

Maturity and temperature stratification affect the germination of *Styrax japonicus* seeds

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(Accepted 25 February 2004)

SUMMARY

The effect of seed maturity, warm (18°C) or cold (5.5°C) temperature, and gibberellic acid (GA₃) on *Styrax japonicus* seed germination was investigated. Morphological changes during fruit development were observed using magnetic resonance imaging (MRI) and to correlate changes in germination behaviour with time. Internal structures of fruits were identified using MRI, which showed tissues that contained water with different mobility in early growth stages. In the pericarp, the seed coat, and the endosperm of fruits harvested 17 weeks after anthesis, spin-lattice relaxation time T₁ decreased with maturation of the fruits. This finding indicated the termination of the physiological role of the pericarp due to a loss of free water, and of water that existed in bound form in the endosperm and the cotyledons as seeds became dry. Magnetic resonance images of *Styrax japonicus* 'Pink Chime' fruits harvested 11 weeks after anthesis showed the formation of cotyledons and endosperm. Fruits were fully developed when harvested 13–15 weeks after anthesis. *S. japonicus* fruits harvested 12 weeks (1999) and 16 weeks (2000) after anthesis were fully developed and matured, and responded to germination-promoting treatments. To ensure good germination, or higher than 80%, seeds should be harvested 12–16 weeks after anthesis, and should be treated with one month of warm stratification (WS) followed by two months of cold stratification (CS). The maximum percent germination was 98%, after two months of WS followed by three months of CS, which is significantly higher than the percentage previously reported.

Styrax japonicus Sieb. et Zucc is a small, low branched tree that produces white flowers with yellow stamens from May to June (Dirr, 1990). It is propagated from cuttings and seeds. Warm stratification (WS) for 3–5 months followed by cold stratification (CS) for 3–4 months has been recommended for breaking dormancy and to make seeds germinate. While newly matured fresh fruits harvested in the fall and sown immediately may germinate the following spring, germination of seeds in the field often does not occur until the second spring (Dirr, 1990; Kwon, 1995).

Stratification of seeds at warm and cold temperatures to break seed dormancy improves germination of many woody plants (Young and Young, 1992). Cold stratification improves the final germination percentage, and also the rate of germination of *Aesculus hippocastanum* L. seeds (Pritchard *et al.*, 1999). Seed dormancy may be imposed by a hard seed coat, testa, or dormant embryonic axis and cotyledons (Bandyopadhyay *et al.*, 1999). Seed storage in moist and warm environments affects the physical structures of the seed coat, and increases water imbibition and gas exchange. Physiological dormancy is broken by seed storage at low temperatures. For example, seeds of *Crataegus mollis* Scheele germinated well after at least 60 d of warm stratification at 18–22°C, followed by 120 d of CS at 2–4°C (Morgenson, 2000).

Nuclear magnetic resonance (NMR) imaging has successfully provided both physiological and anatomical information on tissues and organs of plants (Faust *et al.*, 1997; Ishida *et al.*, 2000; Ratcliffe *et al.*, 2001; Roh *et al.*, 1996). The mobility of water is related to the physical state of cell-associated water and to the physiological condition of tissues or organs. This water mobility is measured by the NMR relaxation times of water molecules as indicated by the spin-lattice relaxation time (T₁) or spin-spin lattice relaxation time (T₂). MRI has been used to simultaneously monitor the state of water (free or bound) in seeds and to determine how tightly water molecules are bound by the anatomically distinct tissues (Gassner, 1989; Faust *et al.*, 1991). During maturation of seeds of soybean (*Glycine max* L.), the tissues lose water, T₁ decreases in these tissues, water exists as a bound form, the mobility of water is restricted, and the metabolic rate of the tissues become low (Kano *et al.*, 1990). In flower buds of blueberry (*Vaccinium corymbosum* L.), water is in a bound form in dormant buds, and in a freer form after the chilling requirements are satisfied. Although the T₂ of the dormant buds were too short to be measured, the T₂ of the chilled buds were between 8–15 ms (Rowland *et al.*, 1992). Seeds with a thick seed coat generally produce moderate signals as compared with liquid phase. Therefore, the interpretation of NMR signals is a complex processes that is greatly affected by cellular heterogeneity (Ishida *et al.*, 2000).

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The best time for harvesting fruits of *S. japonicus* to ensure good seed germination has not been fully defined. If fruits, commonly referred to as seeds, are not fully developed and mature when harvested, they will fail to germinate. Determination of the best time for fruit harvest as revealed by physiological or structural changes using MRI would be useful for harvesting fully developed and mature seeds which will germinate when seeds receive the best treatments to promote germination. The objectives of this research were to determine (1) the influence of WS, CS, and gibberellic acid (GA_3) treatments on *S. japonicus* seed germination and (2) to correlate the internal structural changes within fruits with the maturity of seeds and with changes in germination behaviour using MRI.

