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Sensitivity of spinach seed germination to moisture is driven by oxygen availability and influenced by seed size and pericarp

Kim J.H. Magnée[1], Olga E. Scholten[1], Joeke Postma[2], Edith T. Lammerts van Bueren[1] and Steven P.C. Groot[3]*

[1] Plant Breeding, Wageningen University & Research, The Netherlands
[2] Biointeractions and Plant Health, Wageningen University & Research, The Netherlands
[3] Bioscience, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands
*Author for correspondence (E-mail: steven.groot@wur.nl)

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Abstract

Uniform seedling emergence is crucial for economically viable spinach (*Spinacia oleracea*) leaf production. However, non-uniform seed germination occurs due to variation in moisture sensitivity between and within spinach seed lots. To test moisture sensitivity, we developed a floating germination system with fixed distances between germination papers and the water table, so that moisture levels could be standardised. We tested germination performance of different cultivar seed lots, with one seed lot fractioned in different seed sizes, and of seeds with an intact, open or removed pericarp. At a high moisture level, smaller seeds germinated better than larger seeds, and seeds without a pericarp or with an open pericarp germinated better than intact seeds. Further, the pericarp of smaller seeds was thinner than the pericarp of larger seeds. A lower temperature or increased oxygen level resulted in higher germination rates for differently-sized seeds at high moisture levels. In conclusion, the sensitivity of spinach seed germination to moisture is influenced by seed size, hence pericarp thickness and intactness, and is driven by the oxygen availability to the seed embryo. To determine the full germination potential of spinach seed lots, we recommend a standardised low moisture level in addition to a temperature of 15 or 20°C.

Keywords: germination, moisture, oxygen, pericarp, seed size, spinach, temperature

Introduction

Over about 50 years, the worldwide production of spinach (*Spinacia oleracea* L.) has increased ten-fold, with a rapid increase of three-fold in the past decade (FAO, 2019). The development of fresh market clipped and bagged spinach products has been the main reason for this rapid increase. Bagged spinach contains either very small young leaves (baby spinach) or slightly older, medium-sized leaves (teenage spinach). Depending on the season, baby spinach can be harvested 21 to 40 days after sowing and teenage spinach can be harvested after 26 to 50 days (Koike *et al.*, 2011). Because of this short growth period, uniform field emergence is necessary for the production of economically viable bagged spinach. Non-uniform or poor seed germination is the main reason for non-uniform field emergence, which is a major problem for spinach production (Simko *et al.*, 2014).

Spinach seeds have a similar structure to sugar beet seeds (*Beta vulgaris* L.), both in the Amaranthaceae family. What the industry calls the 'seed' is botanically an indehiscent fruit, consisting of a true seed loosely surrounded by a fruit wall, called the pericarp (Sifton, 1927; Coumans *et al.*, 1976). In this paper, we refer to a spinach fruit with intact pericarp as an 'intact seed', and when the pericarp is absent, we call it a 'true seed' (figure 1). The loose structure that can fall out from the upper part of the pericarp we call the operculum. Due to the indeterminate flowering pattern and once-over seed harvesting of spinach, a seed lot varies in seed size and maturity level (Dolya, 1975; Deleuran *et al.*, 2013). In general, less mature seeds have lower seed vigour (Jalink *et al.*, 1998). Seed vigour is the sum of those seed properties that determine the potential of a seed lot in terms of seed germination and seedling establishment in a wide range of environments (ISTA, 2020). Variation in seed vigour between and within seed lots causes non-uniform germination and emergence in the field. In addition, spinach seed germination is highly sensitive to temperature and moisture. Germination percentages decrease when the temperature rises above 12°C (Røeggen, 1984), and may drop to 50% when it rises to 30°C (Atherton and Farooque, 1983). At 35°C, germination does not occur (Leskovar *et al.*, 1999). Therefore, the International Seed Testing Association (ISTA) advises to use temperatures of 10 or 15°C for testing spinach seed germination (ISTA, 2020). Spinach seed germination is also sensitive to moisture levels when sown in substrate (Kear *et al.*, 2005) or on germination paper (Heydecker and Orphanos, 1968). Germination on paper with high moisture content improved when temperatures were lowered (Heydecker and Orphanos, 1968). In the past, ISTA rules (ISTA, 1976) recommended to use prechilling in addition to a low moisture level in the spinach germination test. In the current ISTA rules (ISTA, 2020), prechilling is still recommended, but a low moisture level is no longer mentioned. In the ISTA Rules on germination, it is mentioned that "The water content of the growing medium should be adjusted to correspond to the needs of the species being tested, based on the maximum water-holding capacity of the medium". In our germination tests with spinach seed lots, we experienced considerable variation in their sensitivity to the moisture level of the germination paper. To determine the effect of the moisture level, we developed a floating germination system with fixed distances between the germination paper and water table, so that the moisture content of the germination paper is standardised.

