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Predicting germination of *Medicago sativa* and *Onobrychis viciifolia* seeds by using image analysis

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Abstract: Image analysis is an accessible method that can convert qualitative variables to quantitative ones. Computer imaging has been used in seed biology in various ways, including seed vigor testing and seed identification. In this paper, the seeds of 2 species, *Medicago sativa* and *Onobrychis viciifolia*, were studied. Laboratory tests and a computerized experiment were conducted to evaluate the effects of accelerated aging on the seed vigor of both species. We measured the rate of germination using a factorial and completely randomized design, with 10 treatment combinations replicated 3 times. The main factors were accelerated aging (6, 12, 18, 24, and 30 h) and species (*Medicago sativa* and *Onobrychis viciifolia*). A CCD color camera and microscope were used to record images of seeds in top views. The images were processed by a computer to generate numerical red-green-blue (RGB) density values. The density value of image analysis was significantly correlated with germination and the results could be used as a measure of seed vigor. Different statistics (root mean square error, coefficient of residual mass, model efficiency, and coefficient of correlation) indicated that selective models did a fair job of predicting germination for *M. sativa* and *O. viciifolia* seeds under varying color density. We conclude that the RGB values of density-imaged seeds are nondestructive, practical, and accurate determinants of *M. sativa* and *O. viciifolia* seed quality and can distinguish between high- and poor-quality seed lots.

Key words: Accelerated aging, multimodel inferences, RGB density value, vigor test

1. Introduction

The ultimate object of testing for germination is to gain information with respect to the field planting value of the seed and to provide results that can be used to compare the value of different seed lots (ISTA, 2005). However, the standard germination test often overestimates actual field emergence (Hampton, 2009). The major reason for this overestimation is that germination tests are conducted under conditions that are more favorable than typically encountered in the field. In order to provide a more accurate appraisal of seed quality for estimating field emergence, the concept of seed vigor was established to measure the quality of a seed lot, based on sampled observations of seedling growth (Sako et al., 2002).

Vigor testing not only measures the percentage of viable seeds in a sample; it also reflects the ability of those seeds to produce normal seedlings under the less-than-optimum or adverse growing conditions that may occur in the field (ISTA, 2005). Low-vigor lots having poor field emergence may not necessarily be detected by standard germination (Demir and Mavi, 2008). Mavi and Demir (2007a, 2007b) identified that vigor test regimens correlated well with field emergence potential.

Although vigor testing provides useful information, most vigor tests are time-consuming and costly, and they produce variable results from laboratory to laboratory (Sako et al., 2002). In plants that have small seeds, such as *Medicago sativa* and *Onobrychis viciifolia*, these problems are serious and have prevented the widespread, standardized use of seed vigor testing. The vigor tests that have been proposed can be grouped into 3 categories (Bennett, 2002): stress tests (e.g., cold test, accelerated aging), biochemical tests (e.g., electric conductivity, tetrazolium test), and germination evaluation and seedling growth tests (e.g., first count of the germination test, normal seedling emergence in peat, image analysis).

Accelerated aging is one of the 2 frequently used ISTA-recommended vigor tests (Hsu et al., 2003; Demir et al., 2004; Mavi and Demir, 2007a, 2007b; Demir and Mavi, 2008). Accelerated aging of seeds, induced by several days of exposure to high temperature and high humidity, is recognized as an accurate indicator of seed vigor and storability (Hsu et al., 2003).

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Recent advances in software development and computer hardware have made possible the use of more sophisticated image analyses and advanced statistical methods to distinguish seeds that are visibly similar to each other but differ in field emergence (Sako et al., 2001). Such a technique would be of potential use in ecological studies that characterize large samples of polymorphic seeds collected from different populations (Sako et al., 2001). In addition, if such software is efficient in distinguishing seed morphs within a species, it can be adapted to the classification of seeds of different species and could be applied to other areas of research involving seed identification, such as seed bank studies and crop seed purity analyses (Sveinsdottir et al., 2009).

