Evaluation of *Eucalyptus* spp. seedling size following germination using computer-aided analysis of digital images

Avaliação do tamanho de plântulas de *Eucalyptus* spp. após a germinação, com utilização de análise de imagens digitais auxiliada por computador

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RESUMO: O crescimento inicial de plântulas pode ser utilizado para se estimar o vigor de sementes, especialmente daquelas espécies de plantas cujas sementes são muito pequenas. No entanto, a medição manual de plântulas de dimensões reduzidas é um processo tedioso e de difícil reprodutibilidade entre analistas. A análise de imagens digitais, auxiliada por métodos computacionais, poderia melhorar a acurácia e a reprodutibilidade destas medições. O uso de um scanner digitalizador de mesa produziu imagens de alta resolução de sementes em germinação de 18 acessos de diferentes espécies de *Eucalyptus* (Myrtaceae) com sementes grandes e pequenas. Imagens puderam ser obtidas de diferentes acessos simultaneamente e analisadas em menos de 2 minutos. Um gel transparente, à base de agar foi utilizado como substrato em ensaios de germinação em placas de petri para facilitar a captura de imagens com um scanner de mesa. O meio transparente permitiu que as imagens das plântulas fossem adquiridas sem a remoção da tampa e possibilitou a repetição das medidas em momentos diferentes para o cálculo de taxas de crescimento. Imagens de sementes germinadas puderam ser obtidas em papel-fitro. As medidas do comprimento de plântulas via análise computacional de imagens digitais foram obtidas para todos os acessos de *Eucalyptus* estudados e mostraram ser similar às médias das medidas feitas separadamente por três analistas.

PALAVRAS-CHAVE: Germinação de sementes, Vigor de sementes, Análise de imagens

ABSTRACT: Early seedling growth rate can be used to estimate seed vigor for small-seeded plants. However, hand measurement of small seedlings is tedious and difficult to reproduce among analysts. Computer-aided analysis digital images of seedlings should improve accuracy and reproducibility. A flat-bed scanner provided high resolution images of germinating seeds of eighteen accessions of large and small-seeded species of *Eucalyptus* spp. (Myrtaceae). Images could be captured for different accessions simultaneously and analyzed in less than 2 minutes. A clear, agar-based gel was used as the germination substrate in petri dish germination assays to facilitate capturing images with a flat-bed scanner. The transparent medium permitted seedlings to be imaged without removal from the petri dish and also allowed for repeated measures of the same seedlings in order to calculate growth rate. Alternatively, images could be captured from inverted petri dishes containing seeds germinated in wet filter paper. Computer analysis of seeding length was possible for all *Eucalyptus* accessions and was statistically similar to hand measurements averaged for three analysts.

KEYWORDS: Seed germination, Seed vigor, Image analysis

INTRODUCTION

Seed vigor is an important aspect of seed quality. Unlike standard germination tests, which are a required for each commercial seed lot, seed vigor tests attempt to provide an assessment of germination performance under field (ASSOCIATION OF OFFICIAL SEED ANALYSTS, 1993) or greenhouse conditions. Although numerous seed vigor tests have been developed, five major ones are used by most seed analysts. These include accelerated aging, cold test, cool test, electrolyte leakage and seedling growth rate (HARTMANN et al., 1997). Of these, accelerated aging and cold test account for the majority of vigor tests for agronomic and forestry crops (FERGUSON-SPEARS, 1995).

Seed vigor tests used to evaluate large-seeded agronomic crops are generally not useful for evaluating smaller-seeded species (JIANHUA and McDONALD, 1996). In a standard accelerated aging test, rapid water uptake by small seeds may result in high mortality unrelated to the vigor of the seed (FERGUSON-SPEARS, 1995).

Seedling growth rate tests can also be used to assess vigor in seeds. A vigor index text has been developed based on the use of a video camera to capture digital images of emerged seedlings in a plug tray to determine cotyledon area (CONRAD, 1999). This has proven to be very successful in a limited number of bedding plant species.

One alternative is to measure seedling or radicle length in plants grown under controlled environments as an indicator of seed vigor. Evaluating seedling growth rate, especially radicle growth under controlled conditions, has been used successfully to test vigor in a number of small-seeded crops, including carrot (*Daucus carota* L.), lettuce (*Lactuca saliva* L.) (McCORMAC et al., 1990; SMITH et al., 1973), radish (*Raphanus sativus* L.), sugar beet (*Beta vulgaris* L.) (PERRY, 1981), cauliflower (*Brassica oleracea* L.), onion (*Allium cepa* L.) and leek (*Allium ampeloprasum* Tausch.) (FINCH-SAVAGE, 1986). Radicle length or growth rate, measured by using a slant-board test, was correlated with field emergence in these crops. These studies and others have established a strong correlation between radicle growth and the vigor level of a seed lot (BINGHAM et al., 1994). One problem with the slant-board test is that it is time consuming for the analyst to evaluate radicle length by hand and it can introduce analyst error in measuring radicle length (McCORMAC et al., 1990).

