

Higher seed maturity levels, darker pericarp, and smaller seed size relate to improved damping-off tolerance in spinach

Kim J.H. Magnée^{a,b}, Olga E. Scholten^a, Jan Kodde^a, Joeke Postma^a, Gerrit Gort^c, Edith T. Lammerts van Bueren^a, Steven P.C. Groot^{a,*}

^a Wageningen Plant Research, Wageningen University & Research, P.O. Box 16, 6700 AA, Wageningen, the Netherlands

^b Certis Belchim B.V., P.O. Box 607, 3500 AP, Utrecht, the Netherlands

^c Biometris, Wageningen University & Research, P.O. Box 16, 6700 HB, Wageningen, the Netherlands

ARTICLE INFO

Keywords:

Chlorophyll fluorescence
Damping off
Multispectral imaging
Light reflectance
Pythium ultimum
Spinacia oleracea
Seed quality

ABSTRACT

With the growing demand for baby-leaf spinach (*Spinacia oleracea* L.) and an increasing ban on chemical seed treatments, problems of damping-off diseases become more apparent. *Pythium ultimum* has been shown to be the most common damping-off pathogen of spinach. Previous studies showed a large variation in pre-emergence damping-off tolerance levels among seed lots of spinach cultivars, which did not correlate with the rate of seedling emergence. In this study, we analysed if individual seed vigor-related traits, such as seed size and maturity, can influence seed tolerance towards pre-emergence infection by *P. ultimum*. Seeds of the same seed lots were measured for their morphological and multispectral properties on the level of a single seed. The seedling emergence from those individual seeds was assessed at different *P. ultimum* doses and for each individual seed, the characteristics were correlated with its emergence success. The results showed that relatively higher levels of chlorophyll fluorescence, an indicator of lesser seed maturity, had a negative association with emergence success. With increasing *P. ultimum* dose, the seed size and light reflectance from the pericarp, particularly with violet-blue light, showed an increasingly negative association with emergence. This indicated that smaller seeds and seeds that reflected less (violet-blue) light were more tolerant towards *P. ultimum* infection. A validation study with seed size and maturity fractions of a single seed lot (excluding potential genotypic and seed production effects) confirmed that seed maturity had a positive association with seedling emergence, and that seed size had a negative association with the emergence in the presence of *P. ultimum*. Seed size and light reflectance accounted for a small part of the variation in *P. ultimum* tolerance among the seed lots. Selecting the most mature seeds with relatively less light reflectance (darker pericarp) or mature seeds with smaller sizes is recommended to obtain seed lots with improved pre-emergence damping-off tolerance levels.

1. Introduction

The production of fresh-market baby-leaf spinach requires uniform seedling establishment since the leaves are harvested within three to six weeks after sowing (Koike et al., 2011). Damping-off diseases can cause non-uniform seedling establishment and severe yield losses. Damping-off symptoms can occur in the seed germination or seedling stages, causing either seed or root rot before the seedling emerges above

soil level (pre-emergence damping off) or seedling root rot and wilting after emergence (post-emergence damping off) (Lamichhane et al., 2017). These symptoms can be caused by multiple soilborne microorganisms, of which *Pythium ultimum* has been shown to be the most prevalent in causing spinach pre-emergence damping off (Koike et al., 2011; Larsson, 1994; Magnée et al., 2022). Management of damping-off includes seed treatments with synthetic fungicides, but their use is scientifically and socially debated because of potential resistance

Abbreviations: AIC, Akaike's information criterion; AU, arbitrary units; Blue, reflectance at 420–480nm; CF, chlorophyll fluorescence; Dose, *P. ultimum* dose; glmm, generalised linear mixed model; Green, reflectance at 535–585nm; Hue, Colour reflectance; Int, light reflection intensity; Red, reflectance at 602.5–677.5nm; RefA, reflectance on a scale between green and red; RefB, reflectance on a scale between blue and yellow; RefL, luminescence; RC, regression coefficient; Sat, saturation; SD, standard deviation.

* Corresponding author

E-mail address: steven.groot@wur.nl (S.P.C. Groot).

<https://doi.org/10.1016/j.scienta.2023.112219>

Received 28 March 2023; Received in revised form 29 April 2023; Accepted 5 June 2023

Available online 22 June 2023

0304-4238/© 2023 Wageningen University & Research. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Table 1

Information on the spinach seed lots used in this study, including the seed size fractions of seed lot D of cultivar Carmel (D.1, D.3, D.5), and subfractions of Carmel-D.1 seeds with low and high chlorophyll fluorescence levels (low CF and high CF).

Experiment	Cultivar	Seed lot	Seed size (mm)	Thousand seed weight (g)	Providing company (country)
I and II	Carmel	A	2.75–3.75	11.2	Pop Vriend Seeds (The Netherlands)
		B	2.75–3.75	12.6	
		C	2.50–3.75	12.7	
III	Carmel	D.1	2.50–2.75	7.4	
		D.1 low CF	2.50–2.75	not determined	
		D.1 high CF	2.50–2.75	not determined	
		D.3	3.50–4.25	20.0	
		D.5	4.50–5.00	29.2	
I and II	Chevelle	A	2.25–2.75	5.5	Enza Zaden/Vitalis (The Netherlands)
		B	2.50–3.75	10.0	
		C	2.50–3.75	10.8	
I and II	Mirage	A	2.75–3.75	9.8	Sakata Seeds (France)
		B	2.50–3.75	9.9	
		C	2.75–3.75	10.5	
I and II	Novico	A	2.50–3.50	9.7	Nunhems/BASF (The Netherlands)
		B	2.75–4.25	12.6	
		C	3.00–5.00	17.2	
I and II	Shelby	A	2.50–3.75	9.5	Enza Zaden/Vitalis (The Netherlands)
		B	2.50–3.75	11.0	
		C	3.30–4.50	16.1	

development by the pathogens and potential side-effects on non-target organisms and the health of operators (Lamichhane et al., 2020). Related to this, synthetic fungicides are increasingly banned worldwide (European Commission, 2021; United Nations Environment Programme (UNEP), 2006). Moreover, they are not allowed in organic crop production. As the demand for non-chemically treated seeds increases, issues with pre-emergence damping off occur more often. This urges for alternative solutions, e.g., the use of seed treatments with biological products (Lamichhane et al., 2022), breeding for cultivars with improved tolerance levels, innovative seed sorting methods or mechanical treatments, or a combination of several of these. This study aimed to find certain seed traits that can improve pre-emergence damping-off tolerance in spinach.

Previous studies on pre-emergence damping-off tolerance demonstrated large variations among seed lots, confounding the search for potential genotypic variation (Magnée et al., 2022). Therefore, we hypothesised that other individual seed vigor-related seed traits may be involved. The spinach seed is botanically an indehiscent, dry fruit with a true seed covered by a fruit coat, the so-called pericarp (Sifton, 1927). Due to the indeterminate flowering habit of spinach and the once-over harvest of the seeds, the produced spinach seeds may differ in seed size and/or maturity levels (Deleuran et al., 2013). Also, seed lots originating from different production environments may differ in certain seed characteristics.

The general observation is that larger seeds have a greater emergence capacity, provide more vigorous seedlings, with higher survival rates, and greater crop yields (i.e., as shown for wheat (Das Gupta and Austenson, 1973), broccoli (Heather and Siczka, 1991), olive (Rey et al., 2004), legumes (Arellano and Peco, 2012), soybean (Vange et al., 2016), and ginseng (Zhang et al., 2018)). Seed size may also relate to biotic stress tolerance, as a study on lucerne seeds showed that seedlings developed from heavier seeds grew more vigorously in *Pythium*-infected soils than seedlings from smaller seeds (Hawthorne, 1988). Also for spinach, a positive correlation between seed size and germination has been reported, although additional factors seemed to be involved (Shetty et al., 2012). Seed size can be influenced by the seed maturity level, the genotype and the environment (Fenner, 1992). Generally, under relatively dry conditions, seeds may become larger when the plant develops fewer branches and can allocate resources to fewer individual seeds, whereas under wet conditions, seeds may remain smaller (Baker, 1972). So, larger seeds are not more mature per se when they originate from different genotypes, seed production environments or from different mother plants with a production. Hence, seed size and maturity

level of individual seeds were both investigated in this study, using seed lots from different genotypes and productions. In addition, seed size and maturity fractions were obtained from a single seed lot, excluding potential genotypic and environmental effects.

Developing seeds are often green, caused by the presence of chlorophyll. During seed maturation, the amount of chlorophyll in the seed or its coat decreases (Steckel et al., 1989; Ward et al., 1995). The chlorophyll content of individual seeds can be determined by its fluorescent properties, and for many crops, including cabbage, pepper, and tomato, chlorophyll fluorescence (CF) level has shown to be a good marker for the seed maturity level, with an inverse correlation with seed germination (Jalink et al., 1998). Also for spinach seeds, when fluorescence of the pericarp was measured, CF has been demonstrated to correlate negatively with seed germination (Deleuran et al., 2013). CF can be measured after emitting light onto the seed at 450 or 660 nm and measuring excitation from the seed at 730 nm (Jalink et al., 1998). This non-destructive technique is highly specific so that CF measurements are not likely to be influenced by other substances colouring the seed coat or pericarp. Several studies showed a relation between seed coat color and seed quality, including abiotic stress tolerance, like salt tolerance in red clover (Atis et al., 2011), and biotic stress tolerance, e.g., damping-off tolerance in pine seeds (Grzywacz and Rosochacka, 1980). Pericarp color, measured by light reflectance, can also serve as a marker for the presence of fungal infection of spinach (Olesen et al., 2011) and barley seeds (Boelt et al., 2018). This has triggered our interest in studying pericarp color in relation to pre-emergence damping-off tolerance.

