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Non-deep simple and deep simple morphophysiological dormancy in seeds of three species of *Ilex* from subtropical and tropical regions of Taiwan

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Abstract

Dormancy-breaking requirements and level of morphophysiological dormancy (MPD) were determined for seeds of *Ilex formosana* and *I. uraiensis* from the subtropical region, and seeds of *I. rotunda* from both the subtropical and tropical regions of Taiwan. We hypothesized that some *Ilex* species would have deep simple MPD broken by warm stratification. Germination of seeds and embryo growth was monitored at 30/20, 25/15, 20/10, 15/5°C and at 25°C. Seeds were cold-stratified and then incubated at 25/15°C, and seeds treated with GA₃ and GA₄ were incubated at 25/15°C. Fresh seeds reached 50% germination after 11–45 weeks of warm stratification. Pre-treatment with GA increased germination percentages of *I. formosana* and *I. rotunda* (tropical) but not *I. uraiensis* and *I. rotunda* (subtropical), while cold stratification did not promote germination of either species but increased the germination rate of *I. rotunda* (tropical). Embryo length in seeds of all species increased $\geq 710\%$ prior to root emergence, and growth occurred during warm stratification. The positive response to GA and relatively short time for beginning of germination and to reach 50% germination indicate non-deep simple MPD in seeds of *I. formosana* and *I. rotunda* (tropical). The negative response to GA and long time for beginning of germination and to reach 50% germination indicate deep simple MPD in seeds of *I. uraiensis* and *I. rotunda* (subtropical). Thus, in both the subtropical and tropical regions of Taiwan, the seeds of *Ilex* species have non-deep simple and deep simple MPD that are broken by warm stratification. Furthermore, GA treatment increases the germination rate and percentage of *Ilex* seeds with non-deep simple MPD, and cold stratification promotes the seed germination rate of *Ilex* species with non-deep simple MPD in tropical region.

Introduction

An important adaptation of a plant species to its habitat is that seed germination occurs at a time when conditions are favourable for establishment and growth of the seedlings. Thus, an understanding of the geographical distribution of a species is enhanced by information on the kind of dormancy in freshly matured seeds and the environmental conditions required for dormancy-break and germination (Baskin and Baskin, 2014). Five classes of seed dormancy are recognized: physiological (PD, low growth potential of the embryo due to a physiological inhibiting mechanism), morphological (MD, small, underdeveloped embryo that needs to grow inside the seed prior to germination), morphophysiological (MPD, a combination of MD and PD), physical (PY, water-impermeable seed/fruit coat) and combinational (PY and PD) (Nikolaeva, 1969; Baskin and Baskin, 2004).

The breaking of MPD (the class of dormancy of concern in this paper) requires exposure of seeds to environmental conditions that promote growth of the embryo and the breaking of PD. For seeds with differentiated small (underdeveloped) embryos, nine levels of MPD have been distinguished based on temperature requirements for growth of the embryo, breaking of physiological dormancy (PD) and emergence of the radicle and shoot and on responses of seeds to gibberellic acid (Baskin and Baskin, 2014).

The genus *Ilex* L. (Aquifoliaceae) consists of about 600 species and is distributed in Asia, Europe, North and South America, northern Australia, a few Islands in the Pacific Ocean, Madagascar and sub-Saharan Africa (Cuénoud et al., 2000; Loizeau et al., 2005). Using two nuclear genes and calibrating with three macrofossil records, Yao et al. (2021) concluded that *Ilex* originated in southeast Asia with a Late Cretaceous stem age of 84.7 million years

ago (Ma) and the crown clade in the Eocene at 50.8 Ma. Furthermore, clades of *Ilex* had become established in South America and North America by 30.4 and 23.1 Ma, respectively.

Seeds of all *Ilex* species studied thus far have MPD (Ives, 1923; Barton and Thornton, 1947; Hu, 1975; Nikolaeva et al., 1985; Ng, 1991; Young and Young, 1992; Tsang and Corlett, 2005; Chien et al., 2011; Tezuka et al., 2013). Non-deep simple, intermediate simple and deep simple levels of MPD have been identified in *Ilex*, and two ways by which deep simple MPD is broken in nature have been identified. Non-deep simple MPD, for example *I. maximowicziana*, is broken by 12–16 weeks of warm ($\geq 15^{\circ}\text{C}$) stratification (Chien et al., 2011), and intermediate simple MPD, for example *I. dumosa*, is broken by a period of dry storage at room temperatures (after-ripening) followed by 18 weeks of warm stratification (Galíndez et al., 2018). Deep simple MPD in temperate regions, for example *I. opaca*, is broken by warm stratification followed by cold (*c.* $0\text{--}10^{\circ}\text{C}$) stratification (Ives, 1923; Barton and Thornton, 1947), while deep simple MPD in subtropical regions, for example *I. paraguariensis*, is broken by warm stratification for 12–40 or more weeks (Galíndez et al., 2018).

Loizeau et al. (2005) noted that additional research is needed to fully understand the distribution patterns of *Ilex*. These authors emphasized the need for more information on species variability and ecological niche, which includes seed dormancy/germination, that is, the level of MPD. In particular, we are interested in deep simple MPD broken by warm followed by cold stratification in temperate regions vs deep simple MPD broken by only warm stratification in tropical/subtropical regions. In the subtropical region of Argentina, seeds of *I. argentina* and *I. paraguariensis* have deep simple MPD that is broken by extended periods of warm stratification (Galíndez et al., 2018). However, this requirement to break deep simple MPD has not been reported for any *Ilex* species in other subtropical/tropical regions.

