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Digital and multivariate analysis of lettuce seed vigor: Impact of hydropriming on physiological potential

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Abstract: Digital image analysis has emerged as a highly precise and efficient methodology for assessing the physiological attributes of seeds. This research aimed to assess the morphological and physiological properties of lettuce seeds subjected to hydropriming using multivariate statistical approaches. Two lettuce genotypes, Roxa and Vanda, were evaluated under hydropriming treatments (primed-dry and primed-stored). Seedlings were digitally scanned, and vigor indices were quantified using the Seed Vigor Imaging System (SVIS®). Data were analyzed by multivariate analysis of variance (MANOVA), with tests of normality and homogeneity of covariance ensuring analytical robustness. The primed-dry treatment resulted in minimal improvement in vigor and uniformity, while the primed-stored treatment promoted a partial recovery of these attributes. The Roxa genotype exhibited greater variability in vigor and seedling length, whereas Vanda demonstrated higher uniformity but slightly reduced seedling growth. A strong positive correlation was observed between the vigor index and seedling length, reinforcing the importance of these parameters in seed quality assessment. These findings underscore the utility of digital image analysis combined with multivariate statistical methods for the accurate assessment of seed vigor, thereby improving seed-lot classification and informing decision-making in lettuce production systems.

1. Introduction

Computerized analysis of seed and seedling images has emerged as an innovative tool for determining the physiological characteristics of these structures. This technology stands out for its objectivity, specificity in detecting subtle traits, efficiency, and potential for standardization (Rahman and Cho, 2016; Xia *et al.*, 2019; Wang *et al.*, 2021; Liu *et al.*, 2023). The use of digital imaging and automated software enables the analysis of a large number of samples in shorter periods, increasing the efficiency and accuracy of evaluations.

Assessing the physiological potential of lettuce seeds through digital image analysis has proven to be a promising approach, particularly as a complement to conventional methods that do not fully reflect seed quality under real field conditions (Waters-Junior and Blanchette, 1983; Marcos-Filho, 1999). The use of the Seed Vigor Imaging System (SVIS®), developed by Sako *et al*. (2001), has been widely adopted to quantify seed vigor in various species, including soybean, corn, melon, sweet corn, castor bean, peanut, okra, common bean, eggplant, tomato, cotton, and sunflower (Hoffmaster *et al.*, 2003; Marcos-Filho *et al*., 2006; Marchi *et al*., 2011; Alvarenga *et al.,* 2013; Caldeira *et al.*, 2014; Gomes-Junior *et al.*, 2014; Rocha *et al.*, 2015). This technology allows for detailed analyses of parameters such as seedling growth uniformity and development, reducing the subjectivity of traditional evaluations. However, to enhance the accuracy of vigor assessment, it is essential to employ multivariate statistical models that enable the simultaneous analysis of multiple interrelated variables.

Multivariate Analysis of Variance (MANOVA) has been used to investigate complex interactions between experimental factors, allowing for the identification of patterns that would be difficult to detect using univariate approaches (Johnson and Wichern, 2002; Nicacio *et al*., 2013). Previous studies have demonstrated that MANOVA is an effective tool for evaluating seed performance under different treatments, ensuring greater robustness in result interpretation (Oliveira *et al*., 2013). This study aimed to analyze the morphological and physiological properties of lettuce seeds subjected to hydropriming using digital imaging of seedlings and a multivariate approach. The study sought to understand the interactions between the physiological attributes of the seeds and the impact of hydropriming on germination potential and early seedling development.

2. Materials and Methods

The research was conducted at the Seed Analysis Laboratories, the 'Professor Silvio Moure Cicero' Image Analysis Laboratory of the Department of Crop Science, and the Department of Math, Chemistry, and Statistics at the Luiz de Queiroz College of Agriculture, University of São Paulo in Piracicaba, SP, Brazil.

Seed material and priming treatment

Lettuce seeds from the genotypes Scarlet Red Crisphead (Roxa) and Vanda Crisphead (Vanda) were used, supplied by Sakata Seed South America Ltd. The selection of the two lettuce genotypes was based on their contrasting physiological and morphological characteristics and on their commercial relevance within Brazilian lettuce production systems. Each genotype was represented by ten seed lots, with germination rates within commercial standards and different vigor levels among the lots. Each seed lot was divided into three treatments: (i) non-primed seeds (control), (ii) hydroprimed dried seeds (dried in an oven at 30°C and 45-55% relative humidity for 96 hours), and (iii) hydroprimed stored seeds (dried and stored in a chamber at 10°C and 30% relative humidity for three months).

