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Factors Affecting Seed Germination of *Ilex latifolia* and *I. rotunda*

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***Abstract*. Mature seeds of *Ilex* species usually contain immature embryos and are extremely difficult to germinate. *Ilex latifolia* and *I. rotunda*, two species that are grown as ornamentals, also produce seeds that are difficult to germinate. In the present study, we investigated some factors affecting seed germination in those species. Although seeds of *I. latifolia* and *I. rotunda* could imbibe water, they did not germinate. When embryos were cultured in vitro, germination was observed in *I. latifolia* but not in *I. rotunda*. Interestingly, a transient decrease in germination frequency occurred in *I. latifolia* embryos isolated from seeds collected in September or October. Among five types of *I. latifolia* seeds that differed in the presence of the endocarp, testa, and endosperm, germinability of isolated embryos was highest. Good germination was also observed in quarter-seeds with or without endocarp followed by half-seeds. Treatment of seeds with sodium hydroxide (NaOH) had no effect on seed germination in *I. latifolia*. Cold stratification at 5 °C increased the germination frequency of *I. latifolia* embryos. In conclusion, the data suggested a mechanical barrier by the endocarp and inhibitors contained in the endosperm, testa, and/or endocarp inhibited seed germination in *I. latifolia*. Although no seeds or embryos of *I. rotunda* germinated, cold stratification in combination with other treatments deserves further investigation. Chemical name: sodium hydroxide (NaOH).**

The genus *Ilex* (Aquifoliaceae) consists of more than 500 species of dioecious trees and shrubs distributed throughout the temperate and tropical regions of the world (Galle, 1997). Some species are grown as ornamentals. In Japan, *Ilex latifolia* is planted in temples and shrines, and *I. rotunda* is grown as garden, park, and street trees. *Ilex latifolia* and *I. rotunda* (Goi et al., 1978) flower in May and June, and their fruits mature to red in the fall (around November). Leaves of *I. latifolia* contain high levels of flavonoids and have been used for beverage tea in China (Liang et al., 2001).

Although vegetative propagation methods such as tissue culture are sometimes used to propagate *Ilex* species (Luna et al., 2003; Mattis et al., 1995; Rey et al., 2002), the simplicity of reproduction through seeds has advantages for mass propagation. However, *Ilex* seeds are difficult to germinate, because embryos in mature seeds are usually at the immature heart or torpedo stage (Chien et al., 2011; Hu et al., 1979; Tsang and Corlett, 2005). This immaturity is thought to be caused by dormancy rather than extremely slow growth (Hu et al., 1979).

Embryo culture techniques have been used to overcome seed dormancy (Biggs et al., 1986; Chee, 1994; Raghavan, 2003; Sharma et al., 1996) and to shorten germination time (Acebedo et al., 1997; Tezuka et al., 2012) in many plant species. Seedlings have also been obtained for several *Ilex* species by culturing immature embryos (Hu, 1975; Mattis et al., 1995; Sansberro et al., 1998, 2001). Hu et al. (1979) reported that when isolated embryos of *I. aquifolium*, *I. cornuta,* and *I. opaca* were cultured in vitro, most of them developed at the mature stage and germinated, whereas embryos with endosperm showed only slight embryonic growth. Based on these results, they suggested that inhibitors responsible for maintaining embryo dormancy in the seed exist in the endosperm and/or membrane-like testa attached to the endosperm. Conversely, in *I. paraguariensis*, no difference was observed in germination frequency between embryos and bisected pyrenes (seeds), suggesting that the endosperm did not inhibit embryo development or germination (Dolce et al., 2010). In *I. dumosa*, germination frequency of cut pyrenes was higher than that of embryos (Dolce et al., 2011). Therefore, different mechanisms may be involved in seed dormancy among *Ilex* species.

