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Storage Behavior of *Chionanthus retusus* Seed and Asynchronous Development of the Radicle and Shoot Apex during Germination in Relation to Germination Inhibitors, Including Abscisic Acid and Four Phenolic Glucosides

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Studies on seed storage of *Chionanthus retusus* Lindl. & Paxt. revealed an orthodox behavior, one which showed both desiccation and freezing tolerance. An epicotyl after-ripening dormancy was expressed in *C. retusus* seeds by slow growth of the shoot apex relative to more rapid growth of the radicle when seeds were germinated at 30/20°C. Although these seeds exhibit radicle protrusion, they must be after-ripened for another 8–10 weeks at 30/20°C in order to obtain normal shoot growth. Removal of the endosperm, however, quickly stimulated cotyledon and shoot emergence without the additional after-ripening. Water-soluble glucoside phenolics, GL-3, Nüzhenide, ligustroside and oleoside dimethyl ester are present at relatively high levels in endosperm of freshly harvested seeds. These glucoside phenolics are excreted from the endosperm during subsequent after-ripening. Embryo and endosperm tissue from seed germinating at 30/20°C (germination being defined by protrusion of the radicle) had a 10 times lower abscisic acid (ABA) content than similar tissues from freshly harvested mature seed. However, no shoot growth occurred even with the 10-fold reduction in ABA and a concomitant increase in endogenous gibberellins A_1, A_4 and A_{20}. Thus, epicotyl dormancy during the first 8 weeks of after-ripening at 30/20°C may be controlled by factors other than high ABA, i.e., the slow development of the shoot apex following radicle protrusion may be controlled more by high levels of glucoside phenolics than by diminished ABA and elevated GA levels.

Keywords: Abscisic acid — *Chionanthus retusus* — Epicotyl dormancy — Glucoside phenolics — Gibberellins — Orthodox seed.

Introduction

Chionanthus retusus Lindl. & Paxt. is a medium-sized deciduous tree in the family Oleaceae which grows up to 40 cm in diameter and 15 m of height in Taiwan. It is a dioecious tree and produces white flowers in the spring and dark purple fruits mature in August, making it a valuable ornamental plant. There are two species in the genus, *C. virginicus* and *C. retusus*. The former is distributed in North America, and the latter in East Asia, from Japan, Taiwan to southern China. In Japan, it is considered an endangered plant because of critical habitat that has been destroyed in two (now restricted) areas of Tsushima Island and the Tôno region (Soejima et al. 1998). In Taiwan we investigated a population in the northern region of the island which occurs at low elevation. Due to urbanization and population pressure, it is difficult to find naturally occurring trees in the native, undisturbed forest. Propagation techniques of *Chionanthus* by seed and stem cuttings were reported earlier (Dirr and Heuser 1987), but delayed germination and the difficulty of rooting cuttings greatly increases the cost of cultivation. Macdonald (1986) reported that *C. retusus*, together with other species such as *Davidia involucrata*, *Paeonia suffruticosa* and *Aesculus parviflora*, exhibited an unusual form of epicotyl dormancy, i.e. the root emerges during first year after sowing, but shoot emergence is extended to the next year after the seed has been through a cold winter. This implies that a period of low temperature may normally be required for epicotyl and shoot growth.

In preliminary experiments (unpublished) we investigated germination of *Chionanthus* seeds in a germination chamber and found that the radicle protruded after the seed was incubated for somewhat more than 3 weeks, but emergence of the cotyledons and shoot did not occur for another 8–10 weeks. However, shoot emergence could be observed within 1 week if radicle-protruding seeds were planted in pots and both endocarp and endosperm were removed. Removing the endocarp only, however, did not promote shoot emergence.This observation implies that the endosperm tissue enclosing the embryo may play an inhibitory role in the extended dormancy of the epicotyl. Hartmann et al. (1990) mentioned briefly that embryo dormancy as well as endosperm inhibition seems to be responsible for this slow germination of *Chionanthus* seeds, and also

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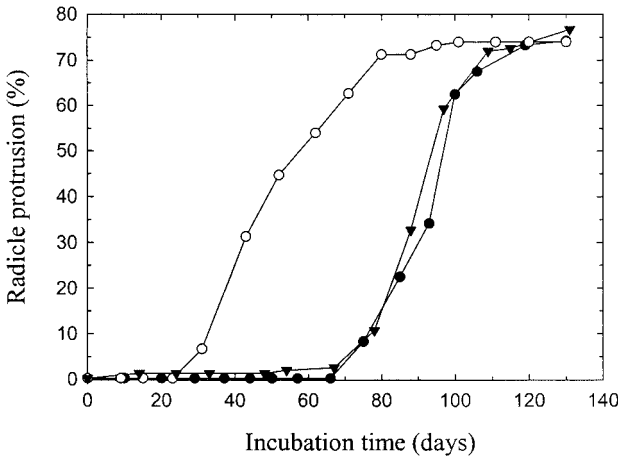


Fig. 1 Radicle protrusion percentage for *C. retusus* seeds after treatment with cold or warm followed by cold stratification. Seeds were harvested in August, 1998 and incubated at 30/20°C to allow for radicle protrusion (there was no epicotyl growth). Open circles, fresh seeds maintained at 30/20°C; closed triangles, fresh seeds stratified at 30/20°C for 3 weeks followed by cold stratification at 5°C for 3 months; closed circles, fresh seeds stratified at 5°C for 3 months.

noted that the dormant seeds normally require a combination of warm and cold moist stratification for germination.

Few studies have investigated epicotyl dormancy of seeds in any detail. One study did show that the roots of freshly harvested seeds of *Quercus alba* and *Q. prinus* are able to protrude in the fall at soil temperatures of 10–15°C, but the epicotyls of these two oak species show partial or complete dormancy, without shoot growth, unless outdoor chilling occurs (Farmer Jr. 1977). Epicotyl dormancy of *Viburnum trilobum* seedlings could be overcome by cotyledon removal, and the presence of a water-soluble inhibitor in the seed cover was associated with slow growth of the radicle and hypocotyl (Knowles and Zalik 1958). The cause of epicotyl dormancy in *C. retusus* seed is not clear.

For other plant species it has been shown that abscisic acid (ABA) and gibberellins (GAs) play important roles in the regulation of seed dormancy and germination. ABA is involved in maintenance of seed dormancy, whereas GAs exhibit an effect that is antagonistic to ABA (reviewed in Bewley and Black 1994). In this study we have characterized seed germination, dormancy and the storage behavior of *C. retusus*, and identified and quantified four water-soluble germination inhibitors from its endosperm. Furthermore, we have assessed shoot apical meristem growth and compared it with hypocotyl growth by electron microscopy during the germination period.