Hydropriming was performed using the drum method, utilizing the S-HIDRO® Control equipment, which allowed for the controlled application of water at regular intervals until reaching the required volume for each lot (Kikuti and Marcos-Filho, 2012). The calculation of the required water volume for this method was based on the water imbibition curve, considering the volume needed for each seed lot before primary root protrusion (Caseiro, 2003). At the beginning of each cycle, the electric pump was activated for 1 second, allowing the intake of a water volume between 0.9 and 1.1 ml, adjusted according to the specific needs of each seed lot and accounting for system losses to ensure 100% efficiency. Water application was carried out at one-hour intervals until the total required volume for each seed lot was reached (ranging from 4.00 to 4.31 ml for the Roxa genotype and from 3.67 to 3.87 ml for Vanda). The entire procedure was conducted under laboratory conditions at a constant temperature of 25°C.

Seed vigor assessment using digital image analysis (SVIS® Software)

Four replicates of 25 seeds per lot for each treatment were arranged in two rows on the upper third of two blotter paper sheets, placed on the lids of transparent plastic boxes (11 × 11 × 3.5 cm). The boxes were covered with transparent plastic bags and incubated in a BOD chamber at 25°C for three days in darkness. To ensure proper seedling development according to natural geotropism, the boxes were positioned at a 70° angle relative to the horizontal plane.

To determine seed vigor, the Seed Vigor Imaging System (SVIS®) (Sako *et al.*, 2001) software was used. The seedlings (and ungerminated seeds) from each replicate were transferred onto a blue ethylene-vinyl acetate (EVA) sheet, providing the necessary contrast for system analysis. The seedlings were then scanned using an HP Scanjet 200 scanner, which was inverted and placed inside an aluminum box (60 × 50 × 12 cm), with the resolution set to 300 dpi and connected to a computer. The scanned images were processed using SVIS® software, including manual corrections when necessary to ensure accurate seedling identification (Fig. 1). The seed vigor index is calculated according to the methodology proposed by Sako *et al.* (2001):

$$Vigor\ index = W_G \times Growth \times W_u \times Uniformity$$

$$Seedling\ length = W_G\ \{W_h \times l_h + W_r \times l_r, 1000\}$$

$$Uniformity\ index = max\ \{1000-(W_{sh} \times S_h + W_{sr} \times S_r + S_{total} + W_{(sr/h)} \times S_{(r/h)} - W_d),0\}$$

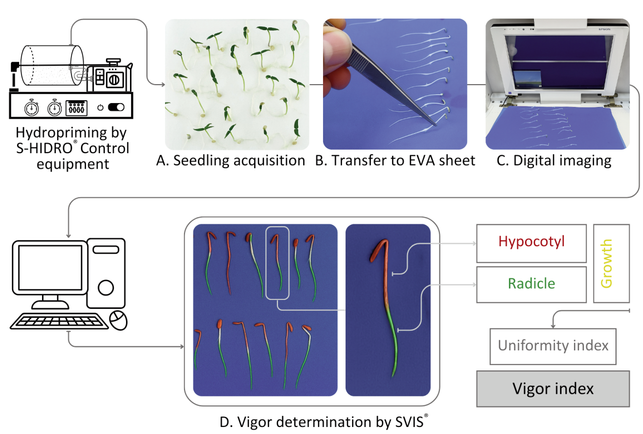

Fig. 1 - Workflow of computerized image analysis of lettuce seedlings, from germination to vigor assessment. Steps include seedling acquisition from the germination test (A), transfer to an EVA sheet (B), digital imaging (C), and vigor determination using the SVIS® software (D). The results include the vigor index (VI) and the uniformity index (UI), both ranging from 0 to 1000 (directly proportional to seedling vigor), as well as the average seedling length, initially measured in pixels and later converted to centimeters.