In this study, we investigated the potential relation between seed traits and pre-emergence damping-off tolerance by measuring for individual seeds the area, length and width, roundness, CF levels, and light reflectance along the visible and near-infrared light spectrum. After these measurements, seeds were individually sown in *P. ultimum*-inoculated or non-inoculated (control) substrate. Seedling emergence was recorded and related to the different seed characteristics. Seeds of three commercially produced seed lots of each of five spinach cultivars were used to determine which seed traits correlate significantly, positive or negative, with emergence success in the presence of different *P. ultimum* inoculum doses. Eventually, we generated a model to study the contribution of each predictor in relation to *P. ultimum* dose. Seed size or maturity fractions of a single seed lot were screened to validate a potential effect of these two traits, without the influence of a potential genotype-by-environment effect. The hypothesis was that the large variation in tolerance levels among seed lots originating from different genotypes and environments could be explained (partly) by one or more

of these seed traits.

2. Material and methods

2.1. Plant material

Untreated seeds of three independently produced seed lots (coded 'A', 'B' or 'C') of five spinach cultivars, 'Carmel', 'Chevelle', 'Mirage', 'Novico', and 'Shelby', were used for Experiments I and II (Table 1). For readability when describing a seed lot of a cultivar, the cultivar name was hyphenated with the seed lot letter, e.g., Carmel-A. Untreated seeds of a fourth Carmel seed production (lot coded 'D') had been fractionated by the supplier into five different seed size ranges (1–5), from which three fractions (1, 3 and 5) were used in Experiment III. All seeds were stored at 30% relative humidity (RH) and 13 °C between receipt in 2015 and use in 2020 (Experiments I and II) and in 2021 (Experiment III). A week before Experiment III, part of size fraction Carmel-D.1 was sorted into maturity classes, based on their chlorophyll fluorescence (CF) level. Low CF seeds had a level below 6,000 CF in arbitrary units (AU) and high CF seeds had a level above 12,000 CF (in AU) (see seed trait measurements).

2.2. Seed trait measurements

Before sowing, seeds were individually measured for several seed traits, including area, roundness, and color, with the VideometerLab instrument (Videometer A/S, Herlev, Denmark, version 3.12.27 with software revision 7311). Fruit (or seed) area was measured by the number of pixels in an image, and roundness was calculated by $\text{circumference}^2 \times (4\pi \times \text{area})^{-1}$ with a number ≥ 1 : the closer to one, the more round the seed. Color was determined by measuring light reflectance in arbitrary units (AU) at 19 unique wavelengths, ranging from 375 (violet light) to 970 nm (near-infrared). Also, total color reflectance (Hue), saturation (Sat), and light reflection intensity (Int) were measured in addition to CIE*LAB (here specified as RefL, RefA, RefB), which represents a 3D color space defined by the International Commission on Illumination (Commission Internationale de l'Éclairage (CIE), www.cie.co.at). RefL stands for luminance, corresponding to a number between 0 (dark) and 100 (bright), RefA stands for reflectance on a scale between green and red, and RefB stands for reflectance on a scale between blue and yellow. In addition, reflectance of red, green, and blue (RGB) light and chlorophyll fluorescence level (CF) were measured with the PathoScreen™ (PhenoVation Life Sciences, Wageningen, the Netherlands). After exposing seeds to white light, RGB reflectance from the seed was measured through a color filter that filtered for specific wavelength ranges: 602.5–677.5 nm ('Red'), 535–585 nm ('Green'), and 420–480 nm ('Blue'). CF, as an indicator of seed maturity (Jalink et al., 1998), was measured after exposing seeds to light of 660 nm and measuring fluorescence at 730 nm. Low CF seeds were selected after setting the CF mask at 6,000 CF in AU. High CF seeds were selected after setting the CF mask at 12,000 CF in AU.

2.3. Pre-emergence damping-off assay

Three experiments were performed with an in-house developed phenotyping assay containing different doses of the cornmeal /sand-based *P. ultimum* inoculum: 0% and 5% (Experiment I), 1.25 and 2.50% (Experiment II), and 0, 1.9 and 2.5% (Experiment III) of inoculum fresh weight versus total substrate weight before the addition of tap water. The preparation of the *P. ultimum* inoculum and the phenotyping assay was based on previously described methods (Magnée et al., 2022), using *P. ultimum* isolate 8b (NCBI accession no. MZ026502 for ITS and MZ152003 for COI). In short, the substrate was a combination of autoclaved dried sand, perlite and vermiculite in an equal volume-based ratio, moistened with tap water to 50% water holding capacity. A cornmeal/sand-based inoculum of *P. ultimum* was added to the substrate

in different doses, as indicated. The prepared substrate was placed in cells in transparent polystyrene dishes (100 × 100 × 18 mm containing 25 cells each). *P. ultimum* has been renamed *Globisporangium ultimum* (Uzuhashi et al., 2010). However, for the sake of continuance, we here use the basionym *Pythium ultimum*. For Experiments I and II, the two *P. ultimum* inoculum doses were positioned in a different stack, but seeds were sown within the same time frame, so that the two stacks together formed a time block, which was performed three times. This resulted in a split-plot design with three complete blocks as replicates, including stacks as main plot units and *P. ultimum* treatment as main plot factor, and trays (within stack) as subplot units with seed lot as subplot factor, and 50 seeds per tray (containing two polystyrene dishes). For Experiment III, all trays were randomly stacked, including the three *P. ultimum* treatments (0, 1.9%, and 2.5% *P. ultimum* dose), resulting in a randomised complete block design with five complete blocks as replicates. For each experiment, the replicates were randomly distributed in a climate-controlled cabinet set at 15 °C in the dark. The positions of the blocks were switched daily, as was the order of the trays within each stack. Spinach seedling emergence, seedling tissue visible above the substrate, was assessed daily until 10 days after sowing. Cells lacking seedling emergence were checked under the substrate and rotten seeds or seedlings were considered sensitive to damping off.

2.4. Statistical analysis

The combined binary data of emergence (1) versus non-emergence (0), and continuous data of the individual seed traits, including the factors representing the experimental design, cultivars, and seed lots, were analysed in R version 4.0.4 and RStudio version 1.2.5042 (RStudio Team, 2020). All seed trait variables showed reasonably symmetrical distributions, except CF, which needed a log-transformation. Seed trait data were standardised by subtracting the overall mean and dividing by the standard deviation. To examine the collinearity among the seed traits, we first calculated Pearson's correlations among the seed traits, measured from a total of 8,950 seeds (50 seeds of a single tray were not measured for RGB). For pre-emergence damping-off tolerance, we applied a generalised linear mixed model (glmm), assuming a binomial distribution of the binary emergence data. A logit link function was used to link the probability of emergence to the diverse explanatory variables, including factors with fixed and with random effects. The fixed factors included block, cultivar, and treatment. The random factors included seed lot, stack (only for the split-plot designs of Experiments I and II) and tray. With Type II Wald χ^2 -tests, we tested for significant effects of the fixed factors.

To determine which seed traits contributed most to the emergence success for Experiments I and II, we first split the data for the four separate *P. ultimum* treatments (0, 1.25, 2.5 or 5% *P. ultimum* dose). Modified glmm's, without the *P. ultimum* treatment factor and with seed lot as a random factor, were fitted to the four datasets. Due to the high collinearity among the measured seed trait variables, we followed a forward regression approach to find those seed traits that associated most with emergence (on a logit scale). With each *P. ultimum* dose, the null model was extended by a single seed trait variable at a time with the "dredge" function of package MuMIn in R. Submodels were sorted by the Akaike's information criterion (AIC) value, which is an indicator of the model fit: The lower the AIC, the better the fit of the model. We labelled the model as improved when the AIC was reduced by at least 2 units ($\Delta\text{AIC} \geq 2$). The variables that improved the model with less than 2 units were also taken into consideration for the selection of regressors (Arnold, 2010).

