J Plant Biotechnol (2018) 45:347–356
DOI:https://doi.org/10.5010/JPB.2018.45.4.347

ISSN 1229-2818 (Print)
ISSN 2384-1397 (Online)

Research Article

Optimization of factors influencing *in vitro* immature seed germination in *Chionanthus retusus*

Khin Yae Kyi Tar · Aung Htay Naing · Trinh Ngoc Ai · Mi Young Chung · Chang Kil Kim

Received: 14 September 2018 / Revised: 19 November 2018 / Accepted: 6 December 2018

Abstract *Chionanthus retusus* is a small deciduous tree that is widely used in landscaping due to its beautiful white spring flowers and ornamental value. Conventional propagation through seeds requires one to two years of breaking dormancy. The objective of this study was to determine the conditions of *in vitro* germination in *C. retusus*. *In vitro* embryo culture was carried out to investigate the effects of six factors: basal media (McCown Woody Plant Medium (WPM) and Murashige and Skoog (MS)); plant growth regulators (different combinations and concentrations of naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BA), and gibberellic acid (GA_3)); embryo age (collected weekly beginning 36 days after fruit setting); low temperature pretreatment (storing 4°C for 1, 2, 3, and 4 weeks); coconut additives (100, 200, and 300 ml·L^{-1}); and genotype (grouping plants depending on their flowering nature). The basal medium used in this study was WPM with 2 mg·L^{-1-1} GA_3, 20 g·L^{-1} sucrose, and 6 g·L^{-1} Agar. WPM medium mixed with GA_3, resulted in higher germination rate as compared to when using a combination of auxin and cytokinin. GA_3 at 2 mg·L^{-1} was the most effective of all combinations and concentrations of PGRs. WPM medium with 2 mg·L^{-1} GA_3 resulted in better and faster germination (75.93%). Embryos collected at 57 days after fruit setting had the highest percent of germinated seeds (87.04%) while low-temperature pretreatment of fruits at 4°C for two weeks produced the highest germination (95.37%). These results of this study could be an open ground for development of an efficient protocol for commercial production of the ornamental tree.

Keywords *Chionanthus retusus*, embryo culture, *in vitro* germination

K. Y. K. Tar · A. H. Naing · C. K. Kim (✉)
Department of Horticultural Science, Kyungpook National University, Daegu, Republic of Korea
e-mail: ckkim@knu.ac.kr

T. N. Ai
School of Agriculture and Aquaculture, Tra Vinh University, Trà Vinh, Vietnam

M. Y. Chung
Department of Agricultural Education, Suncheon National University, Suncheon, Korea

Introduction

The Chinese fringe tree (*Chionanthus retusus*) is a small deciduous tree in the family Oleaceae and is native to China, Korea, Japan, and Taiwan (Nicholson 1990). In Korea, the Forest Research Institute reports that *C. retusus* can be found growing near sea level to over 900 meters inland (Nicholson 1990). The genus *Chionanthus* consists of nearly 100 species distributed worldwide (Lombardi 2006), the majority of which are tropical to subtropical and native to parts of Africa, Asia, the Americas, and Australia (Green 1994). The morphological characters and reproductive processes of the Chinese fringe tree are similar to those of both *C. virginicus* and *C. pygmaeus*, which are prized for their attractive ornamental features (Fagan, A. E. and Dirr, M. A. 1980). The growth of *C. retusus* is very slow, usually growing 4 to 10 inches per year and reaching 12 inches in rich, moist, well-fertilized soil (Gilman and Watson 1993). It is used widely in landscaping owing to its ornamental value. Its leaves have been used in folk medicine for the treatment of diarrhea, palsy, and stomachache (Kwak, et al. 2009). In China, the flowers are used to prepare tea owing to their content of various flavonoids and triterpenoids and for their medicinal value; the young shoots and leaves are used in cooking and young leaves are used as an alternative for tea (Nicholson 1990). *C. retusus* can grow well in full sun to partial shade, with moderate summer drought, and in a wide range of soil conditions (Dirr, 1998) (Gilman and Watson 1993). It can reach 25 m (80 feet)

in height and 70 cm (2 feet) in girth under full sun and high moisture conditions (Kurata 1973). The flowering time is from late April through mid-May, and full, showy, white flowers are produced. Fruits are produced at the end of May. *Chionanthus* species have double dormancy and are conventionally propagated via warm/cold stratification of the seed. They are considered difficult to propagate vegetatively (Nicholson 1990) because of their lack of rooting. Other than for *Chionanthus*, the vegetative propagation of other members of Oleaceae is nearly impossible, for example with stem cuttings of *Fraxinus* spp. (ash) (Dirr and Heuser 1987).

