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Swingle citrumelo seed vigor and storability associated with fruit maturity classes based on RGB parameters

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ABSTRACT: Citrus seeds normally have low storability. Identifying an optimal fruit harvest time for production of high vigor seeds is important for nurseries; however, identifying this stage for Swingle citrumelo fruits has been based only on visual color examination, and research related to this parameter has been inconsistent. The main objective of this study was to evaluate a red-green-blue (RGB) color measurement system for successful identification of Swingle citrumelo fruits possessing seeds with maximum physiological potential and storability. Fruits were harvested at three ripening stages identified as green (G), greenish-yellow (GY), and yellow (Y) pericarp, photographed, and the images processed using ImageJ software. Data were expressed as the average pixel value of R, G and B color components and a mean RGB pixel value (R+G+B/3). After harvest, seeds were evaluated for water content, germination, seedling emergence, length, dry mass and vigor as measured by the Seed Vigor Imaging System - SVIS (uniformity and vigor indexes) after 0 and 5 months storage (5 °C and 65 % relative humidity). Percentage of ruptured coat seeds was also evaluated after extraction. The R color component provided the best identification of each G, GY and Y fruits. Seeds extracted from GY fruits had higher storage potential compared with seeds from G and Y fruits. Thus, precise fruit ripening classification can be generated using the RGB color system to identify the best time for harvest to obtain seeds with greater physiological and storage potential.
Keywords: image analysis, computer vision, fruit maturation, citrus rootstock, seedling vigor

Introduction

Swingle citrumelo [*Citrus paradisi* Macfad. Duncan grapefruit. × *Poncirus trifoliata* (L.) Raf.] was hybridized by Walter. S. Swingle at Eustis, FL (USA) in 1907, producing hybrids tolerant to "tristeza" virus and root-rot organisms (Hutchison, 1974) that are now used in all citrus production areas. In Brazil, Swingle citrumelo is one of the preferred rootstocks due to its tolerance to citrus sudden death, a severe disease found in the late 1990s in the Southwest of Minas Gerais State and the North of São Paulo State (Gimenes-Fernandes and Bassanezi, 2003). However, nurseries have faced problems in the production of seedling rootstocks such as poor germination and low seed storage potential (Carvalho et al., 2002; Roberts, 1973; Rodrigues et al., 2010), as well as difficulties to identify the optimum fruit ripening stage to obtain high quality seeds (Medina et al., 2005; Silva et al., 2011).

Previous studies on Swingle citrumelo seeds focused on standardization of germination conditions and increased seed storability. Examples include seed coat removal (Rodrigues et al., 2010; Zucareli et al., 2009), priming treatments and biostimulant applications to enhance seed germination (Chilembwe et al., 1992; Ono et al., 1995; Rodrigues et al., 2010).

The fruit ripening stage at harvest is one of the major factors affecting citrus seed storage potential (Silva et al., 2011). However, variations in pericarp color make it difficult to consistently select Swingle citrumelo fruits for high physiological quality seed production (Rena et al., 2005). Moreover, characterization of fruit ripening stages based on visual color make standardization of fruit selections problematic (Hamilton et al., 2007; Silva et al., 2011). Color is a perceptual phenomenon that depends on the observer and the conditions in which the color is detected (Pathare et al., 2013). The use of color measurement instrumentation has potential to be a more reliable and consistent method to classify ripening stages (Léon et al., 2006).

The primary colors, red, green and blue (RGB), provide the most common color model used in computer vision systems (Vidal et al., 2013), where sensors capture the intensity of light in the red, green and blue spectrum (Léon et al., 2006). Vidal et al. (2013) developed a rapid, reliable computer vision system based on average RGB values to examine citrus fruit color according to the amount of degreening. However, association between citrus fruit color and seed physiological quality is still not fully understood and more reliable information is needed to link fruit color at harvest with seed germination and vigor. The objective of this research was to evaluate the potential of a RGB color system to classify Swingle citrumelo fruit stages and identify the optimal ripening stage to harvest seeds with greater physiological potential and storability.

Materials and Methods

Location

The experiment was carried out in Piracicaba, São Paulo State, Brazil (22°42′11.2″ S and 47°37′56.8″ W, altitude 542 m) and in a rural area of Gavião Peixoto, São Paulo State, Brazil (21°44′29.5″ S and 48°27′21.0″ W, altitude 560 m).