McCormac et al. (1990) attempted to use digital image analysis of radicle length of small-seeded vegetable crops using the slant-board test. Although this was an accurate measure of radicle length, problems with lighting and background color made it difficult to evaluate the small roots of these crops. Attempts to automate vigor tests by using machine vision (HOWARTH and STANWOOD, 1993), time-sequence photography (TOMAS et al., 1992), and computerized automated seed analysis with a hand potentiometric caliper (KEYS et al., 1984) have been reported. The results of these tests have correlated well with those of hand measurements of radicle length, but have failed to become routine tests used by commercial analysts for a variety of reasons related to standardized conditions for collected images used by the computer.

A good vigor test should be: 1) reproducible from lab to lab and analyst to analyst; 2) correlated with field or greenhouse emergence tests; 3) be rapid, objective and simple; and 4) inexpensive. The objective of this research was to develop a computer-aided evaluation system using digital images of radicle emergence that would be suitable for use as a vigor test. Key aspects developed for this research include the use of a flat-bed scanner to obtain digital images and evaluation of a transparent germination medium to facilitate scanning.

MATERIALS AND METHODS

Plant material

Seeds of eighteen accessions of different *Eucalyptus* species and hybrids (Table 1), obtained at the Instituto de Pesquisas e Estudos Florestais (IPEF/ESALQ/USP) were evaluated for germination and seed vigor on two different germination media.

Germination conditions

Twenty seeds per species were sown in 8.5-cm-diameter plastic sterile petri dishes containing either one piece of filter paper (Wattman Paper , 3M, no.3), or an autoclaved solution of 2% (w/v) agar. The filter paper was cut to fit the petri dish, soaked in distilled water for 30 min, and autoclaved for 15 min at 1 atm (120psi). Sterile water (1.5 ml) was added to the dish containing the filter paper prior to placing the seeds. Petri dishes were sealed with Parafilm (American National Can, USA) and placed in a single germination chamber held at 25 ± 2 °C at 40 $\mu mol.s^{-1}.m^{-2}$ from cool-white fluorescent lamps.

Evaluation of seedling growth

Seedling growth was evaluated either by hand using a ruler to measure radicle lenght, or using computer-aided analysis of digital images. Three analysts separately evaluated the same set of seeds. Petri dishes were refrigerated (5 °C) between hand and digital image acquisition. Each accession was evaluated 2 days after initial radicle emergence (generally 4 days after sowing). Alternatively, growth rates were obtained by sequentially measuring radicle-hypocotyl axis length of digital images taken every 12 hours for E. grandis, E. urophylla and E. saligna germinating seeds. Digital images were acquired using a flatbed scanner (Hewlett Packard ScanJet, USA) that included both base and top lighting. For the seeds on the agar gel, scans were made through the petri dish bottom in an upright position. For the seeds germinated on filter paper, the dishes were placed upside-down on the scan table, with the lids removed. Each scan created a gray-scale 300 dpi (118 dots per cm) resolution image. Seedling length was measured automatically using the Scion Image software (version Beta 4.02; Scion Corporation, USA). The images were adjusted for sharpness using the LUT option set with a threshold between 180 and 205 depending on the size of each species.

A total of 60 seeds (three petri dishes) were evaluated per treatment, for each accession. Each treatment consisted of a combination of germination medium, seed accession and the method of data acquisition. The results were analyzed using ANOVA and the Tukey's test, considering each petri dish one experimental replicate.

RESULTS AND DISCUSSION

Agar gel and wet filter paper were effective media for the germination of seedlings of all *Eucalyptus* species studied. The percentages of seed germination on each germination medium were not statistically different (Table 1). These data and the seedling measurement results observed on the two media indicate that the agar gel could be a suitable substitute for germination paper or blotter when imaging for vigor is important. The use of germination paper or blotter is strictly recommended by the AOSA (ASSOCIATION OF OFFICIAL SEED ANALYSTS, 1993).

Table 1. *Eucalyptus* accessions used in this study and their respective seed germination percentages in each germination medium tested.
(Acessos de *Eucalyptus* utilizados neste estudo e suas respectivas porcentagens de germinação em cada um dos meios testados.)