MATERIALS AND METHODS

Seed germination as influenced by seed maturity

S. japonicus plants reached anthesis by the last week of May, 1999. Fruits from a plant at the Woody Landscape Plant Germplasm Repository at the US National Arboretum (NA) (NA accession No. 60191) were harvested three weeks (23 June), six weeks (14 July), eight weeks (27 July), ten weeks (10 August), 12 weeks (25 August), 14 weeks (8 September), 16 weeks (22 September), 18 weeks (5 October), and 21 weeks (26 October) after anthesis. At each harvest date, 30 seeds were sown per 15 cm pots filled with ProMix BM (Stamford, Conn., USA) and watered. Pots were placed in an air-conditioned greenhouse with chilled air and maintained at 18°C (Lee and Roh, 2001) for two months for WS, and then moved to a cooler maintained at 5.5°C for three months for CS. Pots were then placed in an air-conditioned greenhouse where the number of germinated seeds was recorded. Two replications, i.e., two pots containing seeds, were tested per harvest date (treatment). The experiment was repeated in 2000, using seeds (NA 60191) collected seven weeks (19 July), 10 weeks (2 August), 12 weeks (16 August), 14 weeks (30 August), 16 weeks (13 September), 19 weeks (4 October), and 21 weeks (19 October) after anthesis. Forty seeds were sown per pot, treated, and germinated as described above. Three replications, i.e., three pots with seeds, were tested per harvest date. Pots were watered every 3–4 d or as needed, until the completion of the experiment. The seeds that germinated were counted weekly. Recording of the number of germinated seeds continued for an additional six weeks from the time that the last germinate seed was counted.

Seed germination as influenced by WS, CS, and gibberellic acid (GA_3)

S. japonicus (NA accession No. 56627) seeds harvested during the last week of September 1998 were stored at 20°C until 3 March, 1999. Seeds were soaked in either 0, 3000, or 6000 ppm GA_3 solutions for 16 h at 20°C. Seeds, 50 per treatment, were placed in 15 cm pots filled with ProMix BM, watered, and placed in an air-conditioned greenhouse for 0, 1, 2, or 3 months for WS, and then moved to a 5.5°C cooler for two months for CS. After CS, pots were transferred to the air-conditioned greenhouse. In another experiment, 50 seeds soaked in either 0, 3000, or 6000 ppm GA_3 for 16 h at 20°C were potted as

described above, and received WS for two months. Pots were then moved to a cooler for CS for 0, 1, 2, or 3 months. After CS, pots were transferred to the greenhouse for WS, and germination of seeds was recorded. In both experiments, germinated seeds were recorded when the hypocotyl emerged above the surface of the medium. Two replications, a pot as a unit of replication, per treatment were tested. The number of seeds that germinated was recorded weekly until no changes in the number was noted.

Magnetic resonance imaging (MRI) of fruits harvested at different times after anthesis

Two container grown *S. japonicus* 'Pink Chimes' plants (30 × 30 × 30 cm wooden pot) were overwintered outdoors and then moved to the air-conditioned greenhouse mentioned above on 19 March. These plants reached anthesis during the week of 16 May, 1999. Fruits were harvested six weeks (23 June), eight weeks (7 July), 11 weeks (27 July), 13 weeks (10 August), 15 weeks (25 August), 17 weeks (8 September), and 19 weeks (22 September) after anthesis. Magnetic resonance images were generated using a Bruker MSL-400 NMR spectrometer (Bruker Instruments, Billerica, MA). The fruit was placed into a 25 mm NMR tube in a detector with a 28 mm saddle coil. Five inversion-recovery spin-echo images were collected with pulse intervals of 100 milliseconds (ms), 250 ms, 600 ms, 1 s, and 2 s, with an echo time of 12 ms. The repetition time was 3 s. Frequency encoded data were collected with a sweep width of 50 kHz and later digitized into 256 complex points. A two-step phase cycle was used. Measurements were carried out with a 64 × 64 matrix, or with 256 data points and 64 encoding steps, and images were created on 256 × 256 matrixes. A 2 ms sine pulse was used for the 90 degree excitation. Non-selective pulses were used for both the initial inversion pulse and for the refocus. The field of view was 25 × 25 mm with a 0.5 mm slice thickness giving a resolution of 98 × 391 × 500 μm.

