An efficient protocol for direct somatic embryogenesis from *in vitro* leaf of *Rhododendron fortunei* L.

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Abstract is too long and reads like result description! Re-write!

Abstract: *Rhododendron fortunei* L. is a multibranched evergreen shrub and an ornamental plant with highly appreciative and useful value. Up to now, tissue culture of several rhododendron species has been reported with an emphasis on *in vitro* microproagation and regeneration. This study reported an improved and efficient protocol for direct somatic embryogenesis of *Rhododendron fortunei* L. Mature seeds of *Rhododendron fortunei* L. were used as explants. The Pre-treatment duration on direct somatic embryosgenesis from leaves was detected. Direct somatic embryogenesis and plant regeneration were carried out on WPM or 1/2 WPM medium supplemented with plant growth regulators (PGRs). For the pre-treatment duration, the frequency of direct somatic embryos was 81.0% at 4-week duration and it had significant difference with the frequency 54.30 % at 2-week of duration. However, the somatic embryo regeneration coefficient from 2-week of duration was much better than 4-week of duration. Furthermore, the best conditioning medium duration was 2

weeks. Cytokinin (TDZ, ZT) significantly affected somatic embryogenesis in WPM medium. The highest frequency (95%) of somatic embryogenesis was obtained on WPM medium with TDZ (0.05 mg L^{-1}) and ZT (1.0 mg L^{-1}). On the optimum treatment, young sterilized leaves were smooth when they were inoculated in the test tube. After 1 week of transfer, the superficial of leaves give rise to bulging. After another week of transfer, the early stage of somatic embryos became apparent. They arose singly or in groups from surface of young leaves. And the global somatic embryos can be removed integrated easily. Heart-shaped somatic embryos were evident. The development of somatic embryos from globular to heart-shaped somatic embryos occurred in 2 weeks of transfer mostly. After 3 weeks of transfer, they went through torpedo and cotyledonary embryos. In most cases, various phases of somatic embryos development were observed on one piece of young leaf after 4 weeks of transfer. Somatic embryo germination percentage gradually increased with increasing concentration of NAA or IBA. The highest germination percentage was 74.67% when the concentration of IBA was 0.1 mg L^{-1}.

Key Words: ~~*Rhododendron fortunei* L.~~; pre-treatment duration; ~~direct somatic embryogenesis~~; plant regeneration

Introduction

The genus Rhododendron is extremely large, with 900 to 1000 species growing in many parts of the world. The largest numbers are native to Asia. One of these,

Syntax

Rhododendron fortunei L., which is native to china. It is popular on 400-1900 meter high mountains of Zhejiang province of China, where the rainy humid environment and mild weather are ideal for their growth. *Rhododendron fortunei* L. is a multibranched evergreen shrub or arbor up to 2 to 7-m tall, with pink flowers and ellipse leaves. It is an excellent ornamental plant with highly appreciative and useful value. At present, the species has been extensively cultivated and used in wild or semi-wild state.

Micropropagation is a much faster and more economical propagation method for producing millions of clonal individuals. Up to now, tissue culture of several rhododendron species has been reported with an emphasis on *in vitro* micropropagation and regeneration (Almeida et al. 2005; Tomsone & Gertnere 2003; Zhu et al., 2006; Shevade & Preece 1993; Tomsone et al., 2004; Hsia & Korban 1997; Liu et al., 2007; Miao et al., 2006). Almeida et al. (2005) evaluated for the effect of IAA ratios and a range of zeatin concentrations on shoot multiplication from apical shoots and nodal segments. It showed that the type of cytokinin and the origin of the explants were the most important factors affecting shoot multiplication. Tomsone & Gertnere (2003) reported that Rhododendron shoot regeneration was accomplished using flower explants. The success of *R. fortunei* cutting and grafting depend on several factors, such as season, vigor and maturity of the ~~parent~~ tree, environmental conditions during cutting (Yu et al., 2004; Tong & Xu 2009). Xu & Ye (2005) reported that the seed of *R. fortunei* is very tiny and can hardly germination naturally. Jin et al. (2007) demonstrated that seed germination ~~percentage~~ only 38.6% on the

medium which consisted of leaf mould and sawdust. Therefore, conventional propagation through seeds and cutting is not sufficient to satisfy the ~~progressive~~ increasing demand. Therefore, there is ~~an exigent~~ a need to develop protocols for rapid propagation of *R. fortunei* through tissue culture which ~~will be useful~~ could be also used for protection and conservation of germplasm resources.