The main objective of the present study was to identify factors affecting seed germination in *I. latifolia* and *I. rotunda*. We investigated the germination capacity of seeds and embryos of those species from fruits collected in different months. We also investigated the effect of the endosperm, testa, and endocarp on germination using cut seeds. In addition, we tested seed water permeability and the effect of several treatments on seed germination, including NaOH (Crisosto and Sutter, 1985; Sun et al., 2006), gibberellic acid (GA_3; Chen et al., 2008, 2010; Nicolás et al., 1996), and a moist, cold storage (cold stratification; Abe and Matsunaga, 2011; Chien et al., 1998; Sun et al., 2006; Tsuyuzaki and Miyoshi, 2009), all of which have been used to break seed dormancy in many plant species.

Materials and Methods

Plant materials. Fruits were harvested from *I. latifolia* and *I. rotunda* plants grown in a field at Osaka Prefecture University, Osaka, Japan. Seeds were washed with water to remove the fruit pulp before use in the experiments.

Germination test of seeds and embryos. Fruits of *I. latifolia* and *I. rotunda* were collected on 6 Feb. 2007. Non-sterilized seeds were sown in petri dishes that contained wet filter papers and were incubated at 15 °C in the dark. After two months (9 Apr.), the seeds were then transferred to a 288-cell tray (10 mL/cell) filled with wet vermiculite and placed in a greenhouse. Seeds were observed for emergence for one year after sowing.

To culture embryos, *I. latifolia* seeds collected on 9 Jan. 2007 and *I. rotunda* seeds collected on 12 Jan. 2007 were sterilized with 70% ethanol for 30 s and then with 5% sodium hypochlorite for 15 min. Embryos were excised from the sterilized seeds, placed in flat-bottomed test tubes (25 mm diameter, 100 mm length) that contained 8 mL of plant growth regulator-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.85% agar (pH 5.8) and that were autoclaved for 15 min at 121 °C, and then incubated at 25 °C in the dark. Embryos were observed for germination for six months.

Effect of seed developmental stage on germination capacity. Fruits collected at monthly intervals in two seasons (July 2006 to Feb. 2007 and July to Dec. 2007; see Table 2) were used to investigate whether the germination capacities of seeds and embryos were correlated with the seed developmental stage. In the 2006–07 season, non-sterilized seeds were sown in petri dishes containing wet filter paper and were incubated at 25 °C in the dark. Other seeds (except *I. latifolia* seeds collected on 18 July) were used for embryo culture at 25 °C in the dark as described previously. We could not isolate embryos from *I. latifolia* seeds collected on 18 July because the embryos were immature and too small to excise. Therefore, those seeds were cut in half, and the half-seeds with embryos (Fig. 1B) were aseptically sown. In the 2007 season, non-sterilized seeds were sown as described previously and incubated at 15 °C in the dark. Embryos were excised from the sterilized seeds (excluding *I. latifolia* seeds collected on 20 July), placed in flat-bottomed test tubes that contained MS medium supplemented with the cytokinin 6-benzyladenine (BA; 0 or 1 $mg \cdot L^{-1}$), the auxin 1-naphthaleneacetic

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Table 1. Germination of seeds and embryos of *Ilex latifolia* and *I. rotunda*.

Species	Method	Emergence or germination (%)	Number of days to emergence or germination[x]
I. latifolia	Seed sowing[z]	0	—
	Embryo culture[y]	100	18.9 ± 1.1
I. rotunda	Seed sowing[z]	0	—
	Embryo culture[y]	0	—

[z]n = 30. Observations were continued for one year after sowing.
[y]n = 10. Observations were continued for six months after incubation.
[x]Data are expressed as mean ± SE.

Table 2. Effect of seed developmental stage on germination capacity of seeds and embryos of *Ilex latifolia* and *I. rotunda*.

