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Image Analysis Techniques to Evaluate *Portulaca* Seed Morphology and Vigor

Vanessa Neumann Silva*, Mark Bennett,
Pablo Jourdan and Silvio Moure Cicero

ABSTRACT

Internal seed morphology, germination and vigor were examined in *Portulaca grandiflora* and *P. oleracea* seeds using image analysis techniques, to assess the role of seed free space on germination and seedling vigor. Five lots of *P. grandiflora* and two lots of *P. oleracea* seeds were first X-rayed, and the radiographs analyzed with Image Pro Plus® to measure the free space between the embryo and endosperm of each seed. Seeds were then germinated either immediately or after exposure to saturated salt accelerated aging conditions for 48 and 72 h. Seedling vigor was assessed with the Seed Vigor Imaging System (SVIS®). Free space between the embryo and endosperm in seeds of *P. grandiflora* ranged from 0 to 4.6% as a proportion of the whole seed area for all lots examined, whereas for seeds of *P. oleracea*, the range was from 0 to 10%. The relationship between seed free space and germination of *P. grandiflora* was variable; two of the five lots showed a reduction in the proportion of normal seedlings as the seed free space increased. While there appeared to be no direct relationship between increased free space and reduction in germination and vigor of *P. grandiflora* seeds, for *P. oleracea* seeds the results indicated a negative effect of extensive free space on germination and vigor.

INTRODUCTION

Image analyses are modern techniques that allow rapid and objective seed quality evaluation. One of these techniques is X-ray image analysis or radiography, a well established tool to examine internal morphology and assess the quality of seed lots. Seed banks often rely on this tool to insure that only clean, healthy-looking seeds are tested for germination and subsequently stored. X-ray imaging of seeds has also been used to predict seedling performance, as for tomato (*Solanum lycopersicon* L.) (van der Burg et al., 1994), papaya (*Carica papaya* L.) (Santos et al., 2009), and *Xylopia aromatica* (Lam.) Mart. (Socolowski et al., 2011). One of the parameters that can be measured by radiography is free space (Liu et al., 1993). The term 'free space' is used to describe the occurrence of empty spaces between the embryo and the endosperm or between the seed contents and the integument. Free space, first determined for tomato seeds (Liu et al., 1993), may occur in different parts of the seed, normally around the embryo, most frequently around the radicle tip, and often around the cotyledons in the center area of the seed.

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Radiographic studies have shown that lots with seeds having a higher proportion of free space tend to have lower germination and quality. Pepper (*Capsicum annuum* L.) seeds with more than 2.7% of free space between the embryo and endosperm expressed progressive reductions in normal seedling development (Dell'Áquila, 2007), and the number of abnormal seedlings increased proportionately with an increase in free space (Gagliardi and Marcos Filho, 2011). For tomato seeds, van der Burg et al. (1994) found that embryo size, morphology, amount of endosperm and area of free space correlated well with the morphology of 14-d-old seedlings. In contrast, Downie et al. (1999) found that tomato mutant seeds with extensive free space completed germination more rapidly than wild type seeds with reduced free space. These authors concluded that free space was not predictive of many aspects of tomato germination physiology, but did not specify the amount of free space leading to extensive or reduced quality levels. It is possible that free space areas between embryo and endosperm could affect other features besides germination itself, like seedling vigor, but there have been no published data on this topic, especially for seeds of herbaceous ornamental plants.

Another modern tool to evaluate seed quality is computer imaging analyses, which can provide seed vigor evaluation and allow the exploration of seed morphological features that may influence vigor. Imaging methods are fast, objective, efficient and applicable to several species, including tomato and eggplant (*Solanum melongena* L.) (Silva et al., 2012), cucumber (*Cucumis sativus* L.) (Chiquito et al., 2012), lettuce (*Lactuca sativa* L.) (Sako et al., 2001; Contreras and Barros, 2005), melon (*Cucumis melo* L.) (Marcos Filho et al., 2006), soybean [*Glycine max* (L.) Merr.] (Hoffmaster et al., 2003; Marcos Filho et al., 2009), sweet corn (*Zea mays* L.) (Gomes Junior et al., 2009) and castor bean (*Ricinus communis* L.) (Kobori et al., 2010).