However, there have been no reports on direct somatic embryogenesis and plant regeneration ~~systematically~~ for *R. fortunei*. The aim of this work was to establish an *in vitro* ~~protection and~~ propagation protocol ~~of the germplasm~~. In this paper, we report a two-step protocol for direct somatic embryogenesis and plant regeneration for *R. fortunei*.

Materials and Methods

Plant material and culture conditions

Leaf explants of *R. fortunei* were initiated from ~~*in vitro*~~ aseptically grown seedlings which germinated from the seeds ~~that~~ sampled in Tianmu Mountain Scenic Area in Zhejiang province, China, in October 2009. Seeds were soaked in water for 2 h, rinsed in 10% liquid dishwasher detergent (P&G Co., Ltd), surface-sterilized in 70% ethanol for 30 s, and then rinsed with sterile water 3 to 4 times. Seeds were then sterilized in 5.25% sodium hypochlorite for 20 min under vacuum, rinsed 5 times with sterile water, blotted dry with sterilized filter paper, and put on WPM (Lloyd and McCown 1980) ~~basal~~ medium containing 3% sucrose and 0.8% Type-A agar (Sigma Chemical Co., St. Louis, Mo., USA) for 30 days. The pH was adjusted to 5.2 with 1N KOH or HCl prior

to autoclaving. Culture vessels were 25*150 mm test tubes containing 15 ml medium. Cultures were incubated in dark in a growth chamber for seed germination in the former 2 wk. In other stages, cultures were grown at 25±2°C with a photon fluence of about 40 μmol m^{-2} s^{-1} and 16-h photoperiod.

PGRs on somatic embryogenesis

Young *in vitro* leaves from the ~~germinated seeds~~ were cut down and placed on pre-treatment induction medium which consisted of WPM medium supplemented with casein hydrolysate (CH) 1.0 g L^{-1} and various combinations of Thidiazuron (TDZ) (0, 0.05, 0.1, or 0.5 mg L^{-1}), Zeatin (ZT) (0, 0.5, 1.0, or 2.0 mg L^{-1}), NAA (0, 0.05, 0.1, or 0.5 mg L^{-1}) in orthogonal design for 1, 2, 4 or 8 weeks. The explants were then transferred to basal WPM salts medium. Additional transfers to fresh medium were made at 4-week intervals.

Pre-treatment duration on somatic embryosgenesis

In order to investigate the effect of physiology state of young leaces on somatic embryogenesis, effect of conditioning medium duration (1, 2, 4 and 8 weeks) was also tested. Young leaves were cultured on WPM medium supplemented with 0.05 mg L^{-1} TDZ, ZT 0.5 mg L^{-1}, NAA 0.5 mg $L^{-1}$and 1.0 g $\cdot l^{-1}$ CH for 1 to 8 weeks' duration. The explants were then transferred to WPM basal medium without hormones. Additional transfers to fresh basal medium were made at 4-week intervals.

Somatic embryos germination and plant regeneration

Individual healthy somatic embryos which picked up from the leaf surface were cultured vertically in half-strength WPM medium supplemented with BAP(0.5 mg L^{-1})

and NAA (0, 0.1, 0.5, 1.0 or 2.0 mg L^{-1}) or IBA (0, 0.1, 0.5, 1.0 or 2.0 mg L^{-1}) respectively. Each treatment consisted of 10 test tubes, with 2 embryos being cultured per tube. Somatic embryos were considered germinated as soon as radicle emergence was observed. The percentage of germination were determined after 8-week of culture.

Hardening of plants and transplanting

After plant regeneration, *in vitro* plantlets were then transferred to an acclimation chamber in culture. After 1 wk, the plantlets were washed gently with tap water to remove traces of agar and nutrients. Plantlets were then transplanted into plastic pots containing a 1:1:1 mixture of peat, perlite, and vermiculite. To retain moisture, the pots and plantlets were covered with a plastic bag; after 2 d, a single cut was made in each bag. One week later, the plastic bag was removed, and the plants were transferred into a greenhouse at 18±2°C, with a photon fluence of 100 μmol m^{-2} s^{-1} and a 16-h photoperiod.