Results

Effect of stratification on seed germination

Freshly harvested seeds were unable to germinate (as defined by protrusion of the radicle by at least 2 mm) until the 4th week of after-ripening at 30/20°C. The time at which 50% germination was reached under these conditions was 47 d (Fig. 1). In this treatment no epicotyl growth occurred. The

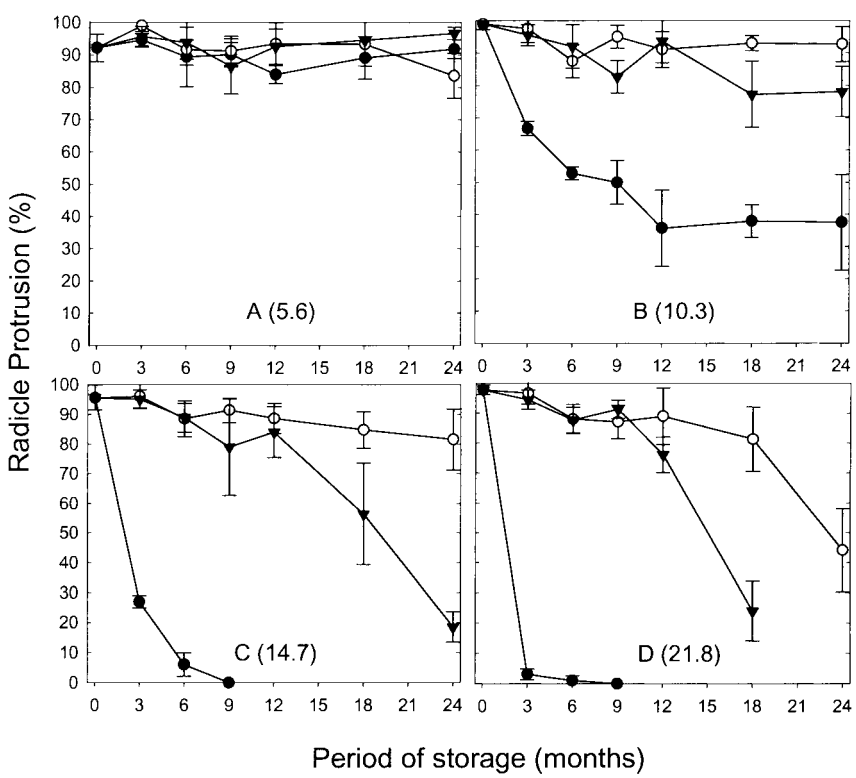


Fig. 2 Radicle protrusion percentage of *C. retusus* seeds subjected to dehydration to achieve the moisture contents (% of FW basis) shown in parentheses. Seeds were subsequently held at that moisture content at –20 (closed circles), 5 (open circles) and 15°C (closed triangles) for a period from 3 to 24 months. The treated seeds were then incubated at 30/20°C to allow for germination. The vertical bars represent means ± SE.

Table 1 Amounts of endogenous GAs and ABA in embryo and endosperm of fresh, mature seeds, seeds with radicle protrusion but arrested shoot emergence, or seeds treated with cold moist stratification which will result in delay of radicle protrusion

	Content (ng $(gDW)^{-1}$)		
	Fresh-mature seeds	Radicle protrusion seeds [a]	Cold-stratified seeds [b]
Embryo (axis + dicots)			
ABA	265.2±22.4	24.4±0.5	21.6±3.2
GA_1	ND	0.97	ND
GA_3	ND	ND	ND
GA_4	0.13	1.65	0.77
GA_7	ND	ND	ND
GA_9	0.47	0.51	0.70
GA_{20}	0.31	2.57	0.33
GA_{24}	43.03	12.85	28.85
ent-kaurene	390.0	1700.0	473.0
Endosperm			
ABA	80.5±6.4	8.8±2.0	16.3±5.5
ABA-GE [c]	317±293	147	324±217
GA_1	ND	ND	ND
GA_3	ND	ND	ND
GA_4	ND	ND	ND
GA_7	ND	ND	ND
GA_9	ND	ND	ND
GA_{20}	ND	1.95	ND
GA_{24}	ND	ND	ND

[a] Radicle-protrusion seeds were obtained from fresh seeds incubated at 30/20°C.
[b] Seeds were stratified at 5°C for 1 year.
[c] Replicate analyses were accomplished 1 year apart and may represent change with time rather than sample-to-sample variation, i.e. the 2nd replicate was ca. 300 ng lower than 1st replicate.
ND, not detectable, but [17,17–$^2H_2$]GA internal standard was detectable.

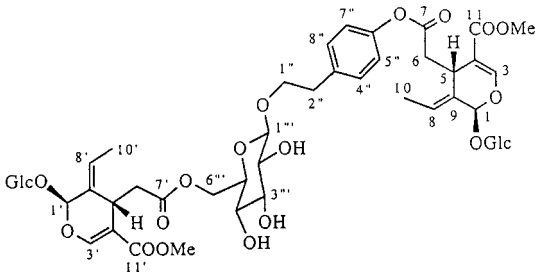
time to reach 50% germination was extended to 90 d when fresh seeds were stratified at 30/20°C for 3 weeks (no radicle protrusion) followed by 5°C for 3 months prior to incubating at 30/20°C. A similar response (50% germination at day 95) when are stratified only at 5°C (no initial warm period) for 3 months.

Seed storage behavior of C. retusus seeds

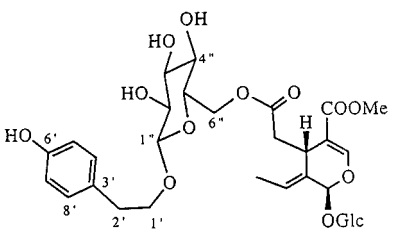
The moisture content of freshly harvested, mature seeds was 30%. The lower the moisture content that was maintained in seeds that were stored –20, 5 or 15°C for various periods, the better was germination (Fig. 2). Seeds at 5.6% moisture content retained their original viability for the entire 24 months test period, whereas seed viability was reduced gradually if moisture content was maintained at 10.3% or higher. A moisture content of 15 or 22% reduced the germination percentage appreciably (and immediately), especially when the storage temperatue was –20°C. Storing seeds with the higher moisture contents at 5°C, however, could extend seed storage life, and good viability for seeds with 10.3% moisture was retained for at least 24 months when stored at 5°C. It should be noted that reducing seed moisture content before storage causes an increased germination percentage, e.g. from 75% to above 90% (Fig. 1, 2). Thus, seeds of *C. retusus* tolerate desiccation and we refer to this as an orthodox type of storage behavior.