Rooting has been attempted in softwood cuttings collected in June to mid-July using 1.0% indole-3-butyric acid (IBA), but success was only around 50% (Dirr and Heuser 1987). Around 95% rooting has been achieved from stem cuttings collected in mid-July using 8000 ppm IBA, but these results are difficult to consistently replicate (Dirr 1998). Attempts to initiate rooting from hardwood cuttings and root cuttings treated with IBA (1000 ppm and 8000 ppm) were unsuccessful for *C. retusus*, *C. virginicus*, and *C. pygmaeus* by Eads, Amanda L. Successful root formation depends on the combination of cutting age and/or the extent of lignification in the cuttings (Cameron, et al. 2003). An experiment comparing the rooting behavior of *C. retusus* and *C. virginicus* was conducted under mist, using half peat and half perlite medium by Arnold Arboretum Propagator Jack Alexander (Nicholson 1990). No root formations were found, even when hormone treatments were used in *C. virginicus*. Rooting occurred at 30% in *C. retusus* using 1% indolebutyric acid in a solution of 50% ethanol and 59% water. Therefore, seed propagation is the most reliable propagation technique for *Chionanthus,* even though the seeds have double dormancy (Nicholson 1990). Carpenter, W. J. observed that endocarp-removed seeds of *C. virginicus L.* needed 10 weeks (26%), while mechanically and acid scarified seeds needed 18 weeks (2%) and 14 weeks (5%), respectively, to germinate to 50% final germination. The highest 50% of germination (82%) was observed after 4.6 weeks at 25°C in endocarp-removed seeds pretreated with 1000 ppm GA_3 for 6 hours. *C. retusus* showed an unusual form of epicotyl dormancy, similar to that in *Davidia involucrata* (dove-tree), *Paeonia suffruticosa,* and *Aesculus parviflora* (bottlebrush buckeye). In epicotyle dormancy, the root comes out during the first year after sowing but shoot appearance is extended to the next year after the seed has been through a cold winter (Chien et al. 2004). According to Baskin's report (Baskin and Baskin 2004), *Chionanthus* seeds, which show epicotyl dormancy, belong to the class of seeds that show deep morphophysiological dormancy, i.e., seeds must be exposed to warm and then cold stratification for radical protrusion and dormancy breaking. In other words, protrusion of the radical of the embryo occurs slowly under favorable temperatures prior to cold treatment, which can cause the embryo to be completely non-dormant.

Embryo culture has been used to overcome seed dormancy in many other species (Arrillaga et al. 1992; Bridgen 1994). Immature embryo culture is the culturing of zygotic embryos cut off from ovules and seeds under sterile conditions in growth regulators. Many factors influence on the successful development of an embryo, important factors include genotype, explant, embryo age, composition of basic growth media, growth regulators, light intensity and quality, photoperiod, temperature and endogenous factors (Umehara et al. 2007; Polanco and Ruiz 2001). Complex media supplemented with a combination of vitamins, amino acids, growth hormones and, in some cases, natural extracts, such as tomato juice and coconut milk, are required for the development of young embryos (Bridgen 1994).

A period of cold, moist conditions (prechill) is required for the germination of certain seeds (Feghahati and Reese 1994). Seeds with morphophysiological dormancy require either treatment with GA_3, abscisic acid (ABA), cytokinins and auxins (Seo et al. 2011), or a combination of warm stratification and moist-chilling to break dormancy (Hilhorst 2011) and initiate the fast multiplication and growth of cells, followed by the protrusion of radicles. However, studies on embryo culture in this species are rare. Charlotte R. Chan (1999) reported that embryo culture in *C. virginicus* started germination within 7 to 10 days and traditional seed propagation (warm /cold stratification) required 8 months for radicle protrusion. The size of 10-month-old cultured plants was comparable with the size of 2-year-old traditionally grown plants. In 2009, I-Ching Huang reported that seed dormancy was broken in *C. retusus* by culturing the embryo on Woody Plant Medium (WPM) containing 2 mg/L GA_3 (Huang et al. 2009).

Therefore, in this study, we investigated several conditions for the *in vitro* germination of *C. retusus* embryos, including medium composition, low-temperature pre-storage, embryo age, plant growth regulators, and genotype. The objective of this study was to identify the conditions most suitable for *in vitro* germination in *C. retusus*.