To enhance our knowledge of MPD in seeds of the large genus *Ilex*, we conducted studies on three species of this genus from Taiwan. Based on the occurrence of *Ilex* in the subtropical/tropical regions of Taiwan (Lu, 1993), we hypothesized that some species of *Ilex* in subtropical/tropical Taiwan have deep simple MPD that is broken only during prolonged periods of warm stratification. To test this hypothesis, we determined the level of MPD in seeds of *I. formosana*, *I. rotunda* and *I. uraiensis* collected in Taiwan. Seeds of *I. formosana* and *I. uraiensis* were collected in the subtropical zone, while those of *I. rotunda* were collected in both the subtropical and tropical zone. To determine the level of MPD in the seeds, we investigated the temperature requirements for embryo growth and germination and the effects of cold stratification and gibberellins on germination.

Materials and methods

Experimental materials

The evergreen trees *I. formosana* and *I. uraiensis* are distributed in subtropical (northern) and central Taiwan at 300–1000 m a.s.l., and those of *I. rotunda* grow throughout Taiwan at 100–1000 m a.s.l. (Hu, 1953; Lu, 1993). Mature, red fruits of *I. formosana* and *I. uraiensis* were harvested in Yangmingshan and Wulai, subtropical (northern) Taiwan, at 700 and 200 m a.s.l., respectively, on 30 January 2012 and 17 January 2013, respectively. Mature, red fruits of *I. rotunda* were harvested on 17 January 2013 in Yangmingshan, subtropical (northern) Taiwan, at the same site where those of *I. formosana* were collected. In addition, seeds of

I. rotunda were collected on 6 December 2012 in Manjhong Township, Pingtung County, the tropical region (southern tip of Taiwan) at 100 m a.s.l. Approximately 1500 matured fruits were harvested from three to four trees each *Ilex* species. All fresh fruits were kept in laboratory (temperature about 25°C) for 2–3 d before the pulp was removed. We examined 50 fruits each of *I. formosana* and *I. uraiensis* and found an average of 3.9 ± 0.2 and 4.0 ± 0 seeds (drupes) per fruit, respectively. Fifty fruits of *I. rotunda* from subtropical and tropical Taiwan had an average of 5.3 ± 0.5 and 4.7 ± 0.5 seeds per fruit, respectively. There were about 75,100 and 19,700 seeds per litre of *I. formosana* and *I. uraiensis*, respectively, and for subtropical and tropical populations of *I. rotunda*, 196,750 and 148,500 seeds per litre, respectively (Fig. 1).

The germination unit (hereafter the seed) is the true seed covered by a hard but water-permeable endocarp. Seeds were extracted from fruits by removing the thin exocarp and fleshy mesocarp, using a sieve and running water. The cleaned seeds that sank in water were retained, while those that floated were discarded. Seeds were air-dried on paper at room temperature for 1 d and then temporarily stored in a sealed polyethylene bag at 5°C for 2–3 d. Moisture content (fresh weight basis) of seeds of *I. formosana*, *I. uraiensis*, *I. rotunda* (subtropical) and *I. rotunda* (tropical) was 6.4 ± 0.5 , 11.5 ± 0.7 , 8.0 ± 0.4 and $7.5 \pm 0.2\%$, respectively, as determined using the oven drying (17 h at 103°C) method (International Seed Testing Association, 2007).

Effect of temperature on germination of fresh seeds

Seeds of *I. formosana*, *I. uraiensis* and *I. rotunda* (subtropical) and *I. rotunda* (tropical) (hereafter, the four collections are referred to as the *Ilex* species) were mixed with small pieces of moist sphagnum moss (about 400% of its dry mass) and sealed inside transparent polyethylene bags (0.04 mm thick). The dry sphagnum moss was washed with running water three times before use. Moist sphagnum moss provides a good germination medium and contains the fungi *Trichoderma* and actinomycetes that are antagonistic to growth of seed-destroying microorganisms (Wang et al., 1998). Seeds were incubated in light/dark (12 h day/12 h night) at 30/20, 25/15, 20/10, 15/5°C and at 25°C for 30–74 weeks. The light source in the incubators (Saint Tien Co., Ltd, Taiwan) was cool white fluorescent tubes, and photon irradiance (400–700 nm) was about $60\text{--}80 \mu\text{mol m}^{-2} \text{s}^{-1}$. Each treatment consisted of three replications of 50 seeds each. Germination (root emergence ≥ 2 mm) was recorded weekly (presented at 1- or 2-week interval). Results were expressed as mean germination percentage and 95% binomial confidence interval (CI).

Effect of GA₃ and GA₄ on germination

Fresh seeds of the *Ilex* species were soaked in double-distilled water (ddH₂O) and in 250, 2500 and 5000 μM solutions of GA₃ (potassium salt, 95% purity, Sigma, St Louis, Missouri, USA) and GA₄ (90% purity, from Professor Lewis N. Mander, Australian National University) for 24 h at room temperature (about 25°C) prior to incubation. After soaking, the seeds were mixed with moist sphagnum moss and incubated at $25/15^{\circ}\text{C}$ for 12–16 weeks. Germination was monitored weekly. Each treatment consisted of three replications of 50 seeds each. Results were expressed as mean germination percentage and 95% binomial CI.