Harvest and fruit color classification

Harvest was performed manually and fruits were classified into three ripening stages according to the Munsell color charts (Munsell, 1976): green (G), greenish-yellow (GY) and yellow (Y) pericarp color (Table 1). The control treatment was defined by harvesting all fruits from 10 trees, which included a blend of 7 % G, 75 % GY and 18 % Y. Afterward, fruits (20 per treatment) were photographed using a charge-coupled device (CCD) digital single-lens reflex camera with 2.7 megapixels resolution connected to a computer. In order to achieve uniform lighting, all photos were acquired under the same lighting conditions without influence of external light. Lighting was achieved with three 55W fluorescent lights (50 cm in length), 4000 K color temperature, and a color rendering index (CRI) approximating 90 %. Images were processed by computer to obtain the red-green-blue (RGB) pixel intensity values. The original RGB image of each fruit was manually outlined using ImageJ software (Schneider et al., 2012) to split into three 8-bit grayscale images containing the R, G and B components of the pixels (channels). The intensity of each color component (brightness) was measured using values ranging from 0 to 255. In addition, a mean pixel intensity value (R + G + B)/3 was calculated for each fruit, displaying 256 (2^8) gray levels.

Seed extraction, treatment and storage

Seeds were extracted manually from 1000 fruits for each ripening stage. After washing in tap water to remove mucilage, seeds were dried naturally in the shade for 72 h until 35 % water content, treated with 500 ppm Captan fungicide (2 g L^{-1} of seed), and packed in a 2 kg permeable paper bag which was then wrapped in a water resistant, 0.01 mm thick polyethylene bag. Seeds were stored for 5 months in a cold chamber at 5 °C and 65 % relative humidity (RH). Evaluations of water content and seed physiological potential were conducted at 0 and 5 months storage, starting in June and Nov 2011, respectively. Seed coats were removed before each evaluation. Seeds were prepared by adding 2 L of water to 1 L of seeds in a plastic bucket; 0.5 L of 12 % sodium hypochlorite (NaClO), 30 g of sodium hydroxide (NaOH) and 3 mL of hydrochloric acid (HCl) were then added to the bucket. This mixture was stirred for 5 min and seeds were macerated for 50 min until they were bleached white. Seeds were then rinsed in a $CaCO_3$ solution (250 g of lime in 5 L of water) and the testa was manually removed.

Table 1 – Characterization of the pericarp color of fruits and its interpretation according to the Munsell color charts.

Pericarp color	Interpretation
Green	7.5GY = greenish Green-Yellow (gGY); 4/6* = value moderately dark/low chromatic color
Greenish-yellow	2.5GY = yellowish Green-Yellow (yGY); 6/8 = value moderately light/moderately chromatic color
Yellow	2.5GY = yellowish Green-Yellow (yGY); 8/8 = value light/ moderately chromatic color

*The first number represents the value that indicates the color lightness on a scale ranging from 0 (dark) to 10 (light); the second number represents the chroma that indicates the color "purity" (related to saturation), where chroma with lower values is less pure (more washed out).

Percentage of ruptured seed coats

Percentage of ruptured seed coats was performed with four replications of 1 L of seeds from each treatment. The percentage of seeds with ruptured seed coats was determined based on the ratio: weight of the seeds with ruptured seed coats/total weight of the seed sample. Seeds were classified as ruptured when they showed any visual opening in the seed coat.

Seed water content

Seed water content was determined by the oven method at 105 ± 3 °C for 24 h (MAPA, 2009), and expressed on a fresh weight basis. Evaluations were performed on duplicates of 5 g for each fruit ripening stage before and after the storage period.

Germination

Germination was determined using four replications of 25 seeds per treatment on paper towel rolls moistened with a volume of water equivalent to 2.5 times their dry mass at 25 °C. Normal seedling evaluation was performed at 21 (germination first count) and 28 days after sowing. Results were expressed as percentage normal seedlings.

Seedling emergence, length and dry mass

Four replications of 25 seeds per treatment were placed in plastic tubes containing coconut fiber as artificial substrate and kept under sprinkler irrigation in a greenhouse. Evaluations were performed 72 days after sowing by counting seedlings with shoots at least 3 cm above the substrate level. The results were expressed as mean percentage of plants for each treatment.