Considering that limited work has been done exploring ornamental plants' internal seed morphology, and that the relationships among internal seed morphology, germination and vigor are not well-established, we have begun to examine these parameters in herbaceous ornamental plants. Such studies can be of value for germplasm preservation activities where many of the accessions stored are not from highly bred and domesticated crops like those of previous studies. Here, we report on the behavior of *Portulaca grandiflora* Hook. and *Portulaca oleracea* L. seed after assessing internal morphology and its relationship to overall germination and seedling vigor.

MATERIALS AND METHODS

Before *Portulaca* was chosen as the genus to study in more detail, we surveyed the internal seed morphology of various herbaceous species housed at the Ornamental Plant Germplasm Center (Columbus, OH, USA). Seeds of *Rudbeckia hirta* L., *Tagetes patula* L., *Tagetes erecta* L., *Viola tricolor* L. and *Petunia* × *atkinsiana* (Sweet) D. Don ex W. H. Baxter were evaluated by X-ray image analyses and standard germination tests; sample results are shown in Fig. 1. Accessions of *Portulaca*, *Petunia*, *Rudbeckia* and *Tagetes* showed internal seed morphology that could be amenable to more detailed studies of free

space. However, only *Portulaca* provided seeds that could be consistently measured for free space and that germinated in a short period of time, so these were used for this study. Following these preliminary studies, five lots of *P. grandiflora* (G1 to G5) and two lots of *P. oleracea* (O1 and O2) from various

FIGURE 1. Radiographs of surveyed species of (top to bottom) *Portulaca oleracea*, *Petunia* × *atkinsiana*, *Tagetes erecta* and *Rudbeckia hirta*, displaying variations in internal seed morphology.