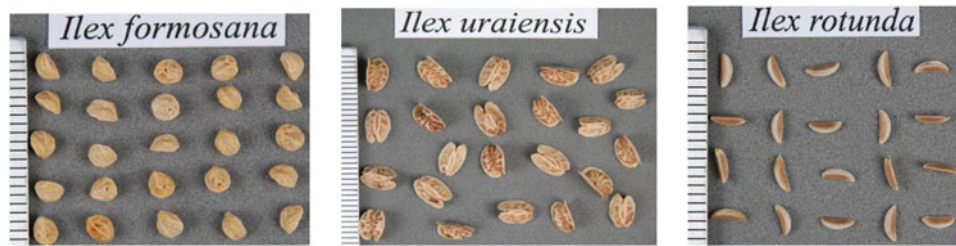


Fig. 1. Intact seeds (endocarp with seed inside) of *Ilex formosana*, *I. uraiensis* and *I. rotunda*. The scale is in mm.

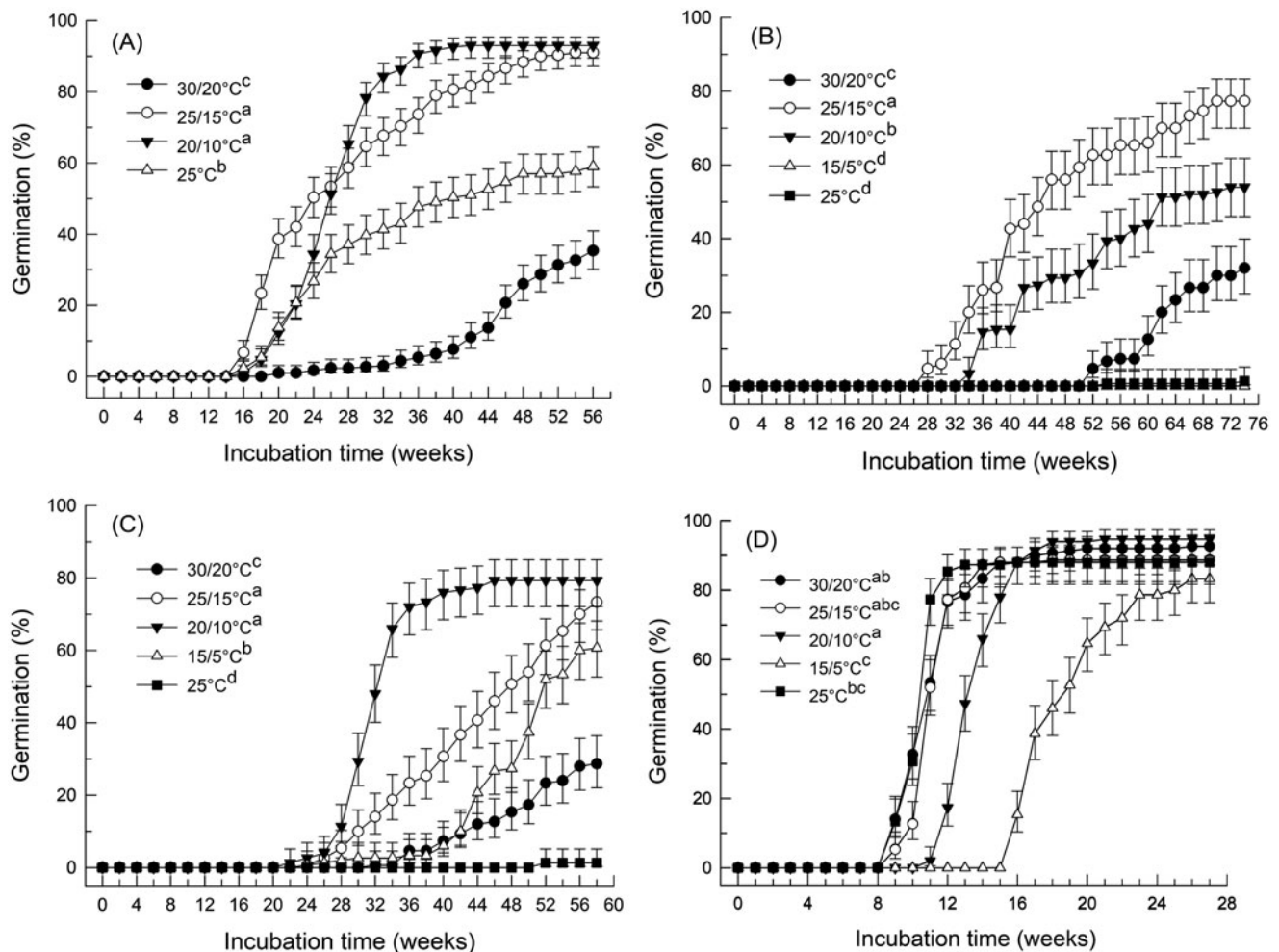


Fig. 2. Cumulative germination percentages (means and 95% binomial confidence intervals) of fresh seeds of *Ilex formosana* (A) and *Ilex uraiensis* (B) from subtropical Taiwan and *Ilex rotunda* from subtropical (C) and tropical (D) Taiwan incubated at various temperatures. Final seed germination percentages among the incubation temperatures followed by different letters differ significantly (Sidak test, $\alpha = 0.05$).

