Tree Physiology 28, 1431–1439

Roles of gibberellins and abscisic acid in dormancy and germination of red bayberry (*Myrica rubra*) seeds

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Received December 22, 2007; accepted April 7, 2008; published online July 1, 2008

Summary Intact seeds from freshly harvested fruits of *Myrica rubra* (Sieb et Zucc.) were dormant and required 8 weeks of warm stratification followed by 12 weeks of cold stratification for germination. Exogenous application of gibberellic acid (GA_3) to intact fresh seeds was effective in breaking dormancy, with > 70% of seeds germinating when treated with 5.2 mM GA_3 and incubated at a day/night temperature of 30/20 °C for 20 weeks. Removing the hard endocarp or endocarp plus seed coat of fresh seeds promoted germination, and addition of GA_3 to the embryo accelerated germination. The gibberellins GA_1 and GA_4 were more effective than GA_3 in promoting germination of seeds with the endocarp removed. Endogenous contents of GA_1, GA_3, GA_4, GA_7 and GA_{20} were quantified by gas chromatography–mass spectrometry–selected ion monitoring in the endocarps, seed coats and embryos of fresh seeds treated with 5.2 mM GA_3. The content of GA_3 decreased in the endocarp during incubation, whereas GA_1 contents increased in the endocarp and seed coat. A high GA_1 content was detected in the endocarps and embryos of newly germinated seeds. We speculate that GA_3 was converted to GA_1 during incubation and that GA_1 is involved in seed germination. Endogenous abscisic acid (ABA) contents were measured in fresh seeds and in warm and cold stratified seeds. The ABA content in fresh seeds was distributed in the order endocarp > seed coat > embryo, with the content in the endocarp being about 132-fold higher than in the seed coat and embryo. Total ABA content of seeds subjected to warm or cold stratification, or both, was 8.7- to 14.0-fold lower than that of fresh seeds. Low contents of endogenous GA_1, GA_3, GA_7 and GA_{20}, but elevated contents of GA_4, were found in the seed coats and endocarps of warm plus cold stratified seeds and in the seed coats and embryos of newly germinated seeds. These observations, coupled with the finding that GA stimulated germination of dormant *Myrica* seeds, provide evidence that endogenous ABA inhibited release of dormancy and that endogenous gibberellins, especially GA_4 or GA_1, or both, are involved in germination.

Keywords: cold stratification, fluridone, paclobutrazol, plant hormone, warm stratification.

Introduction

The roles of abscisic acid (ABA) and gibberellins in seed dormancy and germination have been the focus of many studies. Maintenance of dormancy is a consequence of a high ABA content in mature seeds, and dormancy release is strongly correlated with a reduction in ABA content. Gibberellins counteract ABA effects and promote seed germination (Kucera et al. 2005). Seeds of most *Myrica* species are dormant at maturity and require prolonged cold stratification (chilling) to break dormancy (Krochmal 1974, Bhatt et al. 2000, Skene et al. 2000). Exogenous application of gibberellic acid (GA_3) to intact, unstratified seeds is effective in breaking dormancy of seeds of *Myrica esculenta* Buch.-Ham. ex D. Don., *M. pensylvanicum* L. and *M. adenophora* Hance (Hamilton and Carpenter 1977, Bhatt et al. 2000, Chien et al. 2000). Chien et al. (2000) reported that a combination of warm plus cold stratification promoted seed germination of *M. rubra* Sieb. et Zucc.

The purpose of our study was to further characterize seed dormancy and germination in *M. rubra*, an evergreen dioecious tree distributed widely in the island of Taiwan and occurring also in southern China, Japan, Korea and the Philippines (Yang and Lu 1996). Specifically, we determined the roles of the endocarp, seed coat and embryo in germination, and monitored changes in amounts of endogenous ABA and gibberellins of the various tissues of seeds during both dormancy breaking and radicle protrusion. Because GA_3 has been shown to enhance seed germination in *M. rubra*, we measured the contents of gibberellins in endocarp, seed coat and embryo after GA_3 treatment and incubation to gain insight into GA_3 metabolism. We also used fluridone and paclobutrazol, inhibitors of ABA and GA biosynthesis, respectively, to examine the roles of these hormones in the maintenance and breaking of seed dormancy.

Materials and methods

Fruit collection

Mature red and dark-red fruits of *M. rubra* were harvested

from trees growing in Nanjuang (24°36′ N, 120°59′ E), Miaoli County, Taiwan in June 2003. Fruits were kneaded by hand in water to remove the pulp, and the seeds (with endocarp) were air-dried at room temperature for 2 days to reduce water content to below 10%, then stored in a sealed container at 5 °C. Mean size of fresh *M. rubra* seeds was 8.89 mm long, 7.51 mm wide and 6.28 mm thick. The seed consists of a large embryo that is surrounded by a thin seed coat and a hard endocarp (Figure 1). Hereafter, the term seed refers to the intact seed with endocarp, which is the germination unit in *Myrica*, and the term true seed refers to a seed from which the endocarp has been removed but the seed coat retained.

Effect of warm and cold stratification on germination

Seeds of *M. rubra* were mixed with moist sphagnum (water content of the sphagnum was about 400% of dry mass), sealed inside polyethylene bags (0.04 mm in thickness) and warm stratified for 1–12 weeks at a day/night temperature of 30/20 °C in a 12-h photoperiod supplied by fluorescent lights (80–100 µmol m^{-2} s^{-1}, 400–700 nm). These warm-stratified seeds were then cold stratified for 12 weeks at 4 °C. In addition, some seeds were warm stratified for 8 weeks without a subsequent cold treatment, and other seeds were cold stratified at 4 °C for 12 weeks without a warm pretreatment.