These steps led to the selection of the most contributing seed traits that were further analysed with the complete dataset with all four *P. ultimum* doses. For this purpose, *P. ultimum* dose was included as a quantitative explanatory variable with a fixed effect, but it was also allowed to interact with the selected seed traits. Again, the model fit was evaluated by the AIC, following a forward regression approach with the

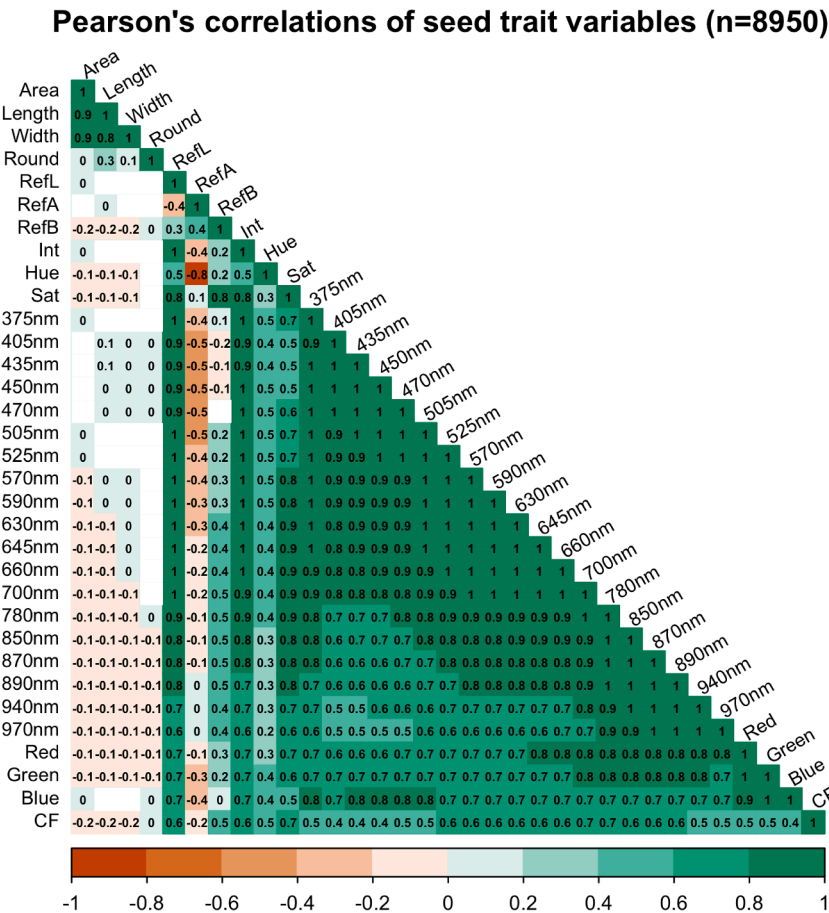


Fig. 1. Visualization of Pearson's correlation coefficients among seed trait variables of individual seeds of 15 spinach seed lots tested ($n = 8,950$). All variables were standardised by subtracting the overall mean and dividing by the standard deviation, and chlorophyll fluorescence (CF) was log-transformed before standardization. Color and color intensity are determined by the value of the correlation coefficients with positive correlations displayed in green and negative correlations displayed in orange. Insignificant correlations ($p < 0.01$) were left blank.

“dredge” function in R. In this way, the most contributing traits with their interactions with *P. ultimum* were selected. Finally, an all-possible subset approach was used for the selected traits and interactions to find the best model fit, according to the AIC. The regression coefficients of these traits and their interactions with *P. ultimum* dose were used to develop a model estimating the emergence from spinach seeds with specific traits, depending on the *P. ultimum* dose. The effects of seed size and CF were validated in Experiment III. The contributions of these two quantitative traits and their interaction with *P. ultimum* treatment were also evaluated using the AIC and the regression coefficients.

As a quantification of the amount of variation among seed lots that could be explained by seed traits, we looked at the reduction in the

variance component of seed lot after the addition of a single seed trait to the model. A reduction in the variance component of seed lot was expected after the inclusion of a seed trait that causes seed lot variance.

3. Results

3.1. Correlations among seed trait variables measured from individual seeds

From all individual seeds used in Experiments I and II ($n = 8,950$), the morphological trait variables Area, Length, and Width showed a large collinearity, with correlations close to 1 (Fig. 1). Roundness

Table 2
Contributions of seed traits to the fitting of a model for seedling emergence for each of the four *Pythium ultimum* doses. The model fit measured by Akaike's information criterion (AIC) of the null models (including fixed effects of cultivar and replicate, and random effects of seed lot) and of the extended models is shown. The coefficients correspond to the variables in bold obtained from the model that showed the best fit with an AIC reduction of ≥ 2 compared to the reduced model. CF was log-transformed, and all trait variables were standardised by subtracting the overall mean and dividing by the standard deviation.

<i>P. ultimum</i> dose	Model	Model fit (AIC)	Coefficient of additional trait variable	
0.00%	Null	1,204.5		
	Null + Area	1,197.0	+0.34	
	Null + Area + 405 nm	1,194.5	+0.19	
1.25%	Null	2,453.1		
	Null + ^e log(CF)	2,442.7	−0.22*	−0.25**
	Null + ^e log(CF) + RefA	2,440.5	−0.11	
	Null + ^e log(CF) + Hue	2,440.6	+0.12	
2.50%	Null	2,654.4		
	Null + ^e log(CF)	2,639.4	−0.22	
5.00%	Null	1,538.7		
	Null + Blue	1,520.8	−0.37	

* coefficient for ^elog(CF) in model with RefA.
** coefficient for ^elog(CF) in model with Hue.

Table 3

Overview of the contributions of selected seed traits to fitting of a model with *Pythium ultimum* dose as a quantitative treatment variable (Dose = *P. ultimum* dose) and fixed effects of experiment, blocks within experiment, cultivar, cultivar-by-treatment interaction; and random effects of seed lot, seed lot-by-treatment interaction and tray. Seed trait variables include seed size (Area), reflectance at 420–480 nm (Blue) and other individual wavelengths, chlorophyll fluorescence (CF), color (Hue), reflectance between green and red (RefA), and their interactions with *P. ultimum* dose. CF was log-transformed and all seed trait variables were standardised by subtracting the overall mean and dividing by the standard deviation. A) First step: evaluating the model fit by Akaike's information criterion (AIC) after the addition of a single trait and trait:Dose interaction, and p-values corresponding to the main seed trait and trait:Dose interaction effects based on the Type II Wald Chi² test. B) Second step: evaluating the model fit by AIC of the best possible combinations of traits in addition to CF. Models were sorted by AIC from high to low. The models indicated in italics are the best fitting models in the first and second steps.

A)	First step full model selection	Model fit (AIC)	p-value trait effect	p-value trait:Dose effect
	Null	7,863.9		
	Null + Area + Area:Dose	7,863.6	0.696	0.040
	Null + Hue + Hue:Dose	7,859.5	0.023	0.088
	Null + RefA + RefA:Dose	7,852.8	0.070	0.001
	Null + 405 nm + 405 nm:Dose	7,843.0	0.011	<0.001
	Null + 435 nm + 435 nm:Dose	7,834.2	<0.001	<0.001
	Null + 450 nm + 450 nm:Dose	7,832.1	<0.001	<0.001
	Null + Blue + Blue:Dose	7,831.9	<0.001	<0.001
	Null + 470 nm + 470 nm:Dose	7,831.8	<0.001	<0.001
	<i>Null + ^elog(CF) + ^elog(CF):Dose</i>	7,828.8	<0.001	0.049
B)	Second step full model selection	model fit (AIC)		
	Null + ^e log(CF) + ^e log(CF):Dose + Area + Area:Dose	7,827.7		
	Null + ^e log(CF) + ^e log(CF):Dose + Blue + Blue:Dose + Area	7,815.5		
	Null + ^e log(CF) + Area + Blue + Blue:Dose	7,813.9		
	Null + ^e log(CF) + ^e log(CF):Dose + Blue + Blue:Dose	7,813.6		
	Null + ^e log(CF) + ^e log(CF):Dose + Area + Area:Dose + Blue + Blue:Dose	7,813.5		
	Null + ^e log(CF) + Blue + Blue:Dose	7,811.9		
	<i>Null + ^elog(CF) + Area + Area:Dose + Blue + Blue:Dose</i>	7,811.6		

(Round) did not correlate with Area but correlated positively with Width and Length. Also, there was strong collinearity among the spectral measurements. RefL and RefA did not show significant correlations with the morphological seed traits, while RefB and CF showed a negative correlation (−0.2) with Area. CF correlated positively with all other spectral measurements (0.4 to 0.7).

3.2. Pre-selection of seed traits that correlated with seedling emergence at the four *P. ultimum* doses

For the development of a model for emergence in relation to the *P. ultimum* dose, pre-selection of seed trait variables was necessary due to the high collinearity among variables. For this purpose, the datasets of the two Experiments I and II were first split with respect to the four separate *P. ultimum* doses. For emergence in the substrate without *P. ultimum*, the addition of Area (seed size) to the null model, which contained fixed effects of cultivar and block, and random effects of seed lot, resulted in the largest model fit improvement, with 7.5 AIC units difference and a positive regression coefficient, compared to the other models with a single trait variable (Supplemental Table 1A and B). The addition of reflection measurements from 405 nm (violet) to 470 nm (blue) resulted in a slight improvement of the model fit compared to the

Table 4

Coefficients for main and interaction effects of each seed trait and seed trait-by-treatment (Dose = *Pythium ultimum* dose, as a quantitative variable) interaction in the model with fixed effects of experiment, blocks within experiment, cultivar, cultivar-by-treatment (as a quantitative variable), and the quantitative trait variables for log-transformed chlorophyll fluorescence (^elog(CF)), seed size (Area), reflectance at 420–480 nm (Blue), and their interaction effects with *P. ultimum* dose. All seed trait variables were standardised by subtracting the overall mean and dividing by the standard deviation.

Fixed effect	Coefficient	p-value
Intercept	+2.43	<0.001
Dose	−1.05	<0.001
^e log(CF)	−0.17	<0.001
Area	+0.11	0.12
Blue	+0.23	<0.001
Area:Dose	−0.05	0.04
Blue:Dose	−0.13	<0.001

null model, also with positive regression coefficients. Only for 405 nm, the ΔAIC was more than 2 units. The addition of any other variable to Area and reflectance at 405 nm did not substantially improve the model (ΔAIC<2) (Supplemental Table 2). The extended model with Area and reflectance at 405 nm demonstrated a positive regression coefficient of 0.34 and 0.19 respectively, with emergence (on a logit scale) in the substrate without *P. ultimum*, as summarized in Table 2.