Vigor results from the combination of two main components: average seedling length and uniformity, both adjusted by weighting factors (*W*) defined by the system, allowing the assignment of greater or lesser relative importance to each characteristic (hypocotyl-to-radicle ratio of 40:60, applied in this research). Growth is estimated from the mean lengths of the hypocotyl (l_h) and radicle (l_r), weighted by their respective coefficients (W_h and W_r), thereby composing the total seedling length. Uniformity, in turn, is calculated based on the standard deviations of hypocotyl length (S_h), radicle length (S_r), total seedling length (S_{total}), and the hypocotyl-to-radicle length ratio ($S_{(r/h)}$), also weighted by their respective coefficients (W_{sh}, W_{sr}, $W_{(sr/h)}$).

Multivariate analysis

Multivariate Analysis of Variance (MANOVA) was used to describe the effects of categorical factors (treatments) on multiple response variables (Huberty and Olejnik, 2006). MANOVA extends ANOVA to the multivariate context, allowing simultaneous testing of multiple dependent variables. In this study, a two-way MANOVA was employed, considering two categorical factors: genotype (Roxa and Vanda) and hydropriming treatment (control, primed dry, primed stored). This factorial approach allows for the evaluation of both the main effects of each factor and their interaction.

The general model for Two-Way MANOVA:

$$X_{lkr} = \mu + \tau_l + \beta_k + \gamma_{lk} + \epsilon_{lkr}$$

X represents the dependent variable, *l* represents the levels of factor 1 (genotype); *k* represents the levels of factor 2 (hydropriming treatment); *r* represents the replications; μ is the overall mean; τ_l representing the interaction effect and β_k representing the treatment or genotype main effects; γ_{lk} is the interaction effect between the factors; represents the random error. The hypothesis tests included:

Interaction effect:

$$H_0: \gamma_{11} = \gamma_{12} = \cdots = \gamma_{gb} = 0 \text{ vs. } H_1: \text{at least one } \gamma_{gb} \neq 0$$

Genotype effect:

$$H_0: \tau_{11} = \tau_{12} = \cdots = \tau_g = 0 \text{ vs. } H_1: \text{at least one } \tau_g \neq 0$$

Hydropriming effect:

$$H_0: \beta_1 = \beta_2 = \beta_3 = 0 \text{ vs. } H_1: \text{at least one } \beta_b \neq 0$$

Statistical significance was determined using Wilks' lambda (Wilks, 1935), Pillai's trace (Hand and Taylor, 1987), Hotelling-Lawley trace (Krzanowsk and

Marriott, 1994; Anderson, 2003), and Roy's largest root (Krzanowski, 2000). When MANOVA indicated significant differences, post hoc univariate ANOVAs were conducted to determine which dependent variables contributed to the observed differences.

Statistical assumptions and data validation

Before conducting MANOVA, the following statistical assumptions were tested: multivariate normality, using the Henze-Zirkler test (Henze and Zirkler, 1990) and homogeneity of covariance matrices using Box's M test (Johnson and Wichern, 2002). In the univariate context, the Anderson-Darling test (Scholz and Stephens, 1987) was used to assess normality. Initial analyses revealed that seedling size did not meet the assumption of normality; therefore, this variable was removed from the final MANOVA model to ensure compliance with statistical assumptions.

Software and data processing

All statistical analyses were performed using the R programming language (R Core Team, 2024), with the packages 'MVN' for normality tests and 'car' for MANOVA. Data visualizations, including boxplots and correlation matrices, were generated using the 'ggplot2' and 'corrplot' packages. Image processing was performed using SVIS® software, ensuring standardization and reproducibility of seed vigor measurements.

3. Results

Descriptive data analysis

The descriptive analysis allowed the identification of the main characteristics of the studied variables. Figure 2 presents boxplots for the three response variables in this research: vigor index, uniformity index, and seedling length. It is observed that the means values of vigor and uniformity are similar between genotypes, while the dispersion of vigor is higher. Seedling length, measured in centimeters, is on a different scale from the other variables and exhibits a lower correlation with them. The Roxa genotype exhibits a higher median and greater variability, suggesting either greater vigor or increased heterogeneity. In contrast, the distribution of the uniformity index between the two genotypes is similar, indicating that growth is uniformly distributed.