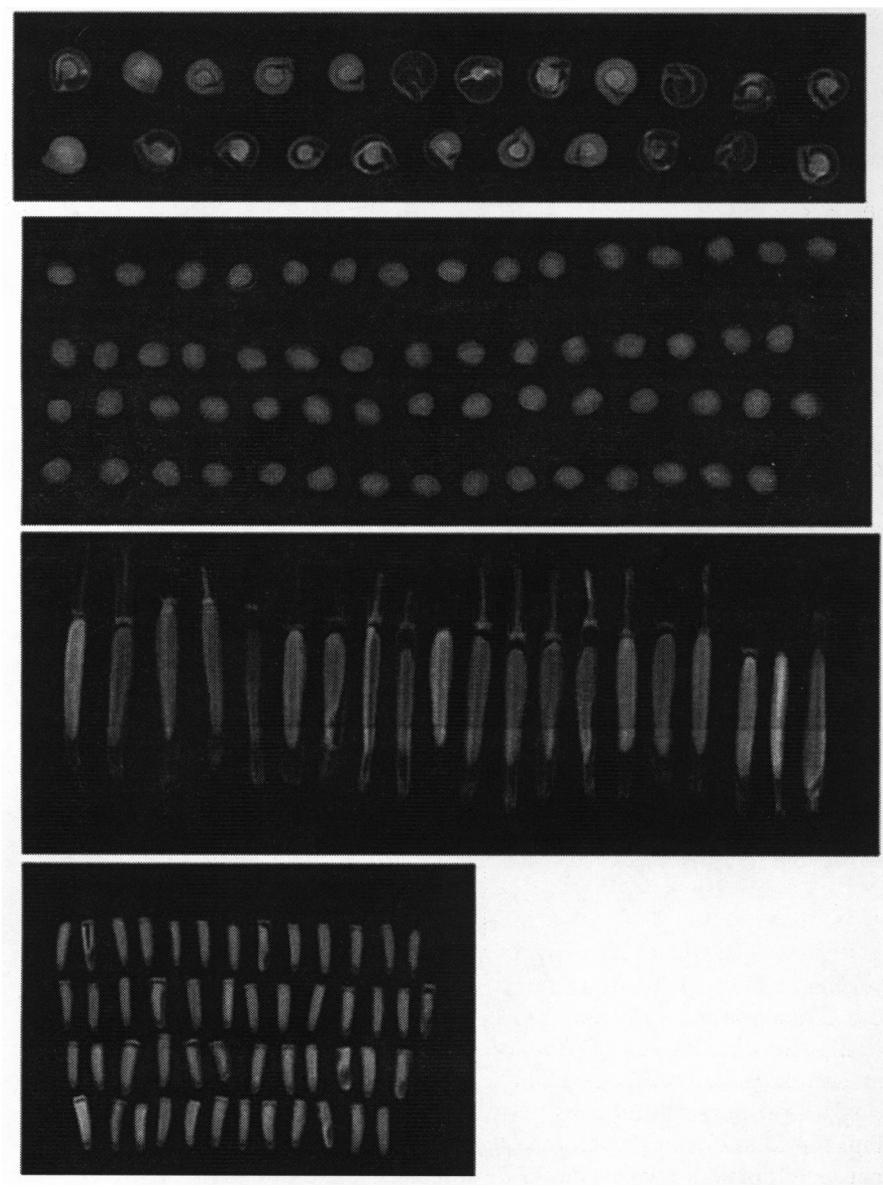


TABLE 1. Sources of *Portulaca grandiflora* and *P. oleracea* seed lots used in this study.

Seed lot	Species	Source
G1	<i>P. grandiflora</i>	Ferry Morse Double Mixed Colors
G2	<i>P. grandiflora</i>	Burpee Magic Carpet* Mix – Holland 1
G3	<i>P. grandiflora</i>	Burpee Magic Carpet* Mix – Holland 2
G4	<i>P. grandiflora</i>	Pan American Seed Margarita Apricot 0020401056
G5	<i>P. grandiflora</i>	Pan American Seed Margarita Apricot 0020401088
O1	<i>P. oleracea</i>	Pan American Seed Toucan Fuchsia 0020185007
O2	<i>P. oleracea</i>	Pan American Seed Toucan Fuchsia 0020185005

seed sources (Table 1) were X-rayed, then germinated immediately or after accelerated aging using standard methodology (AOSA, 2004), including assessment of normal and abnormal seedlings. Seedling vigor was measured and characterized by image analysis, as described below.

X-ray imaging. Four replicates of 25 seeds from each seed lot were taped onto double-sided transparent plastic film, labeled and radiographed on a Faxitron MX-20 (Faxitron Bioptics, Tucson, AZ) for 20 sec at 20 kV. The seeds were then transferred to a plastic tray containing individual cells, and kept in the same order as in the X-ray image until germination. The digital radiographs were analyzed by Image Pro Plus 7.0[®] software (Media Cybernetics), and free space between the embryo and the endosperm measured in mm then calculated as a proportion of the whole seed area. Free space data underwent a frequency analysis and results compared to those from the germination and vigor tests.

Germination and seedling vigor (SVIS). Each of the four 25-seed replicates was carefully laid in two straight lines on a 15 × 22 cm sheet of blue germination paper (Anchor Paper, St. Paul, MN) saturated with deionized water in a plastic germination box (Gomes Junior et al., 2009). Seeds were then covered with 3 moistened sheets of the same paper and the germination box placed in an incubator maintained at 20–30 °C with 8 h of daily light. After four days, the covering sheets were removed and the seedlings on the paper sheet placed on a scanner for vigor index measurements using SVIS[®] (Seed Vigor Imaging System) computer software developed by Sako et al. (2001). This scanner/software system generates two indices on a scale from 0 to 1000; one is a growth index based on seedling size after 4 d (speed of growth) and the other is a uniformity index based on the uniformity of hypocotyl and radicle length. The vigor index was the sum of 70% of the growth index and 30% of the uniformity index, and seedling length was measured by software for each seedling (Sako et al., 2001).

Saturated salt accelerated aging (SSAA). A sample of 200 seeds of each lot were distributed in a single layer on a mesh shelf placed inside an 8.5 × 8.0 cm plastic box containing 40 mL of a saturated NaCl solution, which produces 76% RH in closed containers; the seeds remained approximately 2 cm above the level of the solution (Jianhua and McDonald, 1996). The boxes were tightly sealed and placed in an incubator at 41 °C (± 2 °C) for 48 and 72 h, before being

removed and opened. Seeds were tested for germination as previously described, except that 50-seed replicates were used per germination box instead of 25.