Based on the five inversion recovery images, the T_1 time images were constructed by using a simplex maximization routine (Press *et al.*, 1988). The image intensities of each pixel in these five inversion-recovery images were used to fit in a single exponential curve to derive T_1 relaxation time. Because the inversion point was not known in advance, the fit was performed five times. Before T_1 image measurement, a set of 16 multi-slice images was obtained to choose the best slice location. The distance between successive image planes was 1 mm. MRI measurement was repeated using four fruits, but only one data set is presented because similar images were obtained for all four fruits. Tentative identification of tissues or organs during fruit development based on MRI was aided by observing hand sectioned pieces of seeds under a stereoscope at 70× magnification and by following the description during various developmental stages of fruits and seeds (Esau, 1965).

Statistical analysis

Peak seed germination was defined as the maximum number of seeds that germinated. This number was calculated as a percentage, i.e., (no. of germinated seeds/total number of seeds per pot) × 100. Number of

TABLE I
Effect of seed maturity on *Styrax* seed germination and regression analysis

| Harvest date | Weeks after germination | Mean peak germination (%) ¹ ± SEM ² | Mean no. of week ³ ± SEM | Slope at midpoint ⁴ | Regression [y = f(x)] | Regression coefficient (R ²) | Probability (P) |
|--------------|-------------------------|---|-------------------------------------|--------------------------------|---|--|-----------------|
| <i>1999</i> | | | | | | | |
| 23 June | 3 | 0 ⁵ | nest ⁶ | nest | nest | nest | nest |
| 14 July | 6 | 0 | nest | nest | nest | nest | nest |
| 27 July | 8 | 0 | nest | nest | nest | nest | nest |
| 10 Aug. | 10 | 7 ± 3 c ⁷ | 3.0 ± 0.74 b | 1.0 | 2.5 + 1.2x - 0.08x ² | 0.91 | 0.0002 |
| 25 Aug. | 12 | 67 ± 3 b | 6.0 ± 0.74 a | 8.2 | 15 + 13.0x - 0.80x ² | 0.99 | <0.0001 |
| 8 Sept. | 14 | 80 ± 3 a | 7.0 ± 0.74 a | 16.4 | -32 + 29.3x - 1.84x ² | 0.97 | <0.0001 |
| 22 Sept. | 16 | 73 ± 3 ab | 4.0 ± 0.74 b | 23.6 | -55 + 59x - 8.52x ² + 0.39x ³ | 0.95 | 0.0003 |
| 5 Oct. | 18 | 68 ± 3 b | 4.5 ± 0.74 ab | 20.0 | -29 + 29x - 1.98x ² | 0.88 | 0.0007 |
| 26 Oct. | 21 | 80 ± 3 a | 3.0 ± 0.74 b | 33.7 | -29 + 59x - 9.54x ² + 0.48x ³ | 0.94 | 0.0005 |
| <i>2000</i> | | | | | | | |
| 19 July | 7 | 0 ⁸ | nest | nest | nest | nest | nest |
| 2 Aug. | 10 | 0 | nest | nest | nest | nest | nest |
| 16 Aug. | 12 | 5 ± 2 c | 2.3 ± 0.56c | 3.0 | -3.2 + 4.6x - 0.76x ² + 0.04x ³ | 0.90 | 0.0022 |
| 30 Aug. | 14 | 16 ± 2 d | 7.0 ± 0.56a | 2.6 | -2.6 + 4.4x - 0.26x ² | 0.97 | <0.0001 |
| 13 Sept. | 16 | 65 ± 2 c | 4.0 ± 0.56a | 11.2 | -23 + 21.4x - 1.27x ² | 0.95 | <0.0001 |
| 4 Oct. | 19 | 73 ± 2 b | 4.7 ± 0.56b | 23.6 | -47 - 54.8x - 7.91x ² + 0.36x ³ | 0.98 | <0.0001 |
| 19 Oct. | 21 | 88 ± 2 a | 6.0 ± 0.56ab | 21.4 | -58 + 60.6x - 8.16x ² + 0.36x ³ | 0.96 | 0.0002 |

¹For mean peak germination: 1999, F = 80.20; d.f. = 5, 11; P < 0.0001; N = 12; 2000, F = 254.17; d.f. = 4, 14; P < 0.0001; N = 15.

²Standard error of the means.

³For mean number of weeks for germination peak: 1999, F = 4.88; d.f. = 5, 11; P = 0.0398; N = 12; 2000, F = 15.61; d.f. = 4, 14; P = 0.0003; N = 15.

⁴Slope was calculated at one-half point of mean no. of weeks that reached maximum peak germination after calculating derivative (dy/dx) of regression equation.