Effects of GA_3 on germination of fresh seeds and of warm-stratified seeds

Freshly harvested seeds and seeds that had been warm stratified at 30/20 °C for 8 weeks were treated with 0 (water control), 1.3, 2.6 and 5.2 mM GA_3 (potassium salt, 95% purity; Sigma) for 20 h at room temperature before germination. Each treatment consisted of three replicates of 50 seeds. Treated seeds were mixed with moist sphagnum and tested for germination.

Effects of endocarp and seed coat on germination

True seeds and isolated embryos from freshly harvested seeds were either placed directly on moist sphagnum in sealable polyethylene bags or treated with 0.13 mM (50 ppm) GA_3 for 20 h and then placed in polyethylene bags for germination at a day/night temperature of 30/20 °C in a 12-h photoperiod from fluorescent lights. Each treatment and the untreated control (true seed plus endocarp) consisted of three replicates of 50 seeds or embryos. Germination was recorded three times a week, and germination percentages were calculated.

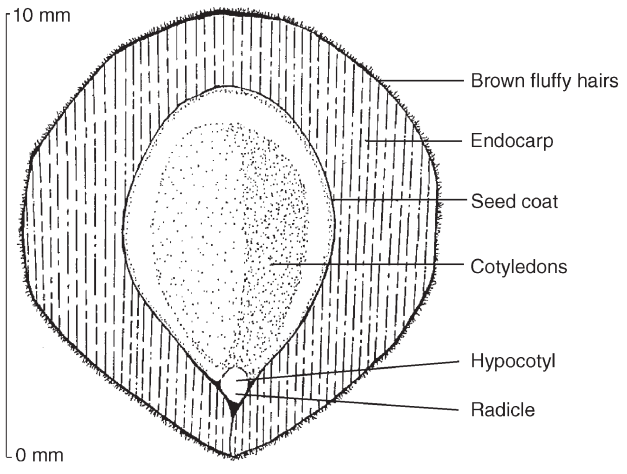

Figure 1. Longitudinal section through an intact seed of *Myrica rubra*.

Effects of fluridone, paclobutrazol, GA_1, GA_3, GA_4 and ABA on germination of true seeds

Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)-phenyl]–4-(1H)-pyridinone) (> 97% purity) was obtained from Olchemim, Czech Republic; paclobutrazol (2RS,3RS)–1-(4-chlorophenyl)–4,4-dimethyl-2-(1,2,4-triazolyl)-pentan-3-ol (97% purity) from Wako Pure Chemical Industries, Japan; (±)-ABA (99% purity); GA_3 from Sigma; and GA_1 and GA_4 (> 90% purity) from Prof. Lewis N. Mander, Australian National University. Because of the inhibitory effect of the hard endocarp on germination, true seeds were soaked for 16 h in double-distilled water, ABA, GA_1, GA_3, GA_4, fluridone or paclobutrazol and in combinations of these hormones. The above-treated true seeds were then mixed with moist sphagnum and germinated at 30/20 °C (12/12 h) with 20 h of fluorescent light beginning with the onset of the higher temperature period. Each treatment consisted of three replicates of 25 seeds. Germination was recorded three times a week, and germination percentages were calculated. The seed lot used in this experiment was harvested in June 2006.

Germination tests

Unless otherwise stated, germination tests were carried out in an incubator for up to 20 weeks at a day/night temperature of 30/20 °C with a 12-h photoperiod (80–100 µmol m^{-2} s^{-1}, 400–700 nm) (i.e., the same conditions as used for warm stratification). Small pieces of sphagnum served as the germination medium. Germination, judged by radicle emergence of at least 5 mm, was recorded weekly. Results were expressed as germination percentage and as mean germination time in days (Naylor 1981), MGT = $(\Sigma n_i t_i)/N$, where n_i is the number of seeds germinated in t_i days from the beginning of the test, and N is the total number of germinated seeds at the end of the test. Mean germination time is a measure of the germination rate and of the sharpness of the germination peak.

Analyses of endogenous ABA and gibberellins

Endogenous ABA, GA_1, GA_3, GA_4, GA_7 and GA_{20} were analyzed by gas chromatography–mass spectrometry–selected ion monitoring (GC–MS–SIM). For these analyses, endocarp, seed coat and embryo were separated individually from freshly mature seeds, from seeds warm-stratified at 30/20 °C for 8 weeks, from seeds cold-stratified at 4 °C for 12 weeks, from seeds warm-stratified at 30/20 °C for 8 weeks followed by cold-stratification at 4 °C for 12 weeks and from newly ger-

minated seeds (radicle-emerged). In addition, other freshly mature seeds were treated with 5.2 mM GA_3 and incubated for 3 and 5 weeks, and then endocarp, seed coat and embryo were separated and analyzed for GA_1, GA_3, GA_4, GA_7 and GA_{20}. The amount of ABA in the pulp (exocarp + mesocarp) was determined. In all cases, parts were separated from 50 seeds, lyophilized and weighed. Fresh masses of endocarp, seed coat and embryo per 50 seeds were 7.85, 0.274 and 1.82 g, respectively, and the dry masses of endocarp, seed coat and embryo per 50 seeds were 5.22, 0.032 and 0.959 g, respectively. The dried parts of two replications were ground separately in liquid N_2 with a mortar and pestle. Internal standards of 100 ng [$^2H_6$]ABA or 50 ng [17,17-$^2H_2$] gibberellins (GA_1, GA_3, GA_4, GA_7 and GA_{20}) were added to each sample after grinding. Tissue extraction was carried out overnight with 15 ml of 80% (v/v) methanol containing 0.4 mg butylated hydroxytoluene and 2 mg ascorbate at 5 °C, and the residue was re-extracted in the same solvent for 2 h at 5 °C. The methanol extracts were combined and reduced to 1–2 ml of aqueous residue with a rotary vacuum evaporator and SpeedVac (Savant Instruments, MA).

