. The highest rooting rate (96.2%) was obtained on medium with 1 mg L⁻¹ NAA and 0.3 mg L⁻¹ AC. Ninety-four percent of rooted plantlets survived and grew normally in the field after acclimatization in greenhouse. The protocol established in this investigation will facilitate future mass propagation and natural resource protection of *E. henryi*.

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