Computer imaging has been used in seed and plant biology in various ways, including seed vigor testing and seed identification (Travis and Draper, 1985), and for estimating woody and herbaceous biomass (Mirik et al., 2007). Travis and Draper (1985) placed a seed on a lighted table and used a monochrome video camera to capture an image of the seed silhouette. Medina et al. (2010) used image analysis to distinguish the geographical provenance of 25 varieties of quinoa seeds cultivated in Europe and South America. Ahmadi Moghaddam et al. (2011) developed a neural network model based on the red-green-blue (RGB) components of the color image captured with a conventional digital camera to estimate leaf chlorophyll content in sugar beet. McCormac et al. (1990) discussed an automated system for assessing the vigor of lettuce seed lots. By growing the seedlings using a slant-board test (Jones and Cobb, 1963; Smith et al., 1973) in which seeds were planted on a blotter and grown vertically in the dark, a gray-scale video camera was able to capture 500 × 500 gray-scale images of germinating seedlings.

Progressive seed color changing in crop seeds may indicate decreasing quality with advancing deterioration. To that end, a computer-aided image analysis package has been developed to analyze RGB color components in seed images. The objectives of this study were to investigate the effects of seed aging treatment on the germination of *M. sativa* and *O. viciifolia* seeds, and to use image analysis because it is reproducible, speedy, and an economical method of vigor testing and would make seed vigor testing more reliable and useful to seed users.

2. Materials and methods

2.1. Plant materials

Medicago sativa and *Onobrychis viciifolia* from the Gene Bank of the Natural Source of Iran, which are commonly cultivated in Iran, were used as seed material. Laboratory tests and a computerized experiment were conducted in 2011 to evaluate the effects of accelerated aging on the seed germination of both species. The seeds were selected randomly from a global sample that had been manually cleaned to remove nongrain matter and damaged seeds.

2.2. Experiment

First, seeds were placed in an accelerated aging chamber that provided a relative humidity of near 100% at 45 °C for 6, 12, 18, 24, and 30 h. The initial moisture content of the seed was determined using the high constant-temperature oven-drying method (ISTA, 2005). Initial seed moisture content was approximately 13% and some seeds deteriorated without further raising the moisture content.

Following the stress period, the seeds were removed and germinated according to the criteria for the standard germination test (Dianati Tilaki et al., 2009; Behtari et al., 2011). We measured the rate of germination in a completely randomized design with 10 treatment combinations replicated 3 times. The main factors were accelerated aging (6, 12, 18, 24, and 30 h) and species (*Medicago sativa* and *Onobrychis viciifolia*). Forty seeds from each treatment were placed on 90-mm-diameter Whatman No. 2 filter papers moistened with distilled water, each of which was placed in a 90-mm-inner-diameter glass petri dish. Seeds were kept at 20 °C air temperature under normal light. Radicle protrusion of 2 mm was scored as germination. Germination of individual seeds was counted at 6-h intervals and counting continued until no further germination occurred.

Estimates of the $G_{50\%}$ taken for cumulative germination to reach 50% of maximum were interpolated from the linear regression of germination (y) on day (x).

Analysis of variance was used to test for differences between species, aging treatments, and their interaction on the final percentage of germination and $G_{50\%}$. Post hoc comparison of the means was made using the LSD test.

The same seed lots and treatments were used for image analyses. First, the proportion of deteriorated seeds was estimated by conducting tetrazolium assays on 7 replications of 40 seeds each (ISTA, 2005). Seed coats were then removed and seeds were placed in a solution and held at 35 °C for complete coloration. After a period in the solution, the length of which varies according to ISTA instructions (ISTA, 2005), the seeds were removed from the tetrazolium solution, rinsed 2–3 times in water, and evaluated under a digital microscope to determine the staining pattern. During evaluation, the seeds were left in a little water to prevent them from drying.