⁵Seeds harvested on 23 June, 14 July and 27 July, 1999 did not germinate, and not included in the analyses.

⁶Non-estimable.

⁷For each year, means within columns followed by the same letter are not significantly different.

⁸Seeds harvested on 19 July and 2 August, 2000 did not germinate.

weeks to peak germination was defined as the number of weeks that elapsed from the start of the experiment, i.e., from the time when the seeds were moved into the greenhouse, until reaching peak seed germination. These two dependent variables were analysed using a one-way ANOVA because of the detached control group, thus treatment combinations were created, after determining that residuals were normally distributed using the Shapiro-Wilk test for normality, and examining homogeneity of residual variances using the Proc Boxplot procedure within SAS (SAS Institute, 1999). Data for each year were analysed separately. Means of the dependent variables and the standard error of the means (SEM) were obtained using the LSMEANS procedure within SAS (SAS Institute, 1999). Means were compared using contrasts at $P < 0.05$.

Germination rate of *Styrax* seeds over time was modelled using the number of week after transfer to the greenhouse after CS (sampling week) as a explanatory (regressor) variable, and thus, as a polynomial function of time. Regression curves were compared using regression techniques with treatment coded as a dummy variable. Parameter estimates were obtained using the Solution option in the Model statement within SAS (SAS Institute, 1999). The regression equation was expressed as $Y = f(x)$, where Y is germination percentage at the time of sampling week (x). The derivative of this regression equation was used to calculate the slope at the midpoint of the sampling period when the germination percentage is being recorded.

RESULTS

Seed germination as influenced by seed maturity

In 1999, seeds did not germinate when fruits were harvested before 10 August, that is ten weeks after anthesis (Table I). When fruits were harvested 12 weeks and 14 weeks after anthesis, 67% and 80% of the seeds

germinated in 6 and 7 weeks, respectively. More than 80% of the seeds germinated in less than 4.5 weeks when harvested 16 weeks or longer after anthesis. Based on the final seed germination rate, seeds were considered fully developed and mature when harvested at least 12 weeks after flowering. In 2000, more than 65% of the seeds germinated in a period of eight weeks when fruits were harvested 16 weeks after anthesis. In 2000, seeds were considered to be fully developed and mature when harvested 16 weeks after flowering. For both years, the mean peak germination, and the mean number of weeks for germination peak were significantly influenced by harvest date, i.e., seed maturity. The germination speed, expressed as a slope at the midpoint of seed germination, was greater than 16.4% on or after 25 August (week 12), 1999, and greater than 21.4% on or after 4 October (week 19) in 2000. At these dates, mean peak germination was higher than 73% in both years. For both years, the slopes for germination percentage, as expressed by the regression equations, were significantly different among harvest dates (Table I). For both years, the mean peak germination, and the mean number of weeks for germination peak were significantly influenced by harvest dates, i.e., seed maturity (Table I). For both years, regression slopes were significantly different among harvest dates (For 1999: F = 37.07, $P < 0.0001$; for 2000: F = 63.26, $P < 0.0001$).

Seed germination as influenced by WS, CS, and GA₃

When seeds received CS immediately after sowing without WS, only 43% of the seeds germinated (Table II). Germination increased after one month of WS. Seed germination was higher than 92% when seeds were treated with 3000 ppm GA₃ regardless of the duration of WS. The mean germination peak and the mean number of weeks to germination peak were significantly influenced by the GA₃ and by the number of months in storage treatment combination. Regression

TABLE II
Effect of warm stratification (WS) and gibberellic acid (GA₃) treatment on seed germination