Embryo growth

To determine if embryos are underdeveloped (i.e. grow inside the seeds prior to root emergence), we measured seed and embryo length of fresh seeds and of seeds with a split seed coat, which indicated that the radicle was about to emerge. Fresh seeds of the *Ilex* species were mixed with moist sphagnum moss in sealable polyethylene bag and incubated at 30/20, 25/15 and 20/10°C. Seeds were examined at 2-week intervals, at which time 10 seeds of the *Ilex* species at each temperature regimes were cut open longitudinally using a razor blade. Length of the seed and

of the embryo was measured using a dissecting microscope equipped with a calibrated micrometer, and photographs were taken. Embryo (E) length:seed (S) length ratio (E:S ratio) was calculated for each seed.

Embryo growth in seeds of *I. uraiensis* was so slow that the seed supply was exhausted in 2013 before embryo growth could be documented. Thus, we harvested more fruits of the species at the same location in 2014. The seeds were incubated in the following sequence: 30/20°C for 8 weeks → 25/15°C for 8 weeks → 20/10°C for 8 weeks → 15/5°C for 4 weeks → 5°C for

Table 1. Effect of GA₃ and GA₄ on germination percentages (mean and 95% binomial confidence interval [CI]) of *Ilex formosana* seeds after 8 and 16 weeks incubation at 25/15°C

Treatments	8 weeks		16 weeks	
	Mean	95% CI	Mean	95% CI
ddH ₂ O (control)	0	0/0	6.7d	4.3/10.1
250 μM GA ₃	0	0/0	12.0c	8.8/16.2
2500 μM GA ₃	0	0/0	31.0b	26.0/36.5
250 μM GA ₄	0	0/0	43.3a	37.8/49.0
2500 μM GA ₄	0	0/0	48.0a	42.4/53.7

Means ($n = 3$) in a column followed by different letters differ significantly (Sidak test, $\alpha = 0.05$).

4 weeks. This temperature sequence occurs in the natural distributional range of the species. Ten seeds were cut open every 2–8 weeks and length of seed and embryo measured and E:S ratio calculated.

Effect of cold stratification on seed germination

Seeds of the *Ilex* species were placed in sealed polyethylene bags with moist sphagnum moss and stratified at 5°C in a dark cold room for 0, 4, 8 and 12 weeks. In addition, the cold stratification period for seeds of *I. rotunda* was extended to 16, 34 and 52 weeks. Cold-stratified seeds of the *Ilex* species were incubated in light at 25/15°C for a maximum of 52 weeks. Germination was recorded weekly (presented at 1- or 2-week interval), and results were expressed as mean germination percentage and 95% binomial CI (three replications with 50 seeds each) after roots grew to ≥ 2 mm long (based on the number of incubated seeds).

Table 2. Effect of GA₃ and GA₄ on germination percentages (mean and 95% binomial confidence interval [CI]) of *Ilex rotunda* seeds from subtropical and tropical regions of Taiwan after 4, 8 and 12 weeks incubation at 25/15°C

Treatments	4 weeks		8 weeks		12 weeks	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
Treatments (Seeds from subtropical region)						
ddH ₂ O (control)	0	0	0	0	0	0
250 μM GA ₃	0	0	0	0	0	0
2500 μM GA ₃	0	0	0	0	0	0
5000 μM GA ₃	0	0	0	0	0	0
250 μM GA ₄	0	0	0	0	0	0
2500 μM GA ₄	0	0	0	0	0	0
5000 μM GA ₄	0	0	0	0	0	0
Treatments (Seeds from tropical region)						
ddH ₂ O (control)	0	0	8.0d	4.6/13.6	50.7c	42.7/58.6
250 μM GA ₃	0	0	13.3cd	8.8/19.8	70.7ab	62.9/77.4
2500 μM GA ₃	0	0	19.3c	13.8/26.4	60.4b	56.0/71.3
250 μM GA ₄	0	0	37.3b	30.0/45.3	74.7a	67.1/81.0
2500 μM GA ₄	0	0	61.3a	53.3/68.8	78.7a	71.4/84.5

Means ($n = 3$) in a column followed by different letters differ significantly (Sidak test, $\alpha = 0.05$).

Climate in northern and southern Taiwan

Average daily temperature regimes in Yangmingshan, Taipei city, subtropical (northern) Taiwan are 28/21 to 30/23°C in summer (June–August) and 16/10 to 17/11°C in winter (December–February) (<https://www.whereandwhen.net/when/east-asia/taiwan/yangmingshan-national-park>). Average daily temperature regimes in Manzhou township, Pingtung county, tropical (southern) Taiwan are 30/25 to 31/26°C in summer and 23/18 to 24/19°C in winter (<https://www.accuweather.com/en/tw/manzhou-township/314395/november-weather/314395>). The distance between Yangmingshan and Manzhou township is about 390 km.

Statistical analysis

Statistical analyses of seed germination percentages from different temperatures, GA₃ and GA₄ treatments, and moist cold stratification were carried out using the generalized linear model (GZLM) with logit link function and binomial distribution. Moreover, the embryo length and E:S ratio data were carried out using the GZLM with identity link function and normal distribution. The statistical significance was tested using Wald test with *post hoc* Sidak test ($\alpha = 0.05$). All statistical analyses were performed using SPSS Statistics 20 (IBM Corp., Armonk, NY, USA).