2.3. Image analysis

A CCD color camera microscope with a resolution of 1280 × 1024 pixels was used to record images of *M. sativa* and *O. viciifolia* seeds in top views. In order to obtain uniform lighting, a black illumination chamber was placed between the samples table and the lens in order to reduce the influence of surrounding light. A video monitor connected

directly to the camera output displayed the image in real time. The image contained one seed of either *M. sativa* or *O. viciifolia* (Figure 1).

The images were processed by a computer to get RGB density values of the imaged seeds. The seed images were manually outlined using ImageTool v.3.0 software point tools to extract, independently, the red, green, and blue components of the pixels representing the seed. The intensity of each color component was measured (values can range from 0 to 254).

2.4. Multimodel inferences

The relation between the RGB density values and the vigor of *M. sativa* and *O. viciifolia* seeds was modeled by combining information obtained from germination experiments and image analysis. Germination values for each accelerated aging treatment (6, 12, 18, 24, and 30 h) were used as dependent variables, while the independent variables were the RGB density values obtained from image analysis. Assumptions of normal distribution of variances were tested using Kolmogorov–Smirnov tests.

Since the RGB data create up to 3 predictor variables, 7 models were constructed for each species ($2^3 - 1$). A comparison of the models was then made using the Akaike information criterion (AIC), which penalizes the addition of parameters (K) and thus selects a model that fits well but has a minimum number of parameters (i.e. simplicity and parsimony) (Akaike, 1974). We used a second-order AIC (AICc), as recommended when the sample size is