| Treatment ¹ | | Mean peak germination ² (%) ± SEM ³ | Mean no. of week ⁴ ± SEM | Slope at midpoint ⁵ | Regression [y = f(x)] | Regression coefficient (R ²) | Probability (P) |
|-------------------------|-----------------------|--|--|-----------------------------------|---|---|-----------------|
| duration (mo.) of WS | GA ₃ (ppm) | | | | | | |
| 0 | 0 | 43 ± 6.5 d ⁶ | 2.0 ± 0.5 b | 20.9 | -14.33 + 28.72x - 4.17x ² + 0.18x ³ | 0.74 | 0.0106 |
| 1 | 0 | 85 ± 6.5 bc | 2.0 ± 0.5 b | 41.3 | -28.33 + 56.77x - 8.25x ² + 0.36x ³ | 0.84 | 0.0106 |
| 2 | 0 | 73 ± 6.5c | 3.5 ± 0.5 b | 20.1 | 6.09 + 32.75x - 4.70x ² + 0.21x ³ | 0.80 | 0.0039 |
| 3 | 0 | 75 ± 6.5 bc | 4.0 ± 0.5 a | 23.3 | -39.06 + 50.59x - 6.82x ² + 0.29x ³ | 0.96 | <0.0001 |
| 1 | 3000 | 92 ± 6.5 ab | 2.5 ± 0.5 b | 41.1 | -31.51 + 61.59x - 8.93x ² + 0.39x ³ | 0.75 | 0.0084 |
| 2 | 3000 | 98 ± 6.5 a | 3.0 ± 0.5 ab | 43.4 | -55.09 + 68.80x - 9.34x ² + 0.39x ³ | 0.93 | <0.0001 |
| 3 | 3000 | 88 ± 6.5 b | 3.5 ± 0.5 a | 33.2 | -40.18 + 57.61x - 7.83x ² + 0.33x ³ | 0.93 | 0.0007 |
| 1 | 6000 | 94 ± 6.5 a | 2.5 ± 0.5 b | 53.3 | -31.76 + 62.85x - 9.12x ² + 0.40x ³ | 0.74 | 0.0094 |
| 2 | 6000 | 87 ± 6.5 b | 4.0 ± 0.5 a | 25.2 | -45.77 + 59.39x - 8.05x ² + 0.34x ³ | 0.94 | <0.0001 |
| 3 | 6000 | 85 ± 6.5 bc | 3.5 ± 0.5a | 29.8 | -28.33 + 52.51x - 7.28x ² + 0.31x ³ | 0.92 | <0.0001 |

¹Seeds after GA₃ treatment were planted and received 0, 1, 2, or 3 months of WS followed by CS for two months.

²For mean peak germination; F = 5.82; d.f. = 9, 19; P = 0.0055, N = 20.

³Standard error of the means.

⁴For mean number of weeks for germination peak, F = 2.58; d.f. = 9, 19; P < 0.078; N = 20.

⁵Slope was calculated at one-half point of mean no. of weeks that reached maximum peak germination after calculating a derivative (dy/dz) of regression equation.

⁶Means followed by the same letter are not significantly different.

slopes for mean peak germination were significantly different among treatments. Germination speeds were greater than or equal to 20.1% per week, regardless of treatment.

Without CS, germination was very low (3%). At least two months of CS was required to improve germination above 81%. GA₃ treatments and one month of CS promoted germination (23–30%), as compared with seeds treated only with CS (3%) (Table III). The highest germination was obtained when seeds were treated with three months of CS with or without 3000 ppm GA₃. Germination was not further improved as GA₃ concentration was increased to 6000 ppm when seeds received three months of CS. Regression slopes for mean peak germination were significantly different among treatments. Germination speeds were greater than 21.1% when mean peak germination percentage was higher than 81%. The mean germination peak and the mean number of weeks for germination peak of seeds, regardless of the two-month storage temperature, were significantly influenced by the GA₃ and number of months in storage treatment combination (Table II, III). For both storage temperatures, regression slopes were significantly different among treatment combinations (For 1999: F = 1.65, P = 0.0043; for 2000: F = 21.78, P < 0.0001).

Magnetic resonance images (MRI) of fruits harvested at different times after anthesis

During fruit development, various anatomical features of *Styrax* fruits and their changes in size and water status were visualized from T₁ images (Figure 1). White at the top of the scale bar represented the longest T₁ value, which indicated water in free form, not bound, and with high mobility, and black the shortest T₁, which indicated water in bound form and low in mobility. Based on the images obtained during fruit development, the pericarp (A) in red, yellow, and green outer tissues of the fruit that surrounds the seed coat (B), which is coded in blue and dark purple. Inside the seed coat, the tissue with red (C) was evident, but the anatomical identity could not be readily made based on MRI. The endosperm in dark blue (D) and cotyledons in bronze red (E) were clearly visible. The nature of the tissue in dark blue present inside the cotyledons (F) was not identified.

Fruits harvested six weeks after anthesis showed very tiny organs of endosperm and cotyledons that were not distinguishable (D and E with blue and purple colour in the centre of the seed), but were encircled with tissue of green color in the image (Figure 1-1). Tissues in the regions of A and B had a short T₁ but an unidentified tissue C had a long T₁. When fruits were harvested eight