Results

Effect of temperature on germination of fresh seeds

Fresh seeds of *I. formosana* began to germinate after 14 weeks of incubation, and germination increased slowly thereafter. After 50 weeks of incubation, 90 and 88% of the seeds had germinated at 25/15 and 20/10°C, respectively, but at 25 and 30/20°C only 55 and 25% of the seeds, respectively, had germinated (Fig. 2A). Fresh seeds of *I. uraiensis* began to germinate after 26 weeks at

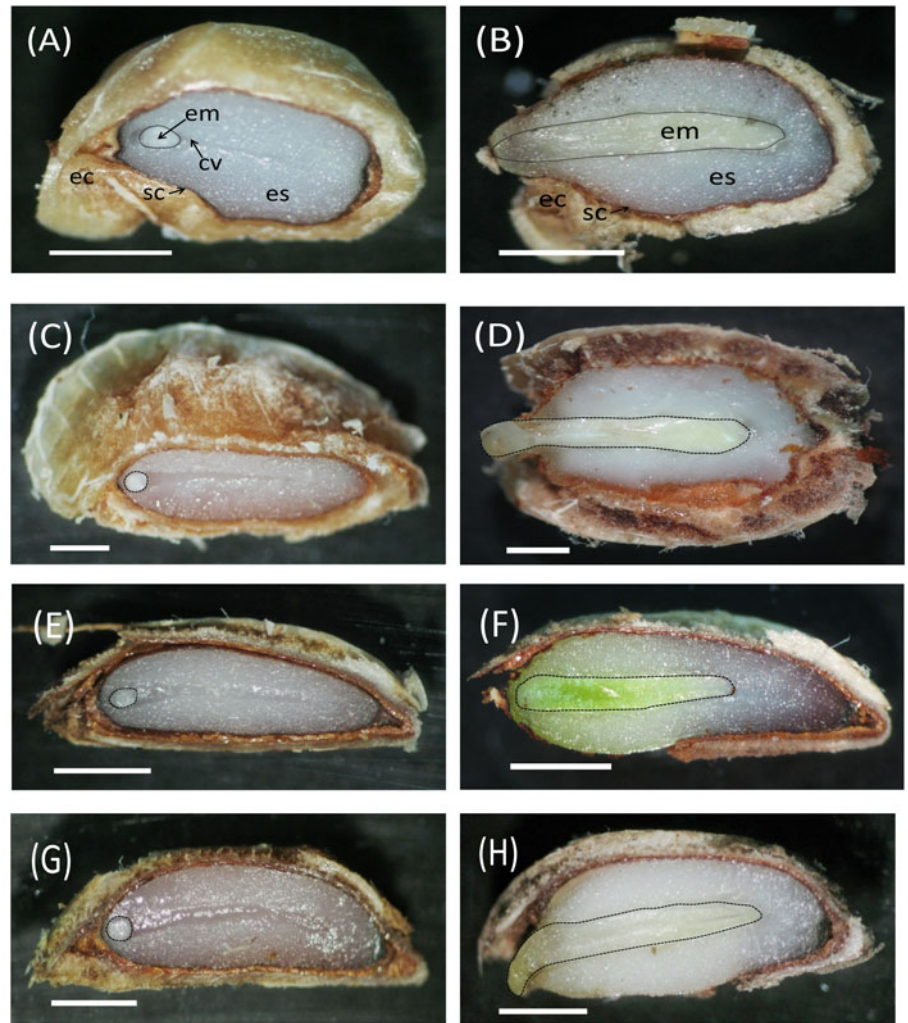


Fig. 3. Embryo growth in seeds of *Ilex formosana* (A, B) and *Ilex uraiensis* (C, D) from subtropical Taiwan and *Ilex rotunda* from subtropical (E, F) and tropical (G, H) Taiwan. Longitudinal sections of fresh seeds with a small, underdeveloped embryo (A, C, E, G) and fully developed embryos (B, D, F, H) are shown. The scale is 1 mm. Embryo (em) in cavity (cv), endosperm (es), seed coat (sc), endocarp (ec).

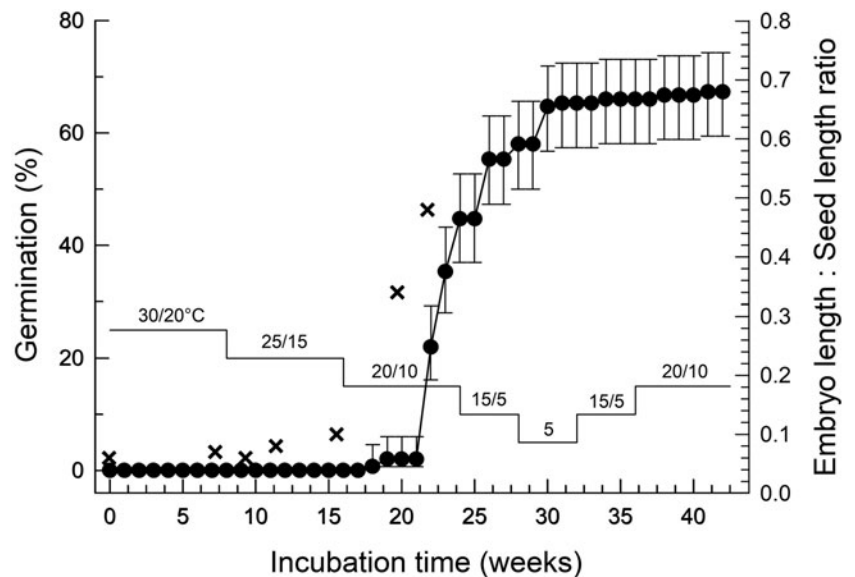


Fig. 4. Cumulative seed germination (●) (mean and 95% binomial confidence interval) and embryo (E):seed (S) length ratio (x) of *Ilex uraiensis* seeds incubated in a sequence of temperature regimes beginning at 30/20°C. The E:S ratio in fresh seeds was 0.06 ± 0.01 , and it reached a maximum of 0.48 ± 0.05 during incubation at 20/10°C.