Observations from spinach seeds on germination paper are that smaller seeds germinate faster and that their total germination is higher compared with larger seeds (Deleuran *et al.*, 2013). However, it was not tested whether differences in germination performance between seeds of different sizes are related to differences in moisture sensitivity. Our first hypothesis is that the germination of spinach seed lots varies in sensitivity towards moisture level due to the variation in seed size. The second hypothesis is that the pericarp determines the difference in moisture sensitivity between small and large seeds. Previous studies indicated that the pericarp can act as a physical barrier to oxygen which hampers germination. Pericarp removal or damage improved total germination on paper at high moisture levels (Heydecker and Orphanos, 1968) and at temperatures higher than 18°C

(Sifton, 1927; Atherton and Farooque, 1983; Suganuma and Ohno, 1984; Leskovar *et al.*, 1999; Katzman *et al.*, 2001). Total germination in substrate was also higher for true seeds (without pericarp) compared with intact seeds at a high moisture level (Kear *et al.*, 2005). However, these studies did not discriminate between seed sizes and they used single seed lots only. We expect that the pericarp of larger seeds, independent of seed lot, leads to a stronger limitation for oxygen diffusion to the embryo than the pericarp of smaller seeds. With respect to this, the third hypothesis is that the availability of oxygen to the spinach seed embryo is critical for germination. Previous research has shown that by increasing the oxygen level, spinach seed germination improved at room temperature (Sifton, 1927) and high moisture (Heydecker and Orphanos, 1968). We performed germination studies with a floating germination system that we developed, using spinach seeds of different cultivars and seed lots, including different seed sizes and seeds with an intact, open or removed pericarp. This allowed us to test whether variation in moisture sensitivity between and within spinach seed lots is due to variation in seed size, pericarp thickness or intactness, and whether oxygen availability to the embryo is the main driver behind germination.

Materials and methods

Plant material

In this study, seed lots of four spinach cultivars, 'Carmel' (Pop Vriend Seeds, Netherlands), 'Chevelle' (Enza Zaden, Netherlands), 'Shelby' (Enza Zaden, Netherlands) and 'Novico' (Nunhems/BASF, Netherlands) were used (table 1). No information was provided by the suppliers on the history or maturity of the seed lots. After arrival, the seeds were stored at 30% RH and 13°C until use. The 'Carmel' seed lots originated from a single harvest and had been sorted by the seed company using sieves into five seed size fractions, which allowed us to analyse the effect of seed size. The seed lots of 'Chevelle', 'Shelby' and 'Novico' originated from different seed productions and varied in seed size ranges and thousand seed weight. Some of the seeds had damage to the pericarp, especially the smaller seeds. To analyse the effect of the pericarp, we separated part of the 'Carmel' seed size fractions into subfractions of seeds with either an intact, open or damaged pericarp (figure 1) using a binocular microscope (10–40 times enlargement). We defined intact seeds as seeds with the pericarp closed or with a small crack only, with the operculum still present and the true seed not visible through the binocular. With open seeds, the operculum is absent or there is a crack around the operculum and the true seed is clearly visible through the crack. With damaged seeds, the true seed is visible from another side than the operculum side, e.g. the placenta side, or from more sides than the operculum side only.

Floating germination system

To standardise the moisture level for spinach seed germination, we developed a germination system with fixed distances between the water table and germination papers on top of polystyrene foam plates (Styrofoam) that float on the water table. Paper wicks (30 mm wide, 235 g m^{-2}) connect the germination papers (100 mm in diameter, 300 g m^{-2},

Table 1. Information on the spinach seed samples used in this study. Seed size is the range covering 95% of the approximately 1200 measured seeds. TSW is the thousand-seed weight in grams, measured by the providing companies.

Cultivar	Lot	Seed size (mm)	TSW (g)
Carmel	1A	2.50–2.75	7.44
Carmel	1B	2.75–3.50	12.60
Carmel	1C	3.50–4.25	19.99
Carmel	1D	4.25–4.50	25.17
Carmel	1E	4.50–5.00	29.24
Chevelle	1	2.25–2.75	5.47
Chevelle	2	2.50–3.75	9.98
Chevelle	3	2.50–3.75	10.81
Novico	1	2.50–3.50	9.68
Novico	2	2.75–4.25	12.85
Novico	3	3.00–5.00	17.17
Shelby	1	2.50–3.75	9.47
Shelby	2	2.50–3.75	11.01
Shelby	3	3.30–4.50	16.13