	Date of seed collection			Germination (%)[y]		
					Embryo	
Species	Season	Date	Length of embryo (μm)[z]	Seed	PGR-free	1 mg·L^{-1} BA and 0.1 mg·L^{-1} NAA
I. latifolia	2006–07	18 July	—	0	0[x]	—
		18 Aug.	373 ± 15 a	0	70	—
		18 Sept.	379 ± 19 a	0	0	—
		12 Oct.	483 ± 18 bc	0	100	—
		16 Nov.	484 ± 22 bc	0	90	—
		12 Dec.	471 ± 11 b	0	100	—
		12 Jan.	544 ± 20 bc	0	100	—
		12 Feb.	551 ± 13 c	0	90	—
	2007	20 July	—	0	40[w]	0[w]
		7 Aug.	309 ± 13 a	0	10	0
		5 Sept.	419 ± 21 b	0	60	40
		4 Oct.	488 ± 11 c	0	20	20
		2 Nov.	465 ± 12 bc	0	100	60
		11 Dec.	456 ± 15 bc	0	80	—
I. rotunda	2006–07	6 Sept.	194 ± 6 a	0	0	—
		6 Oct.	261 ± 10 b	0	0	—
		6 Nov.	292 ± 6 bc	0	0	—
		6 Dec.	295 ± 10 bc	0	0	—
		7 Jan.	318 ± 11 c	0	0	—
		7 Feb.	289 ± 14 bc	0	0	—
	2007	5 Sept.	282 ± 6 a	0	0	0
		4 Oct.	300 ± 8 ab	0	0	0
		2 Nov.	302 ± 5 ab	0	0	0
		11 Dec.	313 ± 6 b	0	0	0

[z]Data are expressed as mean ±SE (n = 10). Means followed by different letters are significantly different ($P < 0.05$, one-way analysis of variance with Tukey's post hoc test) within a season in each species.
[y]Seeds and embryos in the 2006–07 and 2007 seasons (n = 10 in each treatment) were incubated at 25 and 15 °C, respectively. Observations of seeds and embryos were continued for one year after sowing and 20 weeks after incubation, respectively.
[x]*Ilex latifolia* seeds harvested on 18 July 2006 were cut in half, and halves containing embryos were sown in vitro, because the embryos were too small to isolate.
[w]*Ilex latifolia* seeds harvested on 20 July 2007 were cut into quarters, the endocarps were removed, and the quarters containing embryos were sown in vitro, because the embryos were too small to isolate.

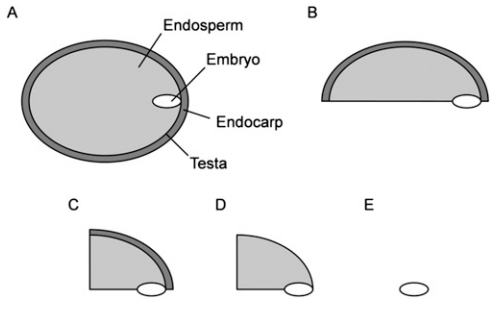

Fig. 1. Schematic illustration of five types of *Ilex latifolia* seeds used to investigate the effects of endosperm, testa, and endocarp on seed germination. (**A**) Intact seed; (**B**) half-seed; (**C**) quarter-seed; (**D**) quarter-seed without endocarp; (**E**) isolated embryo (seed without endocarp, testa, or endosperm).

acid (NAA; 0 or 0.1 mg·L^{-1}), 3% sucrose, and 0.85% agar (pH 5.8), and then incubated at 15 °C in the dark. *Ilex latifolia* seeds harvested on 20 July were cut into quarters, and the endocarps were removed. The quarter-seeds with embryos but not endocarps (Fig. 1D) were aseptically sown. Embryo length was measured using a stereomicroscope (SZ60; Olympus, Tokyo, Japan) equipped with an ocular micrometer, when embryos were cultured. Germination of seeds and embryos were observed for one year after sowing and 20 weeks after incubation, respectively.

Water permeability test. Methylene blue has been used to determine the water permeability of seeds (Orozco-Segovia et al., 2007). Seeds of *I. latifolia* (collected 14 Nov. 2007) and *I. rotunda* (collected 16 Nov. 2007) were immersed for 48 h in 1% methylene blue or distilled water (control). The seeds were washed with water, cut, and observed with a stereomicroscope (SZ60; Olympus).

Effects of endosperm and endocarp on seed germination. Ilex latifolia seeds (collected 8 Nov. 2007) were sterilized with 70% ethanol for 30 s and then with 5% sodium hypochlorite for 15 min. Five types of explants were prepared: intact seeds (Fig. 1A), half-seeds (Fig. 1B), quarter-seeds (Fig. 1C), quarter-seeds without endocarp (Fig. 1D), and isolated embryos (seeds without endocarp, testa, or endosperm; Fig. 1E). These explants were placed in flat-bottomed test tubes containing 8 mL of MS medium supplemented with 3% sucrose and 0.85% agar (pH 5.8) and then incubated at 15 °C in the dark. Seed germination was observed for 10 weeks after incubation.