Seedling growth (length and dry mass) was evaluated 72 days after sowing by removing the seedlings from the plastic tube, washing them in tap water to remove remaining substrate and measuring the seedling length with a ruler. Evaluations were performed by measuring the distance between the apical bud and the tip of the primary root of each seedling (in the event of more than one seedling per tube, only the most developed were measured); mean data were expressed in centimeters. Seedling dry mass was determined after 24 h in an oven with air circulation at 80 °C. Results were expressed in g per seedling.

Seedling analysis

Seeds were treated as described for the germination test. After 21 days, seedlings were transferred from the paper towel to a black paper sheet and digital images were obtained using a flatbed scanner A4 size installed upside down inside an aluminum box (60 cm × 50 cm × 12 cm) set on a resolution of 100 dpi and connected to a computer. Images were analyzed by the Seed Vigor Imaging System (SVIS) as described by Sako et al. (2001). In this system, the software automatically generates numerical values related to a vigor index (values from 0 to 1000 directly proportional to vigor), seedling growth, and uniformity of seedling development (also ranging from 0 to 1000). Vigor index was calculated using the proportion growth/uniformity of 70/30, and 15 cm maximum size was estimated for 21-day-old seedlings.

Experimental design and statistical analysis

A completely randomized design in a factorial arrangement 4 × 2 with four fruit categories for seed extraction (green, greenish-yellow and yellow, plus control) and two evaluation times (0 and 5 months storage) was used. Data were analyzed using ANOVA and the means compared by the Tukey test ($p < 0.05$) using the Assistant software version 7.7 beta.

Results

Color classification of fruits by RGB pixel intensity

In the visual classification by Munsell color charts, fruits were characterized as green (7.5GY 4/6), greenish-yellow (2.5GY 6/8), and yellow (2.5GY 8/8). Visual differences (brightness) were observed among R, G and B color components for the same fruit color and among fruit colors within the same color component (Figure 1).

Based on the pixel intensity histograms, all individual color components and mean RGB, identified differences between the fruit ripening stages. Fruits classified as GY and Y showed a similar pattern for R and G color components and mean RGB pixel value, decreasing in the central portion of 0 to 255 levels (Figures 2B, C, E, F, K and L). Green fruits showed a pattern of pixel intensity to the left (values closer to 0) in each color component and mean RGB pixel value (Figures 2A, D, G and J). Fruits classified as GY and Y showed a similar pattern of G fruits when based on B color component (Figures 2H and I). The mean values of pixel intensity in G, GY and Y fruits using the R color component were 67.3, 126.4 and 147.1, respectively (Figure 3), providing a wide range of pixel intensity values and allowing successful classification of fruit ripening stages. The G color component did not sort GY and Y fruits, as shown

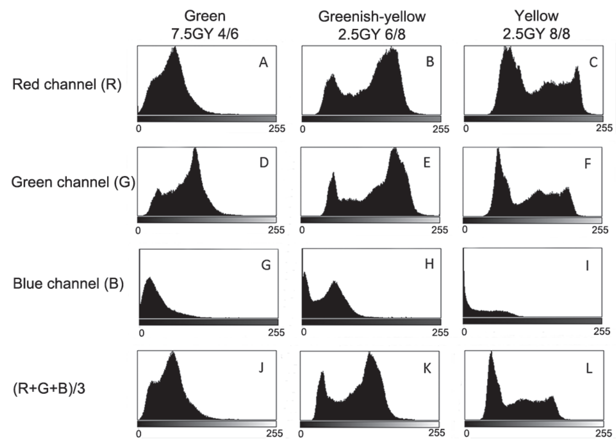

Figure 2 – Histogram of pixel intensity for each red (R) (A, B and C), green (G) (D, E and F) and blue (B) (G, H and I) color component (channel) and mean pixel value (R+G+B)/3 (J, K and L) for the green, greenish-yellow and yellow fruits shown in Figure 1.