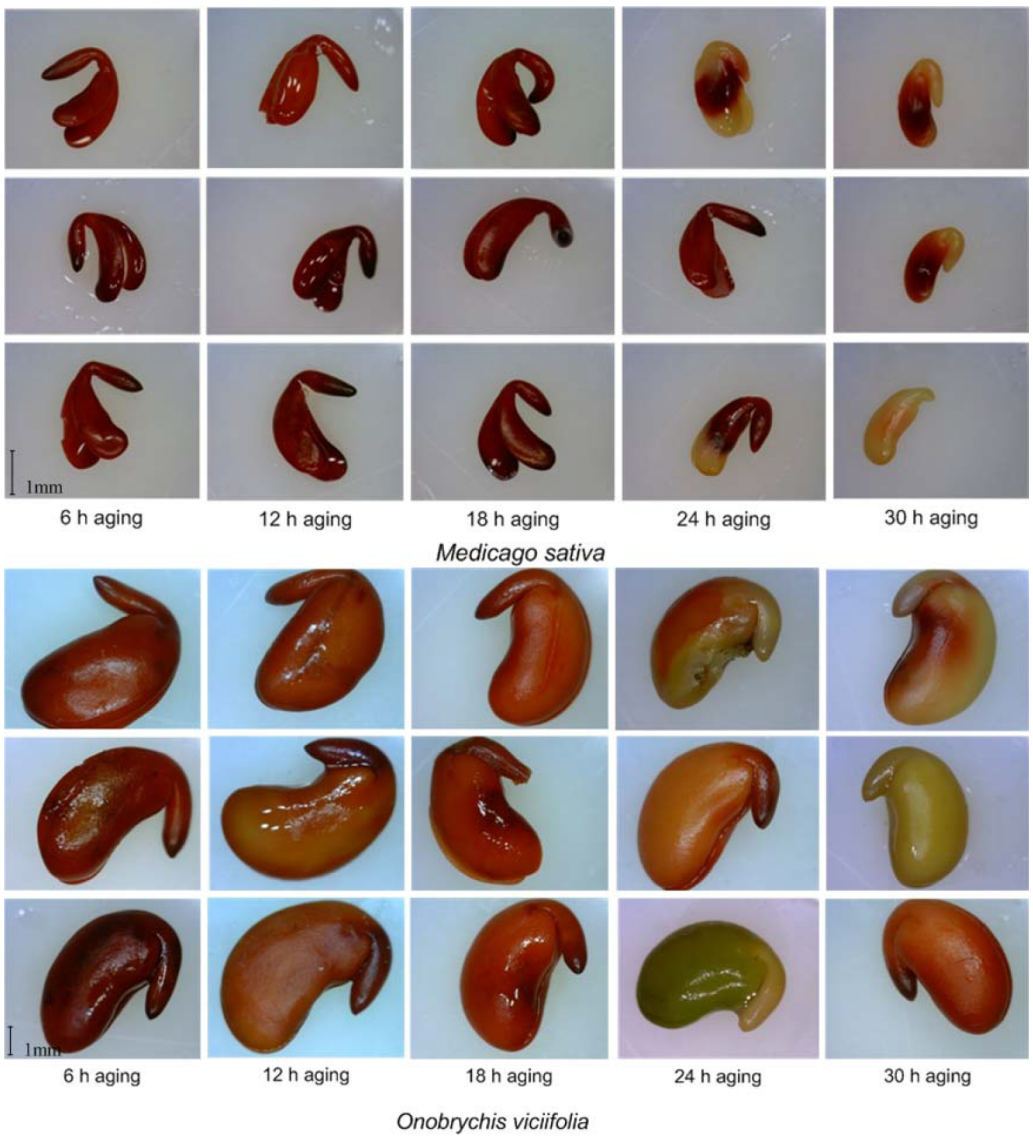

Figure 1. Tetrazolium test images obtained from *M. sativa* and *O. viciifolia* species as affected by accelerated aging.

small in relation to the number of model parameters to be estimated (K) (Burnham and Anderson, 2002). VBA programming in Excel was used to compute the RSS and the LS estimates of the parameters and the standard errors of the parameter estimates for each model.

2.5. Validation

The original sample was randomly partitioned into K subsamples. Of the K subsamples, a single subsample was retained as the validation data for testing the model, and the remaining K – 1 subsamples were used as training data. The goal of cross-validation is to find out whether the result is replicable or just a matter of random fluctuations (Behtari and de Luis, 2012). After fitting the models to the data, a comparison was made between the model-averaged prediction value ($\hat{\bar{P}}_i$) and the nonused observation value (O_i) used in the following model evaluation criteria (Jalota et al., 2010):

Root mean square error (RMSE):

$$RMSE = \frac{\sqrt{\sum_{i=1}^{i=n}(\hat{\bar{P}}_i - O_i)^2 / n}}{\bar{O}}$$

Here, $\overline{O}$ is the average of the observed data and i is the number of observations ranging from 1 to n. A value equal to 0 for a model indicates a perfect fit between the observed and predicted data.

Modeling efficiency (EF):

$$EF = 1 - \frac{\sum_{i=1}^{i=n}(\hat{\bar{P}}i - Oi)^2}{\sum_{i=1}^{i=n}(Oi - \bar{O})^2}$$

An EF value of 1.0 indicates a perfect agreement of model predictions with direct measurement of the parameter in question. Zero or a negative value indicates that the average value is a better predictor than the model.

Coefficient of residual mass (CRM):

$$CRM = \frac{\sum_{i=1}^{i=n} Oi - \sum_{i=1}^{i=n} \hat{\bar{P}}i}{\sum_{i=1}^{i=n} Oi}$$

This indicator shows the difference in observed and predicted data relative to the observed data. Its 0 value indicates a perfect fit, and positive and negative values indicate under- and overprediction, respectively.

3. Results

3.1. Germination test

Results for the germination test conducted on accelerated aging seeds indicated that 84.17%, 87.5%, 67.5%, 58.33%, and 34.17% of the *M. sativa* seeds and 89.17%, 83.34%, 83.33%, 28.33%, and 1.67% of *O. viciifolia* seeds in the aging treatment (6, 12, 18, 24, and 30 h) were viable, respectively.