The residue was adjusted to pH 8.5 with 0.05 M potassium phosphate buffer and passed through a polyvinylpolypyrrolidone column (about 5 g). The eluate was partitioned with ethyl acetate (3 × 15 ml). The aqueous fraction was then adjusted to pH 3.0 with 0.5 M potassium phosphate (pH 2.0) and partitioned with ethyl acetate (3 × 15 ml). The pooled ethyl acetate fraction was taken to dryness with a SpeedVac. The residue was dissolved in 0.05 M potassium phosphate buffer (pH 3.0) and loaded onto a preconditioned ODS-silica column (about 3 g). The ODS-silica column was washed eight times with ddH_2O in 0.1% acetic acid and eluted with 80% aqueous methanol containing 0.1% acetic acid.

After drying under vacuum, the sample was dissolved in 30% aqueous methanol containing 0.1% acetic acid and injected into a Beckman System Gold HPLC with LiChrosphere RP-18 column (250 × 4 mm i.d. × 5 µm particle size; Merck, Germany). The fraction of ABA from this column was dried under vacuum and derivatized by adding ethereal diazomethane, then dried with N_2. The gibberellins were further trimethylsilated and the derivatized samples were analyzed using Agilent Technologies 6890N GC and 5975 MSD with a DB-1 capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, USA). Operating conditions for high performance liquid chromatography and GC–MS–SIM were as described by Chen et al. (2007). For ABA, GA_1, GA_3, GA_4, GA_7 and GA_{20}, the *m/z* ratios of 190/194, 506/508, 504/506, 284/286, 222/224 and 418/420, respectively, were used for quantification.

Statistical analysis

Contents of gibberellins and ABA were calculated from two replicates and expressed as the mean ± SD. Germination percentages (mean ± SE) were calculated, and means were compared by analysis of variance (ANOVA) and by least significant difference (LSD) test at the 5% level of significance.

Results

Effect of warm plus cold stratification on germination

Germination of fresh seeds from mature fruits reached 31.3% during incubation at 30/20 °C for 20 weeks. Cold stratification at 4 °C for 12 weeks increased seed germination to 42.7% in the same incubation period (Table 1). Warm stratification for 8 or 12 weeks followed by cold stratification at 4 °C for 12 weeks significantly increased germination to 67.3 and 69.3%, respectively, and decreased mean germination time (i.e., increased germination rate) from 101 days in unstratified seeds to 48.5 and 46.2 days, respectively.

Effects of GA_3 on germination of fresh seeds and of warm-stratified seeds

Germination percentage of fresh seeds was increased by GA_3 treatment (Figure 2A). During a 20-week incubation, germination of seeds treated with 1.3, 2.6 and 5.2 mM GA_3 reached 46.5, 57.6 and 75.5%, respectively, compared with 31.3% for control seeds treated with water. Furthermore, time to initiation of germination was decreased from 10 weeks for control seeds to 4 weeks for seeds in the 5.2 mM GA_3 treatment.

When fresh seeds were warm stratified and then treated with GA_3, germination rate increased, especially for seeds treated with 2.6 and 5.2 mM GA_3 (Figure 2B), with germination of seeds treated with 5.2 mM GA_3 increasing to 70% after 7 weeks of incubation. The warm stratification plus GA_3 treatment decreased the number of the days for initiation of germination compared with the GA_3 treatment only.

Effect of endocarp and seed coat on germination

Seeds from freshly collected fruits remained dormant during incubation for 28 days or longer. However, removal of the endocarp (seed coat retained) increased germination to 25% within 28 days, and germination of isolated embryos (endocarp and seed coat removed) was 58% during the same incuba-

Table 1. Effects of warm stratification at 30/20 °C followed by cold stratification at 4 °C on germination percentage and mean germination time (MGT; days) of *Myrica rubra* seeds.

Stratification (weeks)		Germination[1]	MGT
Warm	Cold		
0	0	31.3 ± 4.2 c	101.0
0	12	42.7 ± 2.5 b	96.1
1	12	41.7 ± 6.8 b	118.0
2	12	43.3 ± 0.9 b	108.0
4	12	48.7 ± 3.4 b	85.0
8	12	67.3 ± 5.7 a	48.5
12	12	69.3 ± 5.0 a	46.2

[1] Germination percentages were calculated for the 20-week incubation. Means (n = 3) of germination followed by different letters differ significantly (LSD, P = 0.05).