Germination at different temperatures. Four replicates of 50 seeds each from one lot of *P. grandiflora* (G2) and one lot of *P. oleracea* (O2) were distributed on a sheet of 10 × 10 cm blue germination paper saturated with deionized water in a plastic germination box, and placed in a thermogradient table maintained at 12, 14, 16, 18, 21, 23, 26, 30 and 32 °C with 16 h of daily light. Every 24 h for 14 d, germination (radicle protrusion) was assessed and a velocity of germination index was determined according to Maguire (1962).

RESULTS AND DISCUSSION

Seeds obtained from commercial sources had reported germination rates of 77–94% for *P. grandiflora* and 62% and 78% for *P. oleracea* (Tables 1 and 2). When we tested the germination under our conditions, we found that in each

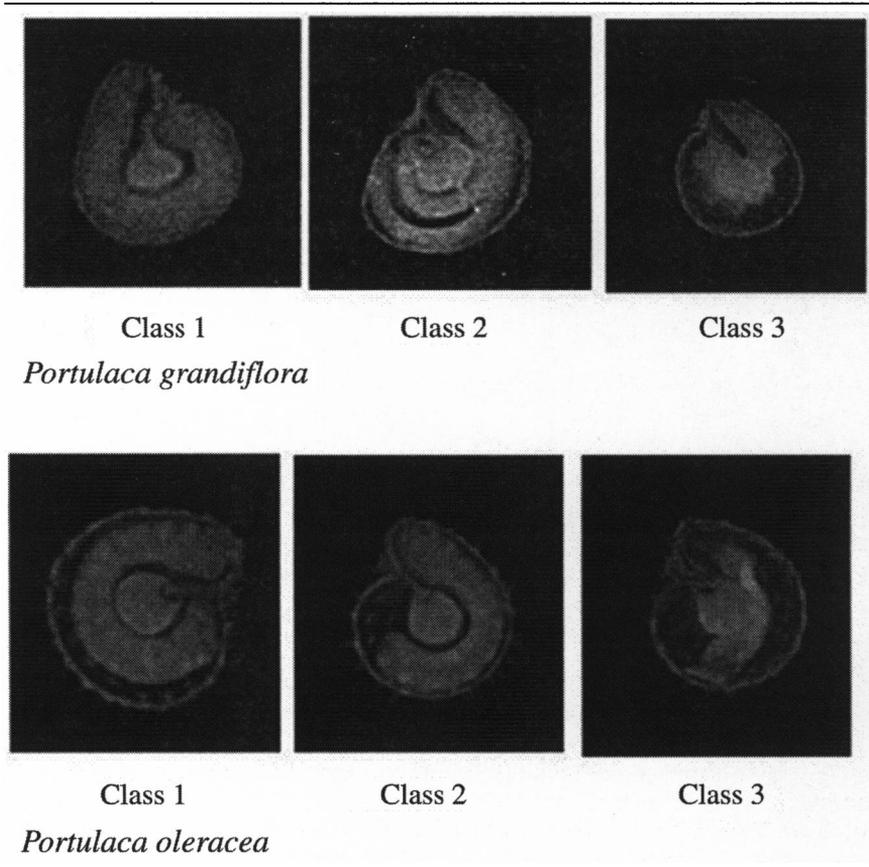
TABLE 2. Percentage germination reported by the seed lot producer (G) and measured in this study (GC), distribution of free space (FE) between embryo and endosperm as a proportion of total seed area organized by class, percentage germination in each class (PGC) and percentage of normal seedlings in each class (N), of five lots of *Portulaca grandiflora* (G1–G5) and two lots of *P. oleracea* (O1 and O2).