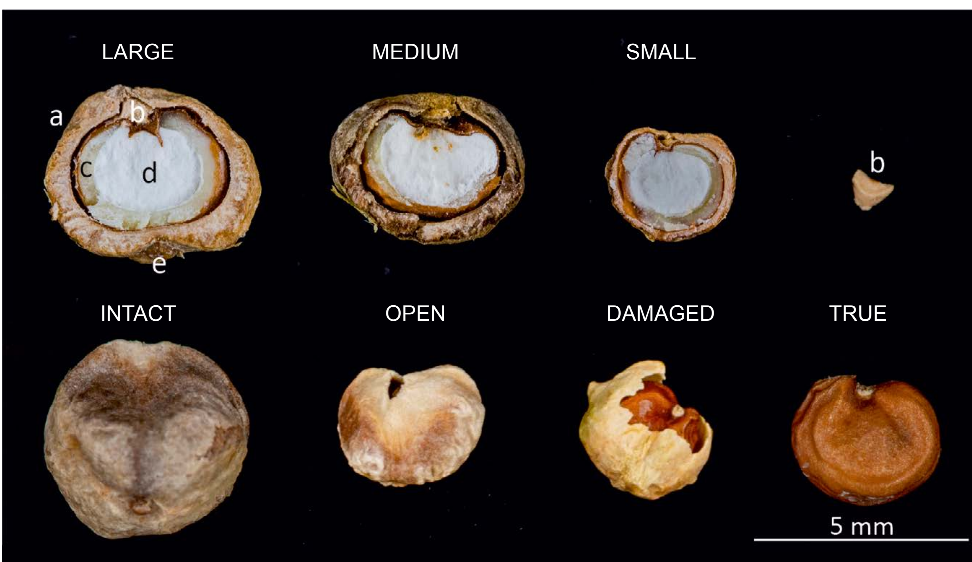

Figure 1. Cross-sections of a large (4.50–5.00 mm), medium (3.50–4.25 mm) and small (2.50–2.75 mm) spinach seed (fruit) with pericarp (**a**), operculum (**b**), embryo (**c**), perisperm (**d**) and placenta (**e**); and side views of intact, open, damaged seeds (fruits) and true spinach seeds.

Allpaper B.V., Zevenaar, NL) through holes in the Styrofoam to the water (figure 2). The height of the Styrofoam regulates the moisture content of the germination papers. Transparent bell-shaped covers with a 10 mm hole in the top were placed over the seeds to maintain a high air humidity level. The seeds had equal distance to the light source (fluorescent tubes). Two days after setting-up the floating germination system, the seeds were placed on the moist papers ('day 0').

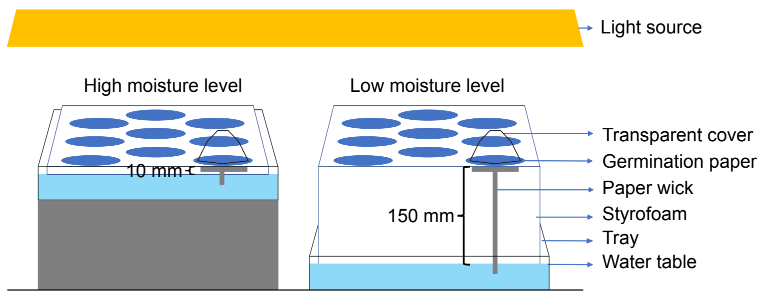

Figure 2. Illustration of germination system with germination papers on top of floating Styrofoam plates with fixed distances to the water table at 10 mm (left) and 150 mm (right) distance.

Experimental conditions
With the use of the described germination system, we tested the germination performance of the 'Carmel' seed size fractions and seed lots of cultivars 'Chevelle', 'Novico' and 'Shelby' at standardised conditions. Germination was assessed daily for 9 to 14 days. A seed was recorded as germinated when the root tip protruded from the pericarp by at least 1 mm. The number of seeds and replicates is indicated in the figure captions.

To test the effect of moisture level, we applied different heights of Styrofoam, including 10 (highest moisture level), 30, 50, 70, 100, 150, 200, 250 and 350 mm (lowest moisture level). During these experiments, the average temperature was 21°C for 12 hours of light and 18°C for 12 hours of darkness. To test the effect of temperature and moisture simultaneously, we placed the system in incubators at 10, 15 and 20°C (± 0.5°C) with three Styrofoam heights (10, 70 and 150 mm) in each incubator in darkness. To test the effect of oxygen level, we placed the system with high moisture level (10 mm Styrofoam) in air tight boxes, and flushed with 21% (air) or 100% oxygen for 20 minutes at a flow of 1 litre per minute after sowing. When sowing open seeds, the pericarp opening was pointing upwards. The germination conditions were an average temperature of 21°C for 12 hours and 18°C for 12 hours, both in darkness. Four days after sowing, germinated seeds were removed and the boxes were flushed again with air or 100% oxygen. The oxygen level in the boxes was measured daily by means of optical oxygen sensor dots (precision ± 1%, PreSens–Precision Sensing GmbH, Regensburg, Germany) placed at the inner side of the transparent lids. With the air treatment, an average level of 19% (± 1%) oxygen was measured, while flushing with 100% oxygen resulted in an average oxygen level of 89% (± 2%).

Data analysis

We determined germination performance by a number of parameters: fitted maximum germination percentage (G_{max}) at the end of the experiment, speed of germination as the time point when 50% of the maximum germinated seeds have germinated (t_{50}) and area under the curve (AUC) when plotting the germination percentages versus time. These parameters were calculated using the GERMINATOR curve fitting tool (Joosen *et al.*, 2010) with the formula:

$$y_x = y_0 + \frac{(a.x^b)}{(c^b + x^b)} ,$$

with y_x = cumulative germination percentage; y_0 = initial germination percentage (intercept on y-axis); x = time point in hours; a = maximum germination percentage (G_{max}); b = shape and steepness of curve; c = time point in hours when 50% of maximum germination has occurred (t_{50}) (El-Kassaby *et al.*, 2008). We performed ANOVA on the G_{max}, t_{50}, and AUC in Genstat 19th edition (VSN International, Hemel Hempstead, UK) to check for treatment main or interaction effects ($\alpha = 0.05$). In case of significant interaction effects, *post hoc* multiple comparisons were carried out with the Fisher's Protected Least Significant Difference (LSD) test ($\alpha = 0.05$).