25/15°C, and after 70 weeks germination was 77, 50, 27, 0.7 and 0% at 25/15, 20/10, 30/20, 25 and 15/5°C, respectively (Fig. 2B). Fresh seeds of *I. rotunda* (subtropical) began to germinate after

20 weeks at 20/10°C, and after 50 weeks germination was 80, 50, 37, 17 and 0% at 20/10, 25/15, 15/5, 30/20 and 25°C, respectively (Fig. 2C). Fresh seeds of *I. rotunda* (tropical) began to

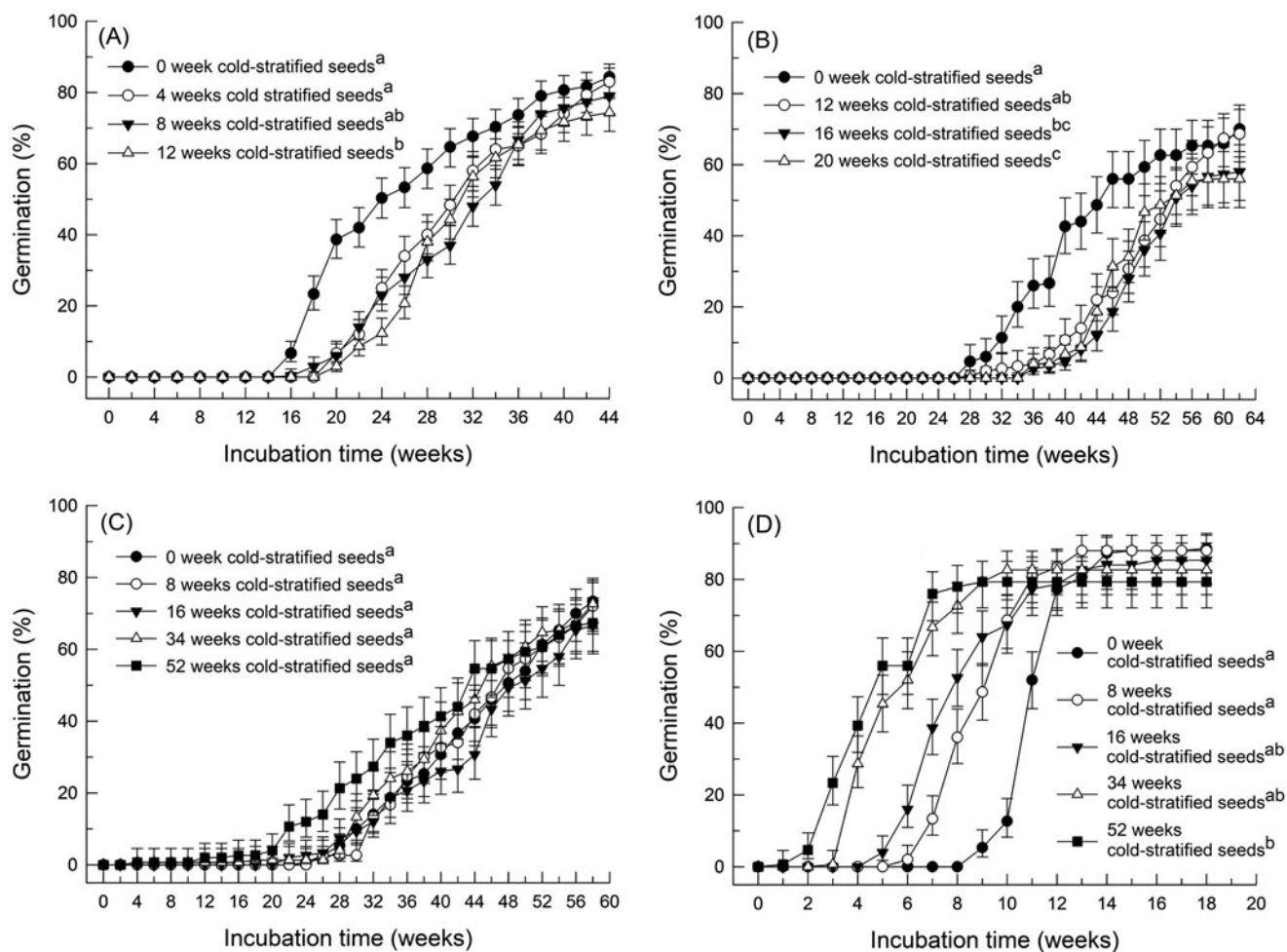


Fig. 5. Effect of moist cold stratification at 5°C on seed germination (means and 95% binomial confidence intervals) of *Ilex formosana* (A) and *Ilex uraiensis* (B) from subtropical Taiwan and *Ilex rotunda* from subtropical (C) and tropical (D) Taiwan. Final seed germination percentages among the different cold stratification times followed by different letters differ significantly (Sidak test, $\alpha = 0.05$).

germinate after 8 weeks at 25, 30/20 and 25/15°C, and after 14 weeks germination was 87, 83, 80, 46 and 0% at 25, 30/20, 25/15, 20/10 and 15/5°C, respectively (Fig. 2D).