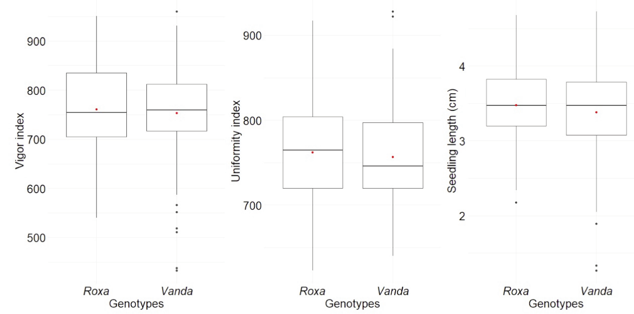

Fig. 2 - Boxplot comparing the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm) between Roxa and Vanda genotypes.

The boxplots for the analyzed variables concerning hydropriming treatments are shown in Figure 3. It is observed that vigor and uniformity vary significantly between treatments. Seeds subjected to the primed dry treatment showed lower means for these variables. Seedling length showed less pronounced differences, with the control treatment presenting the highest mean.

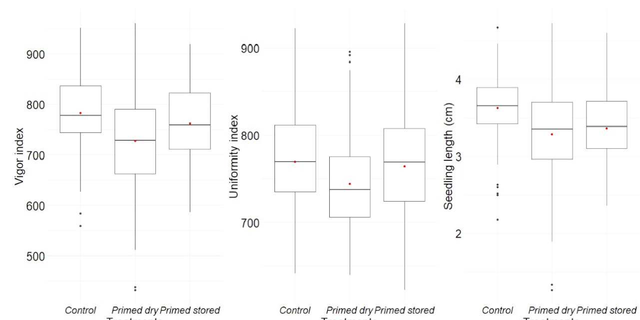

Fig. 3 - Boxplot comparing the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm) across control, primed dry, and primed stored treatments.

The mean values of the variables for each genotype are shown in Table 1, while Table 2 shows the corresponding values for each hydropriming treatment.

Table 1 - Mean values of vigor index, uniformity index, and seedling length for Roxa and Vanda genotypes

Genotype	Vigor index	Uniformity index	Seedling length (cm)
Roxa	761	762	3.47
Vanda	754	757	3.38

Table 2 - Mean values of vigor index, uniformity index, and seedling length for control, primed dry, and primed stored treatments

Hydropriming	Vigor index	Uniformity index	Seedling length (cm)
Control	783	770	3.63
Primed dry	727	745	3.29
Primed stored	762	765	3.37

The distribution, correlation, and dispersion of the vigor, uniformity, and seedling length, their correlations, and dispersion are shown in figure 4. The vigor index is strongly correlated with seedling length (r= 0.903), suggesting that more vigorous seedlings tend to be longer. The correlation between uniformity and length is moderate (r= 0.447), indicating a weaker association between these variables.

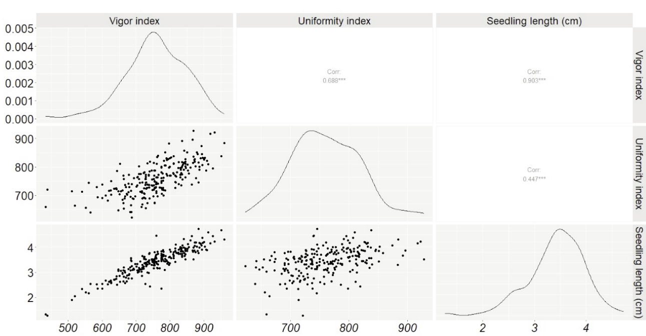

Fig. 4 - Distribution, correlation, and dispersion analysis of the vigor index, uniformity index, and seedling length (cm).

Interaction between genotypes and treatments

The interactions among hydropriming treatments within each genotype are illustrated in figure 5. The primed dry treatment had the least pronounced effect on vigor and uniformity, while primed stored allowed a partial recovery of these parameters. The interaction between genotypes within each treatment is shown in figure 6. Roxa exhibited a better response to the primed stored treatment, while Vanda demonstrated greater sensitivity. Seedling length was more affected in Vanda under the primed stored condition, whereas Roxa showed a tendency toward increased growth.

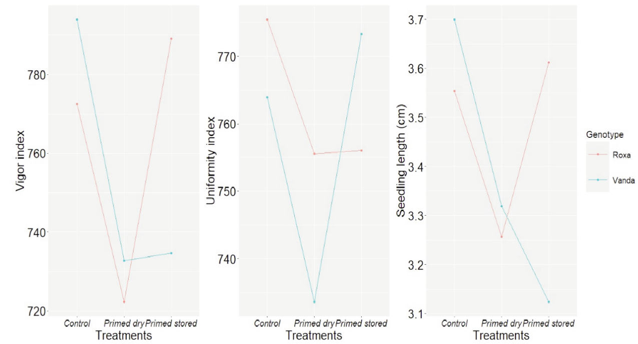

Fig. 5 - Interactions plot of hydropriming treatments within the Roxa and Vanda genotypes for the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm).

