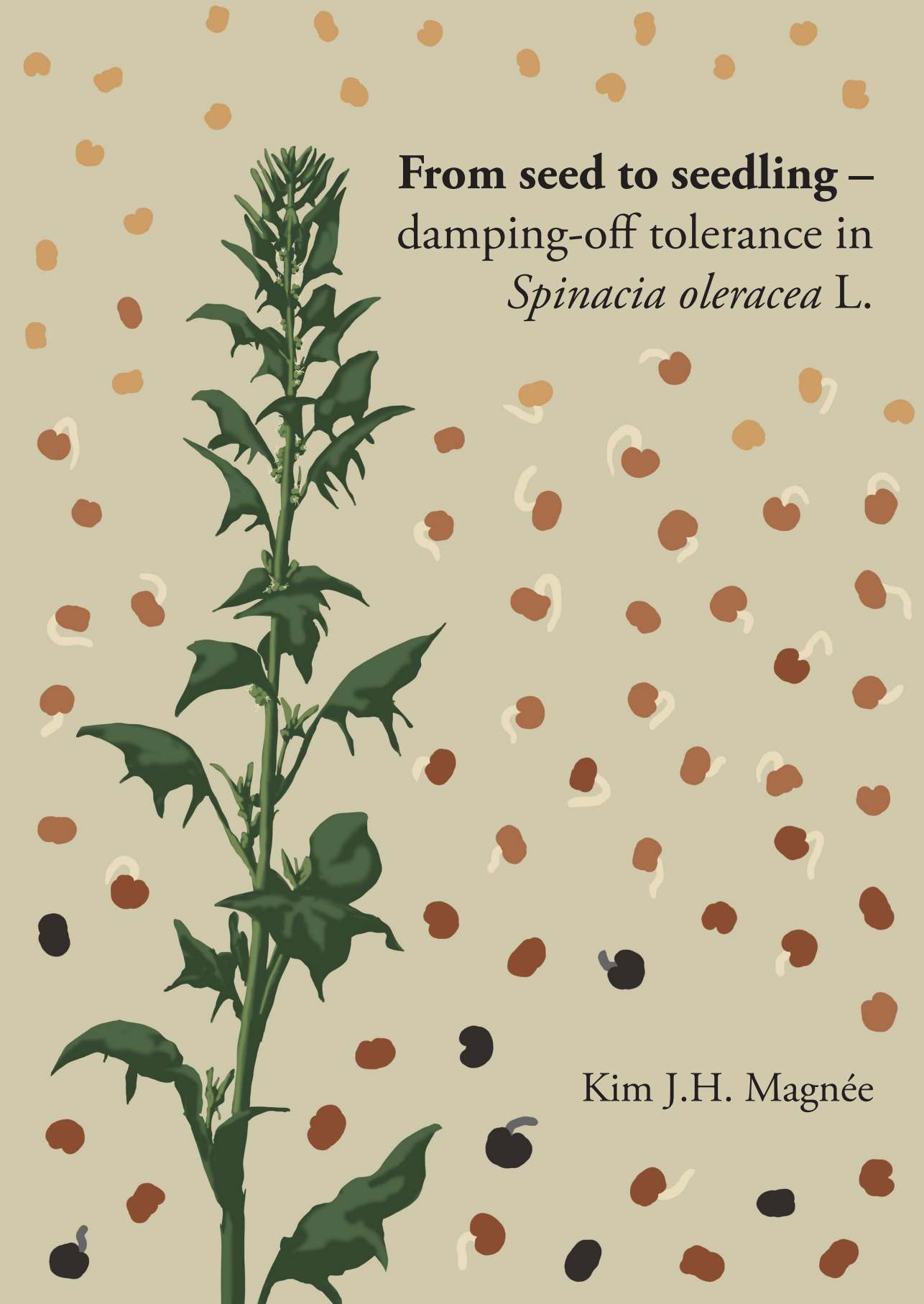


From seed to seedling – damping-off tolerance in *Spinacia oleracea* L.

Kim J.H. Magnée

Propositions

1. Seed vigour is key to pre-emergence damping-off tolerance in spinach.
   (this thesis)

2. Management of damping-off diseases requires seed sorting for improved tolerance levels in spinach seed lots.
   (this thesis)

3. Limited knowledge exchange among companies and research institutions hinders the achievement of global food security.

4. Using the outcomes of existing climate studies is more urgent than initiating new climate studies.

5. Playing music is just as important for human health as doing sports.

6. Teaching children to grow vegetables increases their appreciation for food and nature.

Propositions belonging to the thesis, entitled
'From seed to seedling — damping-off tolerance in *Spinacia oleracea* L.'

Kim J.H. Magnée

Wageningen, 11 May 2022

From seed to seedling – damping-off tolerance in *Spinacia oleracea* L.

Kim Jacqueline Hubertine Magnée

Thesis committee

Promotor
Prof. Dr Edith T. Lammerts van Bueren
Emeritus professor Organic Plant Breeding
Wageningen University & Research

Co-promotors
Dr Olga E. Scholten
Senior researcher, Plant Breeding
Wageningen University & Research

Dr Steven P.C. Groot
Senior researcher, Bioscience
Wageningen University & Research

Other members
Prof. Dr Leonie Bentsink, Wageningen University & Research
Prof. Dr Gerlinde B. de Deyn, Wageningen University & Research
Dr Liesbeth van der Heijden, Bejo Zaden B.V., Warmenhuizen
Dr Lindsey J. du Toit, Washington State University, Mount Vernon, Washington, USA

This research was conducted under the auspices of the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC).

From seed to seedling
–
damping-off tolerance in *Spinacia oleracea* L.