Effect of GA₃ and GA₄ on germination

Pre-treatment with 250, 2500 μ M GA₃ and GA₄ significantly increased the germination percentages of *I. formosana* seeds during 16 weeks of incubation, and seeds treated with GA₄ germinated to 43.3–48.0%, which is significantly higher than that of seeds treated with GA₃ (Table 1). Regardless of GA treatment, no seeds of *I. uraiensis* had germinated after 16 weeks (data not shown). After 12 weeks of incubation, no GA-treated seeds of *I. rotunda* (subtropical) had germinated, while seeds of *I. rotunda* (tropical) had germinated to 50.7% in the control and to 60.4–78.7% in the GA treatments (Table 2).

Embryo growth

In fresh seeds of *I. formosana*, mean (\pm SE) embryo length was 0.27 \pm 0.04 mm and E:S ratio was 0.09 \pm 0.01 (Fig. 3A and see supplementary Table S1). After 18 weeks when the endocarps split just prior to radicle emergence, length of the embryo was

1.45 \pm 0.52 mm and the E:S ratio was 0.44 \pm 0.15 mm (Fig. 3B and see supplementary Table S1). In fresh seeds of *I. uraiensis*, mean (\pm SE) embryo length was 0.43 \pm 0.09 mm, and growth did not occur until seeds were transferred to 20/10°C (Figs 3C and 4). After 8 weeks at 25/15°C, embryos grew slowly, and embryo length reached its maximum of 3.14 \pm 0.28 mm (730% increase in length) with an E:S ratio of 0.48 \pm 0.05 mm at 20/10°C (Figs 3D and 4). Seed germination also occurred at 20/10°C and rapidly increased to 45%. When seeds were moved from 20/10 to 15/5°C, germination increased to 58%, and it increase to 65% when seeds were moved from 15/5 to 5°C (Fig. 4).

In fresh seeds of *I. rotunda* (subtropical), mean (\pm SE) embryo length was 0.27 \pm 0.02 mm and the E:S ratio was 0.07 \pm 0.01 (Fig. 3E and see supplementary Table S2). After 28 weeks when the endocarps split, length of the embryo was 1.38 \pm 0.35 mm, and the E:S ratio was 0.35 \pm 0.10 mm (Fig. 3F and see supplementary Table S2). For *I. rotunda* (tropical), mean (\pm SE) embryo length in fresh seeds was 0.27 \pm 0.04 mm and the E:S ratio was 0.06 \pm 0.01 (Fig. 3G and see supplementary Table S3). At 16 weeks when the endocarps split, length of the embryos was 2.17 \pm 0.33 mm, and the E:S ratio was 0.48 \pm 0.09 mm (Fig. 3H and see supplementary Table S3).

Table 3. Summary of the dormancy-breaking and germination requirements of seeds of three *Ilex* species from Taiwan

Responses of seeds	<i>I. formosana</i>	<i>I. uraiensis</i>	<i>I. rotunda</i> (subtropical)	<i>I. rotunda</i> (tropical)
Fresh seeds				
Optimum germination temp. (°C)	25/15, 20/10	25/15	20/10 ^a	25/15, 30/20
Weeks to first germination at optimum temperature	14	26	20	8
Weeks to 50% germination	24	45	32	11
Embryo growth at $\geq 15^{\circ}\text{C}^{\text{b}}$	Yes	Yes	Yes	Yes
Dormancy class	MPD	MPD	MPD	MPD
Simple MPD	Yes	Yes	Yes	Yes
Complex MPD	No	No	No	No
GA promoted germination at 12 or 16 weeks	Yes	No	No	Yes
Warm stratification at 25/15°C				
Increased germination %	Yes	Yes	Yes	Yes
Cold stratification (12 or 16 weeks)				
Increased germination %	No	No	No	No
Increased germination speed	No	No	No	Yes
Level of MPD	Non-deep	Deep	Deep	Non-deep

^aReached 50% germination at 25/15°C at week 48.

^bAs shown by seed germination at 25/15°C.

Effect of cold stratification on seed germination

Germination of *I. formosana* seeds cold-stratified at 5°C for 4, 8 and 12 weeks was delayed compared with seeds without moist cold stratification (Fig. 5A). Similar results were found for seeds of *I. uraiensis* and those of *I. rotunda* (subtropical) (Fig. 5B, C). However, cold stratification increased the speed of germination of *I. rotunda* (tropical) (Fig. 5D).

Discussion

Seeds of *I. formosana*, *I. uraiensis* and *I. rotunda* required >30 d to germinate, and embryos in seeds of each species increased in length by 710% or more before radicle emergence. Thus, seeds of the three *Ilex* species have MPD, as expected. The question we need to answer is: do seeds have a simple or complex level of MPD? In seeds with a simple level of MPD, embryo growth occurs at $\geq 15^{\circ}\text{C}$, that is, during warm stratification, while in those with a complex level of MPD embryo growth occurs at $\leq 10^{\circ}\text{C}$, that is, during cold stratification (Baskin and Baskin, 2014). Since embryo growth and germination occurred at warm-stratifying temperatures (e.g. 25/15°C), seeds of the three species have a simple level of MPD.