Effect of chemical scarification on seed germination. Ilex latifolia seeds (collected 12 Oct. 2006) were immersed in 1 M NaOH for 3 h. These seeds were washed with water, sown in petri dishes that contained wet filter papers, and incubated at 25 °C in the dark. Observations of seed germination were recorded for six months after sowing.

Effect of gibberellic acid on seed germination. Ilex latifolia and *I. rotunda* seeds (collected 6 Feb. 2007) were immersed in 100 mg·L^{-1} GA_3 for 24 h and then placed in petri dishes containing wet filter papers. These seeds were incubated at 15 °C in the dark and observed for six months. After incubation, embryo length was measured using a stereomicroscope (SZ60; Olympus) equipped with an ocular micrometer.

Effect of cold stratification on seed germination. Ilex latifolia and *I. rotunda* seeds (collected 6 Feb. 2007) were placed in petri dishes that contained wet filter papers, incubated at 15 °C in the dark for 14 weeks (until 15 May), and then transferred to 5 °C in the dark. Finally, the seeds were transferred to an incubator maintained at 15 °C in the dark at ≈6-week intervals (22 June, 7 Aug., 18 Sept., 30 Oct., and 4 Dec.; see Table 3). After 44 weeks of treatment, some seeds were continuously incubated at 15 °C in the dark and other seeds were used for embryo culture at 15 °C in the dark. Embryo length was measured using a stereomicroscope (SZ60; Olympus) equipped with an ocular micrometer, when embryos were cultured. Germination of seeds and embryos was observed for an additional 10 and 8 weeks, respectively.

Statistical analysis. For the analysis of embryo length, one-way analysis of variance with Tukey's honestly significant difference

post hoc test (SPSS Statistics 20; IBM Japan, Tokyo, Japan) was performed.

Results

Germination test of seeds and embryos. When non-sterilized seeds of *I. latifolia* and *I. rotunda* were sown on wet filter paper at 15 °C and then transferred to a greenhouse, they did not germinate even one year after sowing. When embryos were cultured in vitro, all *I. latifolia* embryos germinated by 18.9 ± 1.1 d after incubation at 25 °C. Meanwhile, *I. rotunda* embryos did not germinate (Table 1).

Effect of seed developmental stage on germination capacity. Embryos of *I. latifolia* in the 2006–07 season were 373 ± 15 µm long on 18 Aug. 2006 and increased to 483 ± 18 µm by 12 Oct. 2006. In 2007 season, embryos also elongated through October, suggesting that *I. latifolia* embryos developed at least until October. The length of *I. rotunda* embryos increased approximately until November in either the 2006–07 or 2007 seasons (Table 2).

When non-sterilized seeds of *I. latifolia* and *I. rotunda* were sown on wet filter paper, germination was not observed in either the 2006–07 or 2007 seasons. In contrast, when cut seeds or embryos were cultured in vitro, quarter-seeds without endocarp (sown on 20 July 2007) and embryos of *I. latifolia* germinated, whereas germination was not observed in *I. rotunda*. Interestingly, although the germination frequency of *I. latifolia* embryos collected on 18 Aug. 2006 was 70%, it decreased to 0% in those collected on 18 Sept. and increased to 100% in those collected on 12 Oct. Similar transient decreases in germination was also observed on 4 Oct. 2007. Adding BA and NAA to MS medium did not improve germination frequency (Table 2).

Water permeability test. Intact seeds of *I. latifolia* and *I. rotunda* were immersed in methylene blue to investigate seed water permeability. In both species, although the embryo, endosperm, and most of the testa were not stained, the endocarp and testa adjacent to the embryo were stained (Fig. 2). These results suggested that seeds of *I. latifolia* and *I. rotunda* could imbibe water.