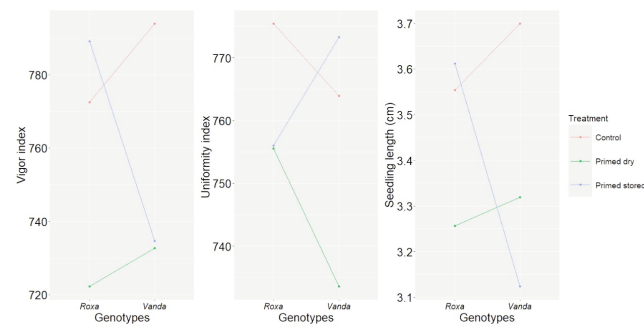

Fig. 6 - Interaction plot between Roxa and Vanda genotypes within each hydropriming treatment for the vigor index, uniformity index (both ranging from 0 to 1000), and seedling length (cm).

Assumptions of MANOVA

The suitability of the data for MANOVA was verified using the Henze-Zirkler and Anderson-Darling tests (Tables 3 and 4). The Henze-Zirkler test indicated that the data did not present multivariate normality due to the length variable. Therefore, this variable was removed for the MANOVA analysis (Table 5). After its removal, multivariate normality was achieved (Table 6). The Box´s M test for equality of covariance matrices confirmed that the data met

Table 3 - Henze-Zirkler test for assessing the multivariate normality of the full dataset

Test	Statistic	p-value	NMV*
Henze-Zirkler	1.84	0.00000005	No

*Not Meeting Validity criteria (normality violated).

Table 4 - Anderson-Darling test assessing the univariate normality of the vigor index, uniformity index, and seedling length variables

Variable	Statistic	p-value	Normality
Vigor	0.6619	0.0830	Yes
Uniformity	0.4255	0.3134	Yes
Seedling length	15.860	0.0004	No

*Not Meeting Validity criteria (normality violated).

Table 5 - Henze-Zirkler test after removal of the seedling length variable, indicating multivariate normality of the remaining variables

Test	Statistic	p-value	NMV*
Henze-Zirkler	0.9217	0.1112	Yes

*Not meeting validity criteria (normality violated).

the required assumptions for MANOVA (Table 7). A two-factor MANOVA was performed to evaluate the interaction effect between genotype and hydropriming treatment. The Roy's largest root test indicated no significant differences between genotypes. However, the main effect of hydropriming treatment, as well as the interaction between genotype and treatment, were highly significant (Table 8). These results suggest that hydropriming treatments significantly influenced the analyzed variables, regardless of genotype.

4. Discussion and Conclusions

The findings of this research can be understood in light of the existing literature on seed physiological potential and vigor. Seed vigor is one of the main factors influencing success seedling establishment in the field and early plant development (Black and Bewley, 2000; Marcos-Filho, 2015). The multivariate analysis showed that the Roxa genotype exhibited greater variability in vigor and seedling length, whereas Vanda demonstrated greater uniformity in growth. These results are consistent with studies indicating that different genotypes may exhibit significant variations in their physiological responses (Hampton and Tekrony, 1995; Elias *et al*., 2012; Rahman and Cho, 2016; Cheng *et al*., 2023).

The significant interaction between hydropriming treatments and genotypes supports the hypothesis that the priming response may be cultivar-specific. The primed dry treatment had a weaker effect on vigor and uniformity, as reported in previous studies, which suggest that osmotic stress generated during the process may compromise seed physiological potential (Raj and Raj, 2019; Lewandowska *et al.*, 2020; Pirasteh-Anosheh and Hashemi, 2020; Rhaman *et al.*, 2020 a, 2020 b), particularly during the period immediately following treatment. Conversely, the primed stored treatment exhibited partial recovery of vigor and uniformity parameters, in agreement with studies highlighting the ability of seeds to

Table 6 - Anderson-Darling test after removal of the seedling length variable, confirming the normality of the vigor index and uniformity index variables

Variable	Statistic	p-value	Normality
Vigor	0.6619	0.0830	Yes
Uniformity	0.4255	0.3134	Yes

*Not Meeting Validity criteria (normality violated).