The non-deep, intermediate and deep levels of simple MPD are distinguished by the depth of the PD component of MPD, that is, non-deep, intermediate and deep PD, respectively. Non-deep PD is broken by warm or cold stratification (depending on the species), seeds usually germinate when treated with GA and may come out of dormancy during dry storage of room temperatures (after-ripening). Intermediate PD is broken by extended periods of cold or warm stratification (depending on species), but a period of after-ripening will decrease the length of the cold or warm stratification period required to break dormancy. GA may, or may not, break intermediate PD. Deep PD is broken by several months of cold or warm stratification (depending on species), and GA does not break dormancy (Nikolaeva, 1969).

A comparison of the dormancy-breaking and germination requirements of the three *Ilex* species (Table 3) leads to the conclusion that seeds of *I. uraiensis* and *I. rotunda* (subtropical) have deep simple MPD that is broken by long periods of warm stratification, like seeds of *I. argentina* and *I. paraguayense* from the subtropical zone of Argentina (Galíndez et al., 2018). Thus, our hypothesis that some *Ilex* species from subtropical zones of Taiwan have deep simple MPD broken only by warm stratification is supported. However, seeds of *I. formosana* (subtropical) and those of *I. rotunda* (tropical) have non-deep simple MPD broken by relatively short periods of warm stratification compared with *I. uraiensis* and *I. rotunda* (subtropical). Yang et al. (2014) reported that seeds of *I. asprella* harvested from northern, central and southern Taiwan had begun to germinate after 10–14 weeks and the suitable temperature was 20/10°C. Moist cold stratification and GA treatment increased seed germination percentage and rate, suggesting that seeds of *I. asprella* have non-deep simple MPD. Seeds of *I. maximowicziana* from both northern and southern regions of Taiwan had non-deep simple MPD (Chien et al., 2011). Thus, in the subtropical and tropical regions of Taiwan seeds, some *Ilex* species have non-deep simple MPD, and seeds of other species have deep simple MPD. The dormancy formula for non-deep simple MPD is $C_{1b}B_b$, and that for deep simple MPD is $C_{1b}B_b-C_{3b}$, where (for both formulas) B is underdeveloped embryo; C, physiological dormancy; subscript b, exposure to warm temperature is required for dormancy break (C_{xa} or C_{xb}) or embryo (B) grows during exposure to warm (B_b) temperatures; and subscripts 1 and 3, non-deep and deep physiological dormancy, respectively (Baskin and Baskin, 2021).

Temperatures of the tropical region of Taiwan are higher than those in the subtropical region. Also, the tropical region has a dry season from October to April or May, but there is some rainfall during this time in the subtropical zone (see Fig. 6 in Chien et al., 2011). Clearly, some *Ilex* species with non-deep simple MPD and deep simple MPD occur in the relatively cool, mesic subtropical

region and others with non-deep simple and deep simple MPD occur in the relatively warm, seasonally dry tropical zone. Thus, the climate of both regions is suitable for the persistence of *Ilex* species with either non-deep simple or deep simple MPD.

In their consideration of phylogeny and biogeography of *Ilex*, Yao et al. (2021) concluded that moisture was more important than temperature in determining the geographical range of *Ilex* clades. Further, these authors suggested that *Ilex* has increased its tolerance of low temperature but not low soil moisture. One possible adaptation to relatively short periods of moist soil is the rapid breaking of the PD component of MPD, that is, non-deep PD. On the other hand, the requirement for warm followed by cold stratification to break the PD component of deep simple MPD in temperate regions (Ives, 1923; Barton and Thornton, 1947; Nikolaeva et al., 1985) likely is an adaptation for growth of some *Ilex* species in climates with cold winters. Furthermore, since *Ilex* originated in subtropical/tropical southeast Asia long before clades of *Ilex* became established in North America with a temperate climate, we might conclude that deep simple MPD broken by warm followed by cold stratification is more recent than deep simple MPD being broken by extended periods of warm stratification. However, it should be noted that when seeds of *I. uraiensis* were given warm stratification at 30/20 and 25/15°C and then moved to 20/10°C they reached c. 45% germination on week 24 of imbibition, whereas seeds incubated continuously at 20/10°C did not reach 45% germination until week 60. These results for *I. uraiensis* seem to suggest that while deep simple MPD of some *Ilex* species in subtropical/tropical regions is broken by extended periods of warm stratification, some cold stratification after several weeks of warm stratification may speed up dormancy break and germination.

Seeds of *I. rotunda* at subtropical Taiwan mature and are dispersed in winter (December through January). No embryo growth or seed germination occurred at the cold temperature but did occur with the onset of warm spring temperatures. However, seeds of *I. rotunda* in tropical (southern) Taiwan also mature and are dispersed in winter (November through December), but they receive warm temperatures after dispersal because the temperature is not below 10°C in this region. Thus, we speculate that seeds of *I. rotunda* from subtropical Taiwan may have adapted to variable winter conditions with the cold stratification to prevent embryo growth and seed germination; however, seeds of *I. rotunda* from tropical Taiwan have not been affected by the cold stratification with embryo growing continuously to final germination.

Supplementary material. To view supplementary material for this article, please visit: <https://doi.org/10.1017/S0960258523000016>.

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