Results

Moisture level of floating germination system over time

The floating germination system allowed the standardisation of moisture availability experienced by the seeds and to vary the moisture level by applying different heights of Styrofoam. With increasing Styrofoam height, the moisture content of the germination paper decreased (figure 3). Over time there was a slight, but significant increase in moisture content. After seven days, there was no significant increase in moisture content, except for 10 mm and 50 mm Styrofoam height.

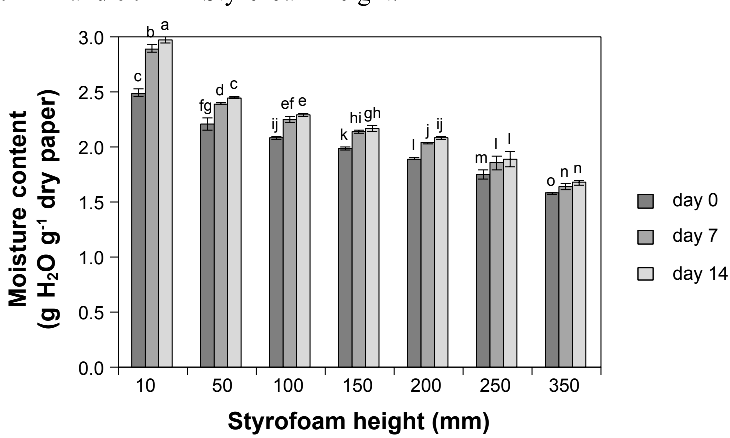

Figure 3. Moisture content of germination paper depending on Styrofoam height on the day of sowing (day 0), day 7 and day 14 of the experiment shown in figure 5. The same letter indicates homogeneous subsets according to Fisher's protected $LSD_{0.05}$. Error bars represent standard deviation (n = 3).

Effect of moisture level on germination performance of differently-sized seeds within a seed lot

To test the effect of moisture level and seed size on germination performance, we conducted an experiment with five seed size fractions of 'Carmel' and two standardised moisture levels: high and medium (10 and 70 mm Styrofoam). Germination performance varied with seed size and moisture level (figure 4). At the high moisture level, the smaller seeds (seed size fractions A and B) germinated better than the larger seeds (seed size fractions C, D and E), with a significantly higher G_{max}, lower t_{50} and higher AUC (after 336 hours). At the medium moisture level, germination of all seed size fractions was significantly better than the germination at the high moisture level. Nonetheless, the smallest seeds germinated best, with a significantly higher G_{max} and AUC, while t_{50} was equal for all seed size fractions.

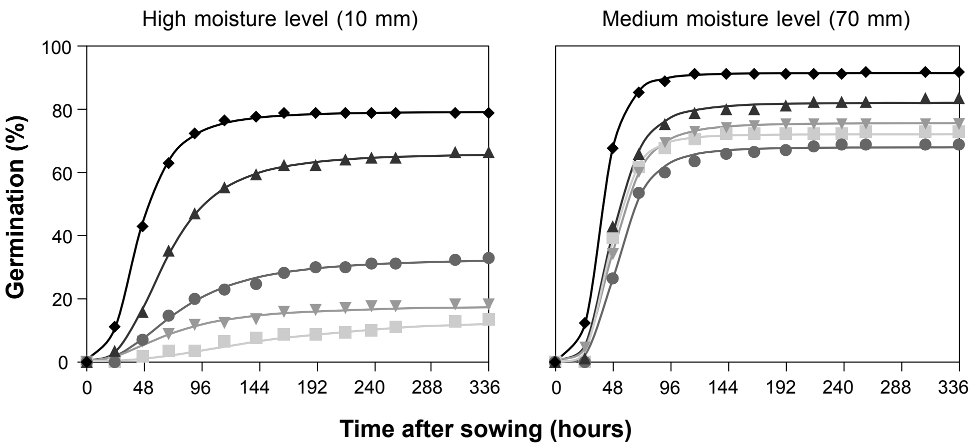

Figure 4. Germination over time of five seed size fractions of spinach cultivar 'Carmel': 2.50–2.75 mm (A, ◆), 2.75–3.50 mm (B, ▲), 3.50–4.25 mm (C, ●), 4.25–4.50 mm (D, ▼) and 4.50–5.00 mm (E, ■). The germination test was performed at high and medium moisture level (10 and 70 mm Styrofoam), with 12/12 hours light/darkness at 21/18°C. (4 × 50 randomly selected seeds).

Optimal moisture level for germination of differently-sized spinach seeds

To find an optimal moisture level for good germination performance of differently-sized spinach seeds, we tested germination performance of the smallest and largest seeds of the 'Carmel' seed size fractions at standardised moisture levels. For both seed sizes, the G_{max} and AUC (after 220 hours) was significantly lower at the highest moisture level (10 mm) compared with all other moisture levels (table 2). The G_{max} of the largest seeds was significantly higher at 150, 200 and 250 mm compared with the other moisture levels and the AUC was significantly higher at 150 mm compared with 10, 50, 100 and 350 mm. The germination performance of the smallest seeds did not differ significantly between the moisture levels, except for a significantly lower G_{max} and AUC at the highest moisture level (10 mm).