TABLE III
Effects of cold stratification (CS) and gibberellic acid (GA₃) treatment on seed germination

| Treatment ¹ | | Mean peak germination ² (%) ± SEM ³ | Mean no. of week ⁴ ± SEM | Slope at midpoint ⁵ | Regression [y = f(x)] | Regression coefficient (R ²) | Probability (P) |
|-------------------------|-----------------------|--|--|-----------------------------------|---|---|-----------------|
| duration (mo.) of CS | GA ₃ (ppm) | | | | | | |
| 0 | 0 | 3 ± 3.1 d ⁶ | 1 ± 0.6 d | nest ⁷ | nest | nest | nest |
| 1 | 0 | 4 ± 3.1 d | 1 ± 0.6 d | nest | nest | nest | nest |
| 2 | 0 | 94 ± 3.1 a | 3 ± 0.6 c | 13.9 | 46.61 + 23.26x - 3.34x ² + 0.15x ³ | 0.78 | 0.0052 |
| 3 | 0 | 98 ± 3.1 a | 4 ± 0.6 c | 35.9 | -67.39 + 60.85x - 7.07x ² + 0.26x ³ | 0.91 | <0.0001 |
| 1 | 3000 | 30 ± 3.1 c | 7 ± 0.6 b | 0.13 | 28.65 - 0.71x + 0.18x ² - 0.01x ³ | 0.95 | <0.0001 |
| 2 | 3000 | 86 ± 3.1 b | 5 ± 0.6 c | 22.3 | 23.62 + 29.12x - 4.08x ² + 0.18x ³ | 0.78 | <0.0001 |
| 3 | 3000 | 97 ± 3.1 a | 4 ± 0.6 c | 37.4 | -69.36 + 66.78x - 8.35x ² + 0.33x ³ | 0.89 | <0.0001 |
| 1 | 6000 | 23 ± 3.1 c | 1 ± 0.6 d | nest | nest | nest | nest |
| 2 | 6000 | 81 ± 3.1 b | 4 ± 0.6 c | 23.0 | -17.84 + 44.12x - 6.0x ² + 0.25x ³ | 0.84 | <0.0001 |
| 3 | 6000 | 82 ± 3.1 b | 9 ± 0.6 a | 21.1 | -51.42 + 49.21x - 5.83x ² + 0.22x ³ | 0.91 | <0.0001 |

¹Seeds treated with GA₃ were planted in pots for two months of WS prior to CS.

²For mean peak germination; F = 164.63; d.f. = 9, 19; P < 0.0001, N = 20.

³Standard error of the means.

⁴For mean number of weeks for germination peak, F = 16.53; d.f. = 9, 19; P < 0.0001; N = 20.

⁵Slope was calculated at one-half point of mean no. of weeks that reached maximum peak germination after calculating a derivative of regression equation (dy/dx).

⁶Means followed by the same letter are not significantly different.

⁷Non-estimable.