Materials and Methods

Immature *C. retusus* fruits were collected in July from the campus of Kyungpook National University, Daegu, and Republic of Korea. Fruits were washed with distilled water three times and put in 70% ethanol for 1 minute. Next, fruits

were rinsed in 1% sodium hypochlorite solution (10 mL·L^{-1}) in the sterile condition and shaken at 200 rpm for 30 minutes, followed by washing three times with distilled water. Thereafter, the stem end of the sterile fruit was cut, removing about one-third of the endocarp, then cut longitudinally to remove the embryo. The embryos were cultured on WPM supplemented with 2 mg·L^{-1} GA_3, 20 g·L^{-1} sucrose, or 6 g·L^{-1} agar, a formulation previously optimized by I-Ching Huang (2009). The petri dishes were then sealed with self-sealing film and incubated in a culture room. All cultures were adjusted to pH 5.8 and incubated at 25 ± 1°C in total darkness for four days. Subsequently, they were transferred to a controlled environment consisting of 32 μmol^{-2}s^{-1} photosynthetic photon flux density under a 16 hour illumination cycle.

Effect of basal medium on germination in *C. retusus*

To study the role of basal salt in *in vitro* germination, we used the medium composition previously described by I-Ching Huang et al (2009), consisting of WPM basal salt, sucrose at 20 g·L^{-1}, GA_3 at 2 mg·L^{-1}, and agar at 6 g·L^{-1}. Also, based on the previous media formulation, MS basal salt (Murashige and Skoog, 1962) was used instead of WPM basal salt; composition with respect to the other components was unchanged. MS and WPM basal salt without plant growth regulators were used as a control. Thus, the formulations studied were as follows: MS control, MS GA_3 (2 mg·L^{-1} GA_3), WPM control, WPM GA_3 (2 mg·L^{-1} GA_3).

Effect of low-temperature pretreatment on germination in *C. retusus*

To investigate the effectiveness of different durations of low-temperature pretreatment to promote germination, the immature fruits were stored at 4°C for 1 week, 2 weeks, 3 weeks and 4 weeks. Each week, the embryos were excised and cultured into the above-mentioned medium. As a result, the cultures of low-temperature pretreatment for *in vitro* germination were as follows: LT1, immature embryos of the fruits that were stored at 4°C for 1 week, LT2 (storage duration 2 weeks), LT3 (storage duration 3 weeks), and LT4 (storage duration 4 weeks).

Effect of embryo maturity on germination in *C. retusus*

In order to examine the embryo conditions in the seed, fruits were regularly cut and checked under a microscope. As a result, the stages of fruit maturity were as follows: EM1, 36 days after fruit set, in which the embryo can be seen under the microscope as the first time; EM2, 43 days; EM3, 50 days and EM4, 57 days after fruit set. Fruits were collected from the same tree weekly and cultured on the medium. For EM1, the embryo was cultured together with the embryo sac onto the culture medium. For EM2, EM3, and EM4, embryos were excised and cultured on the medium in order to assess the effect of the maturity of the embryo on germination.

Effect of plant growth regulators on germination in *C. retusus*

To examine the role of plant growth regulators on *in vitro* germination, the excised immature embryos were cultured on WPM containing 20 g·L^{-1} sucrose and 6 g·L^{-1} agar and supplemented with different concentrations of NAA (0, 0.1, 0.3 mg·L^{-1}) and BA (0, 2, 4 mg·L^{-1}) combined with GA_3 (0, 1, 2, 3 mg·L^{-1}) (Table 4). The media without hormones and the media with NAA and BA were sterilized by autoclaving (121°C for 16 min). Then, micro-filtrated gibberellic acid was added to the warm (35 ~ 40°C) autoclaved medium before it was allowed to solidify under sterile conditions to avoid denaturation.

Acclimatization

Seedlings obtained from above experiment were grown on the MS basal medium containing 10 g·L^{-1} sucrose for plant growth. After 2 months of culture when the seedlings reached the size 4 ~ 5 cm, they were planted in a cell tray with a vermiculite soil, and were covered with a plastic sheet for acclimation to the greenhouse at 25°C. After 3 weeks, the plantlets were transferred to the pots containing a soil mixture of peat and perlite (1:1) and were grown in the greenhouse.

Statistical analysis

Eighteen explants were used for one treatment with three replications of each treatment. For each treatment, data on the percentage of germinating embryos, data on the percentage of surviving explants, and shoot length and root length of germinated explants were recorded. Germination percentage and survival percentage were calculated using the following formula: Germination Percentage = Total number of germinated plants / total number of survival plants × 100, Survival Percentage = Total number of survived plants / total number of plants × 100.

