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New Methods to Prepare Squashes to Study Microsporogenesis in Pinus resinosa Ait. II. The Effects of Boiling Tissue Pieces in Various Fluids

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Abstract

Squashes of microsporophylls of Pinus resinosa were prepared as follows: The material was fixed in ethanol-acetic acid (3 : 1 v/v) (EA), and stored in ethanol-glycerin-water (1 : 1 : 1 v/v) (EGW). The best treatment for leptotene to diakinesis was heating in glycerin-water-dimethyl sulfoxide (2 : 1 : 7 v/v) with sodium bicarbonate 0.05 g/100 ml (GWDS) at 170° C for 30 min, followed by heating in glycerin-water (9 : 1 v/v) with sodium bicarbonate 0.25 g/100 ml (GWS) at 2600 C for 2—8 min. This resulted in untangling of prophase chromosome masses, and thus in an unobstructed view of chromosome details. The best treatment for metaphase to tetrads was heating in GWS at 260° C for 2–8 min without prior heating in GWDS.

Key words: ***Pinus resinosa,*** microsporogenesis, cytology, squashes, meiosis.

Zusammenfassung

Es wurde eine neue Methode zur Herstellung von Quetschpräparaten von Mikrosporophyllen bei Pinus resinosa AIT. entwickelt, durch welche eine gute Entwirrung der Prophase Chromosomenmassen und damit ein ungehinderter Einblick in die Feinstruktur der Chromosomen erreicht wird.

Introduction

During earlier experiments to develop new squash techniques for cytological studies of conifers (BONGA, 1978), it was observed that prolonged boiling of small pieces of male cone of Pinus resinosa in ethanol-glycerin-water (1 : 1 : 1 v/v) resulted in well-separated sporophyte cells and meiocytes with darkly stained, slightly swollen chromosomes and a well-cleared cytoplasm. This suggested that boiling of specimens could play an important role in the development of new squash techniques for conifers. The objective of the present study was to determine if the boiling technique could be improved so that less swelling of the chromosomes would occur, while maintaining the high degree of cell separation and clearing of the cytoplasm.

Material and Methods

Male reproductive shoots of Pinus resinosa AIT. were fixed in ethanol-acetic acid (3 : 1 v/v) (EA) for 24 h and then stored in ethanol-glycerin-water (1 : 1 : 1 v/v) (EGW) (GERLACH, 1969) at room temperature.

To obtain rapid and controlled heating of the material, the heating unit shown in Figure 1 was constructed. It consisted of a clay disc (14 X 4 cm) with four vertical holes for the specimen containers and one small hole in the center for a therrnometer. This disc was placed on top of a thermostatically controlled hotplate located inside a fume hood. Specimens were boiled in aluminium cups made by folding a piece of aluminium foil over the end of a glass tube. Aluminium foil cups were preferred to glass cups because aluminium foil transmits heat faster than glass. The mass of clay of the disc, having a large capacity to store heat, served to reduce temperature fluctuations.

Small pieces of specimen (about 2 X 1 X 1 mm) were transferred to the aluminium foil cups and a specific amount (usually 8 drops) of the fluid used for boiling the tissues (referred to as the boiling fluid), was added. The

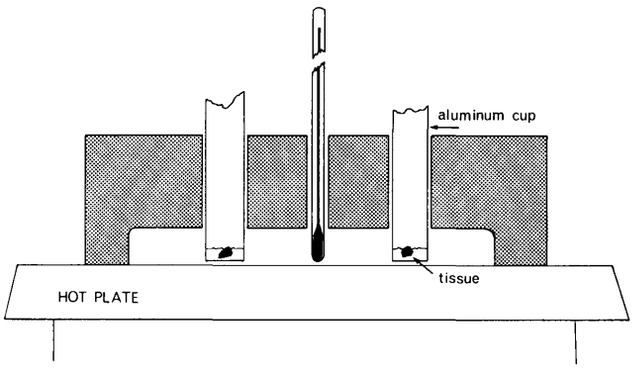

Fig. 1. — A diagram of the oven used to rapidly heat the fluids with small tissue pieces.

cup was then lowered into one of the holes in the hot clay disc. After the desired length of time, the cup was removed with forceps and left to cool to room temperature. The tissue was transferred to a microslide and squashed under a coverglass in either a drop of the cooled boiling fluid or the toluidine blue (GWDt) stain described earlier (BONGA, 1978). Green light (Kodak Wratten B filter 58) was used for all microscopic observations and photography.