Effects of endosperm and endocarp on seed germination. To test the hypothesis that inhibitors present in the endosperm, testa, and/or endocarp interfere with seed germination, five types of seeds that differed in the presence of the endocarp, testa, and endosperm (Fig. 1) were cultured on MS medium. The results (Fig. 3A) showed that among these seeds, germinability of isolated embryos was highest; 100% of embryos germinated within 8 weeks of incubation. Likewise, good germination was observed in quarter-seeds with or without endocarp (70% germination within 8 to 10 weeks) followed by half-seeds (50% germination within 8 to 10 weeks). No germination was observed when intact seeds were cultured, even 10 weeks after incubation.

When seeds germinated, a radicle and hypocotyl usually appeared on the cut surface of seeds (Fig. 3B). In some quarter-seeds without endocarp, the radicle or hypocotyl penetrated the testa and emerged (Fig. 3C).

Effect of chemical scarification on seed germination. The endocarp of *I. latifolia* seeds were softened by NaOH treatment. However, the seeds did not germinate within six months of sowing on wet filter paper and incubating at 25 °C.

Effect of gibberellic acid on seed germination. Seeds of *I. latifolia* and *I. rotunda* treated with GA_3 did not germinate within six months of sowing on wet filter paper and incubating at 15 °C. No change in embryo length was observed after incubation.

Effect of cold stratification on seed germination. After cold stratification, no change in embryo length was observed in *I. latifolia* (Table 3). Neither cold-stratified nor non-stratified seeds germinated. When embryos were cultured in vitro, 50% of non-stratified embryos germinated, whereas the germination frequencies of embryos from seeds stratified for 18 to 30 weeks were 70% to 80%. In *I. rotunda*, although the lengths of embryos from seeds stratified for 6 weeks increased to 361 µm, no change in embryo length was observed in other seeds stratified for 12 to 30 weeks. No seeds or embryos germinated.

Table 3. Effect of cold stratification on germination of seeds and embryos of *Ilex latifolia* and *I. rotunda*.

	Treatment period (weeks)				Germination (%)	
Species	15 °C	5 °C	15 °C	Length of embryo (µm)[x]	Seed	Embryo
I. latifolia[z]	14	0	30	529 ± 15 a	0	50
	14	6	24	506 ± 19 a	0	50
	14	12	18	549 ± 16 a	0	60
	14	18	12	517 ± 24 a	0	70
	14	24	6	522 ± 15 a	0	80
	14	30	0	532 ± 21 a	0	70
I. rotunda[y]	14	0	30	294 ± 10 a	0	0
	14	6	24	361 ± 32 b	0	0
	14	12	18	294 ± 9 a	0	0
	14	18	12	306 ± 9 ab	0	0
	14	24	6	305 ± 6 ab	0	0
	14	30	0	271 ± 13 a	0	0

Observations were continued for 10 (seeds) and 8 (embryos) weeks after 15–5–15 °C temperature treatments.
[z]n = 10.
[y]n = 8.
[x]Data are expressed as mean ± SE. Means followed by different letters within a species are significantly different ($P < 0.05$, one-way analysis of variance with Tukey's post hoc test).

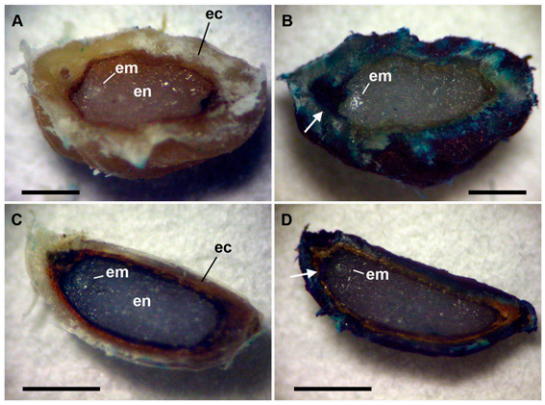

Fig. 2. Water permeability of seeds of *Ilex latifolia* (**A–B**) and *I. rotunda* (**C–D**). Intact seeds were immersed in distilled water (**A, C**) or 1% methylene blue (**B, D**) for 48 h, then longitudinally cut and photographed. The stained testa adjacent to the embryo is indicated by arrows (**B, D**). Em = embryo; en = endosperm; ec = endocarp. Scale bars = 1 mm.