Results and Discussion

Effect of basal medium on germination in *C. retusus*

Figure 1 and Table 1 show the effect of basal medium on

Fig. 1 Plantlets germinated on MS and WPM media on the 30th day of culture. (A) WPM with GA_3, (B) WPM control, (C) MS with GA_3, (D) MS control

Table 1 Effect of basal medium on the germination of immature embryos of *Chionanthus retusus in vitro* culture

Basal media composition	Germination (%)	Survival (%)	Average Length (mm)	
			Shoot	Root
MS C	59.26 ± 4.89 b	96.30 ± 3.70 a	0.47 ± 0.40 ab	7.09 ± 3.91 b
MS GA_3	75.93 ± 6.67 ab	87.04 ± 6.67 a	0.87 ± 0.80 ab	10.70 ± 0.94 b
WPM C	71.30 ± 14.01 ab	79.63 ± 17.66 a	0.20 ± 0.20 b	19.97 ± 1.85 a
WPM GA_3	94.44 ± 5.56 a	90.74 ± 3.70 a	2.54 ± 0.80 a	15.16 ± 3.05 ab

Germination and survival data were collected on the 21st day (3 weeks) after culture. Shoot and root length data were collected on the 30th day after culture. Data were analyzed with $p<0.05$, Duncan's Multiple Range Test. Bars represent ± SE. Different letters (a, b, c) indicate significant differences at $p<0.05$. MS C, MS control; WPM C, WPM control; MS GA_3, MS medium supplemented with 2 mg·L^{-1} GA_3; WPM GA_3, WPM medium supplemented with 2 mg·L^{-1} GA_3.

germination in *C. retusus*. All treatments affected germination to differing extents. The overall final germination percentage was significantly different in all treatments, with $p<0.01$. WPM media supplemented with GA_3 resulted in the highest germination percentage (94.44%). For survival, MS C and WPM GA_3 media resulted in survival over 90%, while WPM C medium resulted in the lowest survival, 79.63%. By comparing the two basal media without GA_3, WPM resulted in higher germination than in MS. Even though shoot lengths were not significantly different, root lengths were better in WPM. The performances of both media were improved by adding gibberellic acid.

GA_3 resulted in higher germination rates. Simsek (2017) reported that WPM medium resulted in the highest rate of micropropagation and rooting in myrtle, compared to MS and Rugini media. Afroz, et al. (2009) reported that the use of GA_3 increased regenerative potential and also reduced the time required for regeneration. Although the developmental response of *in vitro* culture in ash embryos was strongly dependent on the medium composition and embryo maturity, supplementing the culture media with GA_3 improved the *in vitro* germination of ash embryos (Wagner and Kafka 1995).

The tallest mean shoot length was observed in WPM medium supplemented with 2 mg·L^{-1} GA_3. The shortest mean shoot length was observed in the WPM control, though this treatment also resulted in the longest mean root length. However, WPM GA_3 and WPM C were not significantly different in average root length. Therefore, WPM media with GA_3 provided the best results in terms of both mean shoot and root lengths.

In the first week after culture, WPM GA_3 cultured embryos germinated at the quickest rate (50% of germination), followed by MS GA_3, MS control, and WPM control with rates of 36.11%, 25.93%, and 23.15%, respectively. Figure 2 shows

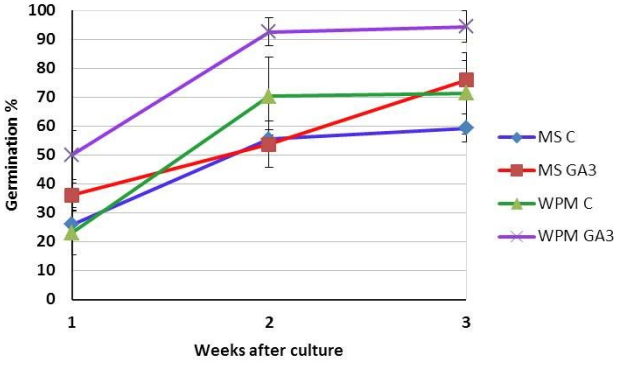

Fig. 2 Effect of basal medium on the cumulative percent germinated in *Chionanthus retusus.* Data were collected weekly after culture and analyzed using Duncan's Multiple Range Test with significance set at $p<0.05$. Bars represent ± SE. MS C, MS control; WPM C, WPM control; MS GA_3, MS medium supplemented with 2mg·L^{-1} GA_3; WPM GA_3, WPM medium supplemented with 2-mg·L^{-1} GA_3

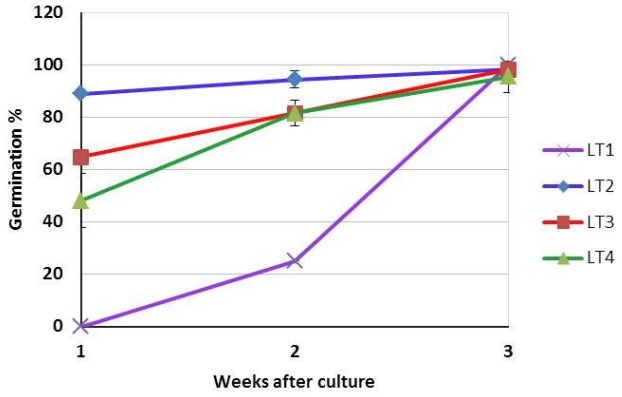

Fig. 3 Effect of low-temperature pre-treatment on the cumulative percent germinated in *Chionanthus retusus*. Data were collected weekly after culture and analyzed using Duncan's Multiple Range Test with significance set at $p<0.05$. Bars represent ± SE. LT1, Low-temperature pretreatment 1 week; LT2, Low-temperature pretreatment 2 week; LT3, Low-temperature pretreatment 3 week; LT4, Low-temperature pretreatment 4 week