Gibberellins, abscisic acid and ABA-glucosyl ester in embryo and endosperm tissues

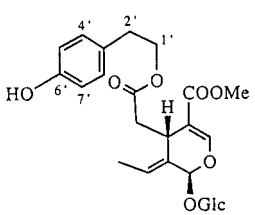
The embryo and endosperm of *C. retusus* seeds showed differences in ABA, GA_1, GA_4, GA_9, GA_{20} and GA_{24} levels (Table 1). Only endosperm tissues were examined for ABA-glucosyl ester (ABA-GE) content. High ABA levels of 249 ng $(g\ DW)^{-1}$ occurred in embryos of freshly harvested seeds (which will not germinate). However, in embryos of seeds treated with periods of cold stratification (which extends the time of germination as defined by radicle protrusion), or under warm stratification conditions that were sufficient to allow for germination, ABA was reduced by ca. 10-fold. Similarly, a high ABA level (80 ng $(g\ DW)^{-1}$) occurred in the endosperms of freshly harvested seeds (which will not germinate) and endosperm ABA level was much lower in stratified seeds. Although ABA-GE levels were very much higher than free ABA levels in the endosperm, there was no obvious trend for ABA-GE with stratification treatment (Table 1).



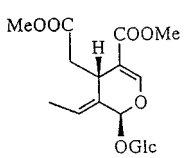
Compound 1: GL-3



Compound 2: Nüzhenide (GL-6)



Compound 3: Ligustroside



Compound 4: Oleoside dimethyl ester

Fig. 3 Structures of compounds 1, GL-3; 2, nüzhenide (GL-6); 3, ligustroside; 4, oleoside dimethyl ester.

The contents of GA_1, GA_4, GA_9, GA_{20} and GA_{24} were assessed by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) for embryos of freshly harvested seeds, and for seeds that had received stratification treatments. GA_1 was detected only in embryos of seeds receiving the 4–5 weeks of warm stratification (a treatment which produces radicle-protrusion). Its immediate precursor, GA_{20}, was also elevated in seeds of this treatment (Table 1). GA_3 was not detected in embryos of *C. retusus* under any treatment. GA_4, the other per se growth-active GA, was found in embryos from all treatments and was highest in warm-stratified seeds (this warm stratification treatment will protrude radicles, but will not elongate epicotyls). GA_4 was also elevated in cold-stratified seeds, a treatment which will, when the seeds are placed in 30/20°C delay radicle protrusion. Interestingly, *ent*-kaurene, a diterpene precursor of GAs, was also elevated (especially) in seeds receiving warm stratification (which allows for radicle protrusion but no epicotyl elongation). GA_9, an immediate precursor of GA_4, and maybe that of GA_{20}, tended to be high in seeds with radicle protrusion. GA_{24}, a C_{20} GA, was elevated in freshly harvested seeds which will not germinate.

In endosperm tissues, there were no GAs detected except for low levels of GA_{20} in seeds that received the warm stratification treatment and were showing radicle-protrusion.

Structures of compounds 1 (GL-3), 2 (nüzhenide), 3 (ligustroside) and 4 (oleoside dimethyl ester)

A crude, highly water-soluble fraction extracted from the endosperm tissue was found to inhibit germination and/or epicotyl elongation of alfalfa seeds/seedlings when applied at a concentration of 5,000 ppm (mg DW liter^{-1}) (data not shown). As detailed in Materials and Methods, four water-soluble compounds that could reduce alfalfa seed epicotyl growth were then purified from these endosperm tissue extracts of *C. retusus* seeds. Compound 1 had a quasi-molecular ion $(M+Na)^+$ at m/z 1095.4 which is consistent with the molecular formula $C_{48}H_{64}O_{27}$. The ^{1}H NMR spectrum exhibited two sets of characteristic signals (oleoside 11-methyl ester): two hemiacetalic protons (H-1, 1′) at δ 5.92 and 6.03, two vinylic protons (H-3, 3′) at δ 7.52 and 7.57, two methyl doublet at δ 1.71 and 1.76 (Me-10, 10′), and two quartets at δ 6.08 and 6.18 (H-8, 8′). Four A_2B_2 aromatic protons (δ 6.98 and 7.28) together with two sets of methylene triplets inferred a phenethyl function. The ^{13}C NMR spectrum of compound 1 also exhibited signals for two oleoside methyl ester units (δ 95.2 and 95.3 for C-1 and 1′; δ 155.2 and 155.3 for C-3 and 3′; δ 13.7 and 13.8 for C-10 and 10′), and one phenethyl moiety at δ 71.4 (C-1″), 36.6 (C-2″), 138.0 (C-3″), 131.0 (C-4″, 8″), 122.5 (C-5″, 7″) and 150.5 (C-6″) together with the third glucosyl moiety (δ 104.4 for Glc-C-1‴). As all the chemical shifts of compounds 1 in D_2O are identical with those of GL-3 (LaLonde et al. 1976), the structure was thus confirmed (Fig. 3).

Compound 2 had the molecular formula $C_{31}H_{42}O_{17}$ that was deduced from a quasi-molecular ion $[M+Na]^+$ at m/z 709 in the FAB-MS, ^{1}H NMR and ^{13}C NMR spectra. The ^{1}H NMR spectrum displayed signals (δ 7.42 s, 5.74 brs, 6.00 q, 3.59 s and 1.59 d) for one oleoside methyl ester unit and signals (δ 6.99 d and 6.70 d) for a phenethyl moiety. The ^{13}C NMR spectrum exhibited signals for only one oleoside methyl ester unit (δ 95.2 for C-1; 155.2 for C-3; 13.7 for C-10) and a phenethyl moiety at δ 71.8 (C-1′), 35.3 (C-2′), 130.5 (C-3′), 130.9 (C-4′, 8′), 116.1 (C-5′, 7′) and 156.8 (C-6′), as well as the second glucosyl moiety (δ 104.4, Glc-C-1″; 64.5, Glc-C-6″) for connecting the *para*-hydroxyphenethyl and oleoside moieties. Finally, a comparison of ^{13}C NMR spectral data with those of authentic Nüzhenide (GL-6) (LaLonde et al. 1976) was used to confirm the identity of compound 2 (Fig. 3). The compound was present in the seeds of all olive cultivars at ripening stage (Servili et al. 1999).