When *P. ultimum* was added at a 1.25% inoculum dose, the addition of CF to the null model resulted in the largest model fit improvement with more than 10 AIC units (Supplemental Table 1B). Reflection measurements ranging from 700 nm (red) to 970 nm (near-infrared) improved the model fit by at least 2 AIC units compared to the null model. In addition to CF, RefA (red-yellow) or Hue contributed to a better fit of the model compared to the model with CF alone (Supplemental Table 2). The extended model had negative coefficients for CF and RefA of −0.22 and −0.11, respectively, with seedling emergence (on a logit scale) in the substrate with a 1.25% *P. ultimum* dose (Table 2). With the extended model containing CF and Hue, CF had a negative coefficient of −0.25 and Hue had a positive coefficient of 0.12 with seedling emergence (on a logit scale).

With a 2.5% *P. ultimum* dose, 27 out of the 32 seed trait variables improved the model fit compared to the null model (ΔAIC≥2) (Supplemental Table 1B). The addition of CF showed the largest model fit improvement with almost 15 AIC units difference. Compared to the null model, all reflectance measurements from 375 nm to 970 nm improved the model individually with negative regression coefficients, except for RefA. Since CF showed the greatest contribution, this trait was added first to the model. In addition to CF, the reflectance measurements ranging from 435 nm to 525 nm, RefA and RefB only slightly improved the model fit by 1.2 AIC units or less (Supplemental Table 2). The model with CF showed a strong negative regression coefficient of −0.22 with seedling emergence (on a logit scale) in the substrate with a 2.5% *P. ultimum* dose (Table 2).

With a 5% *P. ultimum* dose, 26 out of the 32 seed trait variables improved the model fit compared to the null model (ΔAIC≥2), including CF with a regression coefficient of −0.28, and all spectral measurements, each with a negative regression coefficient, except RefA (Supplemental Table 1A). The addition of Blue (420–480 nm analysed with the PathoScreen™RGB camera) resulted in the largest model fit improvement (ΔAIC=17.8) compared to the null model (Supplemental Table

1B). However, the addition of 470 nm and 450 nm (two blue wavelengths analysed with the VideometerLab) showed almost equal improvements (ΔAIC around 16), indicating an equal contribution to the model fit as Blue. When Blue was added to the model at first, no other trait variable further improved the model fit. The model extended with Blue showed a negative coefficient of -0.37 with seedling emergence (on a logit scale) in the substrate with a 5% *P. ultimum* dose (Table 2).

Based on these results, Area, reflectance at 405 nm (violet), 435 nm (indigo), 450 nm (blue), 470 nm (blue), Blue (420–480 nm), RefA, Hue, and CF were selected as potential candidate seed traits for estimating emergence from a spinach seed, depending on *P. ultimum* dose.

3.3. Generating a model for spinach seedling emergence depending on selected seed traits and *P. ultimum* dose

To study the effects of the selected seed trait variables in interaction with *P. ultimum* inoculum dose, the next step in the analyses concerned combining the individual seed trait and emergence data from the two experiments while treating *P. ultimum* dose as a quantitative explanatory variable (=Dose) with four values. The pre-selected traits were first

evaluated individually for the main effects on emergence and the interaction with *P. ultimum*. The model with the addition of a main effect of CF and a CF-by-Dose interaction effect showed the greatest improvement of the model fit (Table 3A, indicated by italics). When adding Area and Area:Dose interaction there was a slight improvement of the model fit, but only the interaction effect was significant. The model fit also improved when RefA, reflectance at 405 nm, 435 nm, 450 nm, 470 nm, or 420–480 nm (Blue), and their interactions with *P. ultimum* dose were included. Since these violet to blue reflection variables gave the greatest improvement of the model fit and showed the strongest interaction and main effects ($p < 0.001$), we chose Blue as overarching trait to be included in the full model with a main and interaction effect. As a second step, an all-possible subset approach with Area, Area:Dose, Blue, Blue:Dose and CF, CF:Dose, with CF as a fixed candidate in the model, was performed. CF, Blue and Area and the interactions for Blue and Area with *P. ultimum* dose resulted in the final model (Table 3B, indicated by italics).

The coefficients for the main and interaction effects of the selected model parameters were determined (Table 4). CF had a significantly negative association with emergence. The positive association between

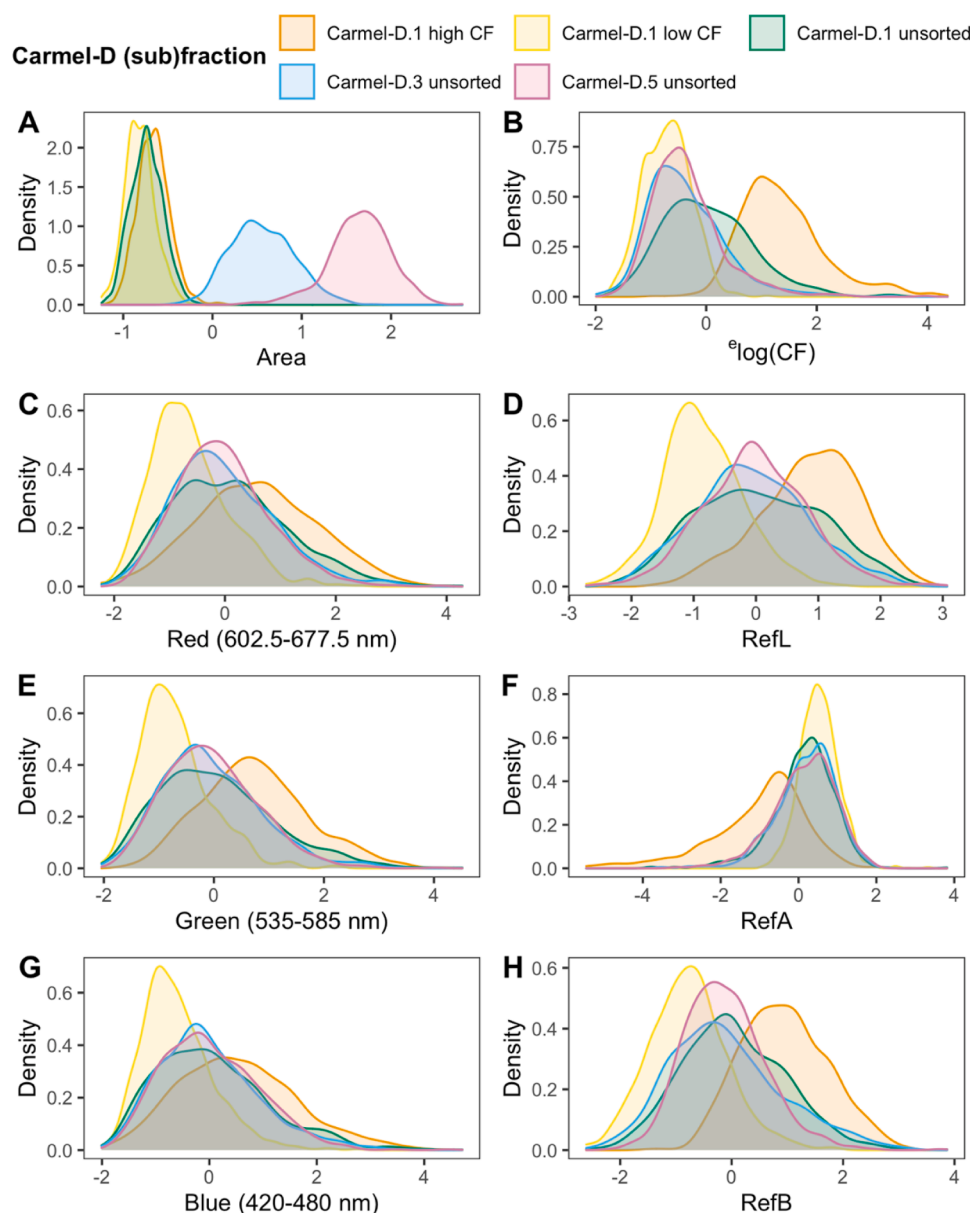


Fig. 2. Distributions of standardised continuous data of (A) seed size (Area), (B) log-transformed chlorophyll fluorescence ($^{\circ}\log(CF)$), (C) reflectance at 602.5–677.5 nm (Red), (D) lightness (RefL), (E) reflectance at 535–585 nm (Green), (F) reflectance from red to green (RefA), (G) reflectance at 420–480 nm (Blue), and (H) reflectance from blue to yellow (RefB) of the five (sub)fractions of Carmel-D spinach seed lot, used in Experiment III: Carmel-D.1 (2.50–2.75 mm); Carmel-D.3 (3.50–4.25 mm); Carmel-D.5 (4.50–5.00 mm); unsorted = randomly selected seeds, not sorted for chlorophyll fluorescence; D.1 high = high CF level (low maturity); and D.1 low = low CF level (high maturity). The area under each curve is 1.



Fig. 3. Seeds from Carmel fraction D.1 sorted by chlorophyll fluorescence (CF) with (A) low CF seeds (<6,000 CF in arbitrary units), and (B) high CF seeds (>12,000 CF in arbitrary units).