All traits in this experiment were normally distributed. Thus, procedures that assume normality can be employed for trait analysis. Results of the germination test indicated that the aging treatment had a significant effect on the final germination percentage in this experiment. The species and species × aging interaction factors were significant for the final germination percent, but not for $G_{50\%}$ (Table 1).

3.2. Density value

The method is based on measuring RGB values that are then separated into 3 fractions, each having a different RGB value. As shown in Figure 2, the RGB value was low at early aging and then increased gradually with increased aging time. In the *M. sativa* seeds, all color fractions increased with increasing aging time; in *O. viciifolia*, the R-value was relatively constant across different aging treatments

Table 1. Analysis of variance for the effects of accelerated aging on germination and number of days it takes to reach 50% of the final germination percentage ($G_{50\%}$) in a petri dish experiment with 2 species (*M. sativa* and *O. viciifolia*).

Source	d.f.	Mean of squares	
		Germination	$G_{50\%}$
Species	1	630.117*	0.705
Aging	4	5442.514**	0.6
Species × aging	4	685.95**	0.185
Error	20	81.731	0.275
CV%		14.64%	22.57%

*, **: Significant at 0.05 and 0.01 probability levels, respectively.

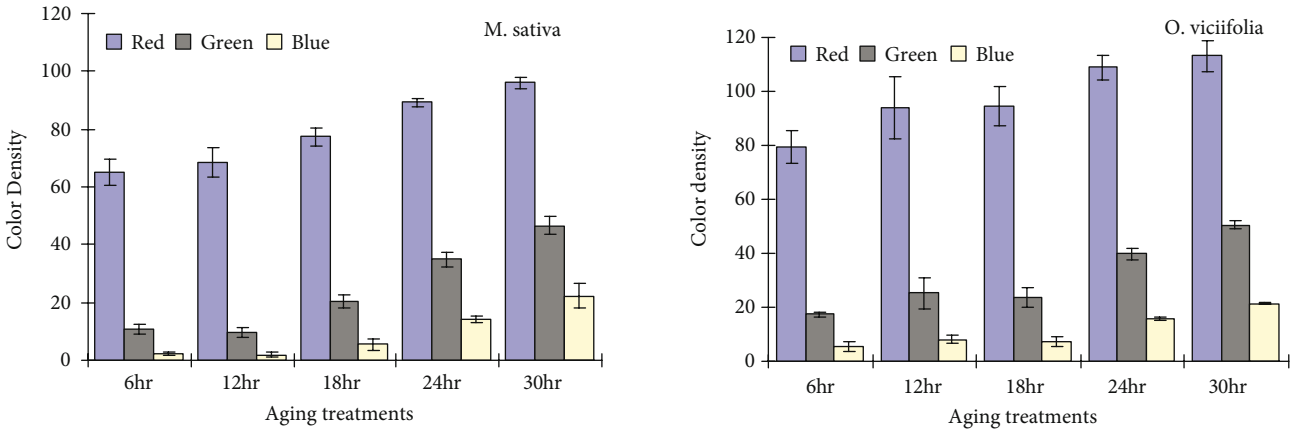

Figure 2. Effect of accelerated aging on mean color density values of *M. sativa* and *O. viciifolia*. Vertical lines represent 95% confidence intervals of mean.

but other fractions increased with aging time, especially the green fraction. The density value of image analysis significantly correlated with germination percentage (R_R = –0.96**, R_G = –0.89*, and R_B = –0.94* in *M. sativa*; R_R = –0.6, R_G = –0.96**, and R_B = –0.99** in *O. viciifolia*) and the suggested results can be used as a measure of seed vigor.

3.3. Prediction models

The models constructed to study the relation between RGB density values and vigor of seeds of *M. sativa* and *O. viciifolia* are shown in Table 2.

