From Forest Nursery Notes, Summer 2008

125. Development of a sequential digital imaging system for evaluating seed germination. Geneve, R. L., Dutt, M., and Downie, A. B. IN: Seeds: biology, development and ecology, p. 315-323. S. Adkins, S. Ashmore and S.C. Navie, eds. CAB International. 2007.

NOTICE: THIS MATERIAL MAY
BE PROTECTED BY COPYRIGHT
LAW (TITLE 17, U.S. CODE)

33 Development of a Sequential Digital Imaging System for Evaluating Seed Germination

R.L. GENEVE, M. DUTT AND A.B. DOWNIE

Department of Horticulture, University of Kentucky, Lexington, KY 40546, USA

Abstract

A sequential imaging system using a flatbed scanner interfaced to a personal computer (PC) was developed as a research tool to study seed germination. The utility of the system was demonstrated in various germination-related studies including: (i) imbibition of physically dormant seeds; (ii) germination rate calculations; (iii) mutant assessment; and (iv) changes in seedling growth rates following dormancy release. Results from these studies revealed changes that were not previously observed or were too tedious to measure using conventional methods. This simple sequential imagery system offers an alternative research tool to study time-sensitive processes related to seed germination.

Introduction

High quality seed lots display rapid, uniform germination under laboratory and commercial conditions. Seed lot quality is typically described by germination percentage, speed and the spread between early and late germinating seeds. Additional quality information can be gained by measuring early seedling growth. The precision of these measurements is limited by the seed analyst's ability to repeatedly monitor the progression of germination and seedling growth. Computer-assisted measurements of digital images can help alleviate potential errors inherent in hand analysis and increase the number of samples an analyst can measure.

Two alternatives have been developed for capturing digital images for analysis of seed germination – charged coupled device (CCD) cameras (Howarth and Stanwood, 1993; Dell'Aquila *et al.*, 2000) and flatbed scanners (Geneve and Kester, 2001; Sako *et al.*, 2001). Both generally capture images with the quality required for computer-assisted analysis. However, flatbed scanners have the advantage of providing consistent lighting and the ability to capture usable images from very small seeds (Geneve and Kester, 2001).

©CAB International 2007. *Seeds: Biology, Development and Ecology* (eds S. Adkins, S. Ashmore and S.C. Navie)

Development of a non-destructive system for capturing sequential digital images for analysis could provide additional precision and insight concerning the aspects of seed germination. It would also provide a way to observe germination and seedling growth of individual seeds making it possible to select seeds displaying a particular behaviour or to monitor seedling growth following germination. Systems developed to take sequential images include time sequence photography (Tomas *et al.*, 1992), machine vision (Howarth and Stanwood, 1993) and computerized automated seed analysis using a hand potentiometric calliper (Keys *et al.*, 1984). We have developed a simple sequential imaging system that uses a flatbed scanner interfaced with a personal computer (PC), which captures images on an hourly basis. The objective of the current contribution is to demonstrate the range of germination-related applications, on which sequential imagery can be applied to using a flatbed scanner system. Applications include an example for imbibition, time to radicle protrusion, analysis of a mutant germination phenotype and seedling growth rate.

Materials and Methods

Sequential imaging system

The flatbed scanner system for capturing images during seed germination and seedling growth has been described in detail earlier (Geneve and Kester, 2001; Oakley *et al.*, 2004). Seeds were sown into 6 cm diameter Petri dishes containing one piece of sterile transparent cellulose acetate film wetted with 1 ml of sterile water or a thin layer of 1% (w/v) agarose. Petri dishes were sealed with Parafilm or Nexcare gentle paper tape (3, St. Paul, Minnesota, USA) and placed on a HP Scanjet 5370 C flatbed scanner with a transparency adapter (Hewlett Packard, Palo Alto, California, USA) inside a growth chamber. The flatbed scanner was interfaced with a PC using Windows 98SE (Microsoft, Seattle, Washington, USA) operating system. A Visual Basic macro in SigmaScan Pro (SPSS, Chicago, Illinois, USA) captured images (300 or 600 dpi, colour TIFF file format) every hour for up to 5 days.