Table 2. Germination performance of intact seeds of two seed size fractions of spinach cultivar 'Carmel': 2.50–2.75 mm (A) and 4.50–5.00 mm (E). The germination test was performed at different Styrofoam heights in the floating germination system, with 12/12 hours light/darkness at 21/18°C. Data are averaged over three replicates of 40 intact seeds. Same letters indicate homogeneous subsets according to Fisher's protected $LSD_{0.05}$. SEM represents standard error of means.

Seed size fraction	Height (m)	G_{max} (%)	t_{50} (hours)	AUC (after 220 hours)
A	0.01	77.5 cd	72.1 a	109.9 f
A	0.05	90.8 ab	50.5 a	148.5 abcd
A	0.10	94.2 a	48.1 a	158.3 ab
A	0.15	93.3 a	48.1 a	158.6 ab
A	0.20	95.0 a	47.7 a	161.1 a
A	0.25	94.2 a	51.8 a	154.4 abc
A	0.35	95.0 a	57.3 a	148.2 abcd
E	0.01	6.7 e	91.8 a	7.4 g
E	0.05	70.0 d	68.1 a	97.3 f
E	0.10	80.8 bcd	52.9 a	130.4 de
E	0.15	92.5 a	52.4 a	152.9 abc
E	0.20	92.5 a	63.3 a	141.1 bcd
E	0.25	95.0 a	68.8 a	138.6 cd
E	0.35	84.2 abc	81.9 a	111.6 ef
SEM		0.04	9.04	6.51

Effect of moisture on germination performance of differently-sized seeds from different seed lots

In addition to the previous results, seeds of different sizes from cultivars 'Chevelle', 'Novico' and 'Shelby' also germinated better at the low moisture level (table 3). They had a significantly higher G_{max} and AUC (after 220 hours) at the low moisture level (150 mm Styrofoam) than at the high moisture level (10 mm). Remarkably, the 'Chevelle' seed lot 1 with relatively small seeds (2.25–2.75 mm) germinated equally well at both moisture levels and at high moisture level they even germinated best compared with all other seed lots. We observed that the 'Chevelle' seed lot 1 contained more seeds with a damaged pericarp than the other seed lots (data not shown). At the high moisture level, all 'Chevelle' seed lots germinated better, with a significantly higher G_{max} and AUC, than the other cultivar seed lots. Even at low moisture level, 'Chevelle' seed lots B and C, with a seed size range between 2.50 and 3.75 mm, germinated significantly better than 'Novico' seed lots B (2.75–4.25 mm) and C (3.00–5.00 mm) and 'Shelby' seed lot C (3.30–4.50 mm).

Effect of temperature and moisture on germination performance of differently-sized seeds

To test the effect of seed size, moisture and temperature simultaneously, we sowed three seed size fractions of 'Carmel' at high (10 mm) or low (15 mm) moisture level and at

Table 3. Germination performance of three seed lots of spinach cultivars ‘Chevelle’, ‘Novico’ or ‘Shelby’ with different seed size ranges (table 1). The germination test was performed at high and low moisture levels (10 and 150 mm Styrofoam), with 12/12 hours light/darkness at 21/18°C. Data are averaged over three replicates of 50 randomly picked seeds. Same letters indicate homogeneous subsets according to Fisher’s protected $LSD_{0.05}$. SEM represents standard error of means.

Seed lot	°C	G_{max} (%)		t_{50} (hours)		AUC (after 220 hours)	
		10 mm	150 mm	10 mm	150 mm	10 mm	150 mm
Chevelle 1	21/18	88.0 abc	92.7 ab	33.3 a	34.3 a	157.6 bc	166.7 ab
Chevelle 2	21/18	82.0 bcd	98.7 a	55.8 bcd	35.5 a	125.1 de	180.1 a
Chevelle 3	21/18	75.3 cd	98.7 a	81.6 f	44.1 ab	99.2 f	169.9 ab
Novico 1	21/18	38.0 gh	91.3 ab	66.7 de	54.2 bcd	53.5 gh	140.1 cd
Novico 2	21/18	22.0 i	83.3 bcd	85.2 f	61.4 cd	27.9 i	120.2 e
Novico 3	21/18	28.7 hi	73.3 de	80.3 f	62.1 cd	37.9 hi	108.0 ef
Shelby 1	21/18	32.0 hi	94.7 ab	77.0 ef	57.0 cd	41.8 hi	146.3 c
Shelby 2	21/18	50.0 fg	91.3 ab	88.0 f	51.3 bc	60.7 g	146.8 c
Shelby 3	21/18	61.3 ef	85.3 bcd	101.9 g	81.4 f	66.7 g	108.3 ef
SEM		0.05		4.57		6.96	

Table 4. Germination performance of three seed size fractions of spinach cultivar ‘Carmel’: 2.50–2.75 mm (A), 3.50–4.25 mm (B) and 4.50–5.00 mm (C). The germination test was performed at high and low moisture levels (10 and 150 mm Styrofoam), at 10, 15 and 20°C, without light. Data are averaged over three replicates of 50 randomly picked seeds. Same letters indicate homogeneous subsets according to Fisher’s protected $LSD_{0.05}$. SEM represents standard error of means.