Experimental design and statistical analysis

An random experimental design was used in this experiment for somatic embryogenesis and plant regeneration. Each treatment consisted of 10 tubes, with 2 explants cultured per tube and experiments were repeated three times. (four experiments in total?)

The frequency of somatic embryogenesis = (number of induced somatic embryos / total number of explants) × 100%. Visual observations were made every day. The data were analyzed by ANOVA using statistical package of SPSS and linear regression analyses were fitted using Sigma Plot 8.0. The means were compared using

↖ Do you mean number of explants with induced somatic embryos ?

Duncan's multiple range test (95% confidence level) and the standard errors of means were calculated.

Results

Effect of pre-treatment duration on ~~callusgenesis~~ and somatic embryogenesis

Conditioning medium duration significantly affected frequency of callus and differentiation of somatic embryos (Fig.1). The greatest frequency of callus was 20.66% at 8 weeks of duration and it had significant difference with the frequency (12.33%) of callus at 4-week duration and other treatments. The frequency of direct somatic embryos was 81.0% at 4-week duration and it had significant difference with the frequency 54.30 %) at 2-week of duration. However, the somatic embryo regeneration coefficient from 2-week of duration was much better than 4-week of duration. In general, the best conditioning medium duration was 2 weeks.

Effect of plant growth regulators on somatic embryogenesis

The plant growth regulators play a prominent role in the induction and proliferation of somatic embryos. Induction response varied with the different combination of PGRs as shown after 8 weeks of culture (Fig.2, 3). When cultured on media supplemented without PGRs, the explants did not produce any somatic embryos, while all media supplemented with cytokinin induced somatic embryogenesis. A significant difference was found in somatic embryogenesis between the treatment (0.05 mg L^{-1}TDZ and 1.0 mg L^{-1} ZT) and other treatments. Among the

different combinations of different PGRs used, the frequency of somatic embryogenesis ranged between 0 to 95%. The highest frequency of somatic embryognesis was obtained on WPM medium with TDZ (0.05 mg L^{-1}) and ZT (1.0 mg L^{-1}). Furthermore, WPM medium supplemented with 0.05 mg L^{-1} TDZ and 1.0 mg L^{-1} ZT was the optimum treatment for somatic embryogenesis.

Cytokinin (TDZ, ZT) significantly affected somatic embryogenesis in WPM medium. The somatic embryogenesis frequency and number gradually increased with increasing concentrations of TDZ from 0 to 0.05 mg L^{-1}, followed by a decrease from 0.05 to 0.5 mg L^{-1}. The highest somatic embryogenesis frequency and number was 71.08% and 10 pieces respectively, when the concentration of TDZ was 0.05 mg L^{-1} (Fig.10, 11). The effects of low TDZ concentration (0.05 mg L^{-1}) on the somatic embryogenesis were significant when compared with the control, which indicated that low TDZ promoted somatic embryogenesis. For ZT, as the concentration of ZT was increased, the somatic embryogenesis frequency and number first increased and then decreased (Fig. 8, 9). The highest somatic embryogenesis frequency was 42% when the concentration of ZT was 0.5 mg L^{-1} and the most somatic embryos (10) was obtained when the concentration of ZT was 1.0 mg L^{-1}. With further increases in the concentration of ZT, the somatic embryogenesis frequency and number decreased. However, no significant differences of somatic embryogenesis were found among NAA treatments (Fig. 4, 5).