The ^{1}H NMR spectrum of compound 4 exhibited typical signals of a secoiridoid nucleus, including a hemiacetalic proton H-1 (δ 5.91) and two vinylic protons, H-3 and H-8 (δ 7.52,

Table 2 Inhibition of alfalfa seedling shoot growth (shoot length) by compounds 1–4 that had been extracted from the endosperm of *C. retusus* seeds

	Control	500 ppm	1,000 ppm	3,000 ppm
Compound 1 (GL-3)	21.5 [a]	15.7 [b]	9.4 [c]	4.3 [d]
Compound 2 (Nüzhenide)	26.1 [a]	15.5 [b]	10.2 [c]	3.5 [d]
Compound 3 (Ligustroside)	25.2 [a]	–	20.4 [b]	15.8 [c]
Compound 4 (Oleoside dimethyl ester)	24.1 [a]	–	19.9 [b]	14.7 [c]
	Control	5 ppm	10 ppm	
ABA	19.5 [a]	7.2 [b]	0	

Means followed by same letter within a row do not differ significantly at $P = 0.05$.
Alfalfa seedling shoot length was measured after 3 d when the seeds were germinated in each of four concentrations of compounds 1–4. Commercial (±)ABA (Sigma) was also used as an "external control" at 5 and 10 ppm.

6.10). In the ^{13}C NMR spectrum signals at δ 155.2 (C-3), 124.8 (C-8), 130.5 (C-9) and the methyl carbon at δ 13.5 (C-10) as well as pyranose functions, indicating a oleoside nucleus. Two carbonyl resonances (δ 173.6 and 168.7) and methoxyl signals (δ 51.9 and 52.2) were attributed to the carbomethoxyl moieties. Spectral comparison of compound 4 with the report data (Gariboldi et al. 1986, Kuwajima et al. 1988, Shen et al. 1996) established its structure as oleoside dimethyl ester (Fig. 3). Compound 3 showed typical signals for oleoside methyl ester and a A_2B_2 spin system (δ 7.05, d, J = 8.4 Hz; 6.69, d, J = 8.4 Hz) in the ^1H NMR spectrum, as well as its correspondent symmetry character of carbon resonances at δ 130.9 d and 116.2 d in the ^{13}C NMR spectrum concluded its structure, ligustroside. The ligustroside was reported by LaLonde et al. (1976) from *Fraxinus americana*, Kuwajima et al. (1988) from *Olea europaea* and Shen et al. (1996) from *Jasminum polyanthum*, the same plant family.

Although accurate estimates of the concentrations of Compounds 1–4 in the endosperm tissue cannot be made, approximate estimates are: Compound 1, 120 ppm; Compound 2, 340 ppm; Compound 3, 280 ppm; Compound 4, 100 ppm; all based on estimates made at the HPLC stage of purification, i.e. micrograms of the purified compound per gram of fresh weight of endosperm tissue.

Effect of compounds 1–4 on alfalfa seed germination

The mean length of seedling shoots after treatment with the four compounds at 500 ppm or greater was significantly decreased in comparison to untreated control seedlings (Table 2). Compounds 1 and 2 were more effective inhibitors than compounds 3 and 4. That said, ABA was the best inhibitor of alfalfa seed germination, strongly inhibiting germination and seedling growth. In fact, no germination was observed with ABA treatment, even at the very low dose of 10 ppm.

Embryo and endosperm structures in different stages of germination

Freshly harvested, mature seeds of *C. retusus* were 9–11 mm in length and contained an embryo surrounded completely by a copious cellular endosperm except for the radicle

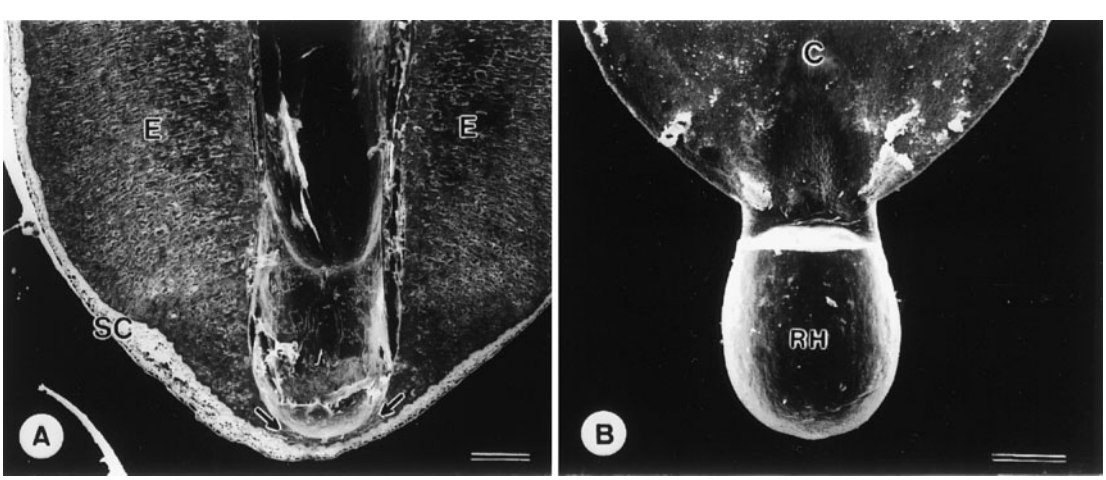


Fig. 4 SEM micrographs of partial embryos and endosperms of fresh, mature *C. retusus* seeds. (A) Hypocotyl and radicle surrounded completely by the endosperm tissue. Arrows indicate the narrow endosperm between the radicle apex and the seedcoat. (B) A shoot apical meristem (embryo axial apex) without the dome-shape. C, cotyledon; E, endosperm; RH, radicle-hypocotyl; SC, seedcoat. Scale bar = 300 μm.