Seed size fraction	°C	G_{max} (%)		t_{50} (hours)		AUC (after 220 hours)	
		10 mm	150 mm	10 mm	150 mm	10 mm	150 mm
A	10	90.7 ab	94.0 a	82.1 de	89.0 d	246.1 de	248.6 de
A	15	91.3 a	94.7 a	59.2 gh	54.6 h	269.0 bc	284.9 ab
A	20	76.0 c	93.3 a	48.1 hi	37.9 i	230.9 ef	295.3 a
C	10	68.0 d	90.7 ab	121.6 b	111.6 bc	155.9 gh	217.7 f
C	15	70.7 cd	90.0 ab	101.5 c	70.8 efg	173.3 g	257.2 cd
C	20	18.7 f	84.0 b	70.1 fg	52.3 h	52.3 j	254.7 cd
E	10	72.7 cd	96.0 a	155.9 a	118.6 b	141.4 hi	225.9 f
E	15	52.0 e	93.3 a	104.6 c	80.5 def	123.6 i	253.9 cd
E	20	17.3 f	92.0 a	51.4 h	53.7 h	50.4 j	279.8 ab
SEM		0.02		4.07		6.53	

constant 20, 15 or 10°C, without light. At the high moisture level, small seeds (2.50–2.75 mm) germinated significantly better than medium-sized (3.50–4.25 mm) and large seeds (4.50–5.00 mm) (table 4). At the high moisture level, total germination (G_{max}) of all seed size fractions was significantly higher when the temperature was 15 or 10°C compared with the total germination at 20°C. However, germination was significantly faster (lower t_{50}) for all fractions at 20°C compared with 15 and 10°C, and significantly faster at 15°C compared with 10°C. Except for the high moisture level and 20°C, small seeds germinated equally well at all conditions. Medium-sized and large seeds germinated significantly better at the low (150 mm) moisture level than at the high moisture level (10 mm). At the low moisture level, the G_{max} did not differ significantly for all seed size fractions, but the small seeds still germinated faster (lower t_{50}) than the medium-sized and large seeds.

Effect of seed pericarp on germination performance at high moisture level

For each 'Carmel' seed size fraction, 200 true seeds were isolated from their pericarp and weighed before and after removing the pericarp. As seed size increased, the pericarp weight increased more than the true seed weight (figure 5A). To test the effect of the pericarp on germination performance at high moisture level, we conducted an experiment with intact and true seeds of three seed size fractions of 'Carmel' at a relatively high moisture level (30 mm Styrofoam). True seeds of all three size fractions germinated equally fast as small intact seeds (figure 5B). With the large and medium-sized seeds, true seeds showed a significantly higher G_{max} and AUC (after 240 hours) compared with intact seeds.

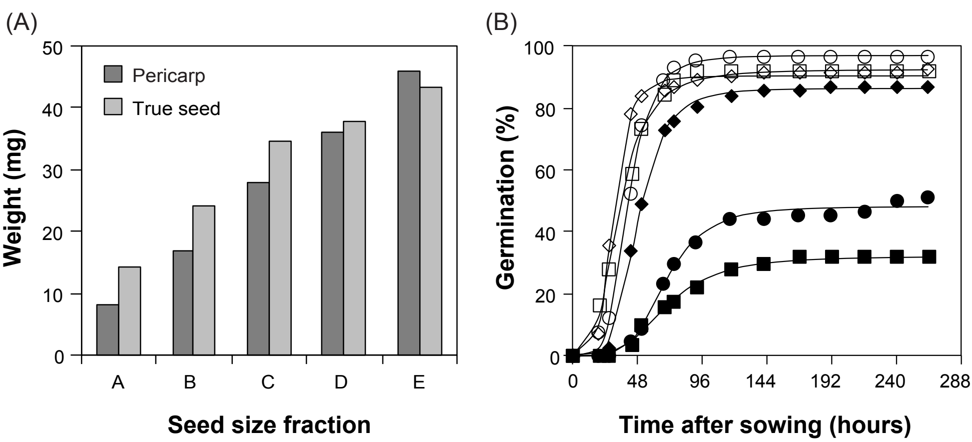

Figure 5. (**A**) Pericarp (dark grey) and true seed (light grey) fresh weight in mg per 200 seeds of the five seed size fractions of spinach cultivar 'Carmel' (no replicates): 2.50–2.75 mm (A), 2.75–3.50 mm (B), 3.50–4.25 mm (C), 4.25–5.00 mm (D) and 4.50–5.00 mm (E). (**B**) Germination over time of seed size fractions A (◆, ◇), C (●, ○) and E (■, □) of 'Carmel'. Closed symbols display measurements of intact seeds (fruits) and open symbols display measurements of true seeds (3 × 30 seeds). The germination test was performed at a relatively high moisture level (30 mm Styrofoam), with 12/12 hours light/darkness at 21/18°C.