The highest frequency of somatic embryogenesis responses on PGRs combination

178 On the optimum treatment (WPM medium supplemented with 0.05 mg L^{-1} TDZ
179 and 1.0 mg L^{-1} ZT), young sterilized leaves were smooth when they were inoculated
180 in the test tube. Early induction response was on the surface of the leaves. After 1
181 week of transfer, the superficial of leaves give rise to bulging (Fig6-A). After another
182 week of transfer, the early stage of somatic embryos became apparent. They arose
183 singly or in groups from surface of young leaves. During the development of the
184 global somatic embryos, the suspensor died and disappeared. And the global somatic
185 embryos can be removed integrated easily (Fig.6-B). Heart-shaped somatic embryos
186 were evident (Fig.6-C,D). In this stage, somatic embryos were polarized and
187 meristematic areas located at either of the end. The development of somatic embryos
188 from globular to heart-shaped somatic embryos occurred in 2 weeks of transfer mostly
189 (Fig.6-E). After 3 weeks of transfer, they went through torpedo (Fig.6-F, G) and
190 cotyledonary embryos (Fig.6-H). In most cases, various phases of somatic embryos
191 development were observed on one piece of young leaf after 4 weeks of transfer
192 (Fig.6-H, J).
193 *The effect of PGRs on somatic embryos germination and plant regeneration*
194 PGRs significantly affected somatic embryo germination in half-strength WPM
195 medium. Germination percentage gradually increased with increasing concentration of
196 NAA from 0 to 0.5 mg L^{-1} followed by a decrease from 0.5 to 2.0 mg L^{-1}. The highest
197 germination percentage was 25.3% when the concentration of NAA was 0.5 mg L^{-1}.
198 For IBA, the same trend of the germination percentage was found as for NAA.
199 Germination percentage gradually increased with increasing concentration of IBA

200 from 0 to 0.1 mg L^{-1} followed by a decrease from 0.1 to 2.0 mg L^{-1}. The highest
201 germination percentage was 74.67% when the concentration of IBA was 0.1 mg L^{-1} .
202 The ANOVA between treatments and multiple comparisons indicated significant
203 difference between IBA 0.1 mg L^{-1} and the other treatments. Furthermore, treatment
204 IBA 0.1 mg L^{-1} was the best treatment for somatic embryo germination (Fig. 4), with
205 a germination percentage of 74.6% (Fig. 7).
206
207 **Discussion**
208 In this study, an effectiveness of somatic embryos development of *R. fortunei* was
209 observed and we found that both pre-treatment duration and PGRs have important
210 influences on somatic embryogenesis. Recent progress to improve *R. fortunei* somatic
211 embryogenesis systems and transformation has been limited. Previous somatic
212 embryogenesis systems and plant regeneration systems of *Rhododendron* have been
213 manipulated (Vejsadova & Pretova, 2003; Zhu et al., 2006).
214 Somatic embryogenesis mostly occurs directly from initial explants or indirectly
215 through an intervening callus phase. In direct somatic embryogenesis, the somatic
216 embryos are formed directly from a cell or a small group of cells without the
217 production of an intervening callus. Compared with indirect somatic embryogenesis,
218 direct somatic embryogenesis is generally rare. In pecan, most somatic embryos
219 developed from the immature embryos directly (Burns and Wetzstein, 1997;
220 McGranahan et al., 1993; Yates, 1990). Similarity to pecan, direct somatic
221 embryogenesis was obtained from the immature hickory embryos and regeneration

222 plant was obtained successfully. This study also presents a detailed comparative
223 morphological description of the initiation and development of embryogenesis of *R.*
224 *fortunei*. The result was similar to the reports on embryogenesis of mango (Xiao et al.,
225 2006), *Quassia amara* L. (Martin & Madassery, 2005) and *Coffea arabica* L. (Gatica
226 et al., 2008), which plant regenerated through direct and indirect somatic
227 embryogenesis from cotyledons.

228 A two-stage system for somatic embryo production is common for
229 embryogenesis in using auxin (Quiroz-Figueroa et al., 2006). In hickory, an increased
230 exposure time (6 to 8 weeks) and higher levels of picloram (1.0 $mg \cdot l^{-1}$) have produced
231 higher frequencies of abnormal somatic embryo formation (Zhang et al., 2011). For
232 *Quercus suber* tree, somatic embryos observed from callus after transferring to the
233 same media with PGRs (Pinto et al., 2002). Similarity, our studies showed that 2
234 weeks duration were the most effective.

235 Earlier reports indicated successful direct or indirect somatic embryogenesis with
236 TDZ and ZT induction. TDZ is a substituted phenylurea (N-phenyl-1,2,3
237 thidiazol-5-ylurea) is a potent bioregulant of in vitro morphogenesis. The nature of
238 TDZ to proliferate and multiply the existing meristematic zone and induce
239 organogenesis was observed in numerous plant species (Singh et al., 2003; Chhabra et
240 al., 2008). In ~~pegeonpea~~ pigeon pea, TDZ induced direct somatic embryogenesis in intact
241 seedlings, and a response usually mediated by an appropriate concentration of high
242 auxin or by auxin-cytokinin ratio (Singh et al., 2003). For ZT, previous investigators
243 typically used 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with