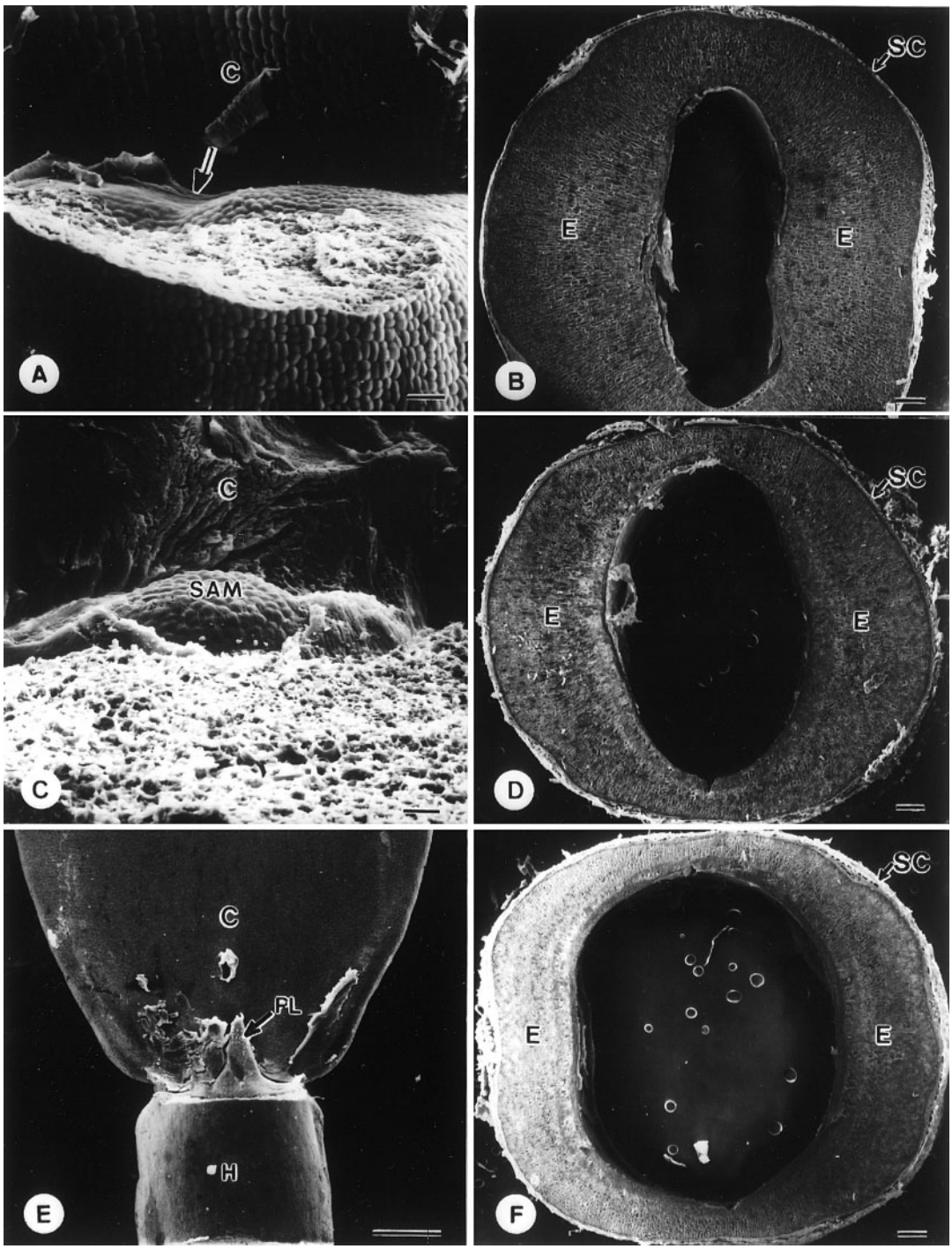


Fig. 5 SEM micrographs of apices of several embryo axes (A, C, E) and transverse section of their endosperms (B, D, F). Apex of embryo axis (A) and endosperm (B) of fresh-matured seed. An emerged dome-shaped shoot apical meristem (C) and diminished amount of endosperm tissue (D) in a germinating seed with a 2 mm protruded radicle. A prominent shoot apex with 2–4 primary leaves (E) and even more diminshed endosperm tissue (F) for a germinating seed with a 10 mm protruded radicle. E, endosperm; H, hypocotyls; PL, primary leaf; SAM, shoot apical meristem; SC, seedcoat. Scale bars = 30 μm (A and C) and 300 μm (B, D, E and F).

apex, where the endosperm layer was quite narrow (Fig. 4A with arrows). The embryo axis and two large cotyledons were about 7–9 mm in length (Fig. 4B). The shoot apical meristem and epicotyl were not detectable in freshly harvested seed (Fig. 4B, 5A). However, a small dome-shaped shoot apical meristem was observed after ca. 3 weeks of 30/20°C stratification, when

the protruded radicle was about 2 mm in length (Fig. 5C). A prominent shoot apex with 2–4 small primary leaves was found when the radicle had extended to about 10 mm (Fig. 5E), during germination after warm stratification. The volume of cellular endosperm decreased gradually during radicle extension (Fig. 5B, D, F).

Discussion

Seeds of *C. retusus* can tolerate desiccation and storage at subzero temperatures, and thus are referred to as exhibiting orthodox storage behavior (Roberts 1973). Although *C. retusus* seeds can be stored over a wide range of temperatures at the relatively low moisture content of 5.6%, their longevity begins to vary greatly as seed moisture content is increased (Fig. 2). Dehydration of fresh seeds increased germination (radicle protrusion) from 75% to above 90% (Fig. 1, 2). Cause of this post-harvest ripening is unknown, though, speculatively, it may be associated with seed maturity and drying effects on seed maturation, phenomena which are known to increase germination (Hay and Probert 1995, Chien and Lin 1999).

Since maintaining seed viability during long-term storage is of the utmost importance, it appears that storage at this low moisture content and at a reduced temperature, instead of ambient temperature, is absolutely necessary. Fortunately, storage at 5 or 15°C does not require a subzero temperature facility and thus long-term storage is feasible even in remote locations. Zheng et al. (1998) reported that a requirement for low or controlled temperature storage of seeds has important cost implications for developing countries. Hence, it is very important to be able to store seeds without use of low temperature (for example, under ultra dry conditions), if seed longevity and vigour can be maintained. However, for ex situ genetic resource conservation, which is typically considered as more than 10 years, further storage testing for this species will be required (our trials only extended 2 years).

In the present study we have demonstrated the phenomenon of asynchronous emergence of the root and shoot in *Chionanthus* seeds. However, the shoot apex is not in a quiescent state as the radicle protrudes. Rather, the shoot apex continues to grow slowly. As a consequence, the cotyledons and shoot (epicotyl) have a greatly delayed emergence which occurs only after 10–12 weeks of incubation. During this period the root system is developing rapidly. Interestingly, removal of the endocarp and endosperm (but not the endocarp only) from a seed where the radicle is protruding promotes shoot apex growth! It thus appears that some inhibitory substance diffusing from the endosperm blocks shoot emergence.

We have provided evidence that ABA occurs in the embryo in high levels, and also that ABA and its glucosyl ester occur in the endosperm. However, even a 10-fold reduction in ABA levels (which occurs after a brief period of after-ripening) does not allow for normal shoot growth. Epicotyl growth may thus be inhibited by levels of ABA which allow for growth of the radicle. However, we have also noted that the endosperm contains water-soluble glucoside phenolics, including GL-3, Nüzhenide, ligustroside and oleoside dimethyl ester. While evidence points toward high endogenous levels of ABA being causally involved in the inhibition of germination (radicle protrusion) in freshly harvested seeds of this species, it is possible that ABA may not be (the primary) inhibitor of epicotyl (shoot) elongation. An alternative or additional inhibitor(s) of shoot growth, given their relatively high levels in the endosperm, are the phenolic glucosides (see above). These compounds thus may function physiologically, either as conjugates or aglycones, with or without the participation of ABA, in the shoot growth inhibition process that occurs during the first 8 or so weeks of after-ripening.

