Immature Embryo Germination and Its Micropropagation of *Ilex crenata* Thunb.

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Abstract. To shorten *Ilex* seed germination time and speed up breeding cycles, immature embryos of *Ilex crenata* ‘Sky Pencil’ seedlings were removed from fruits at their heart-shape stage and cultured in vitro on Murashige and Skoog (MS) medium or Woody Plant Medium (WPM) with 3% sucrose and 0.65% agar. Cultures were incubated at 27 °C for 2 weeks in darkness and subsequently moved to a growth chamber with 14-hour photoperiod (115 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Embryos began to germinate 2–3 weeks after culture. The highest germination rate was 91.67% under 1/4 MS medium. Embryos cultured on MS medium also had high germination rates and produced the longest seedlings to 8.02 mm. Nodal segments with one axillary bud taken from embryo germination seedlings were cultured on MS medium with various concentrations of cytokinins and auxins for micropropagation. Zeatin (ZT; 4-hydroxy-3-methyl-trans-2-butenylaminopurine) increased the number of shoots and shoot lengths significantly more than 6-benzylaminopurine (6-BA). The recommended ZT concentration should be 2.28 μM . Rooting induction could be established on 1/4 MS medium with various concentrations of indole-3-butyric acid (IBA) or 1-naphthaleneacetic acid (NAA). IBA at 4.14 μM produced the best rooting percentage (91.67%) and good-root quality. All rooted plantlets were transplanted into a mixture of peatmoss and perlite (1:1 v/v) and acclimatized in a mist system. The average survival rate was 88.8%. The rapid embryo germination protocol for *Ilex crenata* could save *Ilex* breeders at least 2 years compared with traditional seed germination.

Ilex (holly) is one of two genera in Aquifoliaceae, which comprises ≈ 400 –600 deciduous and evergreen species. The genus is cultivated as an important medicinal and ornamental plant in the temperate and subtropical regions (Galle, 1997; Hu, 1989). The great diversity and adaptability of hollies make them indispensable in gardens and landscapes. These plants can be used as shade trees, screening plants, specimens, mass-plantings, hedges, and groundcovers. They have beautiful, showy fruits in autumn and winter, and most have evergreen foliage which gives them year round interest (Robinson,

1984). *Ilex crenata* is native to eastern China, Japan, Korea, Kuril, Sakhalin, the Philippines, and the Himalayas and is widely planted as an ornamental plant in the southeastern United States for its dense evergreen foliage and various forms (Dirr, 2009). Many cultivars, such as *Ilex crenata* ‘Sky Pencil’, have been released for commercial production. ‘Sky Pencil’ is a popular plant in landscaping for its strong upright habit and lustrous, dark evergreen foliage (Dirr, 2011).

Vegetative propagation using nodal segments and leaves in vitro has already been applied to propagate *Ilex* species, such as *Ilex khasiana* (Dang et al., 2011), *Ilex glabra* (Sun et al., 2010), *Ilex dumosa* (Luna et al., 2003), *Ilex paraguariensis* (Sansberro et al., 1999), *Ilex opaca* (Mattis et al., 1995), and *Ilex aquifolium* (Majada et al., 2000). For plant breeders, clonal propagation usually does not yield genetic variation or new plants. Seeds from open pollination and artificial crosses

have a wide genetic base and they are the main source of new cultivars. Like most *Ilex* taxa, seed germination of ‘Sky Pencil’ is inefficient as a result of low germination rate and long germination time. It usually takes 2–3 years to overcome the double dormancy of the hard, impermeable seedcoat and immature embryos (Dirr and Heuser, 2006; Hu et al., 1979). Pretreatment at room temperature for 2 months followed by 3 months cold stratification at 4 °C produced 90% germination (Dirr and Heuser, 2006). Breeding programs using this protocol usually last many years. For more than 50 years, embryo culture techniques have been successfully applied to many crops to overcome seed dormancy and shorten seed germination time (Sharma et al., 1996). In vitro germination of immature embryos might be helpful to shorten breeding cycles and accelerate the breeding process. In vitro cultures, plant embryos are used as an important research tool for rescuing rare hybrids (Li et al., 2014); genetic manipulation (Udomdee et al., 2014); propagation of elite and disease-free germplasm and physiological, morphological and anatomical studies (Abdolmohammadi et al., 2014; Germanà et al., 2014; Raomai et al., 2014). Immature embryos were also cultured successfully in vitro in several *Ilex* species (Hu, 1975; Sansberro et al., 2001).