6-benzyladenine (BA) and ZT to induce somatic embryogenesis (Zhang et al., 2001; Fei et al., 2002). Sun et al. (2013) reported that the highest number of embryos (50%) was observed on 1/2 MS medium supplemented with TDZ (1.0 mg L^{-1}) and ZT (0.2 mg L^{-1}) in *Schisandra chinensis* (Turez.) Baillon (Sun et al., 2013). In this study, The highest frequency (95%) of somatic embryogenesis was obtained on WPM medium with TDZ (0.05 mg L^{-1}) and ZT (1.0 mg L^{-1}).

In summary, an efficient plant regeneration system was developed for *Rhododendron fortunei*, which can be used for rapid propagation ~~as well as facilitate genetic transformation by particle bombardment or the~~ *Agrobacterium*-mediated ~~method.~~ irrelevant

Acknowledgments

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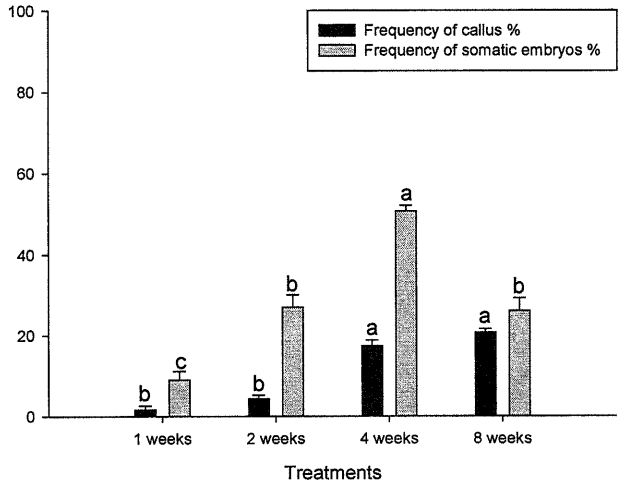
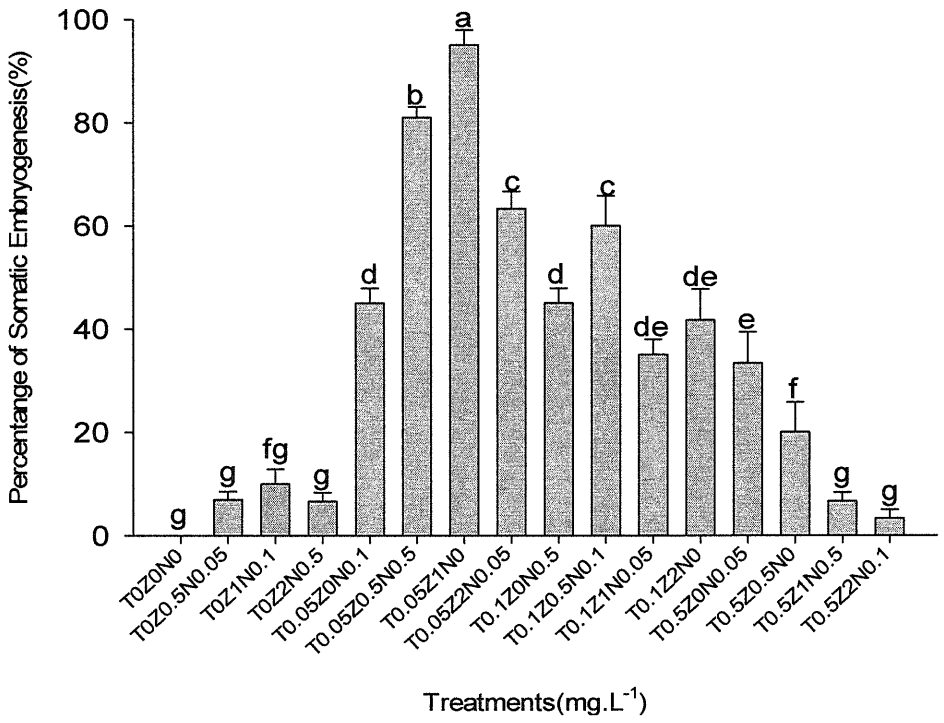


Fig.1 Effect of pre-treatment duration on somatic embryogenesis of *R. fortunei*

The values indicated by the same letter are not significantly different at $p \leq 0.05$. Data are expressed as mean $\pm$SD.