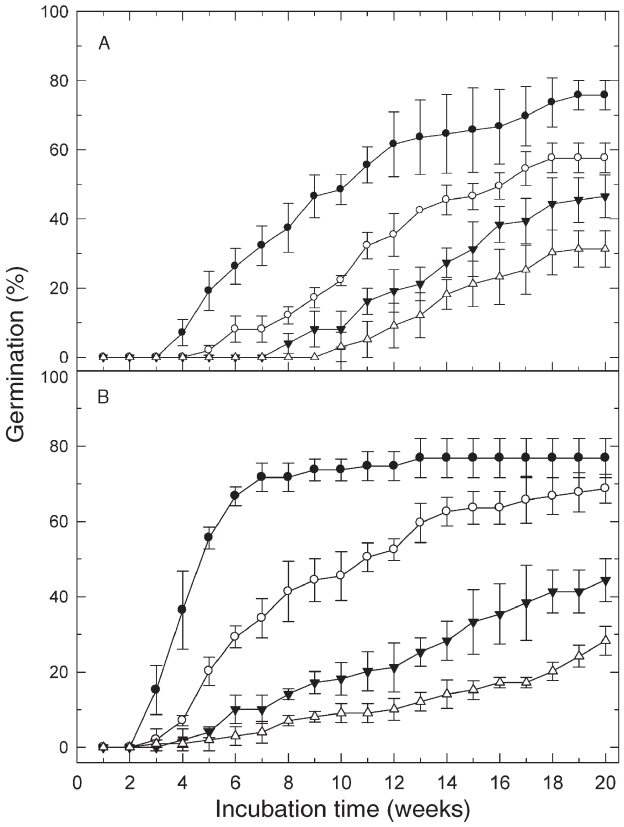

Figure 2. Effects of GA_3 treatment on germination percentages of *Myrica rubra* seeds. (A) Fresh seeds and (B) fresh seeds warm-stratified at a day/night temperature of 30/20 °C for 8 weeks treated with 5.2 mM GA_3 (●), 2.6 mM GA_3 (○), 1.3 mM GA_3 (▼) or ddH_2O (△). Each value is the mean of three replicates of 50 seeds. Vertical bars indicate ± 1 SE.

tion period (Figure 3). Of the seeds with only the endocarp removed (true seeds) treated with 0.13 mM GA_3, 88% germinated, and mean germination time decreased. The most significant decrease in germination time was for isolated embryos treated with 0.13 mM GA_3, where germination percentage reached > 85% by 12 days of incubation (Figure 3).

Effects of fluridone, paclobutrazol, GA_1, GA_3, GA_4 and ABA on germination of true seeds

Intact seeds did not germinate, and 21% of true seeds germinated in 28 days (Table 2). The gibberellins GA_1, GA_3 and GA_4 stimulated germination of true seeds. Germination percentages of seeds treated with GA_1 and GA_4 were > 60%, and mean germination time decreased. These gibberellins were more effective than GA_3 in promoting germination. Germination of true seeds was prevented by 50 µM of ABA, fluridone or paclobutrazol. However, a combination of GA_3 and ABA or of GA_3 and fluridone increased seed germination percentage, and the effectiveness was similar to that of GA_3 alone. Paclobutrazol plus GA_3 also was effective in increasing seed germination percentage (Table 2).

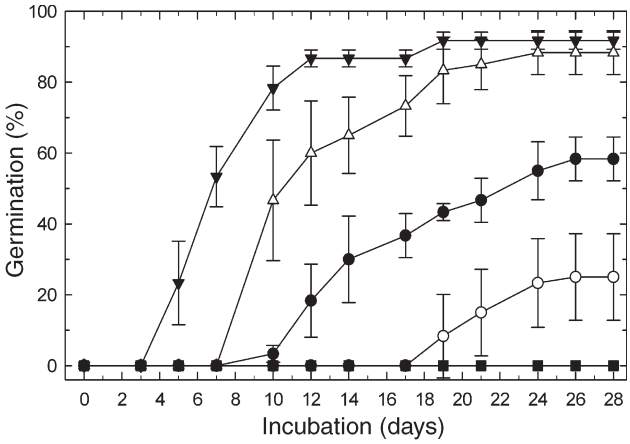

Figure 3. Germination percentages of fresh *Myrica rubra* seeds with endocarp removed (seed coat retained) (○), both endocarp and seed coat removed (●), endocarp removed (seed coat retained) and then treated with 0.13 mM GA_3 (△), both endocarp and seed coat removed and then treated with 0.13 mM GA_3 (▼) and intact seed (with endocarp) (■). Each value is the mean of three replicates of 50 seeds. Vertical bars indicate ± 1 SE.

Abscisic acid content of seed parts in freshly collected and in stratified seeds

The ABA content of *M. rubra* seeds differed among endocarp, seed coat and embryo tissues (Figure 4). It was 866.24 ng per endocarp, 4.54 ng per seed coat and 2.02 ng per embryo in fresh seeds. The ABA content of the endocarp was about 132-fold higher than that of the seed coat and embryo combined. Warm stratification for 8 weeks, cold stratification for 12 weeks and warm stratification for 8 weeks plus cold stratification for 12 weeks drastically decreased ABA contents to 58.74, 99.39 and 71.64 ng per endocarp, respectively (Figure 4). Total ABA content of seeds following warm or cold stratification or both was about 8.7–14.0-fold lower than that of fresh seeds. The ABA content was 33.74 ng per endocarp, 0 (not detectable) ng per seed coat and 0.397 ng per embryo in newly germinated seeds. The ABA content of the pulp (exocarp + mesocarp) was 1.49 ng per seed pulp.