Imbibition patterns in honey locust seeds

Honey locust (*Gleditsia triacanthos* L.) seeds were acid scarified for 2 h in concentrated H_2SO_4 or hot water (100°C) for 1 min. The pattern of water uptake was determined as the portion of the seed swelling upon initial imbibition. Changes in seed size were measured using SigmaScan Pro by creating an overlay and using its area function. There were six seeds per Petri dish and 60 seeds were evaluated per treatment. Time to 50% germination was compared by single degree of freedom *F*-test.

Germination rate

Time to radicle protrusion was determined in petunia (*Petunia* × *hybrida* hort. ex E. Vilm.) seeds. Twenty-five seeds were used in five replicate Petri dishes contain-

ing cellulose film and were placed on the flatbed scanner in a growth chamber set at 25°C. Seeds were considered to have germinated when the radicle was visible on the digital image. Actual time to radicle protrusion was compared with germination rate calculated as the time for 50% of the germinating seed population to reach radicle protrusion (i.e. T_{50}). Germination rates (T_{50}) were calculated using sigmoidal equations and compared against the actual time to radicle protrusion using increasing evaluation intervals between 1 and 16 h beginning after seeds had imbibed for 24 h.

Characterization of cold temperature mutants

Four replicates of ~50 seeds from several overexpressing mutants of *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.), that complete germination faster than the Columbia wild type (Col), were surface sterilized along with wild-type control seeds, and sown on 1% (w/v) agarose solid media in a Petri dish. Each of the four 60 × 15 mm Petri dishes was bifurcated by a line and, in each of the four plates, one replicate of 50 wild-type seeds was sown on one side of the line and 50 mutant seeds were sown on the other side. The plates were sealed and the four dishes were arranged side by side on the flatbed scanner inside a germinator maintaining a constant temperature at 10°C ± 1°C and constant light (135 μmol/m^2/s). Images were captured every hour starting on the third day of the experiment (Salaita *et al.*, 2005). Careful evaluation of the exact timing of radicle protrusion between the mutant putatively exhibiting faster completion of germination and the wild type was conducted from the chronology of images.

Seedling growth rate

Redbud (*Cercis canadensis* L.) seeds were treated with concentrated H_2SO_4 for 30 min and stratified at 4°C for 4 weeks then fully imbibed (i.e. for 48 h). Non-stratified seeds were acid scarified and fully imbibed, but not chilled. Embryos were surgically removed from non-stratified and stratified seeds and evaluated for radicle elongation for 115 and 70 h, respectively. The growth chamber was set at 25°C with a 16 h photoperiod supplied by cool-white fluorescent lamps (providing ~80 μmol/m^2/s). Images were captured every 5 h and the data were compared on the basis of analysis after 5 or 24 h intervals. Time taken by the radicle to reach a length of 10 mm was compared by a single degree of freedom *F*-test.

Results and Discussion

Imbibition patterns in honey locust seeds

Scarified honey locust seeds showed a typical triphasic increase in seed size that was indicative of water uptake during imbibition (Figs 33.1 and 33.2). Acid-scarified seeds had a faster imbibition rate compared with hot water-treated seeds. Acid-treated

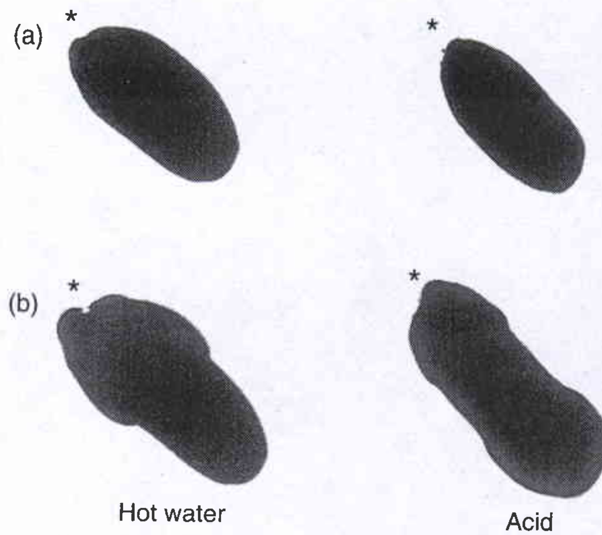

Fig. 33.1. Increase in seed size in honey locust seeds treated with acid for 2 h or hot water for 1 min. (a) is initial seed size; (b) is seed shape after 50% imbibition; and * indicates the micropylar end of the seed.