To shorten the germination time and select new cultivars efficiently, we investigated the optimum basic medium and appropriate harvesting time for in vitro embryo germination and micropropagation of *Ilex crenata* ‘Sky Pencil’ seedlings.

Materials and Methods

Plant materials and culture conditions. Fruits from an 8-year-old plant of *Ilex crenata* ‘Sky Pencil’ seedling in the University of Georgia Horticulture Farm (Watkinsville, GA) were collected in Nov. 2013. They were washed with running tap water for 20 min and then rinsed with distilled water. The seeds were surface disinfected with 75% ethanol for 5 min and then immersed in Clorox (8.3% sodium hypochlorite; The Clorox Company, Oakland, CA) for 15 min. They were washed five times with autoclaved water and kept in sterile water until the embryos were excised under a STEREOMASTER stereomicroscope (Fisher Scientific Education, Nazareth, PA) in a laminar flow hood. The isolated embryos were transferred into 6-cm petri dishes containing various basic culture media plus 3% sucrose (Sigma-Aldrich, Co., Louis, MO) and 0.65% agar (Fisher Science Education, Nazareth, PA) for germination. Full-strength MS basic medium (Murashige and Skoog, 1962), half-strength MS (1/2 MS), quarter-strength MS (1/4 MS), and WPM (Lloyd and McCown, 1981) were tested. The pH of the media was adjusted to 5.8 with NaOH or HCl before adding agar. A total of 12 mL of the media was transferred by pipette into every autoclaved dish. All dishes with embryos were cultured in a dark growth chamber at room temperature. After 2 weeks, all

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embryos were moved to a growth chamber with cool-white fluorescent lamps ($115 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 14-h photoperiod. Embryo germination rate and the seedling length (mm) were recorded after 2 weeks in the dark and 1 additional week under the light. One portion of the seedlings with at least two true leaves was transplanted for acclimation. The rest was cut as nodal segments (≈ 1.0 -cm long without roots) and used for the following experiments.

Effect of the fruit harvest time for in vitro embryo germination. *Ilex* fruits with immature embryos were harvested on 15 July, 15 Aug., 15 Sept., and 15 Oct. 2014. The seeds were harvested and surface disinfected using the protocol mentioned above. Seeds were dissected and cultured without any plant growth regulator on MS culture medium in darkness in the same culture room. Each experiment was replicated three times and each treatment had nine embryos. A total of 27 embryos were excised for the experiment and germination and contamination rates were recorded.

Effect of cytokinins on shoot proliferation. To figure out the optimal conditions for axillary bud proliferation and shoot elongation, 6-BA at 2.22, 4.44, 8.88, or $17.76 \mu\text{M}$ and ZT (Sigma Chemical Co., Perth, WA) at 2.28, 4.56, 9.12, or $18.24 \mu\text{M}$ were compared in this study. Two-month-old seedlings with totally expanded leaves from embryos germinated in vitro were the explants in this experiment. Nodal segments ≈ 1.0 -cm long were excised and cultured on MS medium with 6-BA or ZT at various concentrations. All cultures were sealed with PARAFILM (Bemis Company, Inc. Neenah, WI) and incubated in a growth room at 25°C with a 14-h photoperiod. A total of 20 glass tubes (5 per replicate) were used for each treatment. Eight weeks later, height (the longest shoot, cm), number of shoots (>5 mm long), and average shoot length (centimeters) were recorded.

Effect of rooting hormone on root induction. Terminal shoots were used to induce roots on 1/4 MS with IBA at 2.07 – $8.29 \mu\text{M}$ or NAA (Sigma Chemical Co.) at 2.23 – $8.92 \mu\text{M}$. Shoots were also placed on 1/4 MS medium without auxin as control. A total of 24 glass tubes (6 per replicate) were used for each treatment. Eight weeks later, rooting rate, the number of roots (>5 mm long) and total root length per seedling (centimeters) were recorded.