LOT	G	GC	Class	FE	PGC	N
	----- (%) -----				----- (%) -----	
G1	85	76	1	0–1.5	85	71
			2	1.6–3.0	13	69
			3	3.01–4.5	2	100
G2	93	84	1	0–1.5	83	93
			2	1.6–3.0	15	73
			3	3.01–4.5	2	0
G3	93	80	1	0–1.5	83	82
			2	1.6–3.0	15	87
			3	3.01–4.6	2	0
G4	77	75	1	0–1.5	94	35
			2	1.6–3.0	4	25
			3	3.01–4.5	2	0
G5	94	80	1	0–1.5	88	44
			2	1.6–3.0	8	25
			3	3.01–4.5	4	50
O1	62	60	1	0–3.5	80	48
			2	3.6–7.0	16	13
			3	7.01–10	4	0
O2	78	75	1	0–1.5	69	62
			2	1.6–3.0	26	46
			3	3.01–4.5	5	80

case there was a decrease from the reported germination rate, more so with *P. grandiflora* (2–14%) than with *P. oleracea* (2–3%). It is commonly observed that seeds, even when stored under favorable conditions and in appropriate packages, still deteriorate, because seed aging is an irreversible and continuous process that starts at physiological maturity and inexorably reduces germination capacity.

We found variation both in the extent of free space between the embryo and endosperm as well as in the proportion of the seeds that had detectable free space (Table 2). In *P. grandiflora*, the free space ranged from 0–4.6% of the whole seed area, whereas for *P. oleracea* the range was greater, 0–10% for lot O1 and 0–4.5% for lot O2. Examples of free space in seeds of each species are shown in Fig. 2. It was clear that for all five lots of *P. grandiflora*, most seeds had less than 2% of free space, with lots G1–G5 having 7, 4, 5, 4 and 8 seeds out of 100 with 2% or greater free space, respectively. In *P. oleracea*, lot O1 had 30 of 100 seeds with free space of 3% or above, while lot O2 had only 7

FIGURE 2. Examples of seed free space observed in seeds of *Portulaca grandiflora*, lot G2, and *P. oleracea*, lot O1.



of 100 seeds at this level. The two lots of *P. oleracea* we examined had more extensive free space than *P. grandiflora* lots, but the two *P. oleracea* lots also differed from each other. The majority of seeds of lot O1 had free space between 0 and 5%, but some seeds reached almost 10%, whereas lot O2 had more seeds with free space ranging from 0 to 3%, but the maximum values were close to 5%. Studies with pepper seeds showed that free space greater than 2.7% of the whole seed area can negatively affect germination (Dell'Áquila, 2007), but ours is the first analysis of this feature for an ornamental like *P. grandiflora*.

The distribution of seed free space values in each lot showed that for *P. grandiflora* the majority of seeds (> 80%) had free space in the lowest end of the size range, from 0 to 1.5%. Similarly, for *P. oleracea*, most seed of lot O1 (80%) had less than 3.5% free space while in lot O2 69% of seeds had less than 1.5% free space. Thus, lot O1 had more extensive free space than lot O2 (Table 2). The occurrence of free space may affect germination due to reduced seed reserves and a less-developed embryo, as was reported for some species like *Handroanthus heptaphyllus* (Vell.) Mattos (syn. *Tabebuia heptaphylla*) (Amaral et al., 2011) and *Lithraea molleoides* (Vell.) Engl. (Machado and Cicero, 2003). In endospermic seeds like *Portulaca*, the substances responsible for the energy necessary to start the germination process are stored in endosperm tissues. When germination starts, the protein synthesis and respiratory activity initially involve components stored within the mature dry seed (Nonogaki et al., 2010). Seeds with more developed endosperm or cotyledons are richer in the energy reserves necessary for germination (Moegenburg, 1996), and can also show higher hormone levels and a larger embryo (Surlés et al., 1993). To elucidate the mechanisms of seed germination, it is essential to analyze the structure of seeds common to many different species (Nonogaki, 2006).

To simplify the analyses of internal morphology, germination and vigor, the free space values were grouped using a system of three classes (Table 2). For *P. grandiflora*, seeds with more than 3% free space (class 3) had not germinated in lots G2, G3 and G4. Seeds from this class in lot G1 had all germinated and seeds of this class in lot G5 had 50% germination. It is important to note that for lots G1–G4, just 2 of 100 seeds in each lot had free spaces greater than 3% and for lot G5 just 4 of 100 seeds did. Such low frequency makes it difficult to draw definite conclusions about the possible influence of free space on subsequent germination and vigor. Comparing seed germination rates from seeds in class 1 and 2 showed no great differences between the five lots of *P. grandiflora*, but again, more than 80% of seeds were in class 1 (less than 1.5% free space).