Area and emergence, at *P. ultimum* dose equal to zero, was not significant, but the negative interaction between Area and *P. ultimum* dose was significant. An even stronger negative interaction was found between Blue and *P. ultimum* dose, whereas Blue associated positively with emergence in substrate without *P. ultimum*. The effect of *P. ultimum* dose increased with increasing seed size and with increasing reflectance at 420–480 nm.

The coefficients from Table 4 were used to generate a formula for estimating the logit of emergence of an individual spinach seed:

$$\log\left(\frac{\text{emergence}}{\text{non-emergence}}\right) = 2.43 + 0.11 \times \text{Area} - 0.17 \times \log(\text{CF}) + 0.23 \times \text{Blue} - (1.05 + 0.05 \times \text{Area} + 0.13 \times \text{Blue}) \times \text{Dose}$$

with:

Area: seed size in standard deviations, with 0 equals mean=9.59 mm² and 1 SD=2.57 mm²,

^elog(CF): natural logarithm of chlorophyll fluorescence, with 0 equals mean=9.05 ^elog(CF) in AU and 1 SD=0.27 ^elog(CF) in AU, Blue: reflectance at 420–480 nm in standard deviations, with 0 equals mean=6230.1 reflectance in AU and 1 SD=1,831.7 reflectance in AU,

Dose: *P. ultimum* dose in %.

In other words, in the substrate without *P. ultimum* and independent of seed trait, the probability of seedling emergence was 0.92 ($=e^{2.43} \times (1 + e^{2.43})^{-1}$). In this situation, all variables were set to zero, which corresponded to the mean values of the seed traits measured in the substrate

Table 5

Mean $\pm$ standard deviations (SD) of the incidences of emergence 10 days after sowing spinach seeds of the seed size fractions of Carmel-D, and subfractions with high and low chlorophyll fluorescence (CF) levels of Carmel-D.1, averaged over five replicates of 50 seeds.

<i>P. ultimum</i> dose (%)	Size fraction	CF level	Mean ($\pm$ SD) emergence (%)	
0	Carmel-D.1	high	72.8	± 5.8
0		low	89.2	± 4.1
0		unsorted	88.0	± 4.0
0	Carmel-D.3	unsorted	89.6	± 3.8
0	Carmel-D.5	unsorted	86.4	± 4.8
1.9	Carmel-D.1	high	23.6	± 9.5
1.9		low	43.0	± 10.1
1.9		unsorted	37.6	± 7.4
1.9	Carmel-D.3	unsorted	32.8	± 11.6
1.9	Carmel-D.5	unsorted	22.0	± 10.9
2.5	Carmel-D.1	high	11.6	± 2.6
2.5		low	29.2	± 8.1
2.5		unsorted	21.2	± 4.6
2.5	Carmel-D.3	unsorted	21.6	± 8.9
2.5	Carmel-D.5	unsorted	12.0	± 5.9

with a 0% *P. ultimum* dose. With an increase of the natural logarithm of CF by 1 standard deviation, the probability of emergence was 0.91 ($=e^{2.43-0.17} \times (1 + e^{2.43-0.17})^{-1}$). With a 3% *P. ultimum* dose, the probability of emergence decreased to 0.33 ($=e^{2.43-1.05 \times 3} \times (1 + e^{2.43-1.05 \times 3})^{-1}$). With a 3% *P. ultimum* dose in addition to an increase in Area or Blue by 1 SD, the probability of emergence further decreased to 0.32 with the larger seed size ($=e^{2.43+0.11-(1.05+0.05) \times 3} \times (1 + e^{2.43+0.11-(1.05+0.05) \times 3})^{-1}$), and to 0.29 with the higher reflectance at violet-blue light ($=e^{2.43+0.23-(1.05+0.13) \times 3} \times (1 + e^{2.43+0.23-(1.05+0.13) \times 3})^{-1}$).

3.4. Validating seed size and maturity effects on tolerance to *P. ultimum*

Since seed size correlated negatively with CF (Fig. 1), but only showed a negative effect on seedling emergence in the presence of *P. ultimum*, a validation study with seed size and CF-sorted fractions was conducted to validate whether seed size or seed maturity contributed the most. The three seed size fractions from seed lot Carmel-D showed little overlap in seed size (Area) (Fig. 2A). The size fractions D.3 and D.5 did not differ in their CF distributions, while fraction D.1, the smaller seeds, contained relatively more seeds with higher CF levels (Fig. 2B). Beside seed size and CF, individual seeds showed considerable overlap among the size fractions in the distributions of RefLAB, and RGB levels (Fig. 2C–H). The two CF-sorted subfractions of the smallest seeds (fraction D.1) showed clear morphological distinctions in pericarp intactness and color (Fig. 3). Most of the high CF seeds showed a damaged pericarp, exposing the true seeds, which was less the case with the low CF seeds. The low CF seeds were darker in appearance (Fig. 3A) and had a lower light reflectance (Refl.) (Fig. 2D), including Red, Green, and Blue light reflectances (Fig. 2C, 2E, 2G).

The Carmel-D (sub)fractions were tested simultaneously in an assay with 0, 1.9, and 2.5% *P. ultimum* doses. On average, emergence was 85.2% ($\pm 7.7\%$) in the control substrate, 31.3% ($\pm 12.1\%$) in the substrate with a 1.9% *P. ultimum* dose, and 19.4% ($\pm 8.9\%$) in the substrate with a 2.5% *P. ultimum* dose. Carmel-D.1 (smallest seeds) showed greater emergence than Carmel-D.5 (largest seeds) in the presence of *P. ultimum* (Table 5). The statistical analysis confirmed significant main effects of the CF fractions, seed size fractions, and *P. ultimum* dose ($p < 0.05$). Seed size had no effect on emergence in the control substrate without *P. ultimum*, but it had a negative effect on emergence in the presence of *P. ultimum*. For Carmel-D.1, the emergence from the low CF seeds was greater in all three substrates compared to the high CF seeds. In the control substrate, these smaller mature seeds emerged comparable to the fractions D.3 and D.5 having larger seeds. In the presence of *P. ultimum*, the smallest seeds showed a greater emergence than the largest seeds.

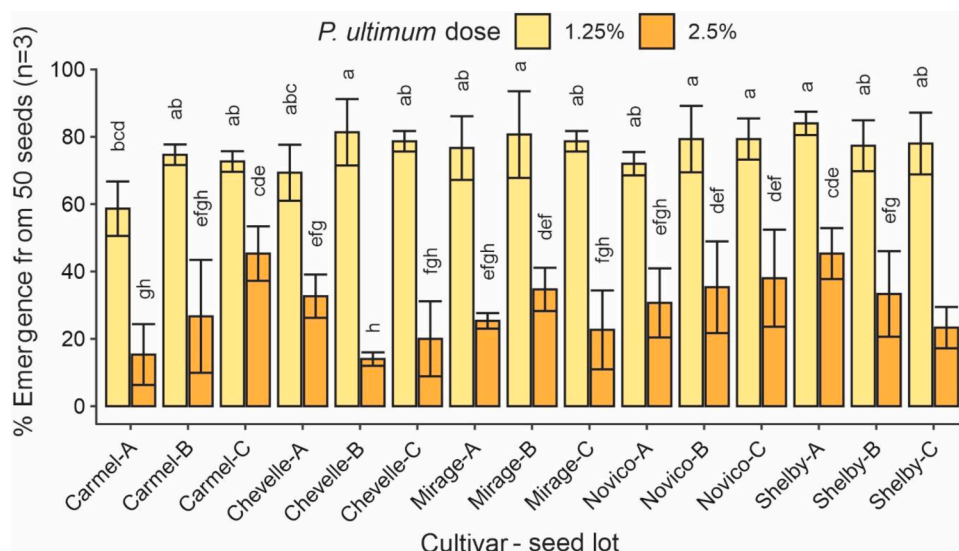


Fig. 4. Mean incidence (%) of emerged seedlings 10 days after sowing three seed lots of five cultivars in a substrate with sand:perlite:vermiculite mixture (1:1:1 vol basis) with 1.25% or 2.5% *Pythium ultimum* dose. Error bars represent standard deviations ($n = 3$, replicated trays with 50 seeds each tray). Bars with same letters indicate no significant differences based on Tukey's pairwise comparisons among the seed lots for emergence on a logit scale, using logistic regression with fixed effects of cultivar-seed lot combinations.

Table 6

Variance components of spinach seed lot effects in the null regression model estimating emergence in the substrate with a 2.5% *Pythium ultimum* dose, and extended models with individual seed traits (including fixed effects for cultivar and replicate, and random effects for seed lot and seed lot-by-replicate interaction). The models indicated in italics caused a reduction in seed lot variance compared to the null model.

Model	Seed lot variance
Null model	0.1253
+ <i>Blue</i>	0.1084
+ <i>Int</i>	0.1086
+ <i>RefL</i>	0.1118
+ <i>Green</i>	0.1143
+ <i>Red</i>	0.1149
+ <i>Area</i>	0.1185
+ <i>Round</i>	0.1246
+ <i>Sat</i>	0.1253
+ *log(CF)	0.1271
+ <i>RefB</i>	0.1306
+ <i>RefA</i>	0.1337
+ <i>Hue</i>	0.1470

Instead of looking at seed size and CF fractions as qualitative factors, the quantitative seed measurements of seed size and CF were evaluated with the model. The results confirmed a significant main effect of CF on the emergence, with a regression coefficient of -0.27 ($p < 0.001$). In addition, there was a significant interaction between seed size and *P. ultimum* dose ($p = 0.007$). Emergence in the absence of *P. ultimum* was not affected by increasing seed size, but in the presence of *P. ultimum*, emergence was affected negatively, with a regression coefficient of -0.30 at the 1.9% inoculum dose, and -0.28 at the 2.5% dose. In accordance with our previously developed model based on the data from 15 seed lots, this result confirmed a negative effect of CF on seedling emergence independent of *P. ultimum* dose, whereas the seed size effect depended on the *P. ultimum* dose and CF level.