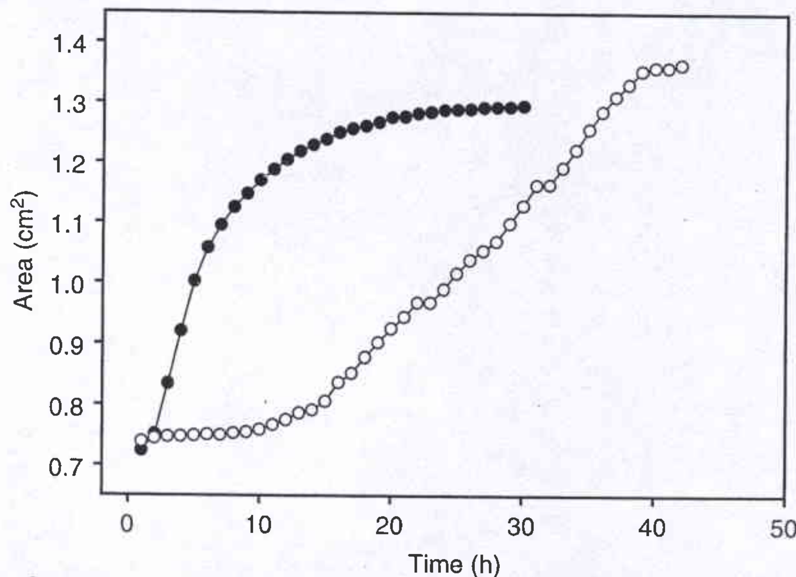

Fig. 33.2. Pattern of seed size increase upon imbibition of honey locust seeds treated with acid for 2 h (•) or hot water for 1 min (○).

seeds reached 50% of their final imbibed size within 7 h following imbibition compared to 28 h for hot water-treated seeds ($P \leq 0.01$). Acid scarification permitted imbibition to be initiated at both ends of the seed and generally resulted in a typical 'dumbbell' shape midway through hydration (Fig. 33.1). In contrast, hot water-treated seeds consistently showed imbibition initiating only at the micropylar end of the seed (Fig. 33.1).

Physical dormancy restricts the ability of seeds to imbibe when exposed to water and is typical of members of the *Fabaceae* (Baskin and Baskin, 1998). Honey locust seeds have a typical palisade epidermal layer with thick-walled macroscleried cells that are responsible for restricting imbibition. Baskin *et al.* (2000) suggested that in legume seeds, the lens is the first place on the seed coat for water entry when hard seeds become permeable under natural conditions.

It is becoming apparent that high temperature is the most probable cause for breaking physical dormancy (Morrison *et al.*, 1998). Sequential images of hot water-treated seeds support the lens as the natural entry point for imbibition in honey locust.

In acid-scarified legume seeds, Liu *et al.* (1981) showed a general reduction in the materials covering macrosclerieds throughout the seed. Therefore, rather than a single entry point for water, it would be anticipated that acid-treated seeds would show uniform water uptake over the entire seed surface. However, when water entry was followed on an hourly basis, acid-treated honey locust seeds showed asymmetric water uptake across the seed with more water initially entering at the polar ends (Fig. 33.1). This suggests that the cells in the polar regions of the seed were more susceptible to acid scarification than cells in the middle of the seed or that they are more adept at allowing water entry. It should also be considered that the extremely rapid increase in seed size suggests that water could be imbibed across the entire surface of the seed in contact with water, but at a lower rate for the non-polar regions of the seed.

Sequential imagery was adequate for determining the general region of the seed where imbibition initiated and provided a compelling case that honey locust seeds exposed to high temperature initiated imbibition at the micropylar end of the seed. However, the specific region of cells (i.e. lens) responsible for initial water entry could not be resolved from these images. Another limitation for strictly observational evaluation of water uptake was that it was not possible to determine if water was directly entering particular cells or if the size increase was due to lateral movement of water from cell to cell. It is anticipated that sequential imagery would provide good insight into specific water movement into seeds if water soluble dyes were used (Wilson and Geneve, 2004).

Germination rate

Germination usually approximates a normal distribution and can be described by total germination percentage, the median (i.e. time to reach 50% germination or T_{50}), and uniformity expressed as the standard deviation or the difference between the upper and lower quartile (Hara, 1999). The Association of Official Seed Analysts (AOSA) considers germination rate to be an indicator of seed vigour (AOSA, 1983). Seed lots with similar total germination percentages often vary in their rate of germination and growth. However, it is difficult to determine the actual time of radicle protrusion accurately because the time interval between evaluations is limited by the researcher's availability and can be as long as 24h.