The overall results suggest that differences in seed color may be used to assess changes in seed vigor. The *M. sativa* model using G density values (model 2) proved to be the best, since the AICc values were the lowest (43.23). For *M. sativa*, models 12 and 23 also had lower AICc values (46.87 and 49.146, respectively). For *O. viciifolia,* substantial model selection uncertainty is evident since models 2, 3, and 23 provided similar results, i.e. all had similar predictive ability. All the selected models in each set were used in averaging.

Table 2. Model selection statistics for 14 models of R, G, and B density data for *M. sativa* and *O. viciifolia.*

M. sativa	Model	α ± SE	Model parameter			Multimodel inference results	
			R ± SE	G ± SE	B ± SE	AICc	Model probability Wi
	1	166.63 ± 18.54	–1.33 ± 0.24			54.251	0.003
	2	**96.00 ± 3.49**		**–1.27 ± 0.12**		**43.231**	**0.800**
	3	83.58 ± 3.79			–2.09 ± –0.93	50.690	0.019
	12	**60.97 ± 25.90**	**0.63 ± 0.46**	**–1.81 ± 0.41**		**46.874**	**0.129**
	13	114.99 ± 28.03	–0.49± 0.43		–1.43 ± 0.65	55.013	0.002
	23	**95.19 ± 4.98**		**–1.18 ± 0.42**	**–0.17 ± 0.72**	**49.146**	**0.042**
	123	60.74 ± 28.21	0.63 ± 0.51	–1.84 ± 0.67	0.04 ± 0.71	55.868	0.001
O. viciifolia							
	1	281.61 ± 58.9	–2.17± 0.56			72.398	0.007
	2	**144.50 ± 13.9**		**–2.69 ± 0.39**		**63.742**	**0.494**
	3	**126.65 ± 11.8**			**–5.92 ± 0.89**	**64.153**	**0.403**
	12	135.92 ± 62.09	0.12 ± 0.83	–2.80 ± 0.90		69.714	0.025
	13	90.752 ± 74.22	0.46 ± 0.94		–6.92 ± 2.24	69.815	0.024
	23	**138.09 ± 15.56**		**–1.48 ± 1.34**	**–2.80 ± 2.96**	**68.540**	**0.045**
	123	92.546 ± 72.77	0.60 ± 0.92	–1.60 ± 1.41	–3.85 ± 3.49	76.875	0.001

R: red (1), G: green (2), B: blue (3).
The selected models are shown in bold.

3.4. Validation

During the validation with the remaining dataset, the simulated germination matched well with the observed results in aging treatments (Figure 3). The results showed that curve-fitting gave efficiency higher than 0.91 in both species. The RMSE, CRM, and r for germination were 0.063, 0.058, and 0.982 for *M. sativa* and 0.116, –0.11, and 0.984 for *O. viciifolia*, respectively (Table 3). The predicted changes in *O. viciifolia* models were slightly greater than the observed values (CRM having negative values), though it fit well with the shape of the experimental data (Figure 4). This showed a slight overprediction in the case of the *O. viciifolia* models.