3.5. Seed lot variation in emergence and other seed traits

With *P. ultimum* doses of 1.25% and 2.5% (Experiment II), the average emergence over the 15 seed lots was 76.1% ($\pm 8.6\%$) and 29.5% ($\pm 12.6\%$), respectively. These averages fell between those of the preceding experiment with 0% and 5% *P. ultimum* doses (Experiment I), as described in the introduction. In the previous analyses, the individual

seed traits and the emergence response to the various doses of *P. ultimum* were studied independently of spinach cultivar, allowing a random variation for seed lots. However, variation in response among the seed lots and cultivars could be related to certain seed traits. When comparing the 15 individual seed lots, significant differences in emergence were found in the presence of 2.5% *P. ultimum* dose (Fig. 4). For instance, Carmel-C showed greater emergence than Carmel-A, Chevelle-A showed greater emergence than Chevelle-B, and Shelby-A showed greater emergence than Shelby-C. With the 1.25% *P. ultimum* dose, no or few differences were found in emergence among the seed lots.

In an attempt to explain the large variation in emergence among the 15 seed lots in the substrate with a 2.5% *P. ultimum* dose, we compared the variance components of seed lot between the models for emergence without and with each individual seed trait. The seed lot variance became smaller when Blue, Intensity, RefL, Green, Red, or Area was added to the null model (Table 6). The inclusion of the individual reflectance measurements (from 375 nm to 970 nm) also caused a reduction in seed lot variance (data not shown), comparable to the reduction when adding Red, Green, or Blue.

The box-and-whiskers plots of the continuous data for seed measurements showed considerable overlap among the 15 seed lots tested (Fig. 5). Light reflectance from the pericarp of Chevelle-A seeds was, on average, lower than that of seeds of Chevelle-B and Chevelle-C. Chevelle-A also showed greater emergence in the presence of 2.5% *P. ultimum* dose (Fig. 4). Light reflectance from the pericarp of Carmel-A seeds was, on average, higher than that of seeds of Carmel-B and Carmel-C. Carmel-A also showed less emergence in the presence of 2.5% *P. ultimum* dose (Fig. 4). Chevelle-A with, on average, smaller seeds, and Novico-C and Shelby-C with, on average, larger seeds, particularly deviated in size from the other seed lots, even within the same cultivar (Fig. 5F). Shelby-C also showed less emergence in the presence of 2.5% *P. ultimum* dose than Shelby-A (Fig. 4). The Novico seed lots showed large variation in seed size, with greater variation among individual seeds when the seed size was, on average, larger. They did not show differences in emergence (Fig. 4).

4. Discussion

4.1. Variation in pre-emergence damping-off tolerance levels and seed traits among seed lots

Since the seedling emergence incidences in substrate without *P. ultimum* did not significantly differ among the 15 spinach seed lots, we presumed that the emergence with 2.5% *P. ultimum* dose was indicative

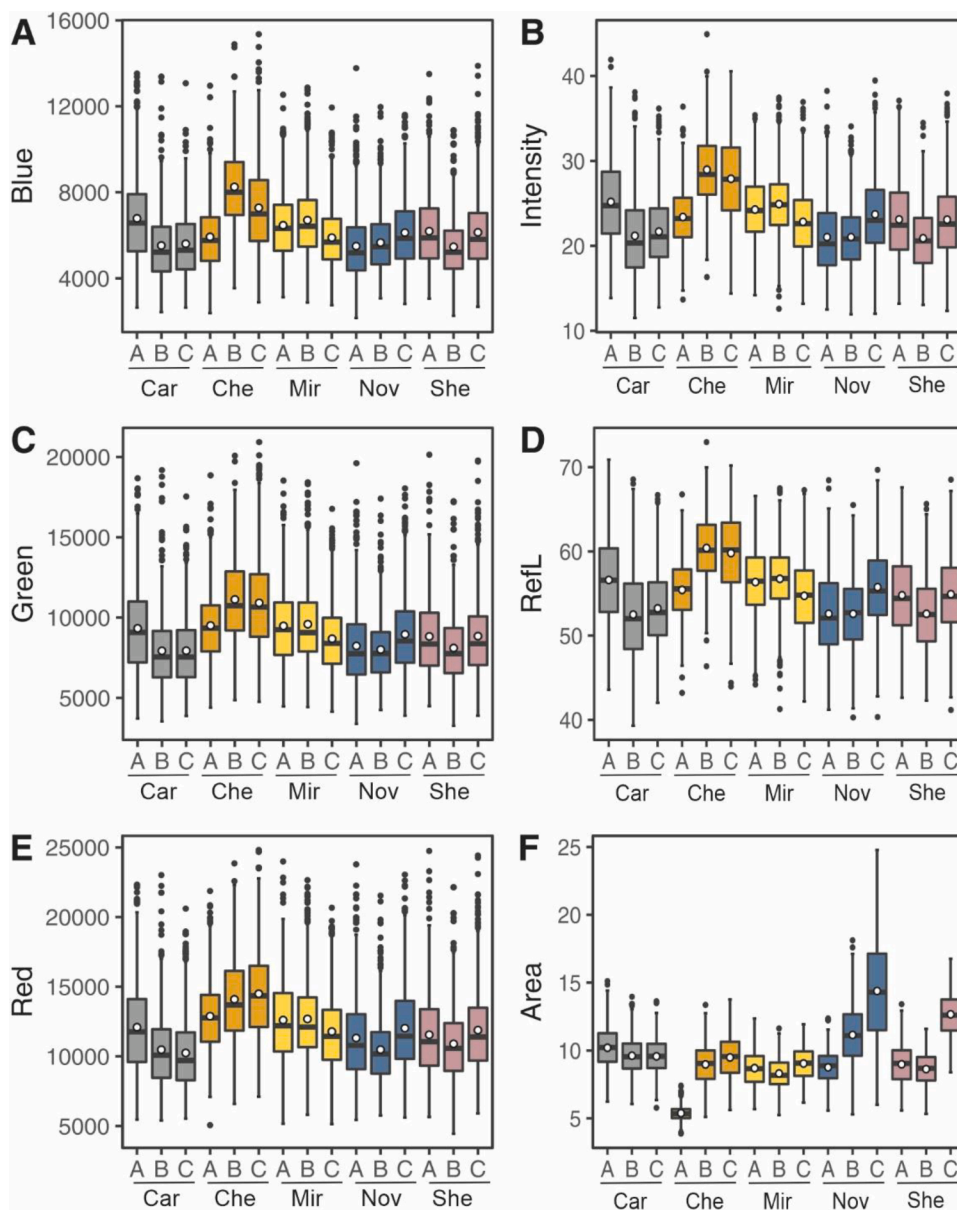


Fig. 5. Box-and-whiskers plots of non-scaled data of (A) light reflectance at 420–480 nm (Blue), (B) light reflectance intensity (Intensity), (C) reflectance at 535–585 nm (Green), (D) lightness (Refl), (E) reflectance at 602.5–677.5 nm (Red), and (F) seed size (Area) of individual seeds ($n = 600$) from each of three seed lots of the same spinach cultivar. The cultivar names are abbreviated: Carmel (Car), Chevelle (Che), Mirage (Mir), Novico (Nov) and Shelby (She). The boxes are the interquartile ranges (IQR). The horizontal lines inside the boxplots display the median, and white dots display the mean. The vertical lines (whiskers) go from the minimum value to the 25% quantile and from the 75% quantile to the maximum value, with minimal and maximum values within the range of $-1.5 \times \text{IQR}$ to $+1.5 \times \text{IQR}$.

for the pre-emergence damping-off tolerance levels, as calculated for the same 15 seed lots before (Magnée et al., 2022). According to the current study, the variation in tolerance levels among the seed lots could only be partly explained by seed size and light reflectance differences, as observed with a few seed lots. For instance, Chevelle-A contained the smallest seeds and showed greater *P. ultimum* tolerance compared with Chevelle-B that contained larger seeds. Also, Shelby-C, which had larger seeds, showed a lower tolerance level than the other Shelby seed lots that had smaller seeds. The Chevelle seed lots also contained relatively more seeds with higher light reflectance compared to other seed lots, and the tolerance level of Chevelle-B was lower. Similarly, Carmel-C seeds had, on average, a lower light reflectance and a greater *P. ultimum* tolerance than Carmel-A, which is in accordance with the results from the analysis on individual seeds. The large variation in tolerance levels among seed lots may have resulted also for a large part from differences in seed production, harvesting, and seed storage conditions. The differences in seed traits among seed lots emphasised the importance of characterizing seed lots before conducting genotypic studies on variation in *P. ultimum* tolerance in spinach. The large variation among and within seed lots from the same cultivar triggered our

interest to zoom in on the individual seed level.