Transplanting and acclimatization. Four weeks after transferring to the light conditions, seedlings with at least two true leaves were transplanted to a tray with 32 cells ($6.5 \times 6.5 \times 9 \text{ cm}^3$) with a 1:1 mixture of AERO-SOIL perlite (Dicalite; Dicapril Minerals, Inc., Bala Cynwyd, PA) and a commercial substrate (Fafard 3L Mix; Sun Gro Horticulture Canada Ltd., Agawam, MA) by volume and kept in the greenhouse. The flats were placed under intermittent mist and misting frequency was controlled by a misting controller (Phytotronics, Inc., Earth City, MO) set at 15 s every 10 min for the first 2 weeks. The mist system was on during the

day and off at night. No additional light was provided. A total of eight flats (32 plants per flat) were placed into four corners (four replicates, two flats per replicate) of a mist bench. After 2 months, plant survival rate was recorded.

Statistical analysis. A randomized complete block design was employed in all experiments. Analysis of variance and mean separation (least significant difference) were performed using statistical analysis systems (SAS Version 9.2; SAS Institute, Inc., Cary, NC).

Results and Discussion

Normally, holly seeds germinate in 2–3 years. Our results indicated that embryos can germinate in 2–3 weeks, which significantly shortened breeding time. Embryos were dissected at their heart shaped stage (Fig. 1A) and the majority of them germinated after 2 weeks (Fig. 1B). The embryo germination rates ranged from 45.83% to 79.17% during a 2-week dark period. After 1 week under the light, embryos under media at 1/4 MS, 1/2 MS, and WPM germinated an additional 12.50, 12.50, and 8.33%, respectively (Table 1). Obviously, the majority of embryos germinated in 2 weeks under darkness and this trend continued in another week under the light. The highest germination rate after 3 weeks (91.67%) was obtained with the treatment of 1/4 MS. This result was significantly higher than that of 1/2 MS at 58.33%. Both MS and WPM had lower germination rates than that of 1/4 MS, but no statistical difference was seen. Seedling growth was also regulated by culture media. The longest seedling length (8.02 mm) was obtained under MS medium and was significantly higher than that of 1/4 and 1/2 MS media. Culture media had significant influence on embryo germination and growth. MS medium is recommended for embryo germination, but WPM medium is also acceptable (Table 1). Our results are in general agreement with Hu's conclusion (1975) that the embryo germination could be higher than 90%. The embryo germination time of *Ilex crenata* is ≈ 1 week shorter than that of *Ilex latifolia* (Tezuka et al., 2013).

When is the best time to collect fruits for embryo germination of *Ilex crenata*? In June, no visible embryo was observed in the fruits, but all embryos collected after June had germination capacity (Table 2). The structure of the *Ilex crenata* seed (Fig. 2) had the same general pattern observed on other *Ilex* (Dolce et al., 2011; Tezuka et al., 2013) and both global and heart shaped embryos were the right stages to harvest for germination. The embryo germination rates ranged from 81.94 to 91.67% and the lowest germination rate occurred in July collection (Table 2). In that month, the immature fruits were green at their expanding stage and embryo emergence rate from immature fruits was not high when the seeds were dissected. After July, full size fruits were green (August), light purple (September), or black (October), with immature embryos

ready for germination (Table 2). Although the fruits were all full size after July, the embryo contamination rates increased significantly from August to October (Table 2). It is possible that the longer the fruits were exposed to field conditions, the higher the infection rates became, which led to the tedious task of sanitizing the seed. The fruit also had a softer seedcoat and endosperm in August, so the embryos were easier to be separated. Therefore, the recommended harvesting time for embryo germination is in August, when the fruits have just reached full size and are still green in color. However, it is still feasible to conduct embryo germination from fruits collected after August.