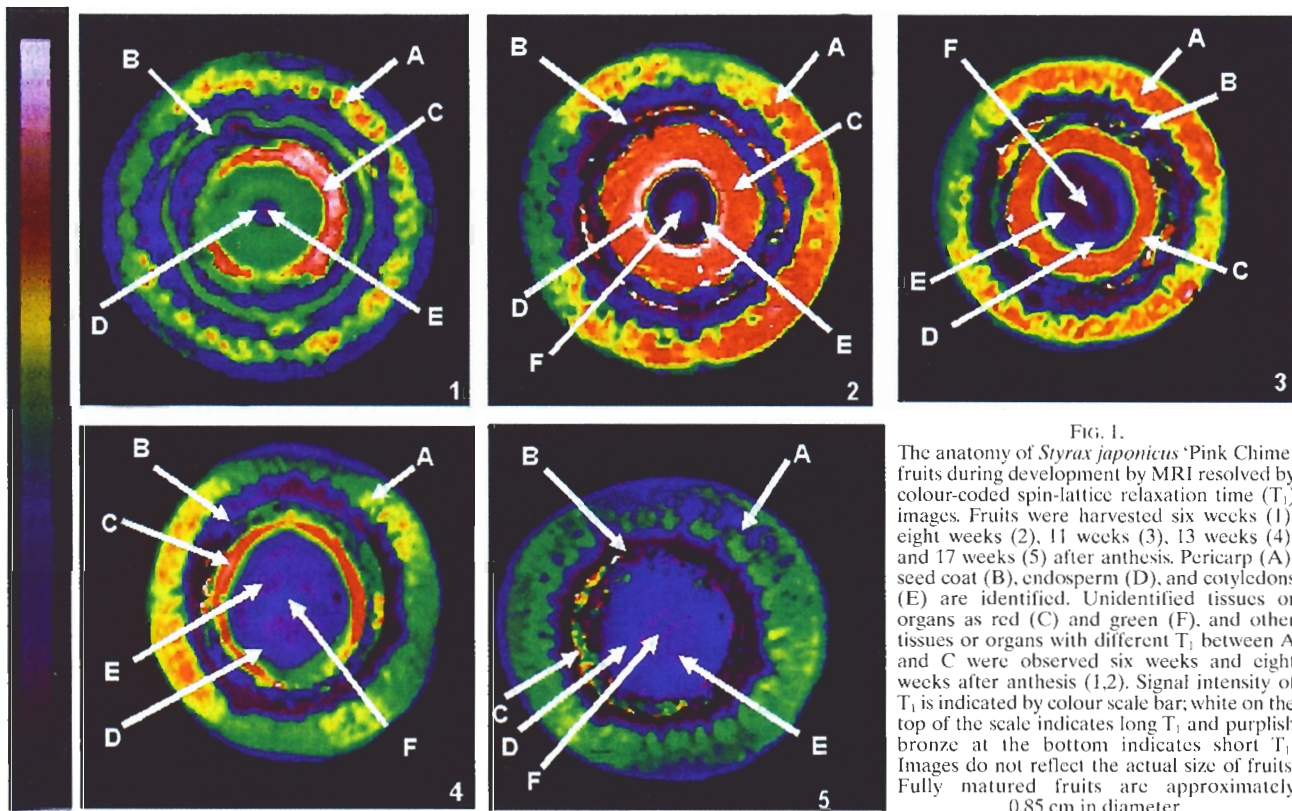


FIG. 1. The anatomy of *Styrax japonicus* 'Pink Chime' fruits during development by MRI resolved by colour-coded spin-lattice relaxation time (T_1) images. Fruits were harvested six weeks (1), eight weeks (2), 11 weeks (3), 13 weeks (4), and 17 weeks (5) after anthesis. Pericarp (A), seed coat (B), endosperm (D), and cotyledons (E) are identified. Unidentified tissues or organs as red (C) and green (F), and other tissues or organs with different T_1 between A and C were observed six weeks and eight weeks after anthesis (1,2). Signal intensity of T_1 is indicated by colour scale bar; white on the top of the scale indicates long T_1 and purplish bronze at the bottom indicates short T_1 . Images do not reflect the actual size of fruits. Fully matured fruits are approximately 0.85 cm in diameter.

weeks after anthesis (Figure 1-2), an unidentified tissue (C) with a long T_1 expanded and was clearly formed surrounding the endosperm and cotyledons (D and E) which started to increase in the size, and this inclination lasted until 11 weeks after anthesis (Figure 1-3). The size of cotyledons and endosperm in the centre of the fruits that were harvested 13 weeks (Figure 1-4), and 17 weeks (Figure 1-5) after anthesis were evident. Fruits harvested 15 weeks after anthesis showed a very similar image to the one observed in fruits harvested 17 weeks after anthesis (data not shown). When fruits were harvested 19 weeks after anthesis, the pericarp dried and became thin, and colour images of these tissues (data not presented) were very similar to seeds (Figure 1-5).

DISCUSSION

Seed germination as influenced by maturity, WS, CS, and GA₃

Styrax seeds were fully developed and mature when harvested 12 weeks (1999) and 16 weeks (2000) after anthesis (Table I). The difference between years could have resulted from differences in environmental factors, such as temperature and light irradiance level. To break their dormancy, mature seeds need to receive at least one month of WS (Table II), prior to CS for two months (Table III). In our study, germination percentages were higher with one month of CS than percentages reported by Dirr (1990) and Kwon (1995) with 3 to 4 months of CS. Seeds of *Crataegus mollis* Scheele and of *C. × anomala* Sarg., did not germinate when cold treatment was given to seeds that had not received previous warm treatment at 18–22°C (Morgenson, 2000).

The *Styrax* seed is considered to have double dormancy (Dirr, 1990), imposed by a seed coat that

resists water absorption and a dormant embryo that prevents growth of the radicle and cotyledons. These appear to be directly responsible for the low germination of seeds that did not receive WS and CS. Physical changes in the seed coat or physiological changes in the embryo that may occur during one month of WS in *Styrax* should be further studied. If seed development or maturation of the embryo is a factor, these physiological changes are completed in one month. No visual differences in seed development, water mobility, and T_1 values at 20°C were observed (data not presented). This suggests that the embryo of *Styrax* seeds is fully developed and capable of germination, and that morphological dormancy (MD) due to an underdeveloped embryo (Nikolaeva, 1977) is, therefore, not involved. Warm treatment in *Nandina domestica* seeds did not promote germination due to a presence of rudimentary embryo (Dehgan, 1984). If *Styrax* seeds were underdeveloped after one month of WS, the development process could have been completed after WS and CS. A study of the development of immature tissue in *Pinus albicaulis*, Engelmann demonstrated that seeds became fully mature after 30 d at 20°C, followed by 60 d at 2°C (Leadem, 1986).