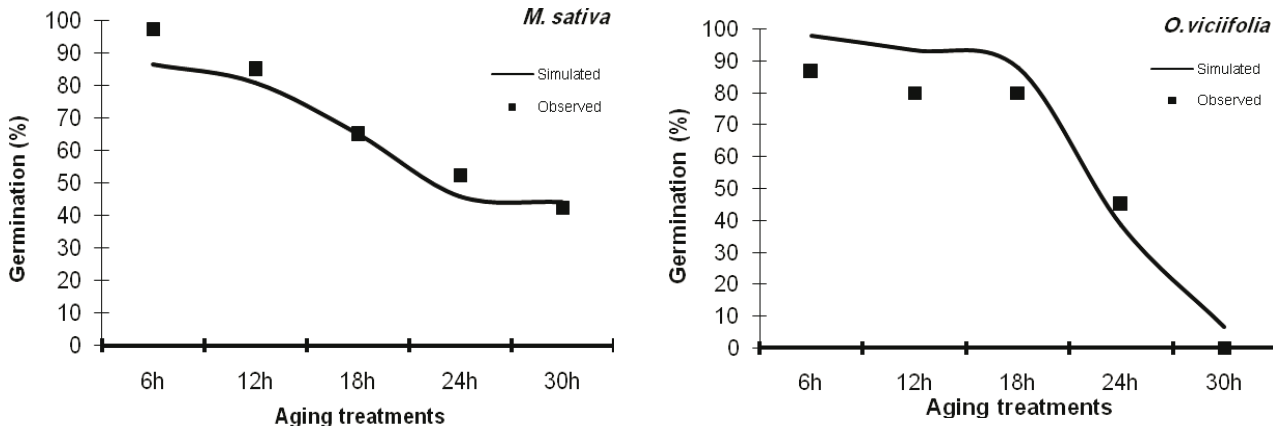

Figure 3. Simulated and observed germination fitted by color density selective models in *M. sativa* and *O. viciifolia*

Table 3. Statistical analysis of observed and predicted germination (averaged) in *M. sativa* and *O. viciifolia* crops.

	RMSE	EF	CRM	r
M. sativa	0.063	0.910	0.058	0.982
O. viciifolia	0.116	0.913	–0.11	0.984

RMSE = relative root mean square error; EF = modeling efficiency; CRM = coefficient of residual mass; r = correlation coefficient.

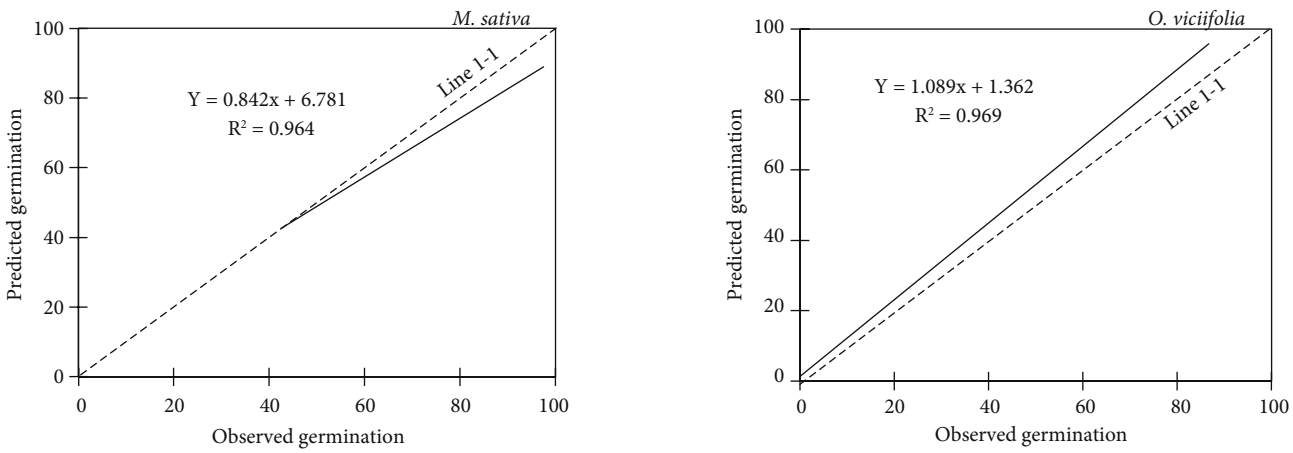

Figure 4. Scatter plot used to fit the regression of observations of the predicted seed germination for the 3 models averaged for *M. sativa* and *O. viciifolia*.

4. Discussion

There is much laboratory and field emergence research on different plant species that demonstrates that both germination velocity and uniformity play a decisive role in field emergence (Behtari et al., 2011). These 2 properties are the most important predictors of seed vigor (Sako et al., 2001). Both germination velocity and uniformity can be obtained easily within a short period of time (Sako et al., 2001).