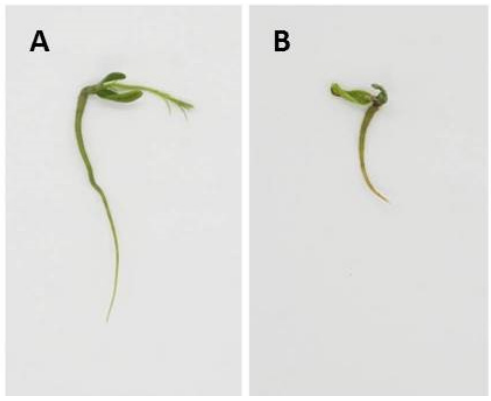

Fig. 4 Effect of low-temperature pretreatment on *in vitro* germination of immature embryos of *Chionanthus retusus* on the 30th day of culture. (A): low-temperature pretreatment for 2 weeks (LT2), (B): low-temperature pretreatment for 3 weeks (LT3)

that both media supplemented with GA_3 resulted in higher germination compared to controls beginning the first week after culture. Among the media treated with GA_3, WPM resulted in the highest percent of germinated embryos beginning in the first week until the end of the period. The media supplemented with GA_3 improved germination speed, as well as the growth of roots and shoots. Therefore, in this study, WPM media supplemented with 2 $mg \cdot L^{-1}$ GA_3 was the best medium for *C. retusus* germination.

Effect of low-temperature pretreatment on germination in *C. retusus*

Figures 3 and 4 and Table 2 show the effect of different pre-storage times at low temperature on germination in *C. retusus*. In this experiment, there was a significant difference ($p<0.01$) in the relationship between treatments and weeks for germination and germination rate. We found that the effects of low-temperature pre-storage treatments on germination and germination rate depend on the week after culture. Varying the pre-storage time at 4°C affected germination. LT2 resulted in the greatest percent germination (88.89%), followed by LT3 (64.82%) and LT4 (48.15%), in the first week after culture, while LT1 did not result in any germination. LT2 took the first position for the whole experiment. LT3 and LT4 had 81.48% germination in the second week after culture. The response of LT1 on germination started two weeks after culture with 25% germination. All treatments reached 90 ~ 100% germination the third week after culture. LT1 resulted in 100% germination (all embryos in this treatment germinated) followed by LT2 and LT3 (98.15%), and LT4 (95.37%).

For survival percentage, LT2 had the highest survival rate (98.89%). For shoot and root length, the maximum mean shoot and root lengths were in LT2 with mean lengths of 10.78 mm and 41.81 mm, respectively, while the minimum mean shoot and root lengths were in LT3 (2.6 mm and 22.3 mm, respectively). Endogenous factors are more directly involved in controlling seed dormancy than exogenous growth promoters. The chilling of seeds activates germination (Kozlowski and Pallardy 1997). High ABA levels, which support the growth of radicles and inhibits the growth of

Table 2 Effect of low temperature pre-treatment on the germination of immature embryos of *Chionanthus retusus* cultured *in vitro*

Low temperature pre-treatment period	Germination (%)	Survival (%)	Average Length (mm)	
			Shoot	Root
LT 1	100 ± 0.0 a	98.89 ± 1.27 a	6.80 ± 1.69 ab	31.88 ± 2.13 b
LT 2	98.15 ± 1.85 a	100 ± 0 a	10.78 ± 1.47 a	41.81 ± 1.37 a
LT 3	98.15 ± 1.85 a	99.38 ± 1.07 a	2.57 ± 1.05 c	22.25 ± 4.74 c
LT 4	95.37 ± 6.07 a	99.38 ± 1.07 a	5.93 ± 0.29bc	35.78 ± 2.41 ab

Germination and survival data were collected on the 21st day (3 weeks) after culture. Shoot and root length data were collected on the 30th day after culture. Data were analyzed with $p<0.05$, Duncan's Multiple Range Test. Bars represent ± SE. Different letters (a, b, c) indicate significant differences at $p<0.05$.