Accession	Description	Seed age (years)	Germination (%)*	
			Wet Paper	**Agar Gel**
IT335	*E. botryoides*	11	60.0 ± 07.1	61.7 ± 05.7
IB03	*E. camaldulensis*	1	27.5 ± 03.5	28.3 ± 03.0
RE16	*E. citriodora*	2	42.5 ± 03.5	41.7 ± 02.8
AN534	*E. cloeziana*	1	12.5 ± 03.5	13.3 ± 02.9
IB033	*E. grandis*	3	35.0 ± 07.0	36.7 ± 05.8
IB039	*E. grandis*	1	67.5 ± 03.4	68.3 ± 02.9
RE18	*E. maculata*	12	12.5 ± 07.1	13.3 ± 02.9
AN474	*E. pellita*	5	87.5 ± 03.5	88.3 ± 03.5
AN524	*E. pellita* x *E. tereticornis*	10	72.5 ± 03.5	71.7 ± 03.5
AN146	*E. phaeotrichia*	12	10.0 ± 14.1	13.3 ± 11.5
AN359	*E. pilularis*	9	12.5 ± 03.5	13.3 ± 03.5
AN361	*E. propinqua*	9	72.5 ± 03.4	73.3 ± 02.8
AN514	*E. resinifera*	2	62.5 ± 03.5	63.3 ± 02.9
AN05	*E. saligna*	1	52.5 ± 03.5	53.3 ± 03.5
IT352	*E. saligna*	2	47.5 ± 31.8	46.6 ± 22.9
AN01	*E. urophylla*	2	90.0 ± 07.0	91.7 ± 05.7
AN499	*E. urophylla*	3	87.5 ± 03.5	88.3 ± 03.5
AN497	*E. urophylla* x *E. grandis*	3	75.0 ± 07.1	73.6 ± 05.5

* mean ± standard deviation. The numeric differences between the germination percentages observed in wet paper or agar gel were not statistically significant according to the Tukey's test ($p < 0.01$)

The advantage of the agar gel as a germination medium is that it is transparent, permitting digital images to be easily taken using a flat-bed scanner. In the present study, a slant board was not necessary to facilitate seedling length measurements as has been reported previously (FINCH-SAVAGE, 1986; McCORMAC *et al*., 1990). Improvements in analytical software allow for accurate measurements of the length of nonlinear objects and even compensate for seedling crossover. The flat-bed scanner used to capture digital images provided uniform lighting and software calibration would provide for repeatable results between labs.

The flat-bed scanner provided images of excellent resolution of even the smallest seeds and radicles (Figure 1A). The seeds of *E. camaldulensis* were the smallest seeds analyzed and hand measurements of early seedling growth in this species could not be performed easily. Capturing digital images of small seedlings is difficult using digital camera technology. This is not a camera resolution problem, as resolution has improved in recent years. Flat-bed scanner resolutions have also improved to resolve more pixels per unit area, but it was not necessary to increase the scanner resolution beyond 300 dpi (118 dots per cm) to obtain usable digital images. The difference is that the digital camera is capturing images from as far away as 1 m from the seedlings (to account to the same number of petri dishes analyzed simultaneously). With the flat-bed scanner, the seedlings are closer to the image-capturing device, accounting for the high-quality images.

Recently, Dell'Aquila *et al*. (2000) studied imbibitions using computer-aided image analysis. They employed a Charged Coupled Device (CCD-camera) rather than a flat-bed scanner and created a clear medium using 20 mL polymerized agarose in a petri dish. Their light source was an illumination table with fluorescent lamps under the petri dish. With this procedure, they were able to follow imbibition every 30 min for individual seeds. Although this system employs many of the same principles for capturing digital images presented in this study, the flat-bed scanner system offers several advantages. First, the light source is consistent and self-contained in the system. More importantly, the dual illumination from the scanner permits acquisition of images with the petri dish lid intact, regardless of condensation. In the previously cited study, condensation had to be controlled to acquire images. This required a double lid system with the air between the lids heated with a tungsten wire.