Discussion

In the present study, intact seeds of *I. latifolia* and *I. rotunda* did not germinate in any of the experiments. When embryos were cultured in vitro, germination was observed in *I. latifolia* but not in *I. rotunda*. Therefore, germinability obviously differed between these species. Embryos of *I. latifolia* elongated until October and showed good germination in October or later, whereas those of *I. rotunda* elongated until around November. Although fruits of both species reached maturity in the fall, embryos were apparently in the heart or late-heart stage even in February. Because immaturity of embryos was also reported in several other *Ilex* species (Chien et al., 2011; Hu et al., 1979; Tsang and Corlett, 2005), immaturity could be a common phenomenon in *Ilex*.

In *I. latifolia*, germination frequency decreased transiently in embryos isolated from seeds collected in Sept. 2006 and Oct. 2007. Although it is unclear why transient decrease

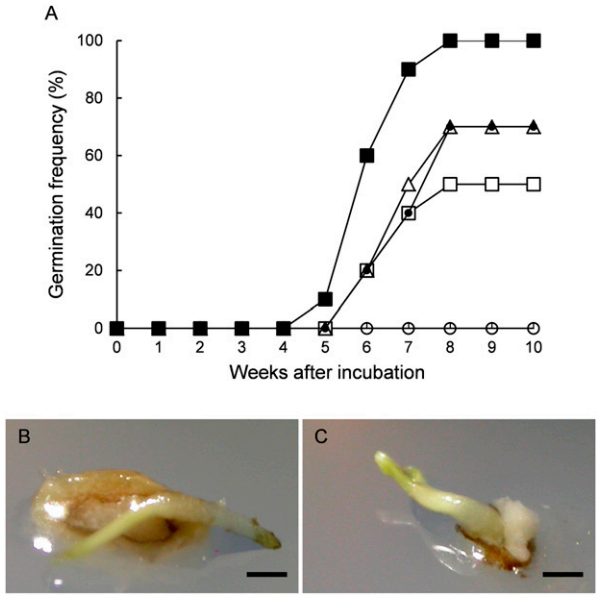

Fig. 3. Germination of *Ilex latifolia* seeds that differed in the presence of the endocarp, testa, and endosperm. (**A**) Germination frequencies. Open circles = intact seeds; open squares = half-seeds; open triangles = quarter-seeds; filled circles = quarter-seeds without endocarp; filled squares = embryos. (**B**–**C**) Seedling emergence from cut seeds. The radicle and hypocotyl appeared on the cut surface of half-seeds (**B**), whereas they appeared after penetrating the testa in quarter-seeds without endocarp (**C**). Scale bars = 1 mm.

of germination frequency was observed, these results implied that during development, embryos increased germinability but underwent transient dormancy.

In *I. paraguariensis* and *I. dumosa*, because cut seeds germinated while intact seeds did not or showed delayed germination, the endocarp was suggested to act as a mechanical barrier interfering with tissue expansion (Dolce et al., 2010, 2011). However, subsequent experiments have incompletely addressed the hypothesis. In the present study, a water permeability test using methylene blue suggested that seeds of *I. latifolia* and *I. rotunda* could imbibe water. In experiments using five types of *I. latifolia* seeds that differed in the presence of the endocarp, testa, and endosperm, the radicle or hypocotyl penetrated the testa and emerged only when quarter-seeds without endocarp were cultured in vitro. These results strongly suggested that the endocarp acted as a mechanical barrier to radicle and hypocotyl emergence rather than avoiding water absorption.

Chemical scarification using NaOH did not allow seed germination in *I. latifolia* even six months after sowing on wet filter paper. Although further investigation of NaOH concentrations and treatment duration may be required, Dolce et al. (2010) also reported that scarification using H_2SO_4 had no effect on seed germination in *I. paraguariensis*.