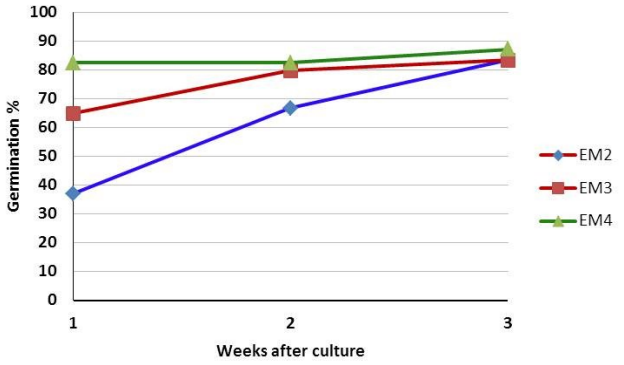

Fig. 5 Effect of embryo maturity on the cumulative percent germinated in *Chionanthus retusus*. Data were collected weekly after culture. Data were analyzed using Duncan's Multiple Range Test with significance set at $p<0.05$. Bars represent ± SE. EM2, 43 days after fruit set; EM3, 50 days after fruit set; EM4, 57 days after fruit set.

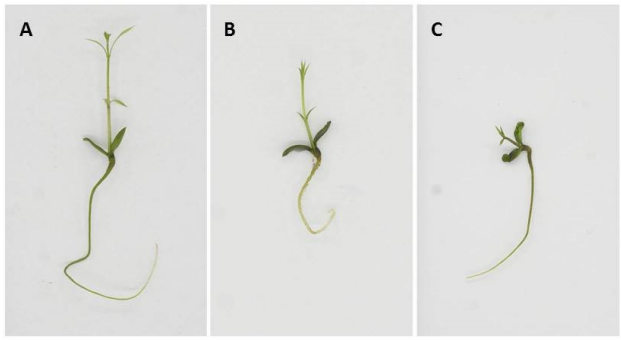

Fig. 6 Effect of embryo maturity on germinated seedlings on the 30th day after culture. (A) 43 days after fruit set, (B) 57 days after fruit set, (C) 64 days after fruit set.

epicotyl, was found in the embryo and endosperm of freshly harvested seeds (Chien et al. 2004). Seeds treated with cold stratification conditions were found to have reduced ABA levels, which were sufficient to allow for germination and stimulate endogenous GA levels (Chien et al. 2004). In this case, endogenous levels of GA and ABA, as well as the moisture content of the seed, might constitute the optimal conditions for germination after cold storage of the seeds for two weeks. Although LT1 resulted in 100% final germination, LT2 showed the highest germination rate at the first week after culture (88.89%), while LT1 did not show any germination. Therefore, according to this study, the pre-storage of seeds for two weeks before culture resulted in the highest percent of germinated seeds, the fastest average germination rate, and the highest survival percent with the maximum lengths of shoots and roots.

Effects of embryo maturity on germination in *C. retusus*

Figures 5 and 6 and Table 3 show the effect of embryo maturity on *in vitro* germination in *C. retusus*. There was no germination when embryos were cultured together with the embryo sac in the first week (EM1; data not shown). There was a significant difference in the relationship between treatment and time (i.e., week after culture), meaning that the effect of treatment was dependent on time after culture. EM4 resulted in the highest germination (82.41%) since the first week after culture, followed by EM3 (64.82%) and EM2 (37.04%). There was an upward trend for these three treatments during the whole period. The final percent of germination for all three treatments was almost the same (80 ~ 90%). The percent germinated in EM4 reached above 80% in the first week after culture and did not vary for the whole period. It can be concluded that the older the embryo, the higher the effect of the germination. Embryo maturity should be considered for *in vitro* embryo germination (Vidhanaarachchi et al. 2016). The key factors for *in vitro* immature embryo culture were the embryo stage and media composition, which had the greatest effect on germination rate in torpedo and cotyledonary embryos in the genus *Capsicum* (Manzur et al. 2013).

In this study, the excised embryos of all treatments responded differently. The oldest embryo had the highest percent germination and the youngest embryo had the lowest,

Table 3 Effect of embryo maturity on the germination of *Chionanthus retusus in vitro* culture

Embryo age	Germination (%)	Survival (%)	Average Length (mm)	
			Shoot	Root
EM 1	0	0	0	0
EM 2	83.33 ± 5.55 a	81.48 ± 3.7 a	35.79 ± 2.02 a	44.14 ± 8.25 a
EM 3	83.33 ± 9.62 a	94.44 ± 5.56 a	17.79 ± 2.63 a	20.49 ± 1.94 a
EM 4	87.04 ± 2.45 a	81.48 ± 3.7 a	5.89 ± 2.68 a	10.36 ± 2.96 a

Germination and survival data were collected on the 21st days (3 weeks) after culture. Shoot and root length data were collected on the 30th day after culture. Data were analyzed with $p<0.05$, Duncan's Multiple Range Test. Different letters (a, b, c) indicate significant differences at $p<0.05$. EM1, 36 days after fruit set; EM2, 43 days after fruit set; EM3, 50 days after fruit set; EM4, 57 days after fruit set.