The length or area of each seedling can be calculated by computer-aided analysis using available software (Figure 1B, C). In the present study, Scion Image was used to measure seedling length. This software is designed to detect and measure length of elements contrasting with the background color. However, the computer could detect and trace the length of small seedlings, even if the roots were white, as the contrast of the gray-scale images can be adjusted to a preestablished threshold using one of the LUT tables provided by the software.
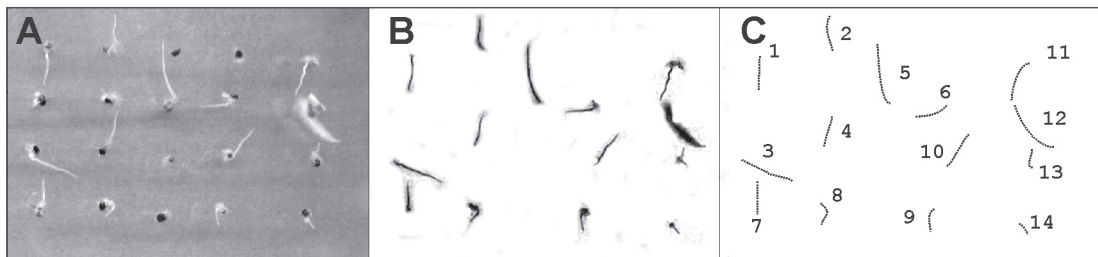

Figure 1. Image analysis of *Eucalyptus* seedling growth. A: Template image taken with a flat-bed scanner of germinated *E. urophylla* (AN499) seeds; B: Processed LUT image with enhanced contrast; C: Linear scaffold of identified particles used for automatic length measurements.
(Análise de imagens do crescimento de plântulas de *Eucalyptus*. A: Imagem-molde obtida com uso de scanner de mesa de sementes germinadas de *E. urophylla* (AN499); B: Imagem processada pelo método LUT e com contraste incrementado; C: Esqueleto linear das partículas identificadas na imagem, usados para obtenção automática das medidas de comprimento.)

There was no difference in seedling length when the same seedlings were repeatedly scanned and measured using computer software, even when the Petri dish was reoriented prior to scanning (data not shown). Therefore, hand measurements of seedling length by three analysts were compared with measurements obtained with the Scion Image software. Computer-assisted and hand measurements were not significantly different (Table 2). More variation was evident among analysts than between analysts and computer-assisted length measurements. These data demonstrated the utility of using computer-aided analysis of seedling length, which also required a minimum of analyst time to perform. Each digital capture and computer-aided analysis required <2 min per petri dish and recorded data in spreadsheet format, saving additional time. Also, digital images can be archived in case a problem with a seed lot arises, and these could be re-evaluated to provide evidence of the seed lot's initial quality.

Additionally, as the method is non-destructive and there is no direct contact with the seedlings, it is possible to obtain sequential data and seedling growth rates can be established (Figure 2).

The system described here is relatively inexpensive to set up and should allow for standardized testing among laboratories. However, it has yet to be determined whether seedling length can be used as a routine measure of seed vigor that has a meaningful relationship to seedling emergence in the field or greenhouse.
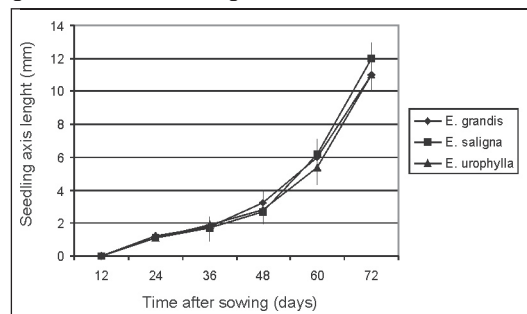

Figure 2. Length of the seedling axis obtained by computer-assisted image analysis of different *Eucalyptus* species during the first 6 days after sowing.
(Medidas de comprimento do eixo hipocótilo-radícula, obtidas pela análise de imagens assistida por computador, de plântulas de *Eucalyptus* de diferentes espécies, durante os primeiros 6 dias de desenvolvimento.)

Table 2. Seedling axis length of different *Eucalyptus* accessions measured by hand and by computer-assisted image analysis, 4 days after sowing.
(Comprimento do eixo hipocótilo-radícula de diferentes acessos de *Eucalyptus*, medido manualmente ou com o uso de análise de imagem assistida por computador, 4 dias após a semeadura)