To improve the effectiveness of the original boiling fluid, several modifications of it were tested, to determine if each of the components (alcohol, glycerin, and water) was essential, and if so, in what proportions; alcohol was deleted first, then glycerin-water combinations (10 : 0, 9 : 1, 8 : 2. . . ., 0 : 10 v/v), were tested. Subsequently, it was investigated if glycerin could be replaced by mineral oil or sucrose-water (1 : 1 w/v) to give a boiling fluid with a high boiling point. The effect of adding other chemicals to the boiling fluid was also tested. Several weak organic acids (lactic, formic, acetic, citric, boric, propionic, trichloroacetic acid, 1 : 20, 3 : 20, and 9 : 20 v/v or w/v) were tried because they often are components of the fixatives commonly used for cytology. Hydrochloric acid (1*N*) was added to the boiling fluid in the ratios 1 : 20, 3 : 20 and 9 : 20 v/v because it has been used for chromosome fixation and for reducing sweling of chromosomes (MARKS, 1973). The effect of several bleaches (chloral hydrate, potasium bisulfate, potassium bisulfite, potassium hydroxide, sodium hypochlorite, 1, 3, and 9 g/100 ml), buffers (citric acid — disodium phosphate, pH 3.0 — 9.0 at 0.5 pH intervals), buffer salts (sodium bicarbonate, sodium borate, mono — and dipotassium phosphate, and sodium carbonate, 0.125, 0.25, 0.5, and 1 g/100 ml), and detergents (Tween 80, and Decon 75 (BDH chemicals) 1, 3, and 9 : 200 v/v), was also investigated. To determine the effect of speed of boiling, the effectiveness of temperatures between 180 and 360° C was checked at 20° intervals for various lengths of time.

It was also determined if the present technique of boiling the tissues could be combined with the earlier technique of heating in GWDMC (BONGA, 1978). The material was either first boiled for 0, 2, 4, 6, 8 or 12 min, followed by GWDMC treatment at 170 or 140° C for 20, 40 ,or 60 min, or the treatments were reversed, i.e. the tissues were first treated with GWDMC, followed by boiling.

Results

Alcohol was not necessary in the boiling fluid, and therefore, was deleted from all subsequent boiling fluid compositions. Water was not essential, but better results were consistently obtained when some water was present, the optimal ratio being glycerin-water (9 : 1 v/v). Replacing the glycerin by oil or by high concentrations of sucrose in water resulted in darkly stained, very hard pieces of tissue, totally unsuitable for squash preparations. Therefore, all further experiments were carried out with glycerin-water (9 : 1 v/v) (GW).

Adding weak acids or 1*N* hydrochloric acid did not improve the chromosome image, reduced staining intensity of the chromosomes, and interfered with clearing of the cytoplasm. Bleaches had no beneficial effect, but adding the detergent Decon 75 (3 : 200, or 9 : 200 v/v) improved staining, separation, and general appearance of the chromosomes. Decon 75 raised the pH of the boiling fluid to about 9.0 and its action could have been a pH rather than a surfactant effect. Using citrate-phosphate buffer, and buffer salts, it was found that a pH between 7.5 and 9 was optimal for cell and chromosome separation, cytoplasm clearing, and chromosome staining and image detail. Best results were obtained with sodium bicarbonate, 0.25 g in 100 ml GW, this combination being referred to as GWS. It was found that 8 drops of GWS per aluminium foil cup was a convenient and effective amount to use.