Kim Jacqueline Hubertine Magnée

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 11 May 2022
at 11 a.m. in the Omnia Auditorium.

Kim J.H. Magnée
From seed to seedling – damping-off tolerance in *Spinacia oleracea* L.,
188 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summaries in English and Dutch

ISBN: 978-94-6447-098-7
DOI: https://doi.org/10.18174/563317

Table of contents

Chapter 1

General introduction

Introduction

The research that was conducted for this dissertation was initiated by the increasing demand for organically produced fresh-market spinach (*Spinacia oleracea* L.) and the increasing ban of chemical seed and field treatments. Consequently, problems with damping-off diseases have become more apparent in spinach production. Those diseases can be caused by multiple soilborne pathogens and are highly influenced by other environmental conditions. Therefore, the demand for damping-off tolerant spinach cultivars has gained more attention by the industry. With the support from Pop Vriend Seeds, Sakata Seeds, and the Dutch government through the Top consortium for Knowledge and Innovation (Horticulture and Starting materials), this has resulted in the current study on damping-off tolerance in spinach. The main goal was to explore the possibilities of improving damping-off tolerance in spinach through plant breeding or through seed vigour enhancement, for instance by selecting for seed traits associated with improved tolerance levels. This chapter introduces spinach as an increasingly popular leafy vegetable, the challenges for its production with focus on damping-off diseases, the spinach seed characteristics, and the potential role of seed vigour in damping-off tolerance. Related to the knowledge gaps, the research questions and hypotheses are formulated thereafter, and the evaluation methods and outline of the research chapters are briefly explained.

The origin of spinach as leafy vegetable

Spinach is an economically important and highly nutritious vegetable that is cultivated for the consumption of its fresh or processed leaves (Morelock & Correll, 2008). The origin of cultivated spinach is probably in ancient Persia (West-Asia), where two wild relatives, *S. tetrandra* Stev. and *S. turkestanica* Iljin, can be found. Both species are valuable genetic resources, e.g., for resistance to common diseases (Andersen & Torp, 2011). The earliest written evidence of cultivated spinach dates back to the 7th century when spinach was introduced to India and via Nepal to China, where it was named the "herb of Persia" (Laufer, 1919). Since at least the 11th century, spinach has been cultivated in southern Spain after its introduction by the Moors from northern Africa. From the end of the 12th century, cultivated spinach was introduced to France and to other European countries (Hallavant & Ruas, 2014). Early colonists from Spain introduced it to the Americas, and by the early 19th century, spinach became cultivated in North America (Ribera et al., 2021).

Worldwide, spinach has become one of the most consumed leafy vegetables due to its high nutritious value and anti-oxidant capacity, containing high levels of folate, beta carotene and lutein (vitamin A), in addition to vitamin C, potassium, sodium, calcium, magnesium, phosphorous, and iron (Prior & Cao, 2000; United States Department of Agriculture (USDA), 2021). In the current market, there are three main types of spinach based on their leaf morphology: savoy (wrinkled), semi-savoy (slightly curled), and smooth (flat) (Morelock & Correll, 2008). The leaves can be consumed fresh (washed and bunched or bagged) or cooked for which mostly processed (sterilised and canned or frozen) spinach is used. The savoy and semi-savoy types are most popular in the fresh market for their leaf structure and taste, while smooth types are more suitable for processing. The popularity of smooth varieties is increasing with the growing demand for fresh-market spinach, in particular baby-leaf spinach (Lucier et al., 2004; Simko et al., 2014).

Spinach (baby-leaf) production and challenges

During the past twenty years, global production of spinach has increased fourfold to reach about 30 million tonnes with the largest production share (91.5%) in China (Food and Agriculture Organization (FAO), 2020). The second and third largest producers, though far behind, are Europe (2.3%) and the United States of America (1.4%). The Netherlands produced 74 thousand tonnes in 2019, which was about 10.6% of the total European production. In the Netherlands, the production of spinach has even increased by 102% since the year 2000 (CBS StatLine, 2021). Worldwide, the spinach production mainly increased as a result of the growing demand for fresh-market leafy vegetables used

in salads (Simko et al., 2014). In the USA, fresh-market spinach production accounts for more than 90% of the total spinach production, and California is the largest spinach producer (73%) (USDA, 2020). Bagged spinach is the dominant fresh-market spinach product, containing small, young leaves, also called baby-leaf spinach. The leaves for this type of spinach are harvested within 21 to 40 days from seeding, whereas the leaves for processed spinach can be harvested up to 90 days from seeding (Koike et al., 2011). The short production cycle requires uniform germination and seedling establishment with uniform leaf sizes. Early harvest allows seeds of baby-leaf spinach to be sown at very high densities, between 8.6 and 9.9 million seeds ha^{-1} (compared to 2.5 to 3.7 million seeds ha^{-1} for processed spinach), requiring relatively high amounts of seeds (Koike et al., 2011). As a result, the cultivation of baby-leaf spinach is more challenging and relatively more expensive than other spinach crop types.

Spinach is produced conventionally and organically, with the largest growth in organic cultivation. Organic production is on the rise due to increasing consumer awareness of potential environmental risks of fungicides use and food safety risks of fungicide residues on freshly consumed crops from conventional production systems. In the USA, 35% of the total acreage of harvested spinach was produced organically in 2019. In comparison, organic production of all vegetables in the US accounts for only 10% of the total acreage of harvested vegetables (USDA, 2019). In the Netherlands, the demand for organically produced crops and vegetables increased by 14.5% from 2019 to 2020 (Bionext, 2020). Currently, Dutch organic spinach cultivation accounts for 27% of total spinach cultivation (for both fresh and frozen products), with harvested spinach processed in the Netherlands, Belgium and Germany, and the end products exported globally (Jan Groen, Green Organics, 2021, *personal communication*).