In *Arabidopsis*, the plant hormone abscisic acid (ABA) synthesized in the endosperm is thought to induce seed dormancy (Lee et al., 2010; Lefebvre et al., 2006). Because germination frequency increased as the sizes of the remaining endosperm and endocarp decreased, we considered that certain inhibitors, such as ABA, contained in the endosperm, testa, and/or endocarp might interfere with seed germination in *I. latifolia*. This hypothesis is consistent with the suggestion that *I. aquifolium*, *I. cornuta,* and *I. opaca* possess inhibitors in the endosperm and/or membrane-like testa attached to the endosperm that are responsible for maintaining embryo dormancy (Hu et al., 1979) but inconsistent with the data for *I. paraguariensis* and *I. dumosa*, in which the endosperm did not inhibit embryo development or germination (Dolce et al., 2010, 2011).

Another plant hormone, gibberellin (GA), is known to break seed dormancy and promote germination. GA and ABA are likely to antagonistically interact to control seed dormancy and germination (Chen et al., 2010; Nicolás et al., 1996). Although we treated seeds of *I. latifolia* and *I. rotunda* with GA_3, they did not germinate. The effects of GA_3 on enhancing seed or embryo germination have been tested in several *Ilex* species. However, GA_3 had only slight effects on the germination of embryos with endosperm in *I. opaca* (Hu et al., 1979) and no effect on germination of intact or bisected seeds in *I. paraguariensis* (Dolce et al., 2010). In *I. maximowicziana*, GA_3 increased germination rate (shortened germination time) but not germination frequency of intact seeds (Chien et al., 2011). To break seed dormancy, other GAs such as GA_1, GA_4,, and GA_{4+7} were reported to be effective in some plant species (Chen et al., 2008, 2010; Chien et al., 1998). In some *Ilex* species, when a mixture of GAs (GA_3, GA_4, and GA_7) was used for embryo culture, germination improved slightly or was unaffected from August to October and inhibited in November and December (Mattis et al., 1995). Nevertheless, GAs other than GA_3 might be effective for breaking seed dormancy in *I. latifolia* and *I. rotunda*.

The effects of cold stratification on germination of cut seeds are likely to differ among *Ilex* species. The germination frequency of cut seeds of *I. paraguariensis* was increased by cold treatment at 4 °C for 60 d (Dolce et al., 2010). Conversely, although germination of cut seeds of *I. dumosa* was not influenced by cold treatment at 4 °C for 30 d, the frequency was decreased by cold treatment at 4 °C for 60 d (Dolce et al., 2011). Cold stratification was not required to break seed dormancy in *I. maximowicziana*, because cold treatment at 5 °C for 4 to 8 weeks increased germination rate (shortened germination time) but not germination frequency of intact seeds (Chien et al., 2011). The present study revealed that the germination frequency of *I. latifolia* embryos was increased by cold stratification at 5 °C for 18 to 30 weeks. Although *I. rotunda* did not germinate, embryos from seeds cold-stratified at 5 °C for 6 weeks increased in length, suggesting that embryos collected in February could develop under appropriate cold stratification conditions.

The use of germinating cut seeds, as compared with embryo culture, has been described for *I. paraguariensis* (Dolce et al., 2010) and *I. dumosa* (Dolce et al., 2011) as follows. Because *Ilex* embryos are quite small and easily damaged during isolation, the internal seed structure must be precisely known. In addition, embryo culture is very laborious. In vitro culture of cut seeds was easier and more effective than embryo culture. Although cut seeds of *I. latifolia* germinated less frequently than embryos in this study, we confirmed the convenience of in vitro culture of cut seeds.

In conclusion, seeds of *I. latifolia* and *I. rotunda* were difficult to germinate. The results obtained in the present study suggested that factors inhibiting seed germination in *I. latifolia* were a mechanical barrier by the endocarp and inhibitors contained in the endosperm, testa, and/or endocarp. In *I. latifolia*, seedlings could be efficiently obtained by culturing embryos and quarter-seeds with or without endocarp from seeds collected in November or later, and the germination frequency of embryos could be increased by cold stratification. Germination was not observed in *I. rotunda*, even when embryos were cold-stratified. Because cold stratification might induce embryo development in *I. rotunda*, it deserves further investigation in combination with other treatments to obtain seedlings from seeds or embryos.

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