4.2. Seed traits in relation to seedling emergence in the absence of *P. ultimum*

An increased seed maturity level (measured by a lower level of chlorophyll in the pericarp) showed a positive effect on emergence of spinach seedlings. This is in accordance with previous studies that described a positive effect of seed maturity on the germination potential for many plant species, including spinach (Deleuran et al., 2013; Jalink et al., 1998; Song et al., 2021). The data from the 15 seed lots sown in the control substrate showed that, under optimal temperature and moisture levels, larger spinach seeds had higher emergence incidence than smaller seeds. A positive correlation between seed size and the germination potential of spinach seeds was also shown in other studies (Shetty et al., 2012). However, when Carmel-D was size-fractionated, seed size did not show an effect on emergence in the control substrate. Also, when analysing the CF-sorted subfractions of the smaller and mature Carmel-D seeds, emergence was comparable with or even higher than emergence from the unsorted, larger seeds of Carmel, indicating

here a negative instead of a positive effect of seed size on emergence. The less mature seeds of the small Carmel-D seeds had lower emergence than the more mature small seeds of the same seed lot in the control substrate. This indicated that the initial seed size effect that we found in the control substrate with the 15 seed lots was more an effect of the seed maturity than of seed size.

4.3. Seed traits in relation to *P. ultimum* tolerance

Overall, a high CF level for a seed had a negative association with seedling emergence in the presence and the absence of *P. ultimum*. The association between seedling emergence and seed size reversed from positive in the control substrate to negative in the presence of *P. ultimum*. These results were confirmed by the validation experiment, except for a positive seed size effect in the control substrate. Even after selecting for high maturity level, the smaller Carmel seeds showed greater *P. ultimum* tolerance than the larger Carmel seeds. This may relate to the pericarp thickness. As smaller seeds have a thinner pericarp, there is less physical obstruction for the radicle to protrude as well as higher oxygen diffusion, allowing for faster germination (Magnée et al., 2020). Such fast germination (initiation of root growth) may enable an 'escape' of the seedling from *P. ultimum* infection (Agrios, 2005). The higher *P. ultimum* tolerance of the low CF seeds compared to the high CF seeds of the smaller seeds was expected to be related to the maturity of the seed, but the effect may also be influenced by the intactness of the pericarp. A large number of the immature, high CF seeds had an open or damaged pericarp with the true seed visible, so these seeds may also be more accessible to infection by *P. ultimum*. A greater sensitivity of the less mature seeds within the fraction of smallest seeds might also be related to lower seed vigor. Although studies on seed vigor in relation to biotic stress tolerance are limited, in some studies seeds with lower vigor were observed to have increased sensitivity towards soilborne pathogens, including *P. ultimum*, for example, seeds of wheat (Das Gupta and Austenson, 1973), soybean (Hamman et al., 2002; Schlub and Schmitthenner, 1978), pea (Perry, 1973; Stasz and Harman, 1980), and lucerne (Hawthorne, 1988).

Light reflectance over a broad range of wavelengths, from 375 nm to 970 nm, showed a negative association with seedling emergence from seeds of the 15 seed lots tested in the presence of *P. ultimum*. This indicated that the darker (less light-reflecting) seeds were more tolerant towards infection. We found the strongest effects with light reflectance at 405 to 480 nm (violet-blue). Without *P. ultimum*, reflectance was correlated positively with emergence, but with an increasing *P. ultimum* dose, the effect was reversed from positive to increasingly negative. The low CF seeds of Carmel-D also had a lower reflectance of light, as they were darker, indicating that a darker pericarp color is associated with higher seed maturity. With taxonomically and morphologically related sugar beet seeds, CF has also been demonstrated to correlate positively with reflectance from the pericarp in the visible to NIR light spectrum (Boelt et al., 2018). Also in another study, the maturity of sugar beet seeds was associated with reduced lightness (darker seeds) and increased germination (Mirzaei and Rajabi, 2021). The color of the pericarp will be associated with the presence and concentration of certain secondary metabolites. With sugar beet seeds, the pericarp contains various secondary metabolites with different biological activities, as regulation of seed germination and seedling emergence, and antimicrobial activity (Oksana and Artur, 2019). Studies on pericarp extracts from sugar beet and red beet seeds showed that they contain phenolic compounds that can inhibit germination by absorbing oxygen (Chiji et al., 1980; Junntila, 1976; Morris et al., 1984). When sugar beet seeds were polished and washed, the removal of water-soluble germination inhibitors resulted in increased germination (Ignatz et al., 2019; Salimi and Boelt, 2019). The pericarp can also contain phenolic compounds with anti-pathogenic effects, such as condensed tannins (Scalbert, 1991). Further studies are needed to unravel which specific secondary metabolites are present in the spinach pericarp that are

associated with violet-blue light absorbance, and their potential positive effects on *P. ultimum* tolerance should be validated.

With the 8,950 seeds measured in this study, we found no correlation between light reflectance and seed size. However, a strong positive correlation was found between CF and (violet-blue) light reflectance (higher CF levels, more reflectance), and a negative correlation was found between CF and seed size (larger seeds, lower CF). High values for CF, violet-blue light reflectance, and seed size had negative effects on the pre-emergence damping-off tolerance level. Apparently, these three parameters have a complex interaction effect on pre-emergence damping-off tolerance of spinach seeds. With this complexity, it should be considered that pericarp light reflection may be associated with a broad range of secondary metabolites with different, and potentially contrasting, biological activities.

5. Conclusion

This study indicated that spinach seed maturity is a main trait for providing better emergence of seedlings from the seeds and greater tolerance to pre-emergence infection by *P. ultimum*. More research is needed to understand the negative effects of violet-blue light reflectance and associated secondary metabolites on pre-emergence damping-off tolerance. Other researchers have suggested that the production of high-quality spinach seed lots could benefit from sorting out the less mature seeds by color-grading (Salimi and Boelt, 2019) or by chlorophyll fluorescence sorting (Deleuran et al., 2013). In line with these suggestions, our results indicated that the production of spinach seed lots with a greater tolerance to *P. ultimum* will benefit from additional sorting of mature seeds in favor of less light-reflecting (darker) seeds and smaller seeds.

CRediT authorship contribution statement

Kim J.H. Magnée: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Olga E. Scholten:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Jan Kodde:** Investigation, Methodology. **Joeke Postma:** Conceptualization, Methodology, Writing – review & editing. **Gerrit Gort:** Formal analysis, Methodology, Writing – review & editing. **Edith T. Lammerts van Bueren:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Steven P.C. Groot:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We gratefully acknowledge the financial and practical support from the seed and breeding companies Pop Vriend Seeds B.V. (The Netherlands) and Sakata Seeds (France, Japan, California). These companies and Enza Zaden/Vitalis Organic Seeds B.V. (The Netherlands), and Nunhems/BASF B.V. (The Netherlands) are also acknowledged for providing the spinach seed lots. This research was funded by the Top Sector Horticulture & Propagation Materials as part of the research program 'Green Breeding' (Grant Number: KV 1409–031).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.scienta.2023.112219](https://doi.org/10.1016/j.scienta.2023.112219).