Better clearing of cytoplasm and chromosome separation were obtained in GWS at temperatures higher than 220° C, than at lower temperatures. However, over 300° C the duration of the heating process became very critical, i.e. there was only a narrow margin between under- and overheating. Furthermore, optimal timing was not the same for all samples. More consistent results were obtained with temperatures between 240 and 280° C, and 260° C was chosen for all further routine heating operations. At 260° C, good results were obtained between 3 and 8 min of heating. Up to 2 min of heating resulted in meiocytes with dense, contracted cytoplasm, and with the nuclei and chromosomes barely visible *(Fig. 2)*. If heated for 3 or 4 min the cytoplasm cleared in most meiocytes and the chromosomes of all meiotic phases had stained dark brown. After 5 or 6 min of heating, the chromosomes of the first division phases of meiosis appeared well stained and well separated, but some nuclei and chromosomes of the second division phases had started to disintegrate *(Fig. 3)*, i.e. the different phases of meiosis required different exposures to heat for optimal results. Heating for more than 8 min resulted in the chromosomes disintegrating progressively more, the longer the heat was applied.

With the appropriate length of exposure to 260° C, all meiotic phases from diakinesis to the tetrad stage showed well *(Figs. 4—7)*. The earlier phases, in particular leptotene and zygotene, had somewhat swollen chromosomes. Treatment in GWS appeared unsuitable for microspores. Even a short exposure to the boiling fluid resulted in the nuclei and cytoplasm being completely dissolved in most microspores, and with insufficient clearing of the cytoplasm in those in which the nuclei had survived. In other words, the GWS method was best suited for the diakinesis to tetrad stages and for these it was more satisfactory than earlier (BONGA, 1978) methods. By squashing the tissues in the toluidine blue stain GWDt (BONGA, 1978), staining of the chromosomes was further intensified.

It was found, that if the material was treated with GWDMC before being heated in GWS marked improvements occurred in the image of the chromosomes of the early phases of meiosis (leptotene-diakinesis); the reverse order of treatments worked less well. The use of GWDMC at 170° C resulted in better chromosome separation than if used at 140° C. Heating duration was not critical for GWDMC, except that 60 min probably was near the upper permissible limit. Therefore, 30 min of heating at 170° C was adopted as standard treatment. The length of heating in GWS was more critical; overheating resulted in degrada-