Gibberellin contents of seed parts in freshly collected and in stratified seeds

There were some differences in GA_1, GA_3, GA_4, GA_7 and GA_{20} contents in endocarp and seed coat of freshly collected seeds and seeds that had been warm or cold stratified, or both, whereas there were low but similar amounts of these gibberellins in embryos of fresh and stratified seeds (Figure 5). However, in embryos of newly germinated seeds, GA_4 content was about 10-fold higher than in embryos of fresh seeds, and GA_1 was also higher in embryos of newly germinated seeds than in embryos of fresh seeds. A higher GA_4 content was found in seed coats of newly germinated seeds than in seed coats of fresh seeds or seeds that had been warm or cold stratified, or both. The contents of GA_1, GA_3 and GA_4 in the endocarp and

Table 2. Effects of ABA, GA_1, GA_3, GA_4, fluridone and paclobutrazol on germination percentages (mean ± SE) of *Myrica rubra* seeds after incubation for 7, 10, 14, 19, 21, 24 and 28 days at a day/night temperature of 30/20 °C. True seed = endocarp removed from intact seed but seed coat retained. The seed lot from the same tree was harvested in June 2006. Within a column, means (n = 3) followed by different letters differ significantly (LSD, P = 0.05).

Treatments	Germination (%)						
	7 days	10 days	14 days	19 days	21 days	24 days	28 days
Intact seed	0	0	0	0	0	0	0
True seed + ddH_2O	0	0	1.3	9.3 ± 5.0 d	14.7 ± 5.0 e	17.3 ± 6.8 eg	21.3 ± 6.8 d
True seed + 10 µM ABA	0	0	0	0	4.0	12.0 ± 6.5 ef	14.7 ± 6.8 def
True seed + 50 µM ABA	0	0	0	0	0	1.3	9.3 ± 5.0 ef
True seed + 50 µM GA_1	16.0 ± 3.3 a	34.7 ± 6.8 a	61.3 ± 8.2 a	65.3 ± 5.0 a	65.3 ± 5.0 a	65.3 ± 5.0 a	65.3 ± 5.0 a
True seed + 10 µM GA_3	1.3	18.7 ± 5.0 c	37.3 ± 6.8 b	38.7 ± 7.5 c	41.3 ± 3.8 d	41.3 ± 3.8 d	42.7 ± 5.0 c
True seed + 50 µM GA_3	0	14.7 ± 5.0 c	36.0 ± 8.6 b	46.7 ± 5.0 c	46.7 ± 5.0 cd	46.7 ± 5.0 cd	49.3 ± 5.0 bc
True seed + 50 µM GA_4	5.3 ± 1.9 c	30.7 ± 1.9 a	53.3 ± 6.8 a	60.0 ± 6.5 ab	60.0 ± 6.5 ab	62.7 ± 7.5 ab	64.0 ± 5.7 a
True seed + 10 µM fluridone	0	0	2.7	12.0 ± 3.3 d	13.3 ± 3.8 e	14.7 ± 5.0 ef	17.3 ± 8.2 def
True seed + 50 µM fluridone	0	0	1.3	2.7	5.3	6.7 ± 3.8 f	8.0 ± 3.3 f
True seed + 10 µM paclobutrazol	0	0	1.3	6.7	13.3 ± 7.5 e	21.3 ± 1.9 e	21.3 ± 1.9 d
True seed + 50 µM paclobutrazol	0	0	0	0	1.3	5.3	5.3
True seed + 10 µM ABA + 10 µM GA_3	0	0	14.7 ± 6.8 c	41.3 ± 10.5 c	50.7 ± 9.4 bcd	53.3 ± 10.5 bc	57.3 ± 7.5 ab
True seed + 50 µM ABA + 50 µM GA_3	0	5.3 ± 1.9 d	26.7 ± 1.9 bc	48.0 ± 3.3 bc	57.3 ± 5.0 abc	64.0 ± 5.7 a	68.0 ± 6.5 a
True seed + 50 µM fluridone + 50 µM GA_3	14.0 ± 2.8 a	34.7 ± 1.9 a	58.0 ± 8.5 a	61.3 ± 9.4 ab	61.3 ± 9.4 ab	61.3 ± 9.4 ab	61.3 ± 9.4 ab
True seed + 50 µM paclobutrazol + 50 µM GA_3	0.6	25.3 ± 1.9 b	64.6 ± 5.7 a	66.7 ± 4.1 a	70.0 ± 7.1 a	70.0 ± 7.1 a	70.0 ± 7.1 a

GA_1 in the seed coat were elevated in warm-stratified seeds that remained dormant. When warm plus cold stratified seeds were ready to germinate, they had large amounts of GA_1 and GA_4 in the endocarp but a low content of gibberellins in the embryo (Figure 5).

Gibberellin contents of seed parts from GA_3-treated seeds

The biologically active GA_1, GA_3, GA_4 and GA_7, and the immediate precursor GA_{20} were detected in the endocarps, seed coats and embryos of fresh seeds treated with 20 mM GA_3 before incubation at 30/20 °C for 3 or 5 weeks (Figure 6). Small amounts of gibberellins were measured in fresh seeds, in which there was 0.183 µg of GA_1 per endocarp. In GA_3-treated seeds during incubation, the content of GA_3 in the endocarp decreased, whereas the contents of GA_1, GA_4, GA_7 and GA_{20} increased in the endocarp, seed coat and embryo. Among the gibberellins, GA_1 content was highest, and increased sharply to 18.5 µg per endocarp, 14.6 µg per seed coat and 1.12 µg per embryo during a 3-week incubation. The GA_1 content increased continuously in embryos during a 5-week incubation period (Figure 6). In newly germinated seeds, GA_1 content was high in the endocarp (29.3 µg per endocarp) and embryo (19.30 µg per embryo), especially when compared with that in embryos of ungerminated seeds.