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348 Fig.2 Effects of interaction between TDZ (T_n), ZT (Z_n) and NAA(N_n) on frequency of somatic

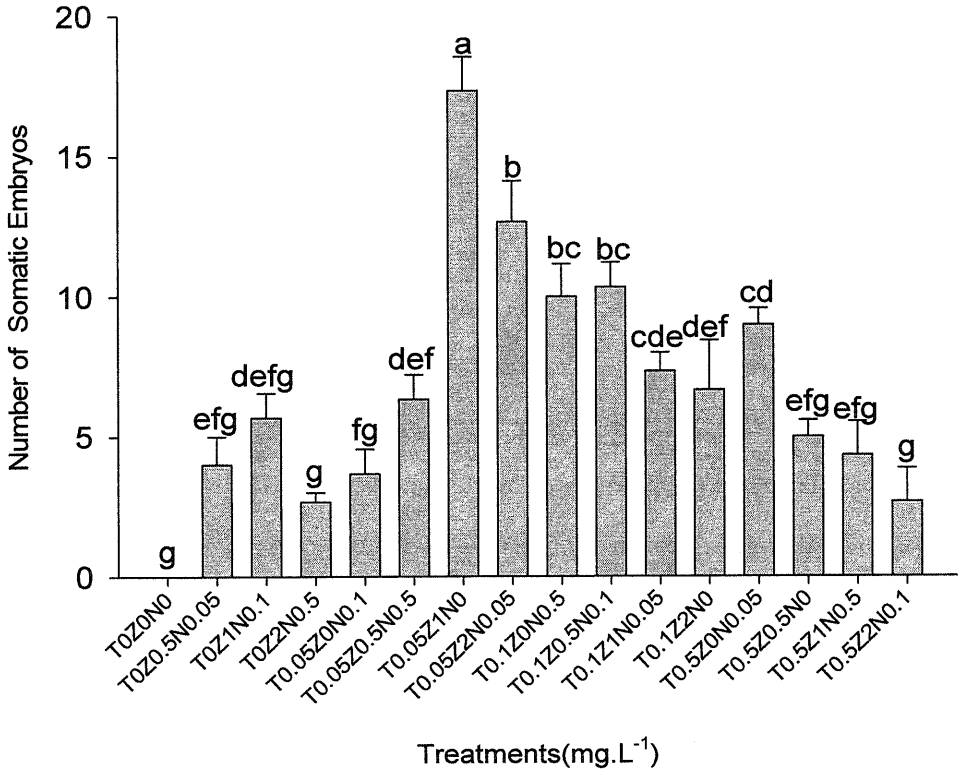
349 embryogenesis. The values indicated by the same letter are not significantly different at $p \leq 0.05$.

350 Data are expressed as mean $\pm$SD.

351 T0, T0.05, T0.1, T0.5 indicate 0, 0.05, 0.1, 0.5 mg·l-1 TDZ, respectively; Z0, Z0.5, Z1 and Z2

352 indicate 0, 0.5, 1.0 and 2.0 mg·l-1 ZT, respectively and N0, N0.05, N0.1, N0.5 indicate 0, 0.05, 0.1,

353 0.5 mg·l-1, respectively. (The same as Fig. 11)



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355 Fig.3 Effects of interaction between TDZ (T_n), ZT (Z_n) and NAA (N_n) on number of somatic

356 embryos. The values indicated by the same letter are not significantly different at $p \leq 0.05$. Data are

357 expressed as mean $\pm$SD. The values indicated by the same letters are not significantly

358 different at $p \leq 0.05$.