In organic agriculture, the use of synthetic chemical inputs, including pesticides and fertilisers, is prohibited and the use of organically produced seeds is required (Meemken & Qaim, 2018). However, the availability of those seeds is limited and, only until 2036, organic spinach growers are allowed to use conventionally produced, untreated spinach seeds (Raaijmakers et al., 2021). Organic growers largely depend on cultivars bred for conventional production with high-external inputs, whereas they need new varieties that are more tolerant to all kinds of environmental stressors (Lammerts van Bueren et al., 2002). The use of chemical treatments is increasingly banned in conventional spinach production as well (United Nations Environment Programme (UNEP), 2006; European Commission, 2021). However, without fungicides, spinach may suffer seriously from diseases (Correll et al., 1994), especially when resistant or tolerant varieties are not yet available. Also, the efficacy of fungicides, like mefenoxam (or the isomer metalaxyl) that are used as seed treatments, can reduce over time due to development of fungicide resistance by pathogens, such as *Pythium* spp. and *Phytophthora* spp. (Taylor et al., 2002; Tekale et

al., 2019). Therefore, alternative solutions are urgently needed to foresee the increasing demand for synthetic chemical-free baby-leaf spinach. ***The aim of the current research was to determine whether it is possible to improve spinach damping-off tolerance through plant breeding and whether specific spinach seed traits underlie the disease tolerance.***

Botanical information of spinach

Spinach belongs to the Amaranthaceae (formerly Chenopodiaceae) family (subfamily Chenopodioideae), which also includes other vegetable crops such as beet (*Beta vulgaris* L.), and pseudograin crops such as amaranth (*Amaranthus* L.) and quinoa (*Chenopodium quinoa* Willd.) (Kadereit et al., 2010; Fuentes-Bazan et al., 2012). Spinach is a diploid species, which contains six pairs of chromosomes per somatic cell (2n=12) (Bemis & Wilson, 1953). The estimated genome size is 989 Mb (Collins et al., 2019).

Spinach plants are mainly dioecious, having separate male and female plants that require wind-pollination for fertilization. A spinach plant can also be monoecious, having male and female flowers on the same plant at various ratios, or the flowers can be hermaphrodite, enabling self-pollination (Wadlington & Ming, 2018). Monoecious spinach plants, particularly strict male plants, are favoured in the efficient production of homozygous lines, which are valuable for breeding goals. Therefore, the closely linked genes for dioecism and monoecism are also spinach breeding targets (Onodera et al., 2011; Yamamoto et al., 2014).

Spinach is able to grow year-round, and in many climates, preferably in the cooler seasons. For successful plant growth, seeds should be planted at temperatures between 5 and 20°C. Seedling growth is optimal at temperatures of 15 to 18°C, and mature spinach plants can withstand frost and temperatures down to -9°C (Koike et al., 2011). Spinach grows best in sandy loam soils, with a loose texture and good drainage, and slightly acidic to alkaline soil pH. Due to the shallow root system, it requires good levels of nitrogen, phosphorous and potassium, and does not tolerate excess levels of soil moisture (Morelock & Correll, 2008). Under short daylengths, the plant forms a rosette of leaves. High temperatures (>20°C) and longer daylengths (between 12.5 and 15 hours photoperiod) promote the formation of flower stalks (bolting) at the expense of leaf formation, which is necessary for seed production (Parlevliet, 1967; Rubatzky & Yamaguchi, 1997)

Since spinach seed development favours long daylength and a relatively cool climate, Denmark has become a market-leader in spinach seed production, covering about 75% of the global spinach seed supply (Correll et al., 2011). Spinach is an annual plant, flowering

in the year of sowing, which is valuable for breeding and seed production. The growth of the plant occurs indeterminately (Figure 1), which means that growth is not terminated genetically, and the plant continues to produce flowers and seeds until environmental conditions terminate growth. This results in a wide variability of seed sizes and maturity levels when the seeds are harvested from the plant (Deleuran et al., 2013). Depending on the environment and genotype, the plant can become up to 1 m tall when bolting. The flowers on the stalk are formed in clusters. Male flowers of dioecious spinach plants have four sepals and four stamens in a spiral pattern in close succession. The female flowers consist of two or four thick, partly fused, perianth parts that surround an ovary with a single ovule, a short style, and four long stigma lobes. Male plants tend to bolt earlier than female plants, but for successful cross-pollination it is required that they flower about the same time (Sneep, 1958; Sherry et al., 1993).

Figure 1. An indeterminately growing monoecious spinach plant that is producing seeds after self-pollination **(A)** with a close-up of immature, green seeds **(B)**. Own pictures taken in the greenhouse of Pop Vriend Seeds, Andijk, the Netherlands (June 2016).

To enhance simultaneous flowering of male and female plants, plants can be (artificially) exposed to low temperatures to enhance flowering, a process called vernalization (Parlevliet, 1967). After successful pollination and fertilization of the ovule, the fruit starts to develop. This fruit is usually called the 'seed' by growers. Depending on the genotype, the two to four sepals may develop into spikes, resulting in prickly (or spiny) seeds. However, for seed processing, the smooth (or round) seed type is preferred and has been selected over the original spiny type by breeding (Sneep, 1958; Sherry et al., 1993). Botanically, the spinach seed is a dry, indehiscent fruit, also called an achene (Hallavant & Ruas, 2014). Other achenes include, e.g., the fruits of amaranth, beet, buckwheat, buttercup, cannabis, lettuce, sunflower, and quinoa. With spinach, the achene consists of a true seed with an embryo surrounding the perisperm, covered with a seed coat (testa) (Figure 2). The true seed is loosely surrounded by a fruit wall, called the pericarp, composed of partly lignified cells (Sifton, 1927). Since the spinach seed anatomy is similar to that of the family-related beet (*Beta vulgaris* L.) seeds (Hermann et al., 2007), studies on beet seeds are particularly relevant for this study on spinach seeds.