It is common to use non-linear equations to estimate germination rate and uniformity. Using images captured every hour, the actual time to radicle protrusion was determined for a petunia seed lot (Table 33.1). Actual germination rate and uniformity was compared to estimates derived from sigmoidal equations using data sampled with increasing intervals. T_{50} was accurately estimated until the sampling interval reached 16h. In contrast, mathematical estimates of uniformity (i.e. T_{75} minus T_{25}) were less accurate and were only statistically similar to actual values when sampled after 1 or 10h (Table 33.1).

Table 33.1. Comparison between actual and calculated germination rates depending on the frequency of observations in a petunia seed lot.

Hours between observations	Visual T_{50}	Sigmoidal T_{50}	Visual T_{75-25}	Sigmoidal T_{75-25}
1	33.0 ± 1.2 b	33.2 ± 1.5 b	10.8 ± 2.2 b	9.3 ± 1.6 ab
2	33.0 ± 1.2 b	33.1 ± 1.6 b	10.5 ± 1.9 b	9.1 ± 1.6 ab*
4	34.0 ± 2.3 b	33.1 ± 2.0 b	10.0 ± 2.3 b	9.1 ± 1.7 ab*
6	35.0 ± 3.5 b	33.7 ± 2.1 b	10.5 ± 3.0 b	9.0 ± 1.6 ab*
8	36.0 ± 0.0 b	33.0 ± 1.6 b	10.0 ± 4.0 b	9.7 ± 1.2 ab
10	40.0 ± 0.0 a	33.5 ± 1.3 b	7.5 ± 5.0 c	10.6 ± 1.2 a
12	38.0 ± 6.9 b	33.2 ± 2.3 b	15.0 ± 2.0 a	8.9 ± 2.6 b*
14	41.0 ± 8.1 a	32.8 ± 0.8 b	10.0 ± 0.0 b	6.9 ± 1.8 c*
16	36.0 ± 0.0 b	34.8 ± 0.6 a*	12.0 ± 8.0 b	4.6 ± 3.4 d*

Observations began after 24 h and, on average, germination was complete after 65 h. Means ± one standard deviation followed by the same letter within a column were not different by Tukey's test ($\alpha = 0.05$).

*Difference between calculated and actual germination according to a single degree of freedom *F*-test ($P \leq 0.05$).

Analysis of cold temperature mutants

After the initial screening, 36 mutant lines putatively resulting in faster than usual germination at 10°C were identified. However, after retesting these lines, only five were found to be demonstrably faster than the wild type. Despite using a suboptimal germination temperature (10°C), which is competent to alleviate *Arabidopsis* seed dormancy (Salaita *et al.*, 2005) and capable of accentuating differences in commencement of radicle protrusion, the longer the seeds had been after-ripened (AR) prior to the analysis, the more restricted became the period in which a measurable difference in germination percentage was observed (compare Fig. 33.3 (6 months AR) and Salaita *et al.* (2005) (~1 month AR)). Statistically significant differences in percentage germination were observed at fewer time points at the optimal (25°C) germination temperature (Salaita *et al.*, 2005). However, while percentage germination has been depicted every 12 h in Fig. 33.3, data was collected every hour. The ability to examine the progression of radicle protrusion every hour facilitated the discrimination between *bona fide ctg* mutants and false positives, particularly at 25°C.

Seedling growth rate

Redbud seeds have intermediate physiological dormancy. Embryos displaying this type of dormancy show an increased growth potential following chilling stratification (Hartmann *et al.*, 2002). Geneve (1991) showed that isolated redbud embryos from chilled seeds grew faster than non-chilled ones. However, these measurements were performed by hand and made every 24 h. In contrast, using the computer-aided imaging system, radicle length could be measured every hour and a precise growth rate calculated with little researcher investment in time. As predicted, radicles of