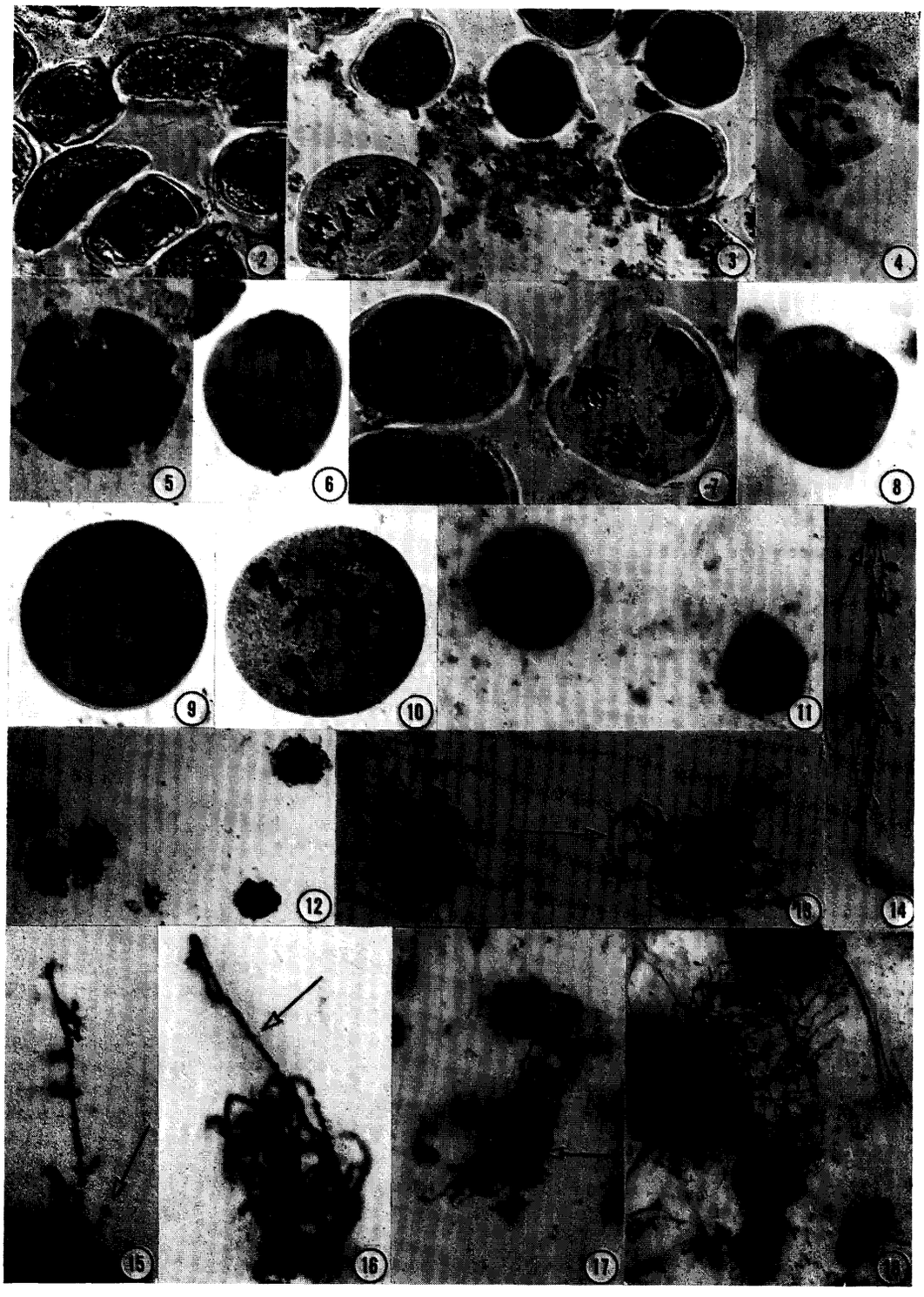

Fig. 2. — Meiocytes heated in GWS at 260° C for 2 min. Heating was too short to ensure clearing of the cytoplasm. × 250.

Fig. 3. — A sample of the same piece of tissue used for *Fig. 2* heated for 6 min. The first metaphase and telophase chromosomes are well fixed and stained. The nuclei of the diads have been overheated and therefore, are partly disintegrated. × 400.

Figs. 4—7. — Tissues heated in GWS for 4—6 min. All × 500.

Fig. 4. — Diakinesis.

Fig. 5. — Metaphase I.

Fig. 6. — Anaphase I.

Fig. 7. — Diad and tetrad formation.

Figs. 8—18. — Tissues heated in GWDS-GWS, squashed in GWDt.

Figs. 8—10. -- Little squashing pressure applied; cells still intact. All × 500.

Fig. 8. — Zygotene.

Fig. 9. — Diplotene.

Fig. 10. — Diakinesis.

Figs. 11—14. — Various stages of untangling of chromosome balls.

Fig. 11. — Two meiocytes. In one, the cell wall is still intact, the cytoplasm is mostly disintegrated and the nuclear membrane has dissappeared. In the other, the cell wall has scattered under pressure and the wall fragments and cytoplasm remnants have washed away. The chromosome ball is still tightly packed. Note nucleoli. × 400.

Fig. 12. — Several free chromosome balls, some starting to untangle along the fringes. × 250.

Fig. 13. — Two partially untangled chromosome balls. Note dark beads (chromomeres) (arrow) on the chromosomes. × 600.

Fig. 14. — A more untangled chromosome ball. Note large nucleous (arrow), and chromomeres. × 400.

Fig. 15. — A chromosome fragment showing paired chromatids and chromomeres (arrow). × 1000.

Fig. 16. — Spiralling chromatids (arrow). × 1000.

Fig. 17. — Paired nucleoli (arrow). Another large nucleolus near upper middle of picture, and several smaller ones in various other positions. × 500.

Fig. 18. — An untangled diploid prophase nucleus. The chromosomes are thinner than those of the haploid nuclei shown in the other figures × 500.

tion of nuclei and chromosomes, and insufficient heating in less than optimal chromosome separation. However, the optimal length of heating in GWS fluctuated considerably from sample to sample, possibly because of variation in size and shape of the tissue pieces. It was decided, therefore, to use 2-, 4-, 6-, and 8-min-heating periods routinely for each tissue sample, thus assuring that at least some squash preparations would receive the right amount of heat.