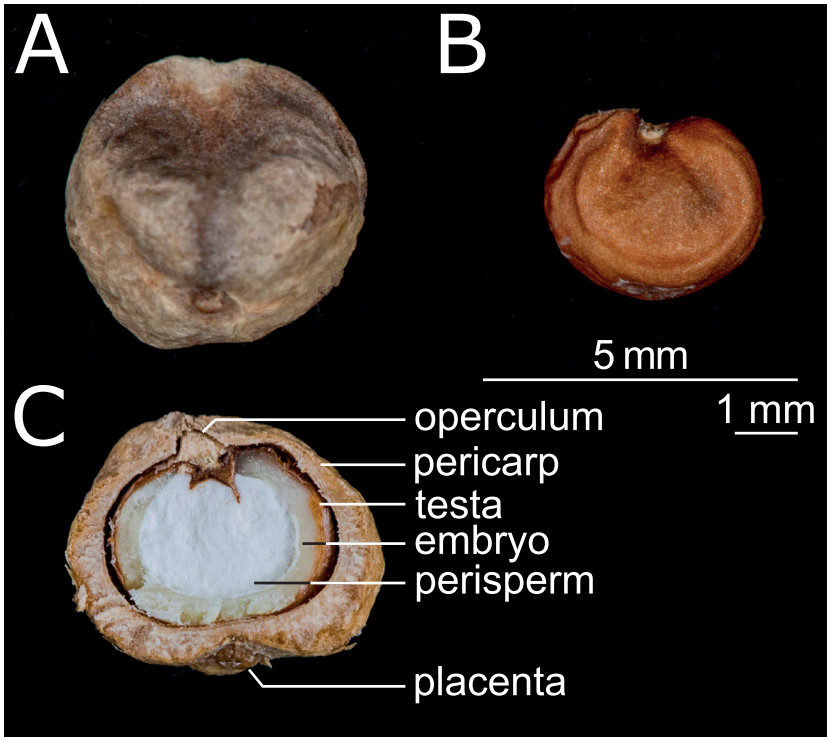


Figure 2. Side views of an intact spinach seed (fruit) **(A)** and true seed without a pericarp **(B)**, and a cross-section with designated structures (C).

Spinach seed sensitivity towards abiotic stress

Temperature and moisture

Previous studies indicated that spinach seed germination is highly sensitive to temperature and to moisture. When tested in the lab on germination paper, the germination of spinach seeds decreased at temperatures above 12°C (Røeggen, 1984), and dropped to 50% at 30°C (Atherton and Farooque, 1983). Temperatures above 35°C could even prevent the seed from germinating (Leskovar et al., 1999). Another study on germination papers showed that the germination of spinach seeds was restricted by high moisture conditions, but could be improved when temperatures were lowered or when oxygen levels were artificially increased (Heydecker and Orphanos, 1968). The International Seed Testing Association (ISTA) advises using temperatures of 10 or 15°C for testing spinach seed germination, whereas they do not give a specific advise on the moisture content of the germination papers (ISTA, 2020). In soils or other substrates, oxygen levels can be reduced by excessive moisture levels or soil compaction. In a study with spinach sown in a substrate with fine peat moss and vermiculite with dolomitic stones, spinach seed germination was hampered at excessive moisture levels (Kear et al., 2005). ***To evaluate the potential of spinach seed germination and emergence, the sensitivity of spinach seeds to high moisture levels needs to be considered, as studied in this dissertation.***

Seed size and maturity

Previous studies on the relationship of spinach seed sensitivity towards abiotic stress conditions did not discriminate among seed sizes, although a large variation in seed sizes can be expected due to the indeterminate growth of spinach and once-over harvest of seeds. Due to the indeterminate flowering and seed development of spinach, the seeds on top of the spinach plants are smaller and less mature than the seeds that were formed earlier and are located lower on the plant. This may result in a large variation in the quality of the seeds harvested at once from a spinach plant (Deleuran et al., 2013). When obtained from the same plant or seed lot, spinach seed size is correlated positively with maturity level. Seed size and maturity can have interactive effects on the rate of germination, depending on the moment when the seeds were harvested. Within an early seed harvest, smaller seeds germinated faster, independent of their maturity level (as the maturity levels were already low). Within a late harvest, seeds with a higher maturity level germinated faster than seeds with a lower maturity level, and this maturity effect was more visible with the smaller seeds (<3.25 mm) than with the larger seeds (>3.25 mm) (Deleuran et al., 2013). ***Hence, in the present study, effects of seed maturity as well as effects of seed size on germination and emergence are evaluated.***