It was observed that the majority of the seeds germinated in about 3–4 weeks, although seeds continued to germinate over nine weeks in one treatment (Tables II, III). After reaching peak germination, one seed every week germinate for the last three weeks of the test. This explains why nine weeks were required to reach the maximum peak of germination. Germination vigour or germination value may not account for this observation (Czabator, 1962). Germination speed gives an indication of how many seeds germinate at that particular point in time. From the derivative equation ($dy/dx = 49.21 - 10.86x + 0.66x^2$), the

germination speed after three months of CS and 6000 ppm GA₃ treatment (Table III) were 38.21, 28.52, 20.17, 23.45, 7.41, 3.27, and -0.19, respectively, at sampling week 1, 2, 3, 4, 5, 6, and 8, and, therefore, we concluded that most of the seeds germinated in one month.

When seeds received two or three months of CS, 6000 ppm GA₃ did not improve germination (Table III). GA₃ treatment improved germination only when seeds received a sub-optimal CS period (Table III). Therefore, GA₃ is not required when *Styrax* seeds received an adequate cold treatment, nor can it completely replace CS. Similar results were reported in *Koelreuteria paniculata* (Rehman and Park, 2000). In contrast, Kwon (1995) reported that sulfuric acid and 1000 ppm GA₃ promote germination of fresh *Styrax japonicus* seeds when seeds subsequently received three months of warm and four months of cold treatment. When gibberellins have been isolated, identified, and quantified from dormant and non-dormant seeds after WS and CS, the mechanism of how gibberellins are involved in *Styrax* seed germination may be better understood, but we have not achieved this yet.

Results obtained in this study exclude the suggestion by Kwon (1995) that, in nature, a radicle from seeds sown in the fall of the previous year emerges from the seed into the surrounding soil around July, and remains dormant during a warm summer season in the first year, and that the epicotyl emerges in the second year above the soil after receiving cold. If a radicle were to emerge after the cold treatment in the first year and then seedlings became dormant, *Styrax* seeds would be showing a hypocotyl dormancy. In this case, seeds would not germinate only after one cycle of WS and CS as we have demonstrated.

Development of seeds using MRI

Internal seed structures, such as endosperm and cotyledons are distinguished easily on the MR images generated during fruit development. These MR images clearly demonstrated the useful application of MRI to study morphological changes during fruit development. Based on the MR images, the fruit has several tissues or organs that contain water with different mobility. Developing young fruits with high water amount, have a strong image signal (Figure 1-1, 1-2). For fruits that are considered mature based on the germination data, T₁ becomes very short (Figure 1-4), suggesting that the

water molecules are bound or constrained with macromolecules and that metabolic activities become low. Water content during fruit maturation was not recorded in this study. However, soybean seeds produced no signal when the water content decreased to approximately 60% and the image signal in the fruits declined with decreased water mobility due to the accumulation of stored materials (Ishida *et al.*, 1987). The longer T₁ values in the early developmental stages suggest that the fruit was actively developing, and therefore not fully developed when harvested on or before 11 weeks after anthesis (Figure 1-3). As fruits grow, T₁ becomes short and water mobility is restricted, indicating the decline of metabolic activity from the accumulation of macromolecules. In the pericarp, T₁ decreased with maturation of the fruits, indicating that active metabolism was over.

The mobility of water and thus the metabolic activity in the pericarp and in the seeds changed inversely with the progression of the developmental stages of the seeds (Figure 1). During fruit development, other structural tissues or organs which could not be identified were observed. When the fruit was immature, the mobility of water was high in the pericarp and in an unidentified tissue (C in Figure 1), as determined by T₁ images. Water is progressively more bound and less free as the harvest dates are extended. When the fruits matured and harvested 15 weeks after anthesis, the water within fruits was reduced and the signal was weak because of the accumulation of macromolecules.

In conclusion, *Styrax japonicus* fruits harvested at 12 weeks in 1999 and at 16 weeks in 2000 after anthesis were fully developed and mature, and responded to germination-promoting treatments. The development of seeds was also verified using non-destructive MR images. One month of WS at 18°C, followed by two months of CS at 5.5°C, resulted in germination higher than 73%. The maximum germination percentage was 98% after two months of WS followed by three months of CS. Gibberellin promoted germination when seeds received a sub-optimal level of WS and CS.

We thank Drs R. Anderson, G. Wulster, B. Maynard, H. Kano, and N. Ishida for their careful and critical reviewing of this manuscript before submitting for publication to the Journal and also an anonymous reader during the review processes after the submission.

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