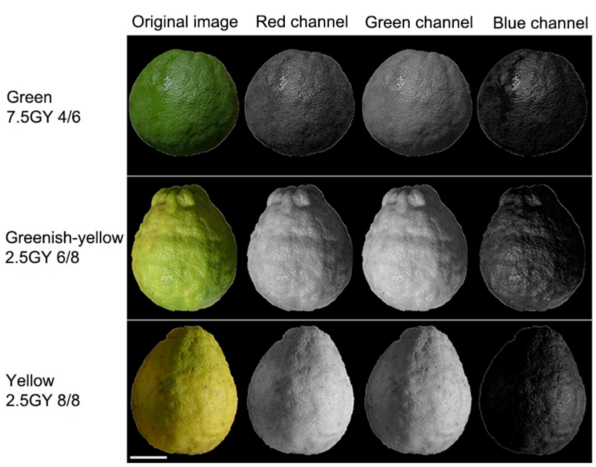

Figure 1 – Characterization of the green, greenish-yellow and yellow Swingle citrumelo [*Citrus paradisi* Macfad. × *Poncirus trifoliata* (L.) Raf.] fruits according to the Munsell color charts (original image) and their respective red, green and blue split color component (channel) using the ImageJ software; Bar corresponds to 2 cm.

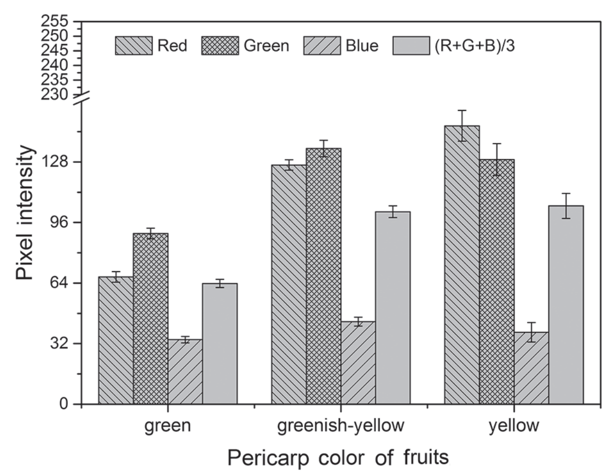

Figure 3 – Pixel intensity for each red (R), green (G) and blue (B) color component and mean pixel value (R+G+B)/3 for the green, greenish-yellow and yellow pericarp color of fruits; Error bars indicate ± standard error of the mean.

previously with the mean RGB pixel value; however, it was possible to classify G fruits. Based on the B color component, a narrow amplitude for the pixel intensity values was observed (34.1 to 43.6) making it difficult to identify any differences between fruit ripening stages.

Effects of the fruit ripening stage on seed physical integrity

A wide variation in the percentage of ruptured seed coats occurred among the fruit ripening stages. In G and GY fruits, values were only 2 % in contrast to 50 % for seeds extracted from Y fruits (Figure 4). The control treatment had 11 % seeds with ruptured seed coats and this value was proportional to the percentage of seeds showing rupture seed coats extracted from G, GY and Y fruits (Figures 5A, B and C).

Seed physiological potential before and after storage

Water content of the seeds extracted from G, GY and Y fruits was 47 %, 44 % and 37 %, respectively. After 5 months of storage at 5 °C and 65 % RH, there was a substantial decrease in water content, e.g. seeds extracted from G, GY and Y fruits had 32 %, 35 % and 28 % water content, respectively. The seed water content in the control treatment decreased from 39 % to 30 % (Table 2).

Before the storage period, seed germination ranged from 82 % to 88 % and differences were not detected between treatments ($p > 0.05$). Seeds from G fruits showed the lowest vigor according to germination first count, plant dry mass and vigor index - SVIS compared with Y fruits. With the exception of seedling emergence and uniformity index - SVIS, the highest performance at 0-month storage was from seeds extracted from Y fruits.

After 5 months of storage, seed germination was significantly reduced ($p \leq 0.05$) in all treatments. However, seeds extracted from GY fruits showed higher germination (58 %) and vigor (germination first count, seedling emergence and vigor index - SVIS) than seeds from G and Y fruits did. All physiological potential evaluations