A similar conclusion was made by Sondheimer et al. (1970), for GL-3 and Nüzhenide isolated from the seeds of species in the genera *Fraxinus*, *Olea* and *Syringa*, (all of these members of the Oleaceae), showing that endosperm and embryo tissue from dormant or cold temperature after-ripened seeds showed little change in levels of the GL-3 and Nüzhenide, but the two glucosides decreased to quite low levels during embryo growth. Speculatively, then, changes of glucoside phenolic levels during embryo emergence (germination) may explain our observation that as shoot emergence begins to occur, there is an appreciable reduction of endosperm tissue and an increase in radicle length (data not shown). Two other phenolics, ligustroside and oleoside dimethyl ester also exhibited an inhibitory effect on alfalfa seed germination, but their inhibitory abilities are lower than GL-3 and Nüzhenide. Thus, ABA or the combination of ABA and the four phenolic compounds from *Chionanthus* endosperm are possible candidates for endogenous inhibitors that exert a retarding influence on shoot apex growth, thereby accounting for the ca. 8–10 week delay between radicle protrusion and the beginning of rapid shoot growth in the germination process.

As shown in Table 1, the GA_1 and GA_4 are probable active forms in this species. The increase in the levels of GA_1, GA_4 and GA_{20} in embryos of radicle-protruding seeds (as opposed to freshly harvested seeds) indicates that GA biosynthesis is likely activated coincidental with radicle protrusion. Even so, growth of the shoot apex still remained slow, despite the fact that ABA levels in these embryos have dropped by 10-fold, relative to freshly harvested seeds which have not been warm stratified. A continued state of seed dormancy has also been reported in seed of *Acer* (Pinfield et al. 1989) and *Taxus* (Chien et al. 1998) species, with the dormant period being coincidental with low levels of ABA, even though the seeds were stratified. Hence, shoot (epicoctyl) growth may be very sensitive to low ABA levels, not only in *C. retusus*, but in seed of other tree species also.

The shoot apical meristem of *C. retusus* could not be seen in freshly harvested mature seed until radicle protrusion had occurred (Fig. 5). After radicle protrusion the shoot apex grew continuously but very slowly, relative to growth of the radicle

and developing root system. Based on Fig. 5B, D and F it appears that the volume decrease in the endosperm is correlated with radicle extension. Thus, once ABA/ABA-GE and the phenolic glucosides become reduced (weeks 8–10 after radicle protrusion) in the endosperm, it is possible that endogenous GA levels are then able to stimulate shoot (epicotyl) growth. For example Sondheimer et al. (1970) reported that the concentrations of GL-3 and Nüzhenide decreased as seed germination proceeded, and this decrease was correlated with shoot growth of *Fraxinus americana*.

The delay in radicle protrusion for several weeks even after the seed had been stratified (Fig. 1) is consistent across several collection years. Such a germination delay from stratified seeds is unusual for most woody plants (Bewley and Black 1994). Our observations in the greenhouse indicate that the cotyledons and shoot can emerge rapidly if seeds with protruding radicles are exposed to 5°C for 3 months and then planted (data not shown). This observation is in agreement with the view that epicotyl dormancy of *Chionanthus* seed requires a cold temperature period to overcome what can be termed a second stage of dormancy (Macdonald 1986). We also note that seeds of some *Viburnum* species in north America exhibit epicotyl dormancy (Gill and Pogge 1974). However, it should be noted that cotyledon and shoot emergence can occur from Taiwanese *Chionanthus* seeds without cold stratification if a very much longer warm temperature (3–3.5 months in total) is given. According to Nikolaeva's description (Nikolaeva 1977) of the six types of morphophysiological dormancy, *Chionanthus* seeds with epicotyl dormancy belong to the class of seeds showing deep morphophysiological dormancy, i.e., where seeds must be exposed to warm for radicle protrusion, followed by cold stratification for dormancy breaking. In other words, the embryo must grow slowly, but continuously in synchrony with radicle protrusion under favorable temperatures prior to the cold treatment causing the embryo to become completely non-dormant.

Materials and Methods

Seed collection and preparation

Mature fruits (drupes) of *C. retusus* with a dark purple color were collected at the campus of National Taiwan University in Taipei (25°03′ N, 121°31′ E), Taiwan in August 1998 and 1999. Fruits were placed in a nylon net and macerated by hand in water. The empty seeds were floated off, with clean filled seeds being separated into three groups. It should be noted that the seed, in longitudinal section, consists of two cotyledons and an axis surrounded by hard endocarp, seedcoat and endosperm. One group of seeds was mixed with moist sphagnum for stratification and a subsequent germination test. The other two groups were utilized for a storage behavior experiment and for analysis of germination inhibitors, respectively.

Warm and/or cold stratification

Each stratification treatment consisted of three replicates of 50 seeds each. For warm → cold stratification, freshly harvested seeds were placed in sealable polyethylene bags (0.04 mm in thickness) with moist sphagnum, and maintained initially at alternating warm temperatures of 30/20°C with 12 h of fluorescent light (80–100 μE m^2 s^{-1}) at 30°C (Chien et al. 1998). Warm stratification was performed for 3 weeks, at which time no radicle protrusion had occurred. The warm stratified seeds were then given 3 months of cold stratification at 5°C. A cold stratification treatment without warm treatment was also carried out on fresh seeds in moist sphagnum for each of 3 and 12 months prior to the seeds being subjected to germination tests. The moisture content of the sphagnum in both warm and cold stratification treatments was about 400% of its dry mass. Finally, some seeds were also air-dried (see below).

Germination tests

Germination (radicle protrusion) tests from fresh seeds, warm → cold stratified seeds, cold stratified seeds or air-dried seeds were performed in 12 h of fluorescent light with an alternating temperature of 30/20°C, e.g. the same temperature as the warm stratification condition. Germination, judged by radicle protrusion of at least 5mm, was recorded weekly. The final germination percentage was calculated after 15 weeks.

Drying treatments

In order to test seed storage behavior, fresh seeds were dried to four different moisture contents, each seed lot then being stored at three different temperatures, –20, 5 and 15°C. The moisture contents were generated keeping seeds over silica gel in a sealed glass desiccator 24 h, 40 h, 70 h and 175 h. Seeds were then taken out and sealed into aluminum foil envelopes for storage, with moisture content of a subsample being measured. As above, each storage treatment consisted of three replicates of 50 seeds each. At intervals of 3 or 6 months seeds were removed from storage, their moisture content assessed, and their germination evaluated. The dry storage lasted for 24 months, e.g. germination was tested at each of 3, 6, 9, 12, 18 and 24 months.