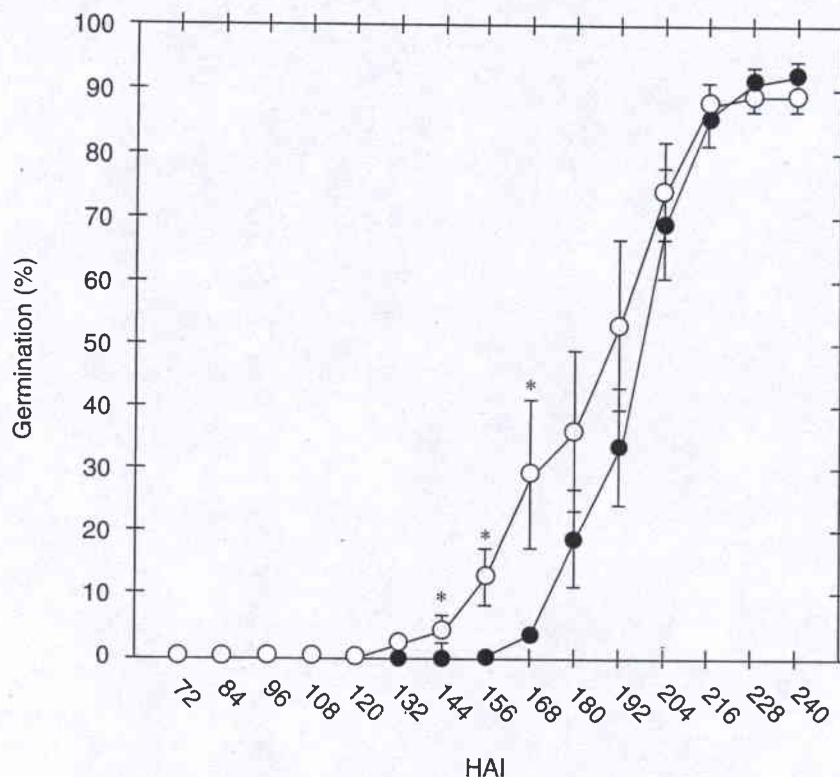

Fig. 33.3. Germination time course of *ctg144-D* (○) and Columbia wild-type (•) *Arabidopsis thaliana* seeds at 10°C after 6 months dry after-ripening (AR) at 25°C. Each symbol represents the mean of four replicates of 50 seeds and the bars represent the standard error. An asterisk above a symbol depicts statistically significant differences in percentage germination determined by a single degree of freedom *F*-test ($P \leq 0.05$). HAI = hours after imbibition.

non-chilled redbud embryos took 90 h to reach 10 mm in length, while embryos chilled for 4 weeks reached a radicle length of 10 mm in only 45 h (Fig. 33.4; $P \leq 0.01$). If the data was evaluated on a 24 h basis, as has been done earlier (Geneve, 1991), a clear quadratic increase in seedling size was seen (Fig. 33.4a). However, using a shorter interval image analysis, redbud embryos showed three distinct phases of growth (Fig. 33.4b). Following removal from the testa, there was a lag period prior to initiation of a slow increase in radicle size. This was followed by a rapid linear increase in size. In non-chilled embryos, growth did not begin until 50 h after removal from the testa. The subsequent slow growth phase required an additional 30 h before embryos entered the rapid linear phase of growth. In contrast, embryos from chilled seeds required only 15 h to initiate growth and began the rapid linear phase only 25 h later. These results show that the major difference between embryos isolated from non-chilled and chilled seeds was the time to initiate radicle growth. After growth was initiated the growth patterns were comparable.

Conclusions

Analysis of sequential digital images offers an alternative research tool to study time-sensitive processes related to seed germination. Analysis of these images revealed