Discussion

Pretreatments of *M. rubra* seeds by prolonged warm plus cold stratification or by exogenous application of GA_3 or by removal of endocarp and seed coat tissues significantly increased seed germination. Sequentially removing the endocarp and seed coat with or without GA_3 treatment revealed that the covering layers of *Myrica* seeds partially prevented radicle emergence. Thus, the embryo exhibited some degree of dormancy that was overcome by gibberellin treatment. The effect of gibberellin in overcoming the inhibition of germination by the embryo-covering layers may involve an effect on the growth potential of the embryo. The embryo of *M. rubra* is fully developed at seed maturity (Figure 1), GA promotes seed germination and warm plus cold stratification breaks dormancy, thus *Myrica* seeds are classified as having physiological dormancy (Nikolaeva, 1977, Baskin and Baskin 2004). Recent physiological and molecular studies have shown that physiological dormancy includes an embryo and coat component, and their sum and interaction determine the degree of whole-seed physiological dormancy (reviewed by Finch-Savage and Leubner-Metzger (2006)). For germination to occur in physiologically dormant seeds, the mechanical constraint of the embryo-covering layers must be overcome by the growth potential of the embryo or the mechanical constraint must be reduced, or both.

Fluridone, an inhibitor in the pathway of carotenoid biosynthesis (Bartels and Watson 1978, Quatrano et al. 1997), did not promote germination of *Myrica* seeds, but it was effective in promoting germination of seeds treated with GA_3 (Table 2).

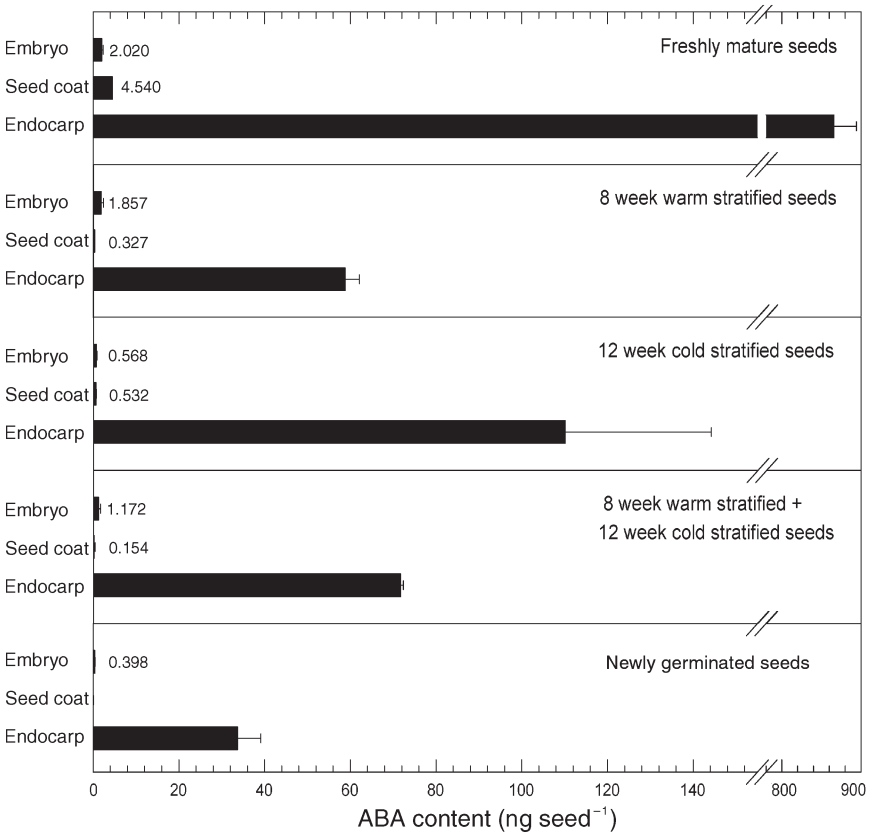

Figure 4. Abscisic acid (ABA) content of seed parts from fresh and stratified *Myrica rubra* seeds. Results are presented as means ± 1 SD from two replicates each of 50 seeds. For clarity, low values are shown as numbers of picograms per seed.

Likewise, paclobutrazol, which inhibits the biosynthesis of gibberellin at the oxidation of *ent*-kaurene to *ent*-karenoic acid step (Coolbaugh and Hamilton 1976, Grossmann 1990), inhibited *Myrica* seed germination, but this inhibition could be overcome by a brief treatment with GA_3. Although fluridone treatment accelerates seed germination in some species (Grappin et al. 2000, Schmitz et al. 2001, Chen et al. 2007), germination of *M. rubra* seeds did not occur in the presence of 10 µM fluridone, implying that inhibition of ABA biosynthesis by fluridone was not the major cause of the stimulatory effect. Furthermore, 50 µM ABA inhibited seed germination, but addition of GA_3 counteracted the ABA effect, implying that GA plays a decisive role in the regulation of *M. rubra* seed germination.

A direct correlation between dormancy and a high endogenous ABA content in tree seeds has been reported (Pinfield et al. 1989, Chien et al. 1998, 2004, Feurtado et al. 2004, Chen et al. 2007), and we found that ABA content decreased during stratification of *M. rubra* seeds (Figure 4). Cold stratification (moist chilling) of western white pine seeds led to a decrease in ABA content and an increase in the ABA catabolites, dihydrophaseic acid and phaseic acid (Feurtado et al. 2004). The amount of ABA in the endocarp of a single fresh seed in our study was about 866 ng, which is 132-fold higher than the ABA content of the seed coat plus embryo. Although the inhibiting effect of ABA on fresh seeds was apparent, the occurrence of dormancy break and germination were not entirely the result of a reduction in the amount of endogenous ABA. For example, warm or cold stratification did not promote *Myrica* seed germination, whereas exogenous application of GA_1, GA_3 or GA_4 promoted seed germination (Table 2) and the addition of GA_3 to warm-stratified seeds caused prompt germination during a short-term incubation (Figure 2). However, the endogenous content of gibberellins in embryos was low when seed dormancy was released by warm stratification for 8 weeks followed by cold stratification for 12 weeks (Figure 5). Based on the increases in GA_4 and GA_1 in embryos of newly germinated seeds, we speculate that the contents of GA_4 and GA_1 increased after the warm plus cold stratified seeds were transferred to germination conditions. Figure 5 shows increased contents of GA_1 and GA_4 in the endocarp and of GA_4 in the seed coat of warm plus cold stratified seeds. These results imply that GA_1 and GA_4 may have diffused into the embryo and stimulated germination.