Our results indicate that the final germination percentage of seeds decreased with increasing accelerated aging. The same results have also been found in wheat seeds by Guy and Black (1998), in *Beta vulgaris* seeds by Song et al. (2001), and in eggplant, cucumber, and melon seeds by Demir et al. (2004).

Studies on seed viability staining by tetrazolium showed that the death of radicle root cells, especially meristematic cells of axes, gradually increased with accelerated aged time. Deterioration began from the root and moved through the embryo in both naturally and artificially aged *Triticum aestivum* seeds (Das and Sen-Mandi, 1988, 1992) and embryonic axes of *A. hypogea* were the most sensitive seed parts to deterioration (Fu et al., 1988). The symptoms observed during accelerated aging can be used to characterize the degree of aging, which is the opposite of storability. Stability against accelerated aging has subsequently been recognized as a useful vigor test for some species (Smith and Berjak, 1995). Physiological and biochemical changes during the deterioration of seeds have been used as indices of aging (Priestley, 1986). The decrease in germinability correlated well with increased accumulations of total peroxide and malondialdehyde content and decreased activity of antioxidant enzymes peroxidase, catalase, ascorbate peroxidase, glutathione reductase, and superoxide dismutase (Goel et al., 2003; Hsu et al., 2003).

The relationship between germination percentage and color density was explored. A statistically significant effect of color density ($P < 0.05$) was observed. This indicates that color density can represent a valid tool, at least for the species tested, for estimating the germination percentage. Changes in seed quality with changes in fruit color were associated as in tomato (Demir and Samit, 2001), pepper (Demir and Ellis 1992), and eggplant (Demir et al., 2002), but not in fruit weight, length, or diameter (Demir et al., 2002). Seed vigor imaging analyses have been proposed as alternatives to traditional vigor tests, such as those used by Sako et al. (2001), Hoffmaster et al. (2003), and Penaloza et al. (2005).

The tetrazolium test provides a rapid (1 to 1.5 h) and objective measurement of *M. sativa* and *O. viciifolia* seed quality. An additional advantage of this method is that the images and vigor indices are stored and a database can be developed for future reference. Although color density values provide the aforementioned advantages in seed quality evaluation compared to traditional vigor tests, there is still no information comparing this method with other vigor tests or with greenhouse performance in *M. sativa* or *O. viciifolia*.

Assessing the RGB index of each individual seed within a large seed sample may allow the development of nondestructive methods in sorting seed subsamples with different germination capabilities (Dell'Aquila, 2006).

New computer-aided image analysis may be extended to the analysis of RGB color components of 2D seed images. Since all visible colors can be represented with varying combinations of these primaries (Fairchild, 1998), related color mapping is represented by a numeric range of RGB values (from 0 - 0 - 0 for black to 254 - 254 - 254 for white). This method was applied to lentil seeds, which were deteriorated under controlled moisture content and temperature conditions and were sorted into 3 fractions with distinct germination potential over the entire period of aging (Dell'Aquila, 2006).

Comparing seed vigor measurement methods showed that a new method of image analysis could be used to identify the results of seeds given the tetrazolium test. In addition, tetrazolium test results obtained by seed analysts cannot be compared and it is not a standard measurement to test seed vigor. In this paper, a new method of modeling, the multimodel inference procedure, has been evaluated. This method has many advantages but none of the shortcomings of earlier methods. We developed original models for estimating germination and compared observed and predicted germination of *M. sativa* and *O. viciifolia*. To our knowledge, these models are the first of their kind to be developed for this purpose. The model was statistically reliable. Its performance was verified by the fact that the regression line fit to the adjusted scatter plot of the observed and predicted germination and almost coincided with the 1:1 line (Figure 4).

We conclude that the RGB values of the density-imaged seed tests are nondestructive, practical, and accurate determinants of *M. sativa* and *O. viciifolia* seed quality and can distinguish between high- and poor-quality seed lots.

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