Accession	Description	Axis lenght (mm)*		Tuckey test
		Hand Measurements	Image Analysis	
IT335	*E. botryoides*	6.0 ± 2.1	6.1 ± 0.4	ns
IB03	*E. camaldulensis*	7.5 ± 0.9	7.3 ± 0.1	ns
RE16	*E. citriodora*	8.3 ± 2.9	8.7 ± 1.8	ns
AN534	*E. cloeziana*	4.5 ± 1.5	4.3 ± 0.1	ns
IB033	*E. grandis*	5.0 ± 1.3	5.2 ± 0.8	ns
IB039	*E. grandis*	7.5 ± 1.4	7.3 ± 0.9	ns
RE18	*E. maculata*	8.5 ± 3.1	8.6 ± 1.7	ns
AN474	*E. pellita*	6.5 ± 0.5	6.3 ± 0.3	ns
AN524	*E. pellita* x *E. tereticornis*	7.5 ± 0.5	7.7 ± 0.2	ns
AN146	*E. phaeotrichia*	5.0 ± 1.1	5.2 ± 0.5	ns
AN359	*E. pilularis*	4.5 ± 1.5	4.3 ± 0.4	ns
AN361	*E. propinqua*	6.5 ± 1.4	6.2 ± 0.8	ns
AN514	*E. resinifera*	6.5 ± 1.3	6.3 ± 0.6	ns
AN05	*E. saligna*	5.5 ± 1.1	5.4 ± 0.3	ns
IT352	*E. saligna*	7.5 ± 1.6	7.6 ± 0.6	ns
AN01	*E. urophylla*	5.0 ± 1.0	5.1 ± 0.7	ns
AN499	*E. urophylla*	4.5 ± 1.5	4.8 ± 0.3	ns
AN497	*E. urophylla* x *E. grandis*	7.0 ± 1.8	7.3 ± 0.5	ns

* mean ± standard deviation of 3 independent mesures

CONCLUSIONS

A system has been developed for routine measurement of seedling length that could be used for seed vigor analysis. The use of a flat-bed scanner required the development of a clear germination medium. The described system can be used for routine measurements of *Eucalyptus* seedling or root length.

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REFERENCES

ASSOCIATION OF OFFICIAL SEED ANALYSTS. Rules for testing seeds. **Journal of Seed Technology**, v.16, n.1, p.1-113, 1993.

BALL SEED COMPAGNY (WEST CHICAGO, IL.). Robert Conrad. **Method and apparatus for assessing the quality of a seed lot US Patent 5901237**. 4 mai.1999.

BINGHAM, I.J.; HARRIS, A.; MACDONALD, L. A comparative study of radicle and coleoptile extension in maize seedlings from aged and unaged seed. **Seed Science and Technology**, Bassersdorf, v.22, n.6, p.127-139, 1994.

DELL'AQUILA, A.; VAN ECK, J.W.; VAN DER HEIJDEN, G.W.A.M. The application of image analysis in monitoring the imbibition process of white cabbage (Brassica oleracea L.) seeds. **Seed Science Research**, v.10, p.163-169, 2000.

FERGUSON-SPEARS, J. An introduction to seed vigour testing. In: VAN DE VENTER, H.A. **Seed vigor testing**. Zurich: International Seed Testing Assessment, 1995. p.1-9.

FINCH-SAVAGE, W.E. A study of the relationship between seedling characters and rate of germination within a seed lot. **Annals of Applied Biology**, v.198, p.441-444, 1986.

HARTMANN, H.T.; KESTER, D.E.; DAVIES JUNIOR, F.T.; GENEVE, R.L. **Plant propagation: principles and practices**. 6.ed. New Jersey: Prentice-Hall, 1997. 770 p.

HOWARTH, M.S.; STANWOOD, P.C. Measurement of seedling growth rate by machine vision. **Transactions of the American Society of Agricultural Engineers**, v.36, p.959-963, 1993.

JIANHUA, Z.; MCDONALD, M.B. The saturated salt accelerated aging test for small-seeded crops. **Seed Science and Technology**, Bassersdorf, v.25, n.8, p.123-131, 1996.

KEYS, R.D.; MARGAPURAM, R.G.; REUSCHE, G.A. Automated seedling length measurement for gennination/vigor estimation using aCASAS (computerized automated seed analysis system. **Journal of Seed Technology**, v. , n.12, p.40-53, 1984.

McCORMAC, A.C.; KEEFE, P.D.; DRAPER, S.R. Automated vigour testing of field vegetables using image analysis. **Seed Science and Technology**, Bassersdorf, v.18, n.3, p.103-112, 1990.

PERRY, D.A. **Handbook of vigour test methods**. Zurich: International Seed Testing Assessment, 1981. 123 p.

SMITH, O.E.; WELCH, N.C.; LITTLE, T.M. Studies on lettuce seed quality: 1. effect of seed size and weight on vigor. **Journal of the American Society of Horticultural Sciences**, Alexandria, v.98, n.4, p.529-533, 1973.

TOMAS, T.N.; TAYLOR, A.G.; ELLERBROCK, L.A. Time-sequence photography to record germination events. **HortScience**, v.27, n.6, p.372, 1992.