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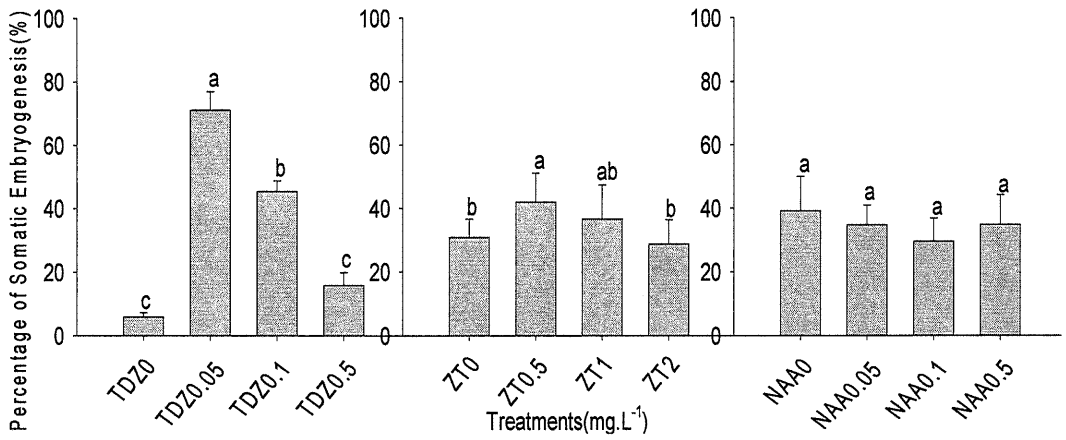

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362 Fig.4 Effects of individual application of TDZ (T_n), ZT (Z_n) and NAA (N_n) on frequency of
363 somatic embryogenesis. The values indicated by the same letter are not significantly different at
364 $p \leq 0.05$. Data are expressed as mean $\pm$SD. The values indicated by the same letters are not
365 significantly different at $p \leq 0.05$.

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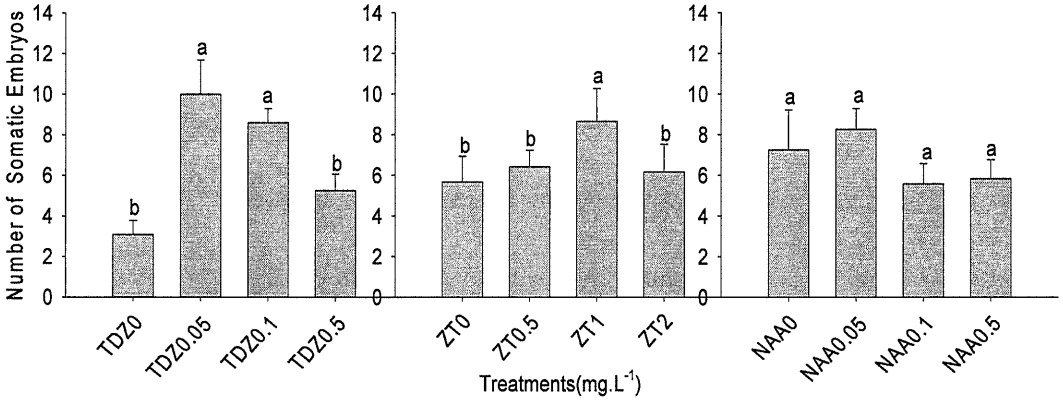

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368

369 Fig.5 Effects of individual application of TDZ (T_n), ZT (Z_n) and NAA(N_n) on number
370 of somatic embryos. The values indicated by the same letter are not significantly different
371 at $p \leq 0.05$. Data are expressed as mean $\pm$SD.

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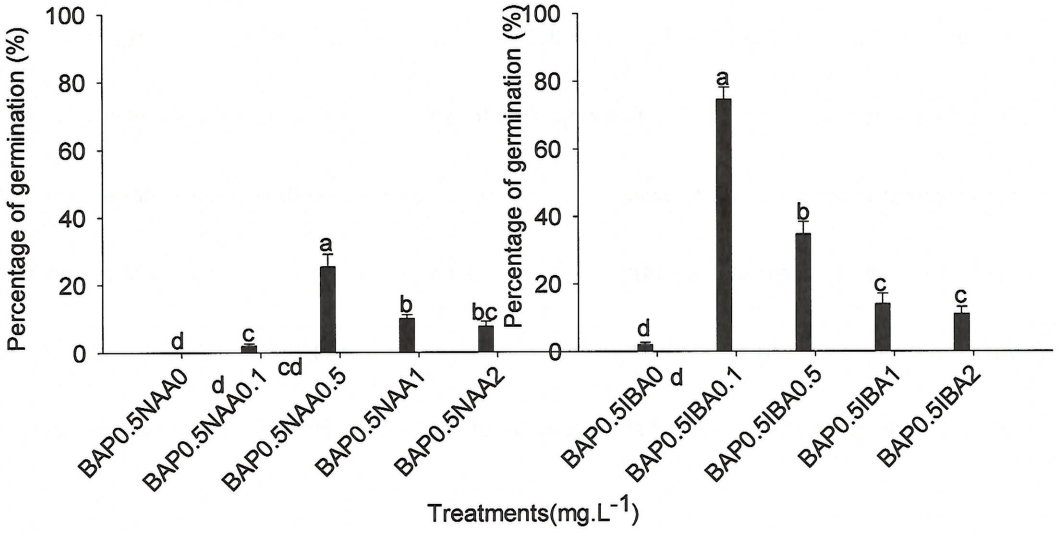