Determination of moisture content

The moisture content (m.c.) of fresh and treated seeds were measured gravimetrically, with three replicates of 10 seeds being oven dried for 17 h at 103°C (ISTA 1999). The seeds were first cut into 4 mm pieces before their fresh weight was determined, then dried. All moisture contents are expressed as a percentage of a fresh mass basis.

Extraction, purification and quantification of GAs and ABA

The endogenous GAs and ABA of embryos and of endosperm tissues dissected from *C. retusus* seeds were first solvent extracted, then purified and finally analyzed by GC-MS-SIM for hormone quantification The endogenous GAs and ABA were quantified by the isotope dilution method (Koshioka et al. 1983) using internal standards of 20 ng of deuterated [17,17–$^2H_2$]GAs (GA_1, GA_3, GA_4, GA_7 GA_9, GA_{20} and GA_{24}) and 300 ng of deuterated [$^2H_6$]-ABA (the deuterated GAs were a gift from Prof. Lewis N. Mander, and the deuterated ABA was a gift from Drs. L. Rivier and M. Saugy, University of Lausanne, Switzerland). The specific extraction, purification and separation procedures follow. The tissue was ground in methanol : water (80 : 20) and extracted overnight in darkness at 4°C. The extract was filtered and the tissue debris was washed twice with the 80% methanol. The combined extract was purified through a C_{18} column (Lichroprep RP18 for liquid chromatography, Merck) to remove the pigments. The eluate was then reduced to the aqueous phase, adjusted to pH 3.0 with 3 M acetic acid, and partitioned four times against equal volumes of water-saturated ethyl acetate. The acidic, ethyl acetate was then dried and chromatographed on a silica gel partition column (SiO_2, ICN Biochemicals) with *n*-hexane (grade for gas chromatography, Sigma). This eluate was subjected to reversed phase C_{18} HPLC (Waters Associates Inc., U.S.A.) and appropriate fractions from the C_{18} column were

grouped, dried in vacuo and then subject to $N(CH_3)_2$ HPLC (Nucleosil, Alltech Associates) (Koshioka et al. 1983). Appropriate fractions of GAs from this column were grouped, dried in vacuo, derivatized by adding etheral diazomethane, then drying with N_2, and silylated with 50 µl pyridine plus 100 µl of *N,N′*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce Chemical Co). For ABA derivatization, methylation with diazomethane without silylation was used. GC-MS-SIM analysis (HP 5790A GC and 5970b MSD) as described by Rood et al. (1989) for GAs and the same condition for ABA and ABA-GE (ABA-glucosyl ester). ABA and ABA-GE values for endosperm were the average of extraction/analysis of two different sets of tissue. The levels of GAs and ABA expressed as ng per gram dry weight (ng $(gDW)^{-1}$) of the lyophilized tissue.

Extraction and isolation of germination inhibitors

Endosperm tissue from *C. retusus* seeds was freeze-dried and after grinding, samples were extracted with equal volumes of chloroform and methanol. The extract was centrifuged at 12,000×*g* for 10 min and the supernatant was transferred into a glass vessel. The pellet was re-extracted one additional time with equal volumes of chloroform and methanol as above. The combined supernatants were taken to dryness in a rotary vacuum evaporator with a vacuum pump, and resuspended in a mixture of H_2O/MeOH/*n*-hexane (1/3/4, v/v) for both H_2O/MeOH soluble fraction and *n*-hexane soluble fraction. The two fractions were concentrated to ca. 5,000 ppm and 1 ml of each fraction was spread on 5 cm diameter filter papers (Whatman no. 1) in sterile Petri dishes and air-dried before a germination test was accomplished. Alfalfa seeds were then used to determine the presence of germination inhibitors. The solvents, H_2O/MeOH or *n*-hexane, were also air-dried on the filter papers in Petri dishes for use as "solvent controls". The filter papers were then watered with double-distilled water and the alfalfa seeds placed on the filter papers for the germination trial. An indication of germination inhibition was obtained by counting the number of germinated seedlings and by measuring the length of the hypocotyls for those seedlings that did germinate. Our results showed that the H_2O/MeOH extract was indeed capable of hindering seed germination. We thus resuspended this fraction residue in water and mixed it with equal volume of chloroform to give both a water fraction and a chloroform fraction. The alfalfa seed test was again utilized and only the water fraction inhibited seed germination. The water fraction was further purified by applying it to a Sephadex LH-20 column which was then eluted with MeOH to obtain four fractions. Each fraction was then tested with alfalfa seeds as above and only one fraction showed activity. So we further purified that fraction by preparative silica gel TLC, developed with $CHCl_3$/MeOH/H_2O, 65/35/10, v/v) and subsequent HPLC using C_{18}-reversed phase column (Beckman Instruments, CA, U.S.A.). From the HPLC eluate seven fractions were tested on the germination assay, but only four of these HPLC fractions were effective inhibitors of germination, e.g. putative compounds **1**, **2**, **3**, and **4**. We then utilized NMR and mass spectroscopy to attempt identification of these four compounds.

NMR and FAB-MS spectroscopy

Purified samples were dissolved in both 300 µl D_2O and CD_3OD, and sealed in NMR tubes. NMR experiments were performed on a Bruker DMX-500 MHz FT-NMR spectrometer. FAB-MS spectra were obtained using a JEOL SX-102A double-focusing mass spectrometer of reversed geometry (JEOL, Japan). Samples in 1 µl methanol were mixed with 1 µl matrix (3-nitrobenzyl alcohol) on a FAB probe tip for subsequent analysis.