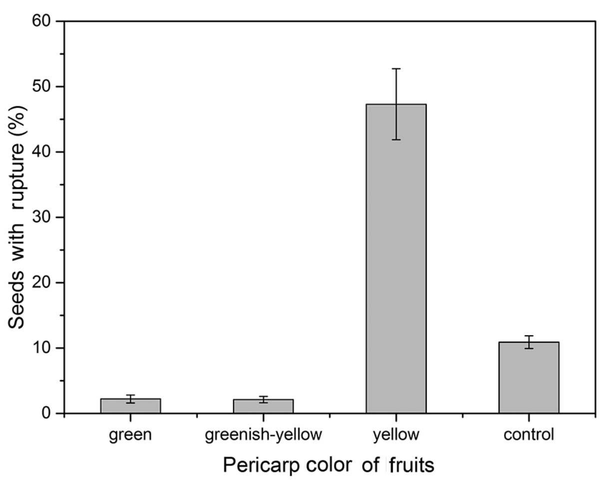

Figure 4 – Percentage of ruptured seed coats for each treatment of green, greenish-yellow and yellow pericarp color of fruits; Error bars indicate ± standard error of the mean.

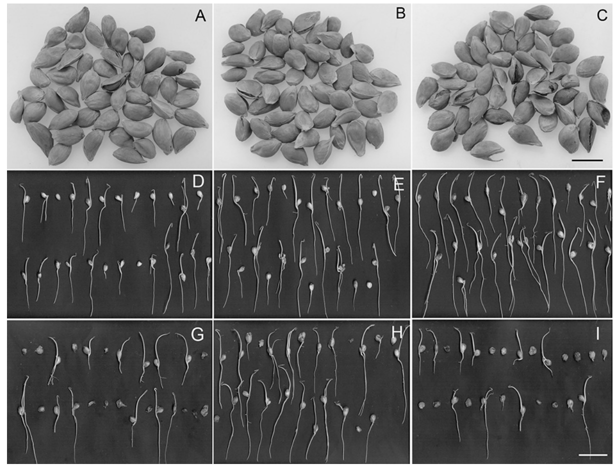

Figure 5 – Characterization of the Swingle citrumelo [*Citrus paradisi* Macfad. × *Poncirus trifoliata* (L.) Raf.] seeds extracted from fruits harvested at three ripening stages identified by green (G), greenish-yellow (GY) and yellow (Y) pericarp color after 5 months of storage (A, B and C) and their respective 21-day-old seedlings at 0 (D, E and F) and 5 months of storage (G, H and I); Bars in C and I correspond to 1 cm and 4 cm, respectively.

Table 2 – Water content and physiological potential evaluations of Swingle citrumelo seeds extracted in different fruit ripening stages at 0 and 5 months storage.

Evaluations	Pericarp color of fruits			Control[1]
	Green	Greenish-yellow	Yellow	
	Seed water content (%)[2]			
0 month	47	44	37	39
5 months	32	35	28	30
	Germination (%)			
0 month	84 Aa	88 Aa	82 Aa	85 Aa
5 months	37 Bc	58 Bab	36 Bc	63 Ba
CV (%) 11.39				
	Germination first count (%)			
0 month	47 Ac	59 Abc	78 Aa	73 Aab
5 months	31 Bb	50 Aa	31 Bb	55 Ba
CV (%) 16.05				
	Seedling emergence (%)			
0 month	56 Ab	60 Bb	62 Ab	79 Aa
5 months	17 Bc	85 Aa	56 Ab	76 Aab
CV (%) 19.48				
	Seedling dry mass (g per seedling)			
0 month	0.063 Bb	0.073 Bb	0.125 Ba	0.135 Ba
5 months	0.143 Ab	0.258 Aa	0.258 Aa	0.255 Aa
CV (%) 15.44				
	Uniformity index - SVIS			
0 month	819 Aa	820 Aa	809 Aa	828 Aa
5 months	572 Bc	712 Bab	603 Bbc	785 Aa
CV (%) 7.58				
	Vigor index - SVIS			
0 month	634 Ac	754 Abc	907 Aa	879 Aab
5 months	485 Bb	696 Aa	363 Bb	719 Aa
CV (%) 11.20				

Means followed by the same lowercase letter within rows and same uppercase letter within columns for each evaluation indicate no significant differences ($p \leq 0.05$). Mean separation by the Tukey-Kramer test; [1]Control: blend of 7 % green, 75 % greenish-yellow, and 18 % yellow; [2]Based on a fresh weight basis.

indicated that the lowest performance came from seeds extracted from G fruits at 0 and 5 months storage. Seedling length results showed differences between treatments (Figure 6); seedling length of seeds from G fruits was more than 2 cm shorter than seeds from Y fruits and control fruits.