376

377 Figure 6 Effects of interaction between BA and NAA or IBA on somatic embryos germination.

378 The values indicated by the same letter are not significantly different at $p \leq 0.05$.

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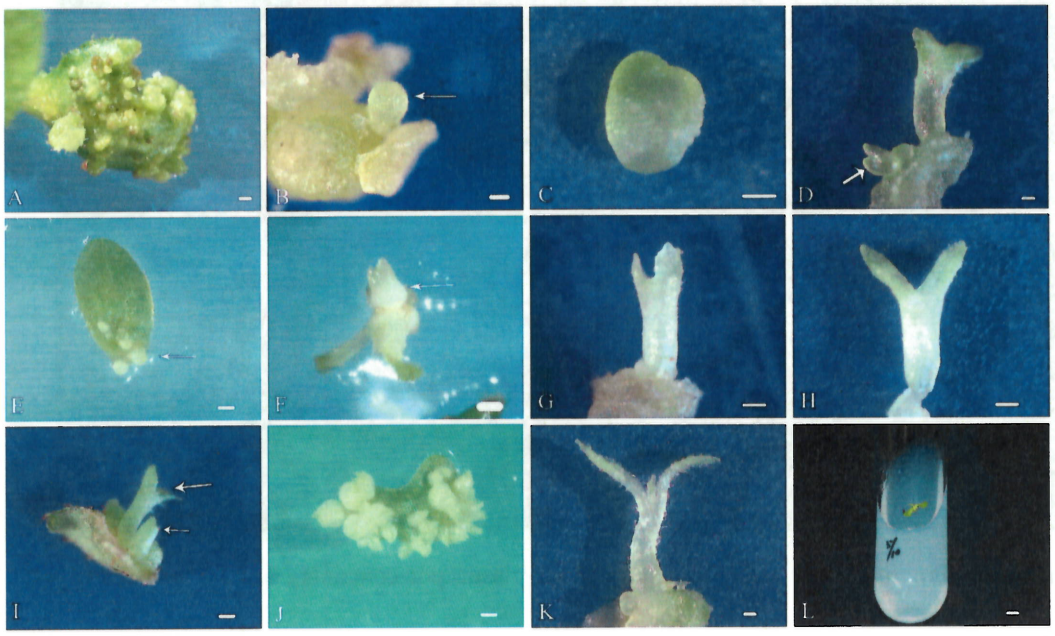

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382 Fig.7 Somatic embryogenesis from young leaves of *Rhododendron fortunei* L.

383 **A** Bulges derived from the superficial of leaves; **B** Globular somatic embryo

384 formation from the superficial of a leaf; **C** Early heart-shaped somatic embryo (arrowheads)

385 formation from embryogenic callus; **D** Heart-shaped somatic embryo (arrowheads) formation

386 from the superficial of a leaf; **E** Several Heart-shaped to torpedo somatic embryo derived

387 from a leaf (arrowheads); **F** Development torpedo somatic embryo (arrowheads); **G**

388 Torpedo somatic embryo (arrowheads); **H** Somatic embryos with expanded cotyledons; **I**

389 Cotyledonary and torpedo somatic embryos derived from one leaf (arrowheads);

390 **J** Various phases of somatic embryos were observed on one piece of young leaf; **K**

391 Germinated somatic embryo; **L** ~~Plant regeneration~~ Regenerated plant (Bars B,C 500μm, L 2mm, others 1mm).

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395 Fig.8 Micropropagated plants transferred to a plastic pot containing a 1:1:1 mixture of peat,

396 perlite, and vermiculite and grown in the greenhouse.

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