Seed pericarp

Studies have demonstrated that the pericarp surrounding beet seeds plays a role in seed dormancy, inhibiting germination until the seed has reached full maturity and until environmental conditions are optimal for germination (Heydecker et al., 1971; Coumans et al., 1976). The pericarp of beet seeds can regulate germination by its chemical composition (e.g., plant hormones like abscisic acid) and physical structure (thickness and intactness) that determine its permeability to water and oxygen (Hermann et al., 2007). The pericarp can also contain phenolic compounds that bind oxygen, reducing oxygen availability to the embryo, which is necessary for most seeds to germinate, as was shown for beet seeds (Heydecker et al., 1971). Another study on the pericarp of sugar beet seeds showed that germination was greater when the operculum (or ovary cap of the fruit, Figure 2) was removed or when seeds were sown with the open basal pore (or placenta, Figure 2) facing up (Coumans et al., 1976). This was probably a result of better oxygen diffusion to the embryo. Complete removal of the pericarp (dehulling) of spinach seeds increased the total germination of seeds on paper at high moisture levels (Heydecker and Orphanos, 1968) and high temperatures (>18°C) (Sifton, 1927; Atherton and Farooque, 1983; Suganuma and Ohno, 1984; Leskovar et al., 1999; Katzman et al., 2001). Also, in a moist substrate, total germination of true seeds (without the pericarp) was greater than total germination of intact seeds (with the pericarp), but in that study the different seed types originated from different genotypes (Kear et al., 2005). Also, these studies did not discriminate among different sizes of spinach seeds, even though seed size can influence germination performance. ***To analyse the influence of seed size and the pericarp of spinach seeds on germination at high moisture levels, experiments with multiple seed lots with different seed size ranges, and a seed lot graded for multiple seed sizes, are included in this study.***

Seed vigour

Seed size and pericarp thickness are positively correlated with seed maturity, which is thought to be related to seed vigour (Finch-Savage & Bassel, 2015). Seed vigour can be defined as the sum of seed properties that determine the ability of viable seeds to germinate fast and uniformly, and to produce healthy seedlings with rapid and uniform emergence under both optimal and suboptimal environmental conditions, based on the combined definitions of the Association of Official Seed Analysts (AOSA) and ISTA (AOSA, 1983; ISTA, 2021). Seed vigour is influenced by seed maturity, genotype, and environment (Finch-Savage & Bassel, 2015). Therefore, inclusion of multiple genotypes with seed lots from different production environments is valuable for studying seed vigour effects. Several methods have been developed to analyse seed vigour, sometimes crop-specific and mostly by testing the response of seeds to stressful conditions, e.g., germination at suboptimal temperatures or moisture levels, or after deteriorating

storage conditions. Other seed vigour tests analyse the rate of germination, membrane damage resulting in leakage of electrolytes as measured by electrical conductivity of the seed soaking water, or using tetrazolium staining (ISTA, 2021). According to Finch-Savage and Bassel (2015), seed vigour is a seed lot characteristic. However, in case of indeterminately produced seeds on spinach plants, large variation in seed vigour among individual seeds within the seed lots may exist. ***In the current study, seed vigour of multiple seed lots is determined by testing their germination and emergence potential under optimal conditions and under stressful conditions (moisture, temperature, pathogen presence). In addition, individual seeds of those seed lots are evaluated, so that their seed characteristics could be directly related to emergence.*** The smaller the difference between germination or emergence under optimal conditions and stressful conditions, the higher the seed vigour. For instance, a seed lot that germinates relatively fast and is less affected by certain stress levels, has a higher seed vigour compared to seed lots that germinate more slowly or have a smaller chance of germinating under the same stress levels (Figure 3).

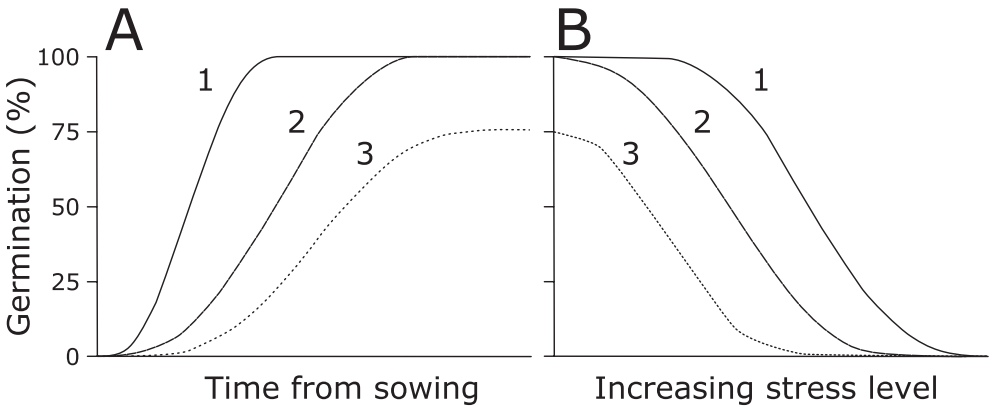


Figure 3. Illustration of the germination probability of three fictive seed lots with high seed vigour (1), medium seed vigour (2), and low seed vigour (3), differing in germination over time **(A)**, and the probability of germination at increasing stress levels **(B)**. Adapted from Finch-Savage and Bassel (2015), Figure 3.D and 3.F on page 571, with permission from Oxford University Press and Copyright Clearance Center.

Non-destructive measurements of seed vigour

In recent years, non-destructive seed measurement techniques have become available for testing and sorting seeds based on maturity, health, and quality. These measurements include multispectral imaging of visible features like seed size, pericarp structure and colour (RGB imaging), combined with light reflectance measurements (spectroscopy) over a wide range of specific wavelengths, including ultraviolet (200 to 380 nm), visible (380 to 750 nm), and near-infrared (>750 nm) light (ElMasry et al., 2019). Light

reflectance from the spinach seed pericarp has been shown to be a good marker for the presence of fungal infection of those seeds (Olesen et al., 2011). In addition, chlorophyll fluorescence (CF) of a seed can be measured by emitting light onto the seed at 450 or 660 nm and measuring excitation from the seed at 730 nm. CF has been demonstrated to be a good marker for the maturity level of seeds of many plant species, with an inverse correlation with germination (Jalink et al., 1998). Also, for sugar beet and spinach seeds, where CF is measured from the pericarp instead of the seed coat, CF correlated negatively with germination (Deleuran et al., 2013; Boelt et al., 2018). ***In this study, spectral and fluorescence measurements in addition to morphological measurements of spinach seeds are performed and, for the first time, related to damping-off tolerance.***