Table 4 Effect of different plant growth regulators on the germination of immature embryos of *Chionanthus retusus in vitro* culture

Treat-ments	Plant Growth Regulators (mg·L^{-1})			Germination (%)	Survival (%)	Average length (mm)	
	NAA	BA	GA_3			Shoot	Root
CM1	0	0	0	71.3 ± 7.91 a	79.63 ± 17.67 a	0.2 ± 0.18 b	19.97 ± 1.85a
CM2	0	0	1	91.42 ± 14.46 a	94.44 ± 1.00 a	5.82 ± 4.67 a	15.42 ± 4.13 ab
CM3	0.1	2	1	75.93 ± 5.56 a	94.44 ± 3.2 a	1.11 ± 0.9 b	8.69 ± 2.83bc
CM4	0	0	2	94.44 ± 1.85 a	90.74 ± 3.7 a	2.54 ± 0.81 ab	15.16 ± 3.05 ab
CM5	0.3	2	1	92.59 ± 9.62 a	94.44 ± 3.2 a	0	5.39 ± 1.39 cd
CM6	0.1	4	1	69.61 ± 11.11 a	92.59 ± 1.85 a	0.2 ± 0.12 b	2.95 ± 1.1 cd
CM7	0.3	4	1	61.11 ± 8.48 a	90.74 ± 6.67 a	1.4 ± 1.3 b	3.78 ± 2.04 cd
CM8	0.1	2	2	72.22 ± 4.81 a	87.03 ±7.4 a	0.5 ± 0.27 b	5.96 ± 1.06 cd
CM9	0.1	4	2	66.67 ± 3.33 a	88.89 ± 3.2 a	0.8 ± 0.7 b	2.78 ± 1.05 cd
CM10	0.3	4	2	74.08 ± 8.48 a	96.29 ± 1.85 a	0	2.59 ± 1.53 cd
CM11	0	0	3	93.7 ± 26.89 a	88.89 ± 5.56 a	4.44 ± 2.6 ab	19.25 ± 2.77 a
CM12	0.1	2	3	67.82 ± 8.07 a	88.89 ± 6.4 a	0.5 ± 0.26 b	8.57 ± 5.32bcd
CM13	0.3	2	3	75.93 ± 22.45 a	96.30 ± 3.7 a	0	5.15 ± 2.74 cd
CM14	0.3	4	3	61.11 ± 14.01 a	94.44 ±3.2 a	0	1.07 ± 1.06 d

Germination and survival data were collected on the 21st day (3 weeks) after culture. Shoot and root length data were collected on the 30th day after culture. Data were analyzed with $p<0.05$, Duncan's Multiple Range Test. Different letters (a, b, c) indicate significant differences at $p<0.05$.

perhaps owing to the different levels of hormones, phenolic compounds, and nutritional requirements in the variously aged embryos. Moreover, it may be the fact that the physiological processes of older embryos are more complete than the younger ones. In the case of EM1, which was cultured together with the embryo sac, there was no germination in this study. This may be because of the endogenous inhibitors of the endosperm in the seed. The possible causes of endogenous inhibitors are ABA or the combination of ABA and the phenolic compounds (glucoside phenolics, including GL3, Nuzhenide, ligustroside, and oleoside dimethyl ester) found in the endosperm of *Chionanthus* (Chien, et al., 2004).

EM4 had a high germination rate, but the lowest survival percentage (85.19%). EM3 resulted in the best survival among the three treatments. The growth of shoots and roots was the opposite from germination. Older embryos germinated better than younger ones, but younger embryos had longer shoots and roots. This may be because the cells and tissues of younger aged embryos are more juvenile and have more cell multiplicity. The potential for embryo growth seems to be controlled by complex interactions between hormonal and other endogenous factors in tissues surrounding the embryo and the embryo itself (Kozlowski and Pallardy 1997).

Effect of plant growth regulators on germination in *C. retusus*

The effects of plant growth regulators on *in vitro* germination in *C. retusus* are described in Table 4, Figures 7 and 8. The effect of gibberellic acid alone on germination was better than the combination of NAA and BA. All treatments with GA_3 alone (CM2, CM4, and CM11) were significantly different from the other treatments. Among them, CM4 provided the highest percent germinated (94.44%), followed by CM11 (93.7%) and CM2 (91.42%). All the combinations of GA_3, NAA, and BA resulted in lower germination compared to GA_3 alone. All treatments had a survival percentage of over 90%, except CM1 which had 80% survival.