Methyl salicylate (M) and chloral hydrate (C) had originally been included in GWDMC to improve cell separation and clearing of the cytoplasm (Bonga, 1978). However in combination with the GWS treatment, better staining and less chromosome breakage were obtained if both salicylate and chloral hydrate were omitted from the GWDMC, leaving just glycerin, water and dimethyl sulfoxide (GWD).

It was found that pH was not only an important factor in the GWS treatment, it also had an effect during the GWD treatment. Adding sodium bicarbonate (0.05 g) to GWD (100 ml) (this formula being called GWDS) further improved chromosome separation, and details of chromosome strucure showed more clearly.

The final procedure for routine investigations was the following: Each tissue piece was heated in 1 ml of GWDS at 170° C for 20—40 min, followed by heating in 8 drops of GWS at 260° C for 2, 4, 6, or 8 min: this treatment being referred to as the GWDS-GWS treatment. Since the toluidine blue stain GWDt (Bonga, 1978) generally intensified staining of the chromosomes, most GWDS-GWS treated tissue pieces were squashed in a drop of this stain.

The GWDS-GWS treatment greatly improved the quality of squashes of the early phases of meiosis (leptotene to diakinesis) *(Figs. 8—17);* chromosome swelling was notably reduced in comparison with that in meiocytes exposed to the earlier treatments. The later phases (metaphase to tetrads) showed better after the GWS treatment alone, i.e. without the preceding GWDS treatment.

The most interesting aspect of the GWDS-GWS squashes of leptotene to pachytene meiocytes was that often the cell walls ruptured, freeing large numbers of nuclei. In these, the nuclear membrane generally broke, releasing chromosome masses which often started to untangle partially *(Figs. 11—13),* or more completely *(Fig. 14),* depending on how much pressure was applied. As a result, large sections of chromosome arms and loops flattened out and could be observed free from interference from other chromosomal material. Many of the chromosome sections distinctly showed very thin paired chromatids and chromomeres *(Fig. 15),* spiralling of chromatids *(Fig. 16),* and paired and single nucleoli *(Fig. 17).*

A similar untangling of chromosomes occasionally occurred in nuclei from the diploid cells of the microstrobili, but generally only after the meiocytes had been destroyed by long heating, i.e. the diploid cells required more heat than the meiocytes for chromosome liberation. These chromosomes *(Fig. 18)* were much thinner and thus, more fragile than those of the prophase meiocytes.

Since nuclei and chromosomes were more fragile after GWDS-GWS treatment than after any of the other treatments, squashing pressure had to be applied very carefully. Best results were obtained if very small pieces of tissue were squashed. The procedure generally used was as follows: First, the tissue was lightly squashed under a coverclass. Then the coverglass was carefully lifted and transferred to a small drop of GWDt on a new microslide, thus transferring about half of the tissue to the new slide. This procedure was repeated till cell density under the coverglasses was reduced to one that allowed good flattening of the cells with only minor needle pressure. Leaving the squash preparations at rest for one to several days often further flattened the cells and chromosomes and intensified the staining of the chromosomes.

Discussion

No single squashing procedure was suitable for all phases of meiosis. Prophases showed best in GWDS-GWS, metaphases to tetrads in GWS, and microspores in GWDMC. These differences in the effects of the various treatments on the different phases of meiosis and microspore formation coincided with the presence of a heavy callose layer during the later stages of meiosis, and of the exine and intine layers in the developing microspores. This suggests a causative relationship between the differences in the treatments and the presence or absence of these layers.

Untangling of prophase chromosomes occurred far less frequently in the GWDMC and GWS treatments than in the GWDS-GWS treatment. This could posibly be explained as follows: Whenever the chromosome masses of the prophase nuclei failed to untangle, remnants of cytoplasm, nucleoplasm, and, or interchromosomal connections (Klasterska, *et al.* 1976) probably were the cause. The nucleoplasm and interchromosomal connections were selectively destroyed by GWDMC or GWDS, but, even though these treatments largely disrupted the cytoplasm, enough of it remained around the nuclei to prevent the chromosome masses from untangling. GWS removed the cytoplasm better than GWDMC or GWDS, but failed to remove the nucleoplasm and interchromosomal connections sufficiently, and therefore, GWS alone did not untangle the chromosome masses. By combining the GWDS and GWS treatments, the cytoplasm as well as the nucleoplasm and inter-

chromosomal connections were sufficiently removed to allow the chromosomes to untangle.