Exogenous application of 5.2 mM GA_3 promoted germination of fresh *M. rubra* seeds. Quantifying endogenous GA_1, GA_3, GA_4, GA_7 and GA_{20} (the immediate precursor of GA_1) of GA_3-treated seeds during incubation showed that GA_3 decreased and GA_1 increased in the endocarp and seed coat. Further, GA_1 in the embryo increased gradually, the greatest amount being detected in newly germinated seeds (Figure 6). This is the first report of a stimulation of tree seed germination by conversion of GA_3 to GA_1. Exogenous application of GA_1 and GA_4 stimulated more seeds to germinate than exogenous application of GA_3 (Table 2). In gibberellin metabolism, the bioactive GA_1 and GA_4 are formed from GA_{20} and GA_9, re-

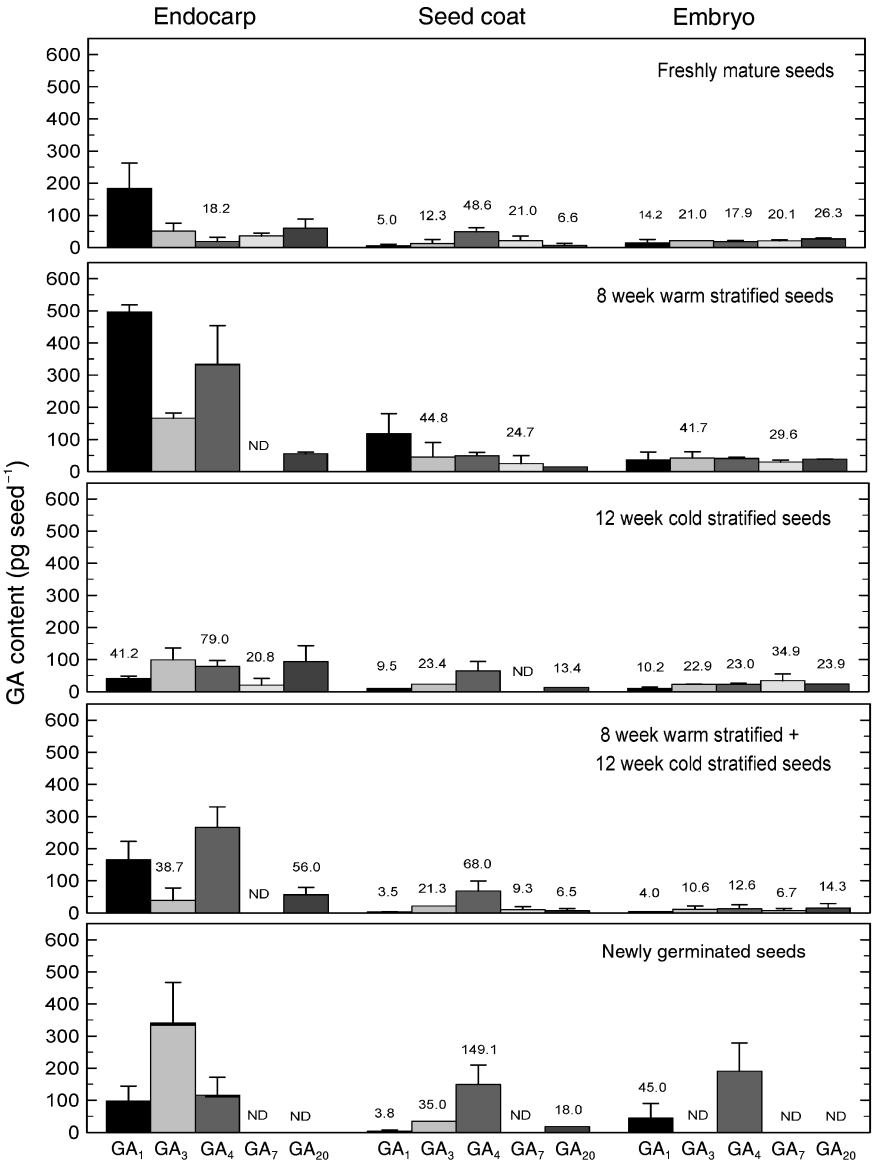

Figure 5. Gibberellic acid (GA) contents of seed parts from fresh and stratified *Myrica rubra* seeds. Results are presented as means ± 1 SD of two replicates of 50 seeds. For clarity, low values are shown as numbers of picograms per seed. Abbreviation: ND = not detectable.

spectively, by the action of GA 3β-hydroxylase, and GA_{20} is converted to GA_3 by the same enzyme in some species (Hedden and Phillips 2000). We speculate that GA 3β-hydroxylase in the endocarp, and perhaps also in the seed coat, catalyzes the conversion of GA_3 to GA_1 during seed germination. Endogenous GA_4 content in embryos of newly germinated seeds was elevated (whereas GA_1 showed only a small increase), which is quiet different from the effect of the exogenous GA_3 pre-treatment. However, both GA_1 and GA_4 are highly active in promoting seed germination.