The two lots of *P. oleracea* (O1 and O2) showed overall lower germination rates than *P. grandiflora* and greater differences in free space between them. For lot O1, even those seeds with the smallest free space (< 3.5%) germinated at less than 50%, suggesting that other seed quality factors may be contributing to the lower overall germination rate of 60% (Table 2). For lot O2, the free space area was smaller than in lot O1, but we observed a decrease in germination from 26 to 5%, as free space increased from 1.6 to 3% (class 2), and seeds with more than 3% free space produced a higher proportion of normal seedlings, although only 5% of seeds belonged to this class.

TABLE 3. Indices of vigor (IV), growth (IG) and uniformity of seedlings growth (IUG), and seedling length (SL) of five lots of *Portulaca grandiflora* (G1–G5) and two lots of *P. oleracea* (O1 and O2), based on Seed Vigor Imaging System (SVIS) analysis, following germination.

Lot	IV	IG	IUG	SL (pixels)
<i>P. grandiflora</i>				
G1	322a†	474ab	823a	116a
G2	389a	527a	850a	150a
G3	366a	506a	832a	124a
G4	210b	353c	687b	50b
G5	235b	356bc	639b	54b
CV (%)	10.1	16	8	25
<i>P. oleracea</i>				
O1	502a	463a	595b	79a
O2	484b	380a	730a	106a
CV (%)	6	8	10	19

†Means followed by the same letter, in the same column, for each species, do not significantly differ ($p \leq 0.05$) according to Tukey's test.

Germination is a complex process influenced by temperature, water, oxygen, light and other factors. Seed quality can affect germination capacity, and many factors can influence seed quality, such as conditions during seed production. There are limited literature sources about *Portulaca* seed features and factors that could affect germination and seed quality. For the two studied species of *Portulaca*, we could not definitively show that seeds with more free space areas had reduced germination capacity. Working with eggplant seeds, Silva et al. (2012) also found no consistent relationship between an increase in free space and reduced germination.

Another aspect of seed quality surveyed by image analysis techniques is seed vigor. Seed vigor evaluation using seedling image analysis provides results that allow seed lots to be differentiated physiologically, and can be comparable to saturated salt accelerated aging (SSAA) results, a commonly used test for evaluating seed quality of several species. Based on vigor and uniformity of growth indices, as well as seedling length, lots G1, G2 and G3 were more vigorous than lots G4 and G5 (Table 3), and this was generally true in the 48 h SSAA test (Table 4). A consistent prediction of seed quality by either SVIS® or accelerated aging tests was demonstrated for melon (Marcos Filho et al., 2006) and soybean (Marcos Filho et al. 2009). It is important to provide more information about seed vigor, especially of flower seeds, because this can impact investments made by plug and bedding plant growers. Even though germination is initiated under relatively controlled environments, successful stand establishment can vary depending on a seed lot's initial vigor (Cantliffe, 1998).

TABLE 4. Seed germination after saturated salt accelerated aging (SSAA) for 48 (AA 48) and 72 (AA 72) h of incubation, indices of vigor (IV), growth (IG) and uniformity of growth (IUG), and seedling length (SL), following accelerated aging, of five seed lots of *Portulaca grandiflora* (G1–G5) and two lots of *P. oleracea* (O1 and O2).

Lot	Germination (%)		IV1†	IG1	IUG1	SL1	IV2	IC2	IU2	SL2
	AA 48	AA 72								
<i>Portulaca grandiflora</i>										
G1	68ab‡	88a	359b	477a	751a	80b	640a	567a	811a	155a
G2	78a	62b	423a	531a	785a	110a	608a	602a	625b	131a
G3	76a	60b	403ab	507a	750a	100a	600a	567a	626b	130a
G4	52c	48b	303c	313c	337c	47c	560a	588a	498b	111ab
G5	60bc	56b	302c	382b	570b	61c	485a	489a	478b	73b
CV(%)	10	16	6	6	9	10	14	17	13	20
<i>Portulaca oleracea</i>										
O1	34b	35b	362a	454a	704a	85a	526b	623b	301b	80a
O2	63a	54a	474a	543a	670a	107a	733a	824a	522a	154a
CV(%)	24	17	21	13	15	22	7	6	22	20

†1 and 2: results following saturated salt accelerated aging for 48 and 72 h, respectively.