Compound **1**: $C_{48}H_{64}O_{27}$; ^{1}H NMR (500MHz, CD_3OD) δ 5.92 (1H, brs, H-1), 6.03 (1H, brs, H-1′), 7.52 (1H, s, H-3), 7.57 (1H, s, H-3′), 6.08 (1H, q, *J* = 7.1 Hz, H-8), 6.18 (1H, q, *J* = 7.0 Hz, H-8′), 1.71 (3H, d, *J* = 7.1 Hz, H-10), 1.76 (3H, d, *J* = 7.0 Hz, H-10′), 3.68, 3.75 (each 3H, s, COOMe), 6.98 (2H, d, *J* = 8.5 Hz, H-5″, 7″), 7.28 (2H, d, *J* = 8.5 Hz, H-4″, 8″), 4.80 (1H, d, *J* = 7.5 Hz, Glc-H-1′), 4.82 (1H, d, *J* = 7.5 Hz, Glc-H-1‘‘), 4.58 (1H, d, *J* = 7.5 Hz, Glc-H-1‴); ^{13}C NMR (125 MHz, CD_3OD) δ 95.2, 95.3 (d, C-1, 1′), 155.2, 155.3 (d, C-3, 3′), 109.3, 109.4 (s, C-4, 4′), 30.5, 30.7 (d, C-5, 5′), 41.1, 41.3 (t, C-6, 6′), 171.7, 173.1 (s, C-7, 7′), 125.0, 125.2 (d, C-8, 8′), 130.5, 130.6 (d, C-9, 9′), 13.7, 13.8 (q, C-10, 10′), 168.6, 168.7 (s, C-11, 11′), 71.4 (t, C-1″), 36.6 (t, C-2″), 138.0 (s, C-3″), 131.0 (d, C-4″, 8″), 122.5 (s, C-5″, 7″), 150.5 (s, C-6″), 52.0 (s, OMe ×2), 100.8 (d, Glc-C-1″), 101.0 (d, Glc-C-1‴), 104.4 (d, Glc-C-1‴), 71.4, 71.5, 71.6 (d, Glc-C-2′, 2″, 2‴), 78.4, 78.0, 77.9, 75.2, 75.0, 74.8 (d, Glc-C-3′, 3″, 3‴, 5′, 5″, 5‴), 71.7, 71.6, 71.5 (d, Glc-C-4′, 4″, 4‴), 72.2 (d, Glc-C-5″), 62.6, 62.7 (t, Glc-C-6′, 6″), 65.0 (t, Glc-C-4′, 4″, 4‴), 72.2 (d, Glc-C-5″), 62.6, 62.7 (t, Glc-C-6′, 6″), 65.0 (t, Glc-C-6‴); FABMS m/z: 1095.4 $[M+Na]^+$.

Compound **2**: $C_{31}H_{42}O_{17}$; ^{1}H NMR (500 MHz, CD_3OD) δ 5.74 (H, brs, H-1), 7.42 (1H, s, H-3), 6.00 (1H, q, *J* = 6.8 Hz, H-8), 1.59 (3H, d, *J* = 6.8 Hz, H-10), 3.59 (3H, s, COOMe), 6.70 (2H, d, *J* = 8.0 Hz, H-5′, 7′), 6.99 (2H, d, *J* = 8.0 Hz, H-4′, 8′), 4.83 (1H, d, *J* = 7.8 Hz, Glc-H-1′); ^{13}C NMR (125 MHz, CD_3OD) δ 95.2 (d, C-1), 155.2 (d, C-3), 109.4 (s, C-4), 31.8 (d, C-5), 41.3 (t, C-6), 173.1 (s, C-7), 125.0 (d, C-8), 1130.7 (s, C-9), 13.7 (q, C-10), 168.7 (s, C-11), 52.0 (q, $COOCH_3$), 71.8 (t, C-1′), 35.3 (t, C-2′), 130.5 (s, C-3′), 130.9 (d, C-4′, 8′), 116.1 (d, C-5′, 7′), 156.8 (s, C-6′), 100.3 (d, Glc-C-1′), 104.4 (d, Glc-C-1″), 72.1, 72.2 (d, Glc-C-2′, 2″), 75.0, 75.1 (d, Glc-C-3′, 3″), 71.5, 71.6 (d, Glc-C-4′, 4″), 77.9, 78.4 (d, Glc-C-5′, 5″), 62.7 (t, Glc-C-6′), 65.0 (t, Glc-C-6″); FABMS m/z: 709.2 $[M+Na]^+$.

Compound **3**: $C_{25}H_{32}O_{12}$; ^{1}H NMR (500 MHz, CD_3OD) δ 5.91 (1H, s, H-1), 7.52 (1H, s, H-3), 6.02 (1H, q, *J* = 6.5 Hz, H-8), 1.71 (3H, d, *J* = 6.5 Hz, H-10), 3.68 (3H, s, $COOCH_3$), 7.05 (2H, d, *J* = 8.4 Hz, H-4′, 8′), 6.69 (2H, d, *J* = 8.4 Hz, H-5′, 7′), 4.83 (1H, d, *J* = 7.8 Hz, Glc-H-1); ^{13}C NMR (125 MHz, CD_3OD) δ 94.6 (d, C-1), 154.3 (d, C-3), 109.3 (s, C-4), 30.6 (d, C-5), 40.8 (t, C-6), 171.7 (s, C-7), 124.2 (d, C-8), 130.5 (s, C-9), 13.6 (q, C-10), 167.3 (s, C-11), 51.6 (q, $COOCH_3$), 66.2 (d, C-1′), 34.9 (t, C-2′), 129.6 (s, C-3′), 130.9 (d, C-4′, 8′), 116.2 (d, C-5′, 7′), 157.0 (s, C-6′), 100.8 (d, C-G1), 74.8 (d, C-G2), 78.5 (d, C-G3), 71.4 (d, C-G4), 78.0 (d, C-G5), 62.8 (t, C-G6).

Compound **4**: $C_{18}H_{26}O_{11}$; ^{1}H NMR (500 MHz, CD_3OD) δ 5.91 (1H, s, H-1), 7.52 (1H, s, H-3), 6.10 (1H, q, *J* = 6.3 Hz, H-8), 1.73 (3H, d, *J* = 6.3 Hz, H-10), 3.71 (3H, s, COOMe), 3.63 (3H, s, COOMe), 4.80 (1H, d, *J* = 7.8 Hz, Glc-H-1); ^{13}C NMR (125 MHz, CD_3OD) É¬ 95.1 (d, C-1), 155.2 (d, C-3), 109.4 (s, C-4), 31.9 (d, C-5), 41.0 (t, C-6), 173.6 (s, C-7), 124.8 (d, C-8), 130.5 (s, C-9), 13.5 (q, C-10), 168.7 (s, C-11), 52.2, 51.9 (q, COOMe), 100.9 (d, Glc-C-1), 74.8 (d, Glc-C-2), 78.4 (d, Glc-C-3), 71.4 (d, Glc-C-4), 77.9 (d, Glc-C-5), 62.7 (t, Glc-C-6).

Scanning electron microscopy

Embryos and endosperms were excised transversely from fresh, mature seeds and from germinating seeds which had protruded radicles of 2 and 10 mm in length. These materials were fixed for 2–4 h in 2.5% glutaraldehyde buffered in 0.1 M phosphate at pH 7.2. After washing in buffer three times, samples were fixed in 1% osmium tetroxide in the same buffer for 6–8 h, dehydrated through an ethanol–acetone series, dried with Hitachi Critical Point Dryer (HCP-1), and then coated with an IB-2 ion coater. Observations and photographs were made with a Hitachi S-550 SEM (Tokyo, Japan).

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