Effect of oxygen level on germination performance of differently-sized intact and open seeds
To test the hypothesis that oxygen shortage is the limiting factor for germination at high moisture levels, we applied normal or high oxygen levels to the floating germination system with high moisture (10 mm Styrofoam). In this experiment with three seed size fractions of 'Carmel', also the effect of pericarp intactness was tested. The three fractions showed variation in the number of intact and open seeds, as the fraction with smaller seeds contained a significantly higher number of open seeds compared to the fractions with medium or larger seeds (figure 6A). The fraction with the larger seeds contained significantly fewer seeds with a damaged pericarp.

Germination performance varied with seed size and pericarp intactness (intact or open) at normal oxygen level (figure 6B). The G_{max} of all seed size fractions was significantly higher at 89% O_2 compared with 19% O_2. Small seeds germinated better, with a significantly higher G_{max} and AUC (after 240 hours) and lower t_{50} at both oxygen levels, compared with medium-sized and large seeds. At normal oxygen availability (19%), open seeds showed a significantly higher G_{max} and AUC than intact seeds, but at high oxygen availability (89%), intact and open seeds performed equally well.

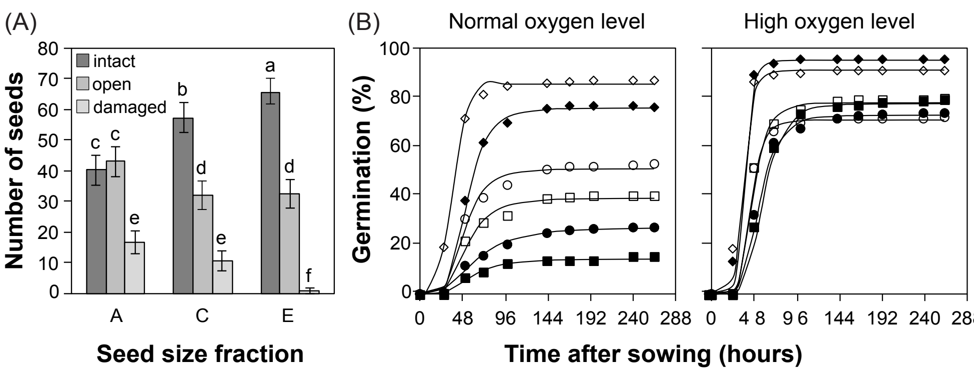

Figure 6. (**A**) Number of intact, open and damaged seeds of three seed size fractions of spinach cultivar 'Carmel': 2.50–2.75 mm (A), 3.50–4.25 mm (C) and 4.50–5.00 mm (E). Data are averaged over three replicates of 100 seeds. Error bars represent approximate standard deviation of the means and same letters indicate homogeneous subsets according to Fisher's protected $LSD_{0.05}$. (**B**) Germination over time of seed size fractions A (◆, ◇), C (●, ○) and E (■, □) of 'Carmel'. Closed symbols display measurements of intact seeds and open symbols display measurements of open seeds (3 × 25 seeds). The germination test was performed at high moisture level (10 mm Styrofoam), at normal oxygen level (19 ± 1% O_2) and high oxygen level (89 ± 2% O_2), at 21°C and without light.

Discussion

This study confirms that the germination of spinach seeds is hampered by high moisture levels, particularly in the case of large intact seeds, and that oxygen availability to the seed embryo is critical for germination. With the use of different seed lots from three different spinach cultivars and one cultivar seed lot fractioned in different seed sizes, we found that spinach seeds show considerable variation in their sensitivity towards high moisture levels. For instance, germination of large seeds varied from 90% at low moisture to 10% at high moisture level, whereas small seeds still germinated to 80% at a high moisture level. To test the variation in moisture sensitivity, a germination system with standardised moisture levels was necessary. Therefore, we developed a floating germination system with fixed distances between the water table and germination papers. By testing a range of moisture levels, we observed that total germination of differently-sized spinach seeds was higher at a relatively low moisture level (100–250 mm Styrofoam) compared with total germination at a high moisture level (10 mm). Total germination was again lower when the moisture level was rather low (350 mm), indicating water shortage at this level. We concluded that 150 mm Styrofoam height in the floating germination system gives the optimal moisture level for germination of spinach seeds with a diameter between 2.50 and 5.00 mm.

This study shows that variation in moisture sensitivity is related to variation in seed size that was observed between and within spinach seed lots, and that pericarp thickness and intactness plays a role. Larger seeds, with a thicker pericarp, were more sensitive to high moisture, with a lower performance than smaller seeds, irrespective of cultivar seed lot. Seed size ranges differed among cultivars, which complicated the analyses of the results. Though, the results of the 'Carmel' seed size fractions together with the results of the other seed lots confirm that relatively smaller seeds (< 3.50 mm) are less sensitive to high moisture level than larger seeds (> 3.50 mm). When comparing the 'Carmel' seed size fractions, we observed that pericarp thickness increased with seed size, which may explain why larger seed size fractions contained more intact seeds and less open or damaged seeds. Results from a previous study (Kear *et al.*, 2005) indicated that intact seeds are more sensitive to high moisture levels than true seeds, but the authors compared intact and true seeds from different cultivars and they could not exclude a cultivar effect. Results from another study, with one seed lot only, showed that pericarp removal resulted in higher germination rates of spinach seeds at high moisture level (Heydecker and Orphanos, 1968). Neither study discriminated between seed sizes or pericarp intactness. With the use of the seed size fractions of 'Carmel', we were able to prove that, in contrast to intact seeds, true seeds obtained from small or large seeds were not sensitive to high moisture levels. In addition, we observed that open seeds obtained from small or large seeds were less sensitive to high moisture level than intact seeds of same size. These results confirm the hypothesis that the pericarp hampers germination at high moisture levels.