‡Means followed by the same letter, in the same column, for each species, do not significantly differ ($p \leq 0.05$) according to Tukey's test.

The other seed vigor test, SSAA, was done at two different durations, 48 and 72 h, because there was no published methodology for *Portulaca* species. Both methodologies were able to evaluate seed vigor, but results following aging for 48 h appear to be correlated to germination and seedling image analysis for *P. grandiflora* seed lots. In this test, lots G4 and G5 again had less vigorous seeds and were the lots most affected by stressful conditions of the SSAA test, evidenced by their reduced seedling development capacity, as evaluated by SVIS (Table 4). For sweet pepper seeds, Kaewnaee et al. (2011) found that a decrease in germination ability was correlated with increased membrane deterioration during accelerated aging. Membrane cell disorganization can cause serious negative effects on germination capacity. A very strong correlation has been demonstrated between the intercellular molecular mobility and seed life span (Buitink and Leprince, 2008). SSAA using 72 h of incubation identified only lot G1 as possessing the highest vigor, perhaps because G1 seeds were more resistant to high temperatures. For *P. oleracea*, both methodologies indicated lot O1 had the lower vigor, similar to SVIS[®] evaluations following SSAA (Table 4).

SVIS imaging analysis following 48 h SSAA could identify differences between *P. grandiflora* lots (Table 4). After 72 h SSAA testing, only two SVIS indices, uniformity of growth and seedling length, detected vigor differences between lots. For *P. oleracea*, indices of vigor, uniformity of seedling growth

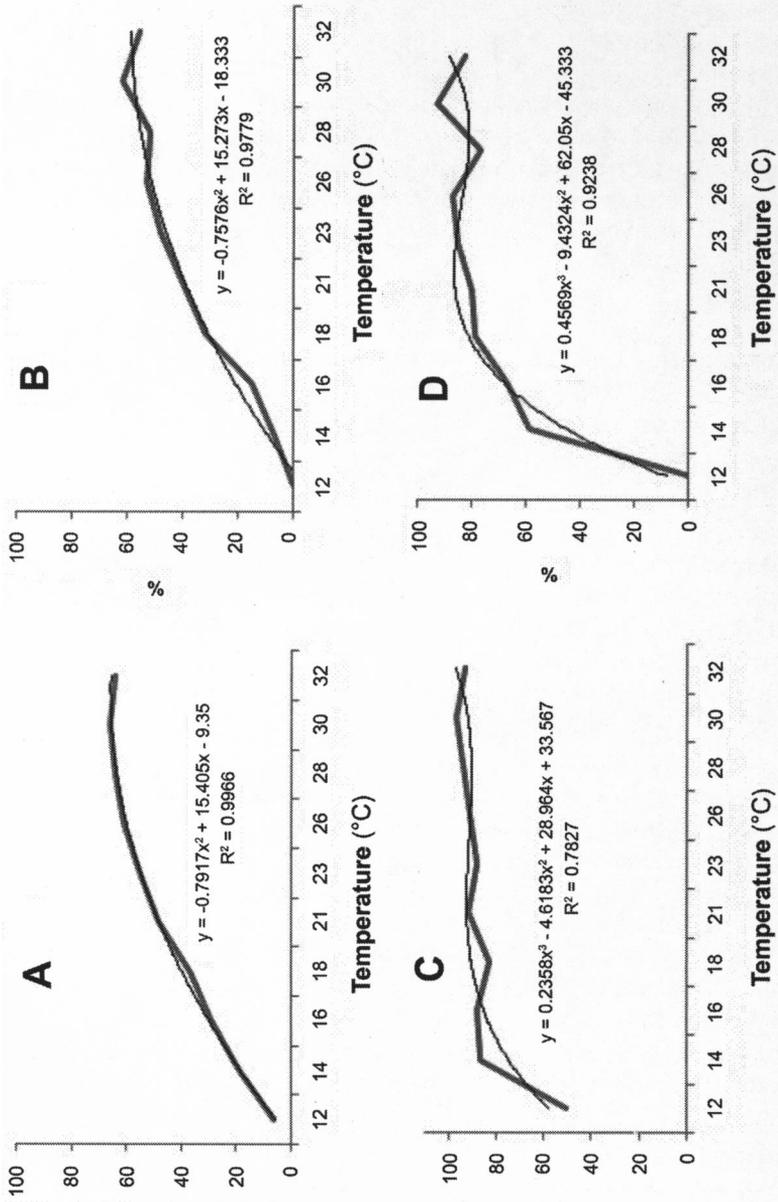
and seedling length were able to verify seed vigor differences between lots (Table 3). Germination results after 48 and 72 h SSAA, as well as all image analysis indices after 72 h SSAA, verified that lot O1 was of lower quality than O2. This could be an effect of extensive free spaces in seeds, with less storage tissue resulting in less nutritional resources for germination. As a consequence, slow germination velocities, seedling growth and seeds that were more susceptible to the adverse conditions of the SSAA tests were noted. Similarly, Peñalosa et al. (2005) found strong relationships between SSAA and SVIS parameters, and these were also correlated to lettuce seedling emergence.