Introduction to the disease: damping off

Spinach crop production faces various diseases, including those caused by airborne pathogens (e.g., *Peronospora* (downy mildew), *Albugo* (white rust), *Cladosporium* and *Stemphylium* (leaf spot pathogens)), soilborne pathogens (e.g., *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia* and nematodes), and virus transmissions (Morelock & Correll, 2008). Downy mildew is probably the most widespread and destructive disease of spinach caused by *Peronospora farinosa* f.sp. *spinaciae*. Resistance to downy mildew is largely based on single dominant genes and is commonly used in hybrid breeding to obtain resistant spinach cultivars. However, due to this approach and year-round spinach production, there is an on-going race between the development of resistant spinach varieties and new races of downy mildew (Morelock & Correll, 2008). Of the soilborne diseases, damping off is the most widely occurring disease, causing empty patches in spinach fields with potentially devastating production losses. The severity of damping off is highly dependent on the specific pathogen(s), host plant, and environment (Lamichhane et al., 2017). Damping-off diseases can be roughly divided in two types based on the developmental stage of the plant at the moment of showing symptoms (Lamichhane et al., 2017). Seed decay before germination and root rot before the seedling emerges above soil or substrate level can be categorised as pre-emergence damping off. Root rot and wilt of seedlings or plants after emergence can be categorised as post-emergence damping off. These symptoms can be caused by a complex of pathogens, including soilborne species from the fungal genera *Fusarium* and *Rhizoctonia*, and oomycete genera *Aphanomyces, Phytophthora* and *Pythium*. Multiple studies on damping-off diseases have characterised *Pythium* spp. as the most prevalent damping-off pathogens in spinach fields in Sweden (Europe) (Larsson, 1994), in California (USA) (Koike et al., 2011), and in Georgia (USA) (Sumner et al., 1976). Generally, damping off occurs more frequently in wet, compacted soils, but the severity of damping off is dependent on a multitude of factors, such as the plant species and genotype, pathogen species, abundance, and pathogenicity,

and management practices (Lamichhane et al., 2017). For spinach, damping off occurs more frequently during warm and wet periods, and after a history of frequent spinach (or other host crop) production (Correll et al., 1994).

The short production cycle and small harvest window in baby-leaf production requires uniform seed germination and leaf production. Suboptimal abiotic and biotic conditions can be devastating during seed germination and seedling emergence. Also, the general observation is that more mature plants can partially withstand infection by *Pythium* spp. (Kamoun et al., 1999). Therefore, the spinach seedling is expected to be most vulnerable in the pre-emergence stage. Since pre-emergence damping off includes non-germination of seeds and non-emergence of seedlings above soil level, it is difficult to assess the occurrence of this type of damping off resulting from pathogen infection rather than suboptimal abiotic conditions. To our knowledge, an assay to evaluate spinach pre-emergence damping off that corrects for non-germination as a result of abiotic influences, is not yet available. For the development of such an assay it is required to identify the pathogen of interest first. ***In the present study, potential damping-off pathogens are isolated from infected seeds and plants grown in field soils where spinach damping off had occurred.***

Characteristics of the pathogen of interest: *Pythium ultimum*

Previous studies have characterised *P. ultimum* as the most prevalent damping-off pathogen in spinach fields in Sweden (Europe) (Larsson, 1994) and California (USA) (Koike et al., 2011). *P. ultimum* Trow, 1901, is one of the over 230 species of the genus *Pythium* Pringsheim, 1858, which is the largest and most comprehensively studied genus in the family of Pythiaceae, order Peronosporales, class Peronosporomycetes, phylum Oomycota, and kingdom Stramenopila (= Chromista) (Plaats-Niterink, 1981; Beakes et al., 2014). This oomycete can cause both types of damping off by infecting the seed, young seedling, or root, mostly at the base of the stem at the soil or substrate level. The pathogen spreads by the formation of mycelium and spores, and the plant tissue becomes infected by germ tubes or hyphae formed by three different structures, encysted zoospores, oospores, or sporangia (Figure 4). The mycelium develops into sporangia that directly produce germ tubes or the sporangium forms a vesicle that produces zoospores that, after release, move to the plant tissue where they encyst and develop germ tubes. The pathogen also forms oospores that each produce a germ tube directly or produce a sporangium in which zoospores that produce a germ tube in presence of a host (Agrios, 2005b). Which infection strategy occurs, depends on the environmental conditions and on the variety of *P. ultimum* (Plaats-Niterink, 1981). The optimum temperature for direct formation of germ tubes from sporangia is above 18°C, and by means of zoospores

is 10 to 18°C with sufficient water availability (Agrios, 2005b). *P. ultimum* var. *ultimum* rarely produces zoospores, only at these lower temperatures, and is more pathogenic than var. *sporangiiferum*, which produces zoospores more regularly (Plaats-Niterink, 1981). The pathogenicity of *P. ultimum* at causing damping off is higher under high soil moisture conditions for a prolonged period (Schmitthenner, 1970; Agrios, 2005c); under soil temperatures that are unfavourable for the host plant (Paulitz & Baker, 1987; Agrios, 2005c), which include lower temperatures than for other *Pythium* species (Klisiewicz, 1968); and at low soil oxygen levels (Schmitthenner, 1970). Optimum growth of *P. ultimum* is at 25-30°C in culture (Leach, 1947) and below 20°C in soil (Munnecke et al., 1971). The thick-walled oospores are the resting structures that may survive for many years (Stanghellini & Hancock, 1971). In addition, *P. ultimum* is a broad host range species, able to infect a wide range of crops, including beet, carrot, soybean, spinach, tomato, and wheat (Abd-Elsalam & Amal-Asran, 2020). As a result, a history of frequent productions of vulnerable crops promotes the development of *P. ultimum* in soils, which makes it very difficult to eliminate the pathogen. This emphasises the need for certain resistance or tolerance levels of a plant to survive in *P. ultimum*-infested soils.