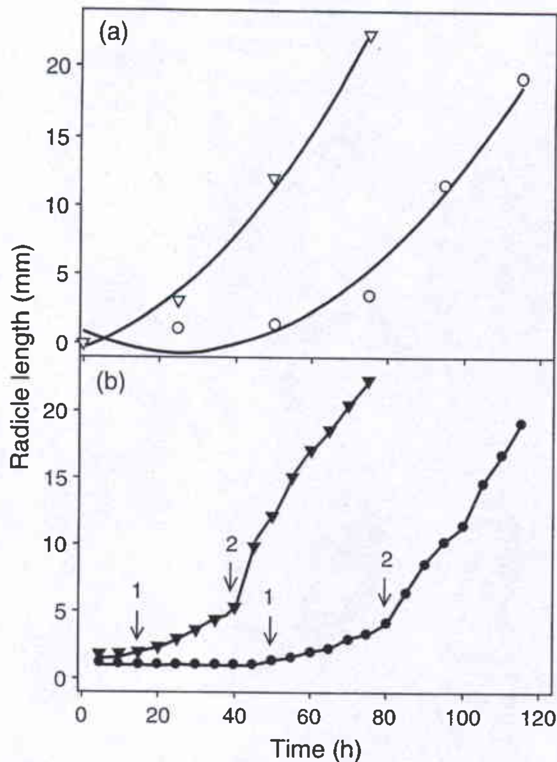

Fig. 33.4. Radicle length in isolated redbud embryos from seeds that have received 4 weeks of chilling stratification (Δ) or no chilling (○). (a) Length measured every 24 h. Regression lines were fitted to a second order polynomial; $r^2 = 0.98$ and 0.99 for chilled and non-chilled embryos, respectively. (b) Length measured every 5 h. Arrows indicate the start of the first and second growth phases.

changes during seed germination that were not previously observed or were too tedious to measure using conventional methods. This technique would allow the researcher to more precisely identify key stages of development during seed germination for future physiological or biochemical analyses and facilitate identification of unique phenotypes during mutant screens.

References

AOSA (1983) *Seed Vigor Testing Handbook, Contribution No. 32.* Association of Official Seed Analysts, Lincoln, Nebraska.

Baskin, C.C. and Baskin, J.M. (1998) *Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination.* Academic Press, New York.

Baskin, J.M., Baskin, C.C. and Li., X. (2000) Taxonomy, anatomy and evolution of physical dormancy in seeds. *Plant Species Biology* 15, 139–152.

Dell'Aquila, A., van Eck, J.W. and van der Heijden, G.W.A.M. (2000) The application of image analysis in monitoring the imbibition process of white cabbage (*Brassica oleracea* L.) seeds. *Seed Science Research* 10, 163–169.

Geneve, R.L. (1991) Seed dormancy in eastern redbud (*Cercis canadensis* L.). *Journal of the American Society for Horticultural Science* 116, 85–88.

Geneve, R.L. and Kester, S.T. (2001) Evaluation of seedling size following germination using computer-aided analysis of digital images from a flat-bed scanner. *HortScience* 36, 1117–1120.

Hara, Y. (1999) Calculation of population parameters using Richards function and application of indices of growth and seed vigor to rice plants. *Plant Production Science* 2, 129–135.

Hartmann, H.T., Kester, D.E., Davies, F.T., Jr and Geneve R.L. (2002) *Hartmann and Kester's Plant Propagation: Principles and Practices*, 7th edn. Prentice-Hall, Englewood Cliffs, New Jersey.

Howarth, M.S. and Stanwood, P.C. (1993) Measurement of seedling growth by machine vision. *Transactions of the American Society of Agricultural Engineers* 36, 959–963.

Keys, R.D., Margapuram, R.G. and Reusche, G.A. (1984) Automated seedling length measurement for germination/vigor estimation using a CASAS (computerized automated seed analysis system). *Journal of Seed Technology* 9, 40–53.

Liu, N.Y., Khatamian, H. and Fretz, T.A. (1981) Seed coat structure of three woody legume species after chemical and physical treatments to increase seed germination. *Journal of the American Society for Horticultural Science* 106, 691–694.

Morrison, D.A., McClay, K. Porter, C. and Rish, S. (1998) The role of the lens in controlling heat-induced breakdown of testa-imposed dormancy in native Australian legumes. *Annals of Botany* 82, 35–40.

Oakley, K., Kester, S.T. and Geneve, R.L. (2004) Computer-aided digital image analysis of seedling size and growth rate for assessing seed vigour in impatiens. *Seed Science and Technology* 32, 907–915.

Sako, Y., McDonald, M.B., Fujimura, K., Evans, A.F. and Bennett, M.A. (2001) A system for automated seed vigour assessment. *Seed Science and Technology* 29, 625–636.

Salaita, L., Kar, R.K., Majee, M. and Downie, A.B. (2005) Identification and characterization of activation tagged Arabidopsis mutants exhibiting rapid seed germination. *Journal of Experimental Botany* 56, 2059–2069.

Tomas, T.N., Taylor, A.G. and Ellerbrock, L.A. (1992) Time-sequence photography to record germination events. *HortScience* 27, 372.

Wilson, T.T. and Geneve, R.L. (2004) The impact of film coating on initial water uptake and imbibitional chilling injury in high and low vigor *sh2* sweet corn seeds. *Seed Science and Technology* 32, 271–281.