Seedling evaluations by SVIS after SSAA testing demonstrated that vigor, growth and seedling length indices were highest after the 72 h SSAA vs. 48 h (Table 4). This outcome was unusual because seed exposed to higher temperatures usually show negative effects, especially as the time of exposure increases. To further explore this finding, we examined the influence of temperature on *Portulaca* seed germination. We confirmed that *Portulaca* exhibits high germination rates at temperatures above 20 °C (Fig. 3). However, we did not observe a reduction in germination with increasing temperatures, even at 32 °C. Others have shown that *P. oleracea* germinates at temperatures near 40 °C and higher (Verdu et al., 2004). Both species appear to have similar speed indices of germination, but *P. oleracea* appears to be more sensitive to cool temperatures than *P. grandiflora*. The germination process involves a programmed sequence of chemical reactions, each with a certain optimal temperature, especially because reactions require specific enzymatic systems to be active (Marcos Filho, 2005). At higher temperatures, water absorption velocity and chemical reactions are increased and seed germination is more rapid (Carvalho and Nakagawa, 2012). One possible reason to explain our results is the tropical origin of these *Portulaca* species (Coelho and Giulietti, 2010), where higher temperatures are common.

Higher free space did not have a negative influence on seed vigor of *P. grandiflora*, considering, for example, that lots G4 and G5 had the lowest proportion of seeds with more than 1.5% free space (classes 2 and 3), yet the lowest vigor. In all vigor tests, these lots had the lowest quality compared to other lots. For *P. oleracea*, however, extensive free space probably had an influence on seed vigor, because lot O1 had seeds with free spaces up to 10%. This affected seed vigor, as reflected in normal seedling percentage reduction after SSAA, vigor indices, and seedling length. In this case, SVIS indices after SSAA (72 h) were higher than after 48 h. This may be linked to the explanation above for *P. grandiflora*, where the highest temperature/duration could be a positive factor in this process, especially considering germination velocity and percentage results at the highest (32 °C) tested temperatures (Fig. 3).

Seed production for the herbaceous ornamental plants industry can be relatively expensive, so it is important that fast and precise methods of evaluating seed quality be available. Seed imaging analyses could facilitate seed quality evaluation, and seed lots with lower potential could be sorted out, avoiding unnecessary investments or reductions in customer satisfaction. The highest priority in seed analysis is to improve germination results using automated

FIGURE 3. Velocity of germination index (A and C) and percentage germination (B and D) of *Portulaca grandiflora* seeds, lot G2, and *Portulaca oleracea* seeds, lot O2, respectively, at different temperatures.



and more accurate techniques that can prevent human errors and be adaptable under different experimental conditions. Partnering X-radiography and computerized image analysis techniques may further automate seed sorting procedures with the development of sophisticated non-destructive methods (Dell'Áquila, 2007).

CONCLUSIONS

X-ray image analysis allowed seed morphology evaluation of *P. grandiflora* and *P. oleracea* seeds. Free spaces inside seeds seemed to have no negative effects on germination and vigor for *P. grandiflora*, although free spaces greater than 3.5% seemed to have a negative effect on seed vigor of *P. oleracea*. Seedling evaluation by SVIS could assess *Portulaca* seed quality when linked with SSAA (48 h) testing.

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