References

- Agrios, G.N., 2005. Chapter 4: Genetics of Plant disease, Plant Pathology, 5th ed. Elsevier Academic Press, Burlington.
- Arellano, G., Peco, B., 2012. Testing the role of seed size in annual legume seedling performance under experimental autumn moisture conditions. *J. Veg. Sci.* 23, 690–697. <https://doi.org/10.1111/j.1654-1103.2012.01394.x>.
- Arnold, T.W., 2010. Uninformative parameters and model selection using Akaike's information criterion. *J. Wildl. Manag.* 74, 1175–1178. <https://doi.org/10.1111/j.1937-2817.2010.tb01236.x>.
- Atis, I., Atak, M., Can, E., Mavi, K., 2011. Seed coat color effects on seed quality and salt tolerance of red clover (*Trifolium pratense*). *Int. J. Agric. Biol.* 13, 363–368.
- Baker, H.G., 1972. Seed weight in relation to environmental conditions in California. *Ecology* 53, 997–1010. <https://doi.org/10.2307/1935413>.
- Boelt, B., Shrestha, S., Salimi, Z., Jørgensen, J.R., Nicolaisen, M., Carstensen, J.M., 2018. Multispectral imaging—a new tool in seed quality assessment? *Seed Sci. Res.* 28, 222–228. <https://doi.org/10.1017/S0960258518000235>.
- Chiji, H., Tanaka, S., Izawa, M., 1980. Phenolic germination inhibitors in the seed balls of red beet (*Beta vulgaris* L. var. *rubra*). *Agric. Biol. Chem.* 44, 205–207. <https://doi.org/10.1080/00021369.1980.10863924>.
- Das Gupta, P.R., Austenson, H.M., 1973. Analysis of Interrelationships among seedling vigor, field emergence, and yield in wheat. *Agron. J.* 65, 417–422. <https://doi.org/10.2134/agronj1973.00021962006500030019x>.
- Deleuran, L.C., Olesen, M.H., Boelt, B., 2013. Spinach seed quality: potential for combining seed size grading and chlorophyll fluorescence sorting. *Seed Sci. Res.* 23, 271–278. <https://doi.org/10.1017/S0960258513000202>.
- European Commission, 2021. European green deal, striving to be the first climate-neutral continent. https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal_en. Accessed on 25-04-2023.
- Fenner, M., 1992. Environmental influences on seed size and composition. *Hortic. Rev. Am. Soc. Hortic. Sci.* 13, 183–213.
- Grzywacz, A.P., Rosochacka, J., 1980. The colour of *Pinus sylvestris* L. seed and their susceptibility to damping-off I. The colour and quality of seeds and fatty acids content of the seed coat. *Eur. J. For. Pathol.* 10, 138–144. <https://doi.org/10.1111/j.1439-0329.1980.tb00019.x>.
- Hamman, B., Egli, D.B., Koning, G., 2002. Seed vigor, soilborne pathogens, preemergent growth, and soybean seedling emergence. *Crop Sci.* 42, 451–457. <https://doi.org/10.2135/cropsci2002.4510>.
- Hawthorne, B.T., 1988. Pathogenicity of *Pythium* spp. to lucerne seedlings and relationships between seed weight, seedling vigour, and infection by *Pythium*. *N.Z. J. Agric. Res.* 31, 359–364. <https://doi.org/10.1080/00288233.1988.10423426>.
- Heather, D.W., Sieczka, J.B., 1991. Effect of seed size and cultivar on emergence and stand establishment of broccoli in crusted soil. *J. Am. Soc. Hortic. Sci.* 116, 946–949. <https://doi.org/10.21273/JASHS.116.6.946>.
- Ignatz, M., Hourston, J.E., Turečková, V., Strnad, M., Meinhard, J., Fischer, U., Steinbrecher, T., Leubner-Metzger, G., 2019. The biochemistry underpinning industrial seed technology and mechanical processing of sugar beet. *Planta* 250, 1717–1729. <https://doi.org/10.1007/s00425-019-03257-5>.
- Jalini, H., van der Schoor, R., Frandas, A., van Pijlen, J.G., Bino, R.J., 1998. Chlorophyll fluorescence of *Brassica oleracea* seeds as a non-destructive marker for seed maturity and seed performance. *Seed Sci. Res.* 8, 437–443. <https://doi.org/10.1017/S0960258500004402>.
- Junttila, O., 1976. Germination inhibitors in fruit extracts of red beet (*Beta vulgaris* cv. *rubra*). *J. Exp. Bot.* 27, 827–836. <https://doi.org/10.1093/jxb/27.4.827>.
- Koike, S.T., Cahn, M., Cantwell, M., Fennimore, S., Lestrangle, M., Natwick, E., Smith, R. F., Takele, E., 2011. Spinach Production in California. University of California, Agriculture and Natural Resources. <https://doi.org/10.3733/ucanr.7212>.
- Lamichhane, J.R., Dürr, C., Schwanck, A.A., Robin, M.H., Sarthou, J.P., Cellier, V., Messéan, A., Aubertot, J.N., 2017. Integrated management of damping-off diseases. A review. *Agron. Sustainable Dev.* 37, 1–25. <https://doi.org/10.1007/s13593-017-0417-y>.
- Lamichhane, J.R., You, M.P., Laudinot, V., Barbetti, M.J., Aubertot, J.N., 2020. Revisiting sustainability of fungicide seed treatments for field crops. *Plant Dis.* 104, 610–623. <https://doi.org/10.1094/pdis-06-19-1157-fe>.
- Lamichhane, J.R., Corrales, D.C., Soltani, E., 2022. Biological seed treatments promote crop establishment and yield: a global meta-analysis. *Agron. Sustainable Dev.* 42 (45) <https://doi.org/10.1007/s13593-022-00761-z>.
- Larsson, M., 1994. Prevalence and pathogenicity of spinach root pathogens of the genus *Pythium* in Sweden. *Plant Pathol.* 43, 261–268. <https://doi.org/10.1111/j.1365-3059.1994.tb02684.x>.
- Magnée, K.J.H., Scholten, O.E., Postma, J., Lammerts van Bueren, E.T., Groot, S.P.C., 2020. Sensitivity of spinach seed germination to moisture is driven by oxygen availability and influenced by seed size and pericarp. *Seed Sci. Technol.* 48, 117–131. <https://doi.org/10.15258/sst.2020.48.1.13>.
- Magnée, K.J.H., Postma, J., Groot, S.P.C., Gort, G., Lammerts van Bueren, E.T., Scholten, O.E., 2022. Evaluation of damping-off tolerance in spinach cultivars in field soils and in a standardized lab assay with *Pythium ultimum*. *Plant Health Prog.* 23, 174–187. <https://doi.org/10.1094/PHP-07-21-0107-RS>.
- Mirzaei, M.R., Rajabi, A., 2021. Relationship of seed pericarp color with seed quality in sugar beet (*Beta vulgaris* L. var. *altissima* Döll). *Genet. Resour. Crop Evol.* 68, 2093–2105. <https://doi.org/10.1007/s10722-021-01120-9>.
- Morris, P.C., Grierson, D., Whittington, W.J., 1984. Endogenous inhibitors and germination of *Beta vulgaris*. *J. Exp. Bot.* 35, 994–1002. <https://doi.org/10.1093/jxb/35.7.994>.
- Oksana, K., Artur, L., 2019. Biological activity of endometabolites from sugar beet (*Beta vulgaris* L.) pericarps. *J. Microbiol. Biotechnol. Food Sci.* 8, 1303–1306. <https://doi.org/10.15414/jmbfs.2019.8.6.1303-1306>.
- Olesen, M.H., Carstensen, J.M., Boelt, B., 2011. Multispectral imaging as a potential tool for seed health testing of spinach (*Spinacia oleracea* L.). *Seed Sci. Technol.* 39, 140–150. <https://doi.org/10.15258/sst.2011.39.1.12>.
- Perry, D.A., 1973. Infection of seeds of *Pisum sativum* by *Pythium ultimum*. *Trans. Br. Mycol. Soc.* 61, 135–144. [https://doi.org/10.1016/S0007-1536\(73\)80095-6](https://doi.org/10.1016/S0007-1536(73)80095-6).
- Rey, P.J., Alcántara, J.M., Sánchez-Lafuente, F., Garrido, A.M., Ramírez, J.L., Manzaneda, A.J., 2004. Seedling establishment in *Olea europaea*: seed size and microhabitat affect growth and survival. *Ecoscience* 11, 310–320. <https://doi.org/10.1080/11956860.2004.11682838>.
- RStudio Team, 2020. RStudio: integrated development environment for R. <http://www.rstudio.com/>. Accessed on 25-04-2023.
- Salimi, Z., Boelt, B., 2019. Optimization of germination inhibitors elimination from sugar beet (*Beta vulgaris* L.) seeds of different maturity classes. *Agronomy* 9, 763. <https://doi.org/10.3390/agronomy9110763>.
- Scalbert, A., 1991. Antimicrobial properties of tannins. *Phytochemistry* 30, 3875–3883. [https://doi.org/10.1016/0031-9422\(91\)83426-L](https://doi.org/10.1016/0031-9422(91)83426-L).
- Schlub, R.L., Schmitthenner, A.F., 1978. Effects of soybean seed coat cracks on seed exudation and seedling quality in soil infested with *Pythium ultimum*. *Phytopathology* 68, 1186–1191.
- Shetty, N., Olesen, M.H., Gislum, R., Deleuran, L.C., Boelt, B., 2012. Use of partial least squares discriminant analysis on visible-near infrared multispectral image data to examine germination ability and germ length in spinach seeds. *J. Chemom.* 26, 462–466. <https://doi.org/10.1002/cem.1415>.
- Sifton, H.B., 1927. On the germination of the seed of *Spinacia oleracea*, L., at low temperatures. *Ann. Bot.* 41, 557–568.
- Song, Y., Tseng, Y.C., Rowland, D.L., Tillman, B.L., Wilson, C.H., Sarnoski, P.J., Zurweller, B.A., 2021. Multiple-generation seed maturity effects on seedling vigour in a production environment. *J. Agron. Crop Sci.* 207, 1024–1040. <https://doi.org/10.1111/jac.12559>.
- Stasz, T.E., Harman, G.E., 1980. Interactions of *Pythium ultimum* with germinating resistant or susceptible pea seeds. *Phytopathology* 70, 27–31.
- Steckel, J.R., Gray, D., Rowse, H.R., 1989. Relationships between indices of seed maturity and carrot seed quality. *Ann. Appl. Biol.* 114, 177–183. <https://doi.org/10.1111/j.1744-7348.1989.tb06798.x>.
- United Nations Environment Programme (UNEP), 2006. Handbook For the Montreal Protocol On Substances That Deplete the Ozone Layer. United Nations Environment Programme/Earthprint, Nairobi, Kenya.
- Uzuhashi, S., Kakishima, M., Tojo, M., 2010. Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience* 51, 337–365. <https://doi.org/10.1007/S10267-010-0046-7>.
- Vange, T., Tamen, K., Okoh, J.O., 2016. Seed size effects on germination and seedling parameters of soybean cultivars. *Seed Technology* 37, 81–87. <https://www.jstor.org/stable/26625375>.
- Ward, K., Scarth, R., Vessey, J.K., Daun, J.K., 1995. Chlorophyll degradation in summer oilseed rape and summer turnip rape during seed ripening. *Can. J. Plant Sci.* 75, 413–420. <https://doi.org/10.4141/cjps95-069>.
- Zhang, H., Xu, S., Pang, S., Piao, X., Wang, Y., 2018. Effect of seed size on seedling performance, yield and ginsenoside content of *Panax ginseng*. *Seed Sci. Technol.* 46, 407–417. <https://doi.org/10.15258/sst.2018.46.2.22>.