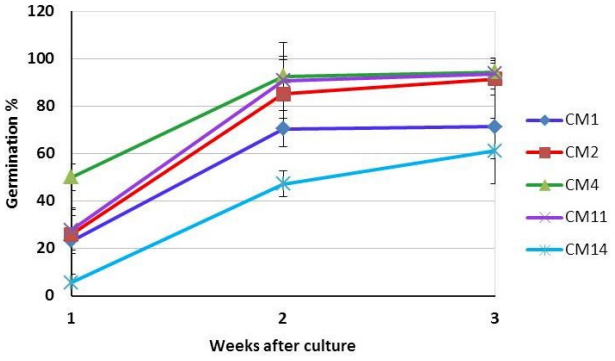

Fig. 7 Effect of plant growth regulators on the cumulative percent germinated in *Chionanthus retusus*. Data were collected weekly after culture. Data were analyzed using Duncan's Multiple Range Test with significance set at $p<0.05$. Bars represent ± SE.

CM4 reached 50% germination the first week of culture.

Fig. 8 Effects of PGRs on *in vitro* germination and seedlings. (A) GA 1 mg·L^{-1}; (B) GA 2 mg·L^{-1}; (C) GA 3 mg·L^{-1}; (D) NAA 0.3 mg·L^{-1} + BA 4 mg·L^{-1} + GA 0.3 mg·L^{-1}

Fig. 9 Showing *in vitro* germinated seedling well growing in the plant growth medium (A) and in the greenhouse (B)

The other treatments showed similar germination (within 20-30%), while CM14 had the fewest germinate in the first week. All of them gradually increased in the second week after culture and then remained stable at the third week after culture. CM2 and CM11 approached CM4 at the second week, then they reached 90-100% germination in the third week. Therefore, in this study, gibberellic acid, especially GA_3 at 2 mg·L^{-1}, encouraged germination of *Chionanthus* embryos.

Mean length of shoots and roots of the germinated *C. retusus* seedlings are shown in Figure 8. The maximum shoot length occurred in CM2, followed by CM11 and CM4. However, the maximum root length occurred in CM11 and CM1 (control), but only CM11 had good results in terms of mean shoot length. In this study, although all the GA_3 treated media resulted in higher length in both shoots and roots, they (CM2, CM4, and CM11) were not significantly different. Therefore, we recommend CM4 for its high germination percentage and fast speed. The growth of the explant combined with GA_3, NAA, and BA was too slow, very small, and hard to form shoots and roots. A combination of auxin and cytokinin promotes growth and differentiation of embryos (Veen 1963). This was not found in our study. In this study, exogenous auxin and cytokinin did not seem to be required for embryo growth and germination. It was reported that embryo culture media with hormone should not be used because they can cause structural abnormalities (Bridgen 1994). The media with GA_3 resulted in a higher percentage of germinated seedlings and longer shoot and root length in this study. GA_3 has been reported to encourage post-germinative development and transformation into plantlets in several species, and also assisted germination of *Zea mays* (seeds, 10-100 μM, White and Rivin 2000) and Malayan Green Dwarf (MGD) and Malayan Yellow Dwarf (MYD) coconut palms (Ake et al. 2007). In *P. radiata* and some other woody species, such as coconut, gibberellic acid increased the percent germinated and improved embryo transformation (Stojicic et al. 2008). In this study, the media which included GA_3 encouraged germination and embryo transformation into plantlets for this species.

The seedlings cultured on the basal MS medium containing 10 g. L^{-1} sucrose grew well and reached the size approximately 4 ~ 5 cm after 2 months of culture (Fig. 9A), and acclimatization of the plants in the greenhouse was achieved with normal plant growth (Fig. 9B).

Conclusion

Based on the results of this study, low-temperature pre-storage treatment was found to be one of the factors with the greatest effect on *in vitro* germination in *C. retusus*. We found that the maturity of embryos also affected the *in vitro* germination of embryos. Older embryos germinated better than younger

ones. However, younger embryos had longer shoots and roots. Therefore, EM3, which is 50 days after fruit set, was suitable for this species not only because it resulted in the best survival percentage, but also because it resulted in a high percent of germinated embryos and long roots and shoots, even though it did not produce the maximum values for either. We also noticed that gibberellic acid encouraged *in vitro* germination in this species. Among the three concentrations of gibberellic acid, GA_3 2 mg·L^{-1} was the most suitable for *C. retusus* because it resulted in a high amount of germination within a short period of time. We did not find any effects from the other controlled factors (coconut additives, plant growth regulators, and genotype) on *in vitro* germination in *C. retusus*. Therefore, the optimal conditions for *in* vitro germination in *C. retusus* included 50-day-old embryos after setting fruit (EM3), a low temperature pretreatment storage for 2 weeks, WPM medium, and GA_3 2 mg·L^{-1}.

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