Both cytokinin type and concentration had a significant effect on height, the number of shoots (more than 5 mm long) and the average shoot length (Table 3). Explants on MS medium without cytokinin (control) yielded no proliferation. ZT had a much better effect than that of 6-BA on shoot proliferation of *Ilex crenata* 'Sky Pencil' seedlings and produced one to two extra shoots. Although Mroginski et al. (1999) and Sansberro et al. (2000) reported that 6-BA worked well with *Ilex paraguariensis* micropropagation, 6-BA had no effect on *Ilex crenata* shoot multiplication at the lower ($2.22 \mu\text{M}$) and higher ($17.76 \mu\text{M}$) concentrations. The concentrations of 4.44 and $8.88 \mu\text{M}$ had some effect, but the proliferation rate was too low to be applied. ZT at $9.12 \mu\text{M}$ is recommended for shoot proliferation (Fig. 1F; Table 3) even though the number of proliferated shoots were lower than the previous reports (four or more shoots) of *Ilex aquifolium* (Majada et al., 2000), *Ilex dumosa* (Luna et al., 2003), *Ilex paraguariensis* (Sansberro et al., 1999), *Ilex khasiana* (Dang et al., 2011), and *Ilex glabra* (Sun et al., 2010). ZT also significantly increased the shoot expansion, while 6-BA reduced the shoot growth. Both height (the longest shoot) and average shoot length under all 6-BA treatments were shorter than that of the control at 0.79 cm. When explants were grown on MS medium supplemented with 2.28, 9.12, or $18.24 \mu\text{M}$ ZT, average shoot growth increased to 1.35, 1.36, and 1.24 cm, respectively. ZT at $9.12 \mu\text{M}$ yielded the longest shoot (2.01 cm), which was significantly longer than all other treatments except ZT at $2.28 \mu\text{M}$ (Table 3). These results agreed with reports for *Ilex aquifolium* (Majada et al., 2000) and *Ilex glabra* (Sun et al., 2010), which also concluded that the length of axillary shoots under treatment of ZT was higher than that with 6-BA. From the above results and the cost effectiveness, $2.28 \mu\text{M}$ ZT (Fig. 1G) is recommended for multiplication of aseptic *Ilex crenata* 'Sky Pencil' seedlings.

Both IBA and NAA had significant impact on rooting of *Ilex crenata* 'Sky Pencil' seedling microshoots. The highest rooting rate (91.67%) was produced under 1/4 MS medium supplemented with IBA at $4.14 \mu\text{M}$. IBA at $8.29 \mu\text{M}$ and NAA at $4.46 \mu\text{M}$ also yielded acceptable rooting rates at 83.33 and

Fig. 1. Immature embryo of *Ilex crenata* 'Sky Pencil' seedling (A). Embryo germination on WPM after 2 weeks (B). Seedlings after growing 4 weeks in chamber (C). Transferring to greenhouse (D). Seedlings after growing in greenhouse for 2 months (E). Shoot proliferation on MS medium for 8 weeks (F, G). Root induction on 1/4 MS for 8 weeks (H, I, K). In vitro rooted plantlets in greenhouse for 4 months (J, L).

Table 1. Effect of basic media on in vitro embryo germination and seedling growth of *Ilex crenata* 'Sky Pencil' seedlings.

Medium	Embryo germination (%)		Seedling length (mm)	
	2 weeks	3 weeks	2 weeks	3 weeks
1/4 MS	79.17 a ^z	91.67 a	3.68 c	5.90 b
1/2 MS	45.83 b	58.33 b	3.71 bc	5.55 b
MS	73.33 ab	73.33 ab	6.09 a	8.02 a
WPM	75.00 a	83.33 ab	5.20 ab	7.29 ab

^zDifferent letters in each column indicate that they are significantly different ($\alpha = 0.05$) according to least significant difference.

MS = Murashige and Skoog; WPM = Woody Plant Medium.

79.17%. Without auxin (the control), the rooting rate was only 33.33%. Higher concentrations of IBA (8.29 μM) and NAA (8.92 μM) could decrease the rooting rate, while lower concentrations of IBA (2.07 μM) and NAA (2.23 μM) had less effectiveness in rooting. The results were in general agreement with previous reports on the rooting of English holly (Majada et al., 2000) and inkberry (Sun et al., 2010) (Table 4). The type and concentration of auxin also had a significant effect on root quality. As IBA concentrations increased, the number of roots increased from 7.07 to 13.30. Under NAA treatments, it increased first to 14.32 roots per microshoot, and then decreased to 6.87 roots (Table 4). The number of roots per microshoot on 1/4 MS with 8.29 μM IBA or 4.46 μM NAA was significantly higher than other treatments: ≈ 4.4 to 4.7 times more roots than the control. Shoots on 1/4 MS medium plus 4.46 μM NAA yielded the longest total root per shoot at 13.67 cm (Fig. 1I). IBA generally had a much more positive effect on the number of roots and total root length than that of NAA. Shoots on all media containing NAA produced a lot of calli before forming roots (Fig. 1K). This behavior was seen previously with inkberry (Sun et al., 2010). As a result of higher rooting rates and rooting quality, 1/4 MS medium with 4.14 μM IBA is most appropriate for in vitro rooting of 'Sky Pencil' seedlings.

All plantlets rooted in vitro were transplanted into a mixture of 3 L: perlite (1:1 by volume) and acclimated under a mist system. Sixty days after transplant, plantlets had well developed shoots with a great root system (Fig. 1J). An 88.8% survival rate was obtained.