We gained insight into the roles of the endocarp and seed coat in *Myrica* seed dormancy. The ABA content of the endocarp is high, but decreases with warm or cold stratification, or both. The endocarp had a mean thickness of 1.90 ± 0.16 mm, which was easier to cut and separate after warm stratification. Weakening the endocarp by warm stratification and increasing the growth potential of the embryo by GA_3 resulted in rapid germination of *Myrica* seeds (Figure 2). Recent reports have shown that ABA inhibits weakening of the endosperms of cress (*Lepidium sativum* L.) and coffee seeds (da Silva et al. 2004, Müller et al. 2006). However, we do not know whether the decrease in endogenous ABA content in the endocarp and seed coat is also involved in the weakening of these embryo covering layers.

Myrica fruit has a pulp layer (exocarp + mesocarp), endocarp and seed coat surrounding an embryo with two large cotyledons. The covering layers of pulp, endocarp and seed coat, which are derived from ovular tissue (maternal origin), contain most of the ABA in intact fruits. In the forest, the fruits are quickly dispersed after they mature. The shed fruits are dormant and do not germinate until the next spring. We found that the endocarp of fresh seed contained a large quantity of ABA but the amount decreased after warm or cold stratification, or both. We assume that the physiological dormancy of *M. rubra*

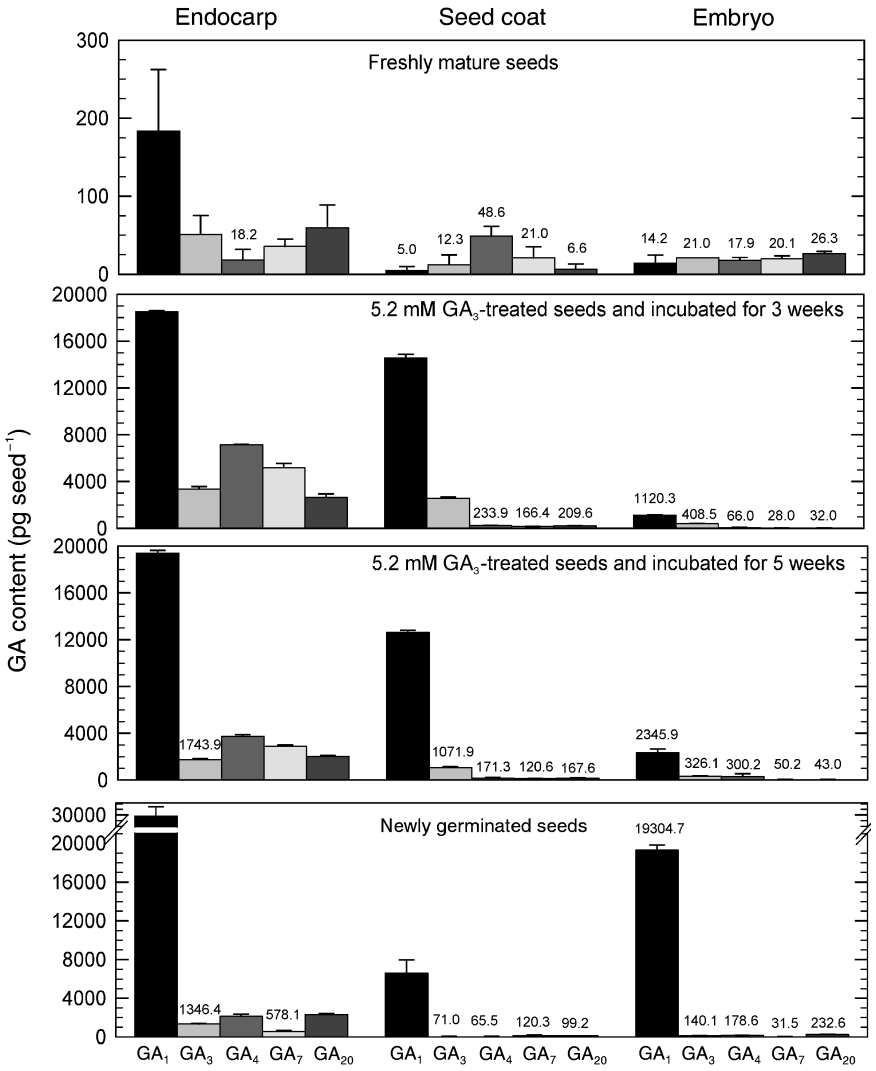

Figure 6. Gibberellic acid contents of the *Myrica rubra* seed parts from fresh seeds and from seeds treated with 5.2 mM GA_3 and incubated at 30/20 °C for a 3- or 5-week period. Results are presented as means ± 1 SD of two replicates of 50 seeds. For clarity, low values are shown as numbers of picograms per seed.

seeds is released over the autumn and winter as warm stratification is followed by cold stratification, with germination occurring in the next warm season. Abscisic acid is closely associated with seed dormancy, and ABA in seeds may be catabolized or leached out, or both, during long-term stratification on forest soil.

Acknowledgments

We are grateful to Professors Jerry M. Baskin and Carol C. Baskin, Department of Biology, University of Kentucky, for comments on the manuscript. We thank Allen Wu, Alice Wu, Chiung-Mei Lee, Kuan-Hung Liu and Wei-Lien Chen for their technical assistance. This study was funded by the National Science Council, Taiwan (NSC 93-2313-B-054-010).

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