Seedling dry mass increased in all treatments after 5-month storage seeds, which was in contrast to the germination results showing higher values before storage. Lower germination first count (Table 2) and higher number of dead seeds was observed for seeds extracted from G and Y fruits (Figures 5D, E, F, G, H and I). Seeds from GY fruits and the control showed similar vigor index - SVIS at 0 and 5 months of storage. However, a significant decrease in vigor index was observed in seeds from G and Y fruits, dropping to 485 and 363, respectively.

Discussion

Although human sight is useful even in the presence of lighting differences, color determination is variable from observer to observer and makes standardization difficult (León et al., 2006). Color is one of the most important visual physical parameters of fresh fruit and can be evaluated automatically using several computer vision systems (Pathare et al., 2013). This study investigated the storage potential of Swingle citrumelo seeds extracted from fruits at different ripening stages as identified by a RGB color system. Previous research in minimally processed tomato fruits showed that individual R, G and B color components were significantly different for three fruit ripening stages and their visual differences could be expressed by variations in RGB values (Lana et al., 2006). In this study, visual differences between GY and Y fruits were not detected, which probably limited fruit separation by individual G and B color components. However, the higher pixel values for the R color component in Y fruits compared with GY fruits indicate that this color spectrum is a valuable parameter for automated classification. Conversely, similar pixel intensities between treatments using B color component indicate that this color spectrum may not be an efficient marker to consistently identify fruit ripening stages.

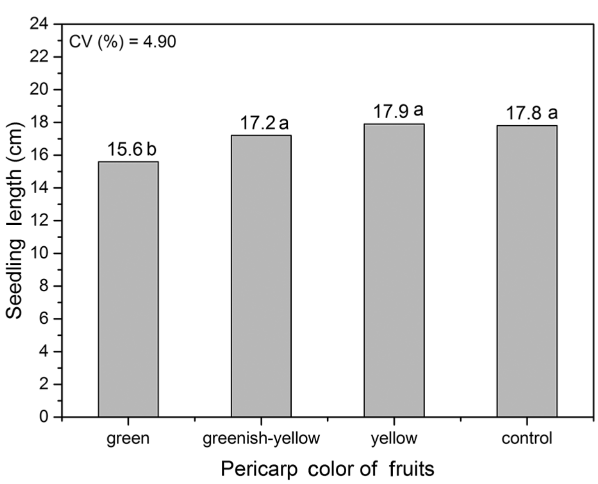

Figure 6 – Seedling length (72-day-old) for each treatment of green (G), greenish-yellow (GY) and yellow (Y) pericarp fruit colors; Mean of two evaluation times (Tukey-Kramer test, $p \leq 0.05$).

Freshly extracted seeds showed wide variations in water content between fruit ripening stages, and drying at 35 % water provided uniformity and favorable storage conditions. According to Silva et al. (2011), this water content is ideal to maintain high viability of Swingle citrumelo seeds. In contrast, von Pinho et al. (2005) reported that Swingle citrumelo seeds should be stored at 44 % water content in a cold room (10 °C and 50 % RH) in impermeable bags due to its recalcitrant behavior. In this research, even using impermeable bags, water content variations from 28 % to 35 % after 5 months of storage were not associated with poor seed germination. These results indicate intermediate seed storage behavior in contrast to that reported by von Pinho et al. (2005). Siqueira et al. (2002) also described intermediate storage behavior of Swingle citrumelo seeds with over 80 % germination, even after seed drying to 21 % water content and significant decrease in germination (5 %) only when moisture content was reduced to 10 %.

Seed germination at 0-month storage was above 80 % in all fruit ripening stages. This germination is comparable to the results from Carvalho and Silva (2013) and Siqueira et al. (2002), and is also near the expected standard germination of citrus, usually less than 85 % (Carvalho et al., 2002; Ono et al., 1995; Rodrigues et al., 2010). Although similar in germination, seeds extracted from G fruits showed significantly lower vigor compared with Y fruits at 0-month storage. Silva et al. (2011) reported higher seed germination from ripe (yellow) fruits compared with unripe (green) fruits just after harvest. In the wild, *Citrus garrawayi* F. M. Bailey, seeds with incomplete embryo and seed tissue differentiation (extracted from unripe fruit coats) produced only 4 % normal seedlings, in contrast to 88 % in ripe fruits (Hamilton et al., 2007). Here, we also found that the least developed seedlings (Figure 5D) and shortest seedling length (Figure 6) occurred with seeds extracted from G fruits. Hamilton et al. (2007) reported similar results when studying seed maturity effects on seedling growth, i.e., an increase in seedling size as seed maturation proceeds.