From a cytological point of view, it is important to have good methods to untangle meiotic prophase chromosomes, so that processes like pairing and genetic recombination can be studied at the chromosome level. A technique often used to untangle prophase chromosomes is to treat specimens with water or hypotonic salt solutions (KLASTERSKA, *et al.* 1976, BEÇAK, *et al.* 1977). These pretreatments were tried in conjunction with the GWDMC technique but were not effective (BONGA, 1978). However, the GWDS-GWS technique is an effective alternative method to untangle prophase chromosomes, at least for those of *Pinus resinosa*.

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Use of the Weibull function to quantify sweetgum germination data

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Summary

The three-parameter Weibull function was used in germination analyses of seeds from four stands of sweetgum *(Liquidambar styraciflua* L.) in central Mississippi. Stratification periods of 4 to 32 days gave equal stimulation of germination; 64 days provided much greater stimulation. There were no significant differences between stands, but there was a significant interaction between stands and stratification period in the "b" parameter which describes the time-scale of germination. Estimates of germination response based on the Weibull parameters were very close to actual germination, and they facilitated interpretation of other components of variance.

Key words: programming (computer), variance analysis, percentiles

Zusammenfassung

Samen von *Liquidambar styraciflua* L. von vier Herkünften aus Zentral-Mississippi wurden unterschiedlich lang stratifiziert. Hierbei zeigten sich erst nach einer Behandlung von 64 Tagen Unterschiede im Keimprozent. Zwischen den Herkünften konnten keine signifikant unterschiedlichen Keimprozente beobachtet werden, jedoch significante Unterschiede im Keimungsverlauf.

Introduction

Germination tests are commonly used to measure seed viability and to describe the relative quality of the seed lot. The pattern of germination frequency in these tests is interpreted as an indicator of seed quality-its vigor. Quantification of differences in the pattern of germination frequency has been a traditional problem in seed research. Recently BONNER and DELL (1976) used the Weibull function for quantifying the trend in the cumulative proportion of seed germinated. The objective of this study was to evaluate the use of the Weibull function to quantify differences in germination between sweetgum *(Liquidambar styraciflua* L.) seed lots from different stands and trees within stands when subjected to various periods of stratification.

Procedure

Four natural stands in eastern Oktibbeha County, Mississippi, were selected for their proximity to Mississippi State University. The stands were separated from each other by a distance of at least 2 km. Within each stand five selected parent trees were an average of 20 meters apart, although this spacing varied between 10 and 25 meters. From a biological standpoint stands and trees within stands were considered as random selections.

Mature fruits were collected from the middle portion of the crown of parent trees (generally at a height of 6 to 9 meters). After air drying seeds were extracted by hand from the fruits, and empty seeds were removed with compressed air. The cleaned seeds were stored at 5.6° C until germination or pretreatment.

Samples of 200 seeds from each parent tree were subjected to each of six stratification treatments. These treatments consisted of chilling the seeds at 4.4° C for periods of 0, 4, 8, 16, 32 or 64 days after soaking for 24 hours at room temperature.

The seeds were germinated in a Stults germinator, on standard blotters at a 20°—30° C regime with a light period of 11 hours during the higher temperature period.[1]) For a period of 15 days germination was scored daily; seeds were scored as germinated when radicles exhibited positive geotropism. At the end of the germination period all ungerminated seeds were cut open to determine the number of seeds that had the capacity to germinate.

DELL and BONNER[2]) provide comprehensive consideration

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[1]) Mention of trade names is solely to identify material used and does not imply endorsement by the U.S. Department of Agriculture.

[2]) DELL, T. R., and F. T. BONNER. Quantifying germination trends over time using the Weibull function. Unpublished manuscript.