Finally, this study led to the conclusion that oxygen shortage at high moisture levels is the cause of germination inhibition. Germination results using air-tight boxes with normal or high oxygen levels, showed that total germination of especially the large intact seeds was higher with a high oxygen level. This indicates that the pericarp of large seeds limits the oxygen diffusion to the seed embryo to a larger extend than the pericarp of small seeds. Lowering the temperature resulted in a higher total germination of all seed size fractions at high moisture level, though small seeds still germinated faster than large seeds. These results are in line with previous studies showing that an increased oxygen pressure and a lower temperature provides higher germination rates (Sifton, 1927; Heydecker and

Orphanos, 1968). The current ISTA rules about spinach seed testing prescribe the use of relatively low temperatures of 10 or 15°C. In our experiments, even at those temperatures, germination of larger seeds was hampered at high moisture levels. We also observed that germination was significantly slower at 10°C than at 15 or 20°C. The slower germination and higher total germination at 10°C can be explained by either a reduction in metabolic activity, hence a lower oxygen consumption, an increased solubility of oxygen in water, or a combination of both. Likely, a thin or open pericarp results in more oxygen diffusion to the embryo and therefore smaller seeds, having a thinner and more frequently open pericarp than larger seeds, germinated better than larger seeds in our experiments with high moisture level. Botanically related sugar beet seeds also germinated better on germination paper with the open basal pore facing upwards rather than downwards (Coumans *et al.*, 1976). These results suggest that air supply through openings or a thin pericarp improves oxygen supply to the seed embryo and stimulates germination.

At present it is not clear whether the physical limitations in oxygen diffusion by the pericarp is the only reason, or that additional factors play a role. Heydecker and Orphanos (1986) stated that formation of mucilage around and within the pericarp at high moisture levels forms a diffusion barrier to oxygen and that the pericarp alone cannot prevent germination as long as no mucilage is formed. They hypothesised that without mucilage formation, the oxygen can diffuse to the embryo via the scar where the pericarp was attached to the mother plant. However, we did not observe mucilage formation with the cultivars used in this study. Moreover, our experiment with a higher oxygen level and intact or open pericarp, showed that the pericarp alone can form a physical diffusion barrier to oxygen at high moisture levels. We cannot exclude that other pericarp factors also play a role. The pericarp of sugar beet seeds contains both abscisic acid, a germination inhibiting plant hormone, and phenolic compounds that bind oxygen (Dolya, 1975; Hermann *et al.*, 2007). Further research on a potential chemical barrier of the spinach seed pericarp is needed for a better understanding of its germination inhibiting effect.

Recommendations

With the knowledge gained from this study, we make some recommendations for spinach production as well as for spinach seed testing. When sown in the field, measures should be taken to prevent high soil moisture conditions. During seed processing, the smallest seeds are generally discarded, because they are expected to be less mature and less vigorous than larger seeds. However, on germination paper we found that smaller seeds are less sensitive to high moisture levels and can germinate even faster than larger seeds. Seed sorting based on chlorophyll fluorescence (the lower the CF, the higher the maturity level) can be used to exclude the less mature small seeds (Deleuran *et al.*, 2013). In addition, the pericarp of larger seeds can be (partly) removed, if commercially feasible, for instance by a seed peeling apparatus (Toshiyuki, 1992). For breeding purposes, it would be worthwhile to analyse whether genetic variation exists for pericarp thickness or intactness. Then, it is also important to take into account that differently-sized spinach seeds vary in their sensitivity to moisture. By using a germination test system with standardised moisture levels, as in this study, the full germination potential of spinach seed lots can be tested. The use of standardised lower moisture levels enables germination testing at higher temperatures, allowing a shorter period for evaluation. Therefore, we suggest ISTA to take moisture level in their considerations for the spinach seed germination testing protocol, using standardised low moisture levels and potentially higher temperatures of constant 15 or 20°C. Our method generates a specific moisture level suitable for testing seeds for germination performance. We found that a distance of 150 mm, with a moisture content of about 2.1 g H_2O g^{-1} germination paper, was optimal for spinach seeds with a diameter between 2.5 and 5.0 mm. When other germination paper is used the optimal moisture content can be easily determined again by using the floating system with different fixed heights. Our method has some similarity with the "Rodewald Method" (FAO, 1985). With this method, a water channel integrated in sand with an adjustable level gives moisture to the filter paper via wicks in the sand and via the capillary effect of the sand (Rubarth Apparate GmbH., 2019). We consider our system simpler and easier to use when it comes to determining the optimal distance between the specific germination paper and the water table for spinach seeds. That knowledge can be used in combination with a Jacobsen apparatus (ISTA, 2020), if this apparatus can be adjusted to the optimal distance.

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