Table 7 - Box´s M test for the equality of covariance matrices among the analyzed groups

Statistic	p-value
2.05	0.56

Table 8 - MANOVA results for genotype and hydropriming treatment factors, considering the vigor index and uniformity index variables

Source	DF	Roy's Statistic	F Approximation	Num. DF	Den. DF	p-value
Genotype	1	0.002587	0.3014	2	233	0.74
Hydropriming	2	0.0726	85.044	2	234	0.0002721 ***
Interaction	2	0.2100	245.726	2	234	0.00002 ***
Residuals	234					

stabilize after hydropriming when stored under appropriate conditions (Farooq *et al*., 2006, 2010; Huang *et al.*, 2015; Souza *et al.*, 2016; Farooq *et al*., 2021). Furthermore, the current results support research indicating that the effectiveness of hydropriming may vary depending on genotype and environmental conditions (Muhie *et al*., 2024). Recent studies emphasize the importance of evaluating each cultivar separately to determine the most suitable seed treatment method (Cheng *et al.*, 2023; Qiu *et al.*, 2023).

The positive correlation between vigor and seedling length reinforces the relevance of these variables in seed quality assessment. The literature suggests that more vigorous seedlings tend to develop stronger root systems and exhibit higher field emergence rates (Kikuti and Marcos-Filho, 2012; Kikuti and Marcos-Filho, 2013; Alvarenga and Marcos-Filho, 2014; Marcos-Filho, 2015; Rego *et al*., 2023). Prior studies indicate that seed vigor is closely associated with early seedling growth and crop establishment (Marcos-Filho, 2015).

Image analysis has proven to be a promising tool for evaluating seed vigor. Technologies such as the Seed Vigor Imaging System (SVIS®) have demonstrated a high degree of precision in classifying lettuce seed lots and those other crops (Gomes-Junior *et al*., 2009; Rodrigues *et al*., 2020). The use of computer vision and machine learning in seed vigor assessment is increasingly being explored, enabling fast and objective analyses (De Medeiros *et al.*, 2020; Wang *et al.*, 2021; Liu *et al*., 2023; Pang *et al.*, 2023).

The statistical methodology adopted in this research was essential to ensure the robustness of the analyses and the reliability of the results. Initially, the descriptive analysis enabled the identification of trends and patterns in the data, facilitating interpretation. To assess relationships among variables, Pearson´s correlation was applied, revealing a strong association between the vigor index and seedling length. The main statistical method used was Multivariate Analysis of Variance (MANOVA), a widely accepted methodology approach studies involving correlated dependent variables (Oliveira *et al.*, 2013; Din and Hayat, 2021; Baumeister *et al.*, 2024).

MANOVA is particularly suitable when response variables are correlated, allowing for the simultaneous evaluation of the effects of experimental factors (Johnson and Wichern, 2002). To ensure the method's applicability, the Henze-Zirkler test was used to assess multivariate normality, and Box's M test was applied to verify the homogeneity of covariance matrices-a key assumption for valid MANOVA results. The significance of main effects and interactions was assessed using Roy's largest root, which is recommended when effects have a strong impact on data variability (Kose *et al.*, 2018).

The MANOVA results were complemented by univariate analyses, allowing for a more detailed interpretation of the individual factor effects, as suggested by Scholz and Stephens (1987). This combined approach improves precision in identifying significant effects and interactions, thereby enhancing the understanding of genotype responses to hydropriming. However, such statistical procedures also have limitations. In this study, seedling length had to be excluded from the final MANOVA due to the violation of normality assumptions, which restricted the scope of multivariate interpretation.

In conclusion, the Roxa genotype performed better under the primed stored treatment than Vanda. Seedling length was influenced by hydropriming, with primed stored proving unsuitable for Vanda. Therefore, the statistical approach adopted in this study enabled a comprehensive and detailed analysis, enhancing our understanding of the effects of hydropriming on the evaluated genotypes. These findings are crucial for understanding genotypes-treatments interactions and may contributing to the optimization of hydropriming strategies for lettuce seeds. Future studies should consider incorporating a broader range of cultivars, extended storage durations, and the integration of machine learning techniques to improve vigor prediction.

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