Environmental conditions during germination and seedling establishment can affect plant performance. Camp et al. (1933) reported that the optimal soil temperature for citrus seed germination was between 31 and 35 °C, and the best rate of seedling growth occurred from 28 to 30 °C. Here, seedling dry mass from 5-month storage seeds was higher than 0-month storage in all fruit ripening treatments. These differences could be attributed to the higher daytime temperatures in the greenhouse (~ 32 °C) from Nov to Jan (spring/summer) when the 5-month storage seeds were germinated. At 0-month storage, the daytime temperatures of approximately 25 °C in June and July (fall/winter) were suboptimal for germination and plant growth.

Except for seedling dry mass, the lowest germination and vigor at 5 months of storage confirmed the poor storability of the Swingle citrumelo seeds reported by Carvalho et al. (2002), Silva et al. (2011) and Siqueira et al. (2002). However, the better performance of seeds extracted from GY fruits, not evaluated in previous studies, represents new information about fruit ripening and seeds with higher physiological and storage potentials. These results showed the importance of fruit color classification to produce vigorous seeds, reducing unnecessary storage costs with low seed performance and with the seedling production process, such as labor, substrates and irrigation. Seeds extracted from G fruits had the greatest decrease in seedling emergence and plant dry mass, showing that this ripening stage was not appropriate for production of seed with high vigor. Hamilton et al. (2007) reported similar results in *C. garrawayi* F. M. Bailey with very low germination and plant growth in seeds extracted from unripe fruits. In contrast, Silva et al. (2011) did not observe differences in germination between Swingle citrumelo seeds extracted from green and yellow fruits up to 6 months of storage. Based on the results of percentage germination, germination first count and SVIS, our research also showed lower storability of seeds extracted from Y fruits (Table 2). Water condensation was observed inside the waterproof package after 5 months of storage, which is considered a high humid condition that could promote seed deterioration. Previous research reported that periodic package opening during storage was necessary to avoid problems with water condensation (Carvalho and Silva, 2013). Here, despite using a similar package during the entire storage period, the highest level of decayed seeds occurred in seeds extracted from Y fruits compared to seeds from G, GY fruits and the control seeds. These results could be attributed to the high percentage of ruptured seed coats (Figure 4), probably related to the higher dry mass accumulation of seeds at the end of the seed maturation process, but without any relation with polyembriony. Preliminary studies based on X-ray images showed 12 %, 18 %, 22 %, and 22 % of polyembriony in seeds extracted from G, GY, Y fruits and the control seeds, respectively; however, the percentage of ruptured seed coats was 2 % (G and GY fruits), 50 % (Y fruits), and 11 % (control). Seed coat integrity is essential for normal germination, and provides protection against microorganisms that causes seed-borne diseases in many cultivated species (Souza and Marcos-Filho, 2001). At 5-month storage, ruptured seed coats showed dark areas inside, which is characteristic of microorganism attack (Figure 5C). Silva et al. (2011) reported that at 9-month storage, seeds extracted from yellow fruits were decayed, moist and with an ethanol odor even after fungicide treatment; however, they did not evaluate seed coat damage.

The results obtained in this research have confirmed that the RGB color system is a valuable approach to identify Swingle citrumelo fruits according to their ripening levels. This computer vision procedure represents an important technological advancement that reduces human errors in classifying fruits, which is useful for the nurserymen producing seeds with high physiological and storage potentials.

Conclusions

The RGB color system is a practical and non-subjective procedure to classify Swingle citrumelo fruits at different ripening stages. The lower seed physiological potential from green (G) fruits and the higher storability of seeds from greenish-yellow (GY) fruits show the importance of using appropriate fruit classification systems. In addition, the low storability of seeds from yellow (Y) fruits suggests the advantage of using an efficient seed sorting method to identify seeds with ruptured seed coats.

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