In vitro embryo germination has great potential in *Ilex* breeding work, especially for those species with the longest duration of seed germination. First, embryo germination could save at least 2 years during the *Ilex* species' breeding cycle and also much better germination rates should be obtained. Second, compared with traditional seed germination, embryo germination techniques could also save labor and allow for better management practices. Third, inclimate weather and potential damage to highly valued hybrid seeds caused by pests and diseases during the long germination process can be eliminated.

In summary, a rapid propagation protocol for *Ilex crenata*, based on our results with seedlings from the cultivar 'Sky Pencil', is the following: 1) Collect embryos in August when fruits are green and have reached their full size. 2) Sterilize fruit surface using the above procedures. 3) Excise and inoculate embryos on MS medium with 3% sucrose and grow them under dark conditions for 2 weeks. 4) Move them to conditions with more light and grow them for 4 more weeks (Fig. 1C) right after embryo germination (Fig. 1B). 5) Wash seedlings and transplant them into growing media (Fig. 1D). 6) Select nodal segments from seedlings and conduct shoot multiplication and elongation of *Ilex crenata* with MS medium supplemented with 2.28

Table 2. Timing effect on in vitro embryo germination of *Ilex crenata* 'Sky Pencil' seedlings.

Treatment	Fruit color	Germination rate (%) ^z	Contamination rate (%)
July	Green	81.94 a ^y	22.22 ab
August	Green	88.89 a	0.00 b
September	Light purple	91.67 a	19.44 ab
October	Purple dark	91.67 a	33.33 a

^yExcluding the contamination embryos.^zDifferent letters in each column indicate that they are significantly different ($\alpha = 0.05$) according to least significant difference. Fig. 2. *Ilex crenata* 'Sky Pencil' seedling seed structure.Table 3. Effect of cytokinin type and concentration on in vitro shoot proliferation of *Ilex crenata* 'Sky Pencil' seedlings.

Hormone	Concn (μ M)	Ht (cm)	No. of shoots ^z	Mean shoot length (cm)
Control	0	0.79 cd ^y	1.00 d	0.79 c
6-BA	2.22	0.68 d	1.08 d	0.67 c
6-BA	4.44	0.73 d	1.31 cd	0.64 c
6-BA	8.88	0.75 cd	1.64 bcd	0.63 c
6-BA	17.76	0.72 d	1.00 d	0.72 c
ZT	2.28	1.82 ab	2.39 ab	1.35 a
ZT	4.56	1.33 bc	2.19 abc	0.98 bc
ZT	9.12	2.01 a	3.00 a	1.36 a
ZT	18.24	1.35 bc	1.40 cd	1.24 ab

^yOnly >5 mm long shoots were counted.^zDifferent letters in each column indicate that they are significantly different ($\alpha = 0.05$) according to least significant difference.

6-BA = 6-benzylaminopurine; ZT = zeatin, 4-hydroxy-3-methyl-trans-2-butenylaminopurine.

Table 4. Effect of auxin type and concentration on in vitro rooting of *Ilex crenata* 'Sky Pencil' seedlings.

Hormone	Concn (μ M)	Rooting rate (%)	No. of roots ^z	Root length (cm)
Control	0	33.33 b ^y	3.00 d	3.28 c
IBA	2.07	62.50 ab	7.07 cd	9.91 ab
IBA	4.14	91.67 a	10.64 abc	10.49 ab
IBA	8.29	83.33 a	13.30 ab	12.87 ab
NAA	2.23	50.00 ab	7.67 bcd	5.88 bc
NAA	4.46	79.17 a	14.32 a	13.67 a
NAA	8.92	62.50 ab	6.87 cd	6.16 bc

^yOnly >5 mm long roots were counted.^zDifferent letters in each column indicate that they are significantly different ($\alpha = 0.05$) according to least significant difference.

IBA = indole-3-butyric acid; NAA = 1-naphthaleneacetic acid.

μ M ZT for 8 weeks (Fig. 1G). 7) Regenerate roots on 1/4 MS medium with 4.14 μ M IBA (Fig. 1H) for 8 weeks. 8) Transplant the plantlets to growing media and grow them under the mist benches in a greenhouse (Fig. 1E and L). Living seedlings can then be transplanted into 1-gallon pots for further

evaluation, therefore saving 2 years of waiting during the breeding process.

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