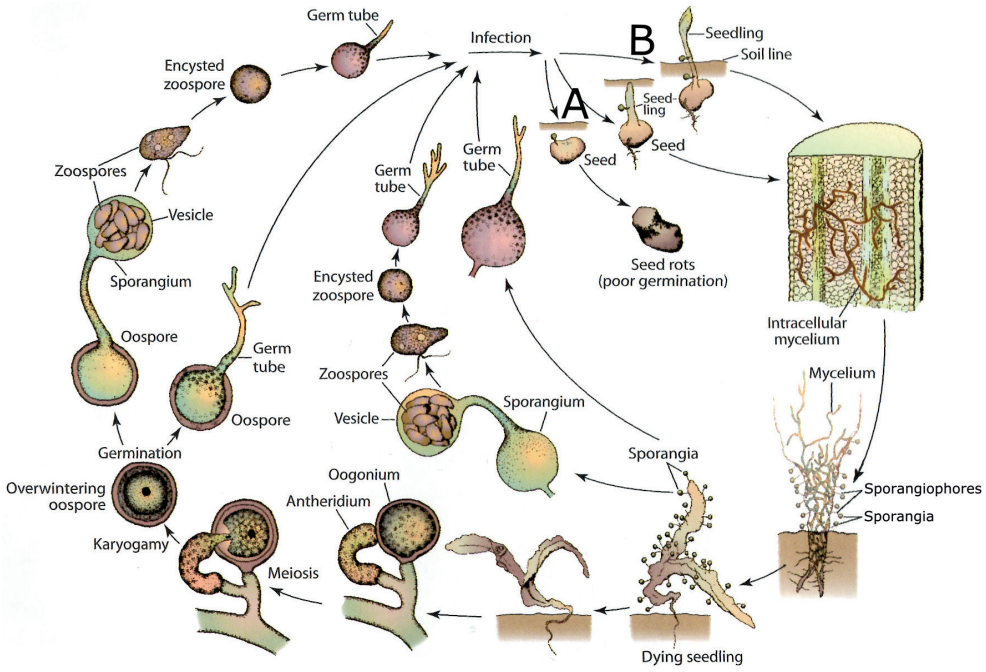


Figure 4. Life cycle of Pythium spp. causing pre-emergence **(A)** and post-emergence **(B)** damping off. Reprinted from Agrios (2005b), Figure 11-18 on page 412, with permission from Elsevier and Copyright Clearance Center.

The potential of spinach seed vigour in damping-off tolerance

Tolerance of seeds and seedlings to biotic and abiotic stress has been suggested to be under the influence of seed vigour (TeKrony & Egli, 1991; Lammerts van Bueren et al., 2003). Some studies have reported positive relationships between seed vigour and biotic stress tolerance. An increased sensitivity towards soilborne pathogens of seeds with a lower vigour that cause delayed emergence has been described for several crop species, such as wheat (Das Gupta & Austenson, 1973), soybean (Schlub & Schmitthenner, 1978; Hamman et al., 2002), pea (Perry, 1973; Stasz & Harman, 1980), and lucerne (Hawthorne, 1988). In these studies, seed vigour was deliberately reduced, for instance by temporary storage at high humidity (Hamman et al., 2002). Delayed emergence is also suggested to increase damping-off sensitivity (Green et al., 2012). Generally, to reduce the severity of damping off in fields, sites are selected with good drainage and well-aerated soils and favourable temperatures for seed germination, promoting rapid, vigorous seedling growth (Lamichhane et al., 2017). Spinach growers in the UK also stated that seed vigour can contribute to the susceptibility of seeds and seedlings to damping-off disease, but the contribution has not been ascertained yet. In a study with 12 spinach cultivars, variation was observed in emergence and damping-off symptoms among cultivars, as well as between two seed lots of three cultivars that were also included (Green et al., 2012). For two of these cultivars, the two seed lots showed a significant difference in germination rate. The other nine cultivars were each represented by a single seed lot. Most of them germinated slower and showed a lower emergence in naturally-infested soils, as well as more post-emergence damping-off (wilting) symptoms. These results suggested that rate of emergence has a positive effect on damping-off tolerance. Green et al. (2012) tested rate of germination in a laboratory environment, whereas damping off was evaluated in naturally-infested soils under unheated, polythene tunnel conditions. The correlation between rate of emergence and damping-off tolerance is more reliable when seeds are sown under the same conditions. In addition, a correction for non-emerged seedlings not due to pathogen infection is needed by including a non-inoculated control substrate. ***For this research, evaluations on emergence rate and damping-off tolerance are performed with three seed lots of each of five spinach cultivars in a newly developed phenotyping assay that includes a control treatment of non-inoculated substrate.***

A positive effect of germination rate on damping-off tolerance could also be tested by enhancing germination rate of seeds through priming. In general, priming methods are based on the process of hydrating seeds in a controlled manner to initiate germination-related processes, while preventing root protrusion by limiting the amount of water uptake that would otherwise result in a loss of desiccation tolerance (Heydecker et al.,

1975). For many different plant species, seed priming has been shown to increase the rate and uniformity of germination by reducing the time for the seeds to germinate. Since priming is known to increase the uniformity of germination, this can be helpful to synchronise the germination of seeds with different seed vigour, i.e., to exclude a potential seed vigour effect on damping-off tolerance and to focus on a potential genotypic effect (*this study*). However, seed vigour can also be genotypic and related to stress tolerance. For instance, in one study, primed spinach seeds performed better under abiotic stress, including high moisture levels and high temperatures (Chen et al., 2010). The priming possibly counteracts germination-inhibitory effects of the pericarp. For sugar beet and spinach seeds, both washing (soaking) and polishing of the pericarp, especially when combined, improved the rate of germination and total germination (Atherton & Farooque, 1983; Ignatz et al., 2019). Positive effects of priming, pericarp polishing or complete pericarp removal on abiotic stress tolerance of spinach seeds may also enhance biotic stress tolerance. To our knowledge, a direct relationship between spinach seed priming or pericarp removal (also called dehulling) on disease tolerance of spinach seeds has not been studied yet. Seed size has been studied in relation to biotic stress tolerance. For instance, in a study with lucerne seeds that do not have a pericarp, seedlings that emerged from heavier seeds were more tolerant towards *Pythium* spp. infection than seedlings from lighter seeds (Hawthorne, 1988). To study the effect of emergence rate on damping-off tolerance, priming and dehulling can be useful techniques. ***Potential effects of the spinach seed pericarp on germination, emergence, and pre-emergence damping-off tolerance are evaluated in this study by comparing dehulled and intact seeds of the same seed lots.***

Research questions and hypotheses

Based on the increasing need for alternative solutions for reducing the incidence of spinach damping-off, the main objective of this research was to find possibilities to improve damping-off tolerance of spinach cultivars or seed lots.

For the evaluation of damping-off tolerance, it is important that germination of seed lots can reach its maximum. Previous studies indicated effects of moisture and temperature on spinach seed germination that interacted with seed size. However, these studies did not include multiple seed lots of the same genotype or seed lots fractionated into different seed sizes. Since spinach seeds in a seed lot can have a large variability in seed size, we were interested to test whether seed size can affect germination, particularly in relation to high moisture conditions, which may also influence damping-off severity. This led to the following question:

Which spinach seed traits underlie germination sensitivity under high moisture conditions? (Chapter 2)

We hypothesised that germination of smaller spinach seeds is less sensitive to high moisture conditions than germination of larger seeds. We expected that the pericarp of the larger seeds is thicker, and that the pericarp inhibits germination at high moisture levels due to lower oxygen availability to the embryo, as oxygen is crucial for seed germination. An increased oxygen level would diminish potential differences between the germination rates of small and large seeds.

Breeding for improved damping-off tolerance requires the existence of genotypic variation for damping-off tolerance, which led to the question:

Does genotypic variation exist for damping-off tolerance in spinach? (Chapter 3)

We hypothesised that genotypic variation for damping-off tolerance exists in spinach. We tested this by evaluating tolerance levels of commercially produced seed lots of multiple cultivars, first in the field and in the greenhouse with field soil. We expected differences among cultivars in both pre-emergence and post-emergence damping-off tolerance. From the literature, we know that abiotic conditions (e.g., moisture and temperature) play a major role in the severity of damping off. To minimise these effects, a standardised phenotyping assay was developed and used for the evaluation of pre-emergence damping-off tolerance levels of seed lots of the same cultivars.

Since damping off can result from early infection of a seed, root, or young seedling, we expected that seed vigour plays an important role in disease tolerance. One of the aspects of vigour is the rate of germination. Based on a previous study using a limited number of spinach seed lots, we evaluated the following question further, using multiple seed lots per genotype:

Can faster emergence improve pre-emergence damping-off tolerance in spinach? (Chapter 4)

We hypothesised that faster germinating seeds show higher rates of seedling emergence and greater tolerance towards pre-emergence infection by damping-off pathogens. Also, faster emerging seed lots were expected to have greater pre-emergence damping-off tolerance than slower emerging seed lots.

Spinach seed lots can show large variability in seed quality due to the indeterminate nature of seed development and once-over harvesting of the seeds. Seed quality consists of various components, of which seed vigour determines the success of a seed to develop into seedlings under both optimal and stressful conditions. Various seed traits are known to influence seed vigour and we were interested in the relation between those traits and pre-emergence damping-off tolerance, which resulted in the following question:

Which specific seed traits relate to pre-emergence damping-off tolerance in spinach? (Chapter 5)

We hypothesised that seed size plays a role in pre-emergence damping-off tolerance as we also expected an effect of seed size on germination performance under abiotic stress. Seed size may correlate with seed maturity and, therefore, seed maturity was also expected to play a role. In addition, other characteristics that relate to the physiological maturity of the seed may play a role. Therefore, various non-destructive morphological and spectral seed measurements were performed.

Research design and evaluation methods

For all experiments, we used commercially produced seed lots of spinach cultivars that originated from different seed productions. For one cultivar, we also obtained a seed lot that was fractionated into five seed size ranges. Thus far, the use of multiple seed lots in a cultivar study is not common, whereas it may strengthen the potential effect of cultivar when different seed lots of the same cultivars show similar results. A large variation in damping-off tolerance levels among seed lots, even within cultivars, triggered our interest to compare seed lots for multiple seed vigour aspects, including the rate of germination and emergence in relation to pre-emergence damping-off tolerance. Also, specific seed traits were studied in relation to pre-emergence damping-off tolerance at the seed lot level (comparing seed lots) and for individual seed lots (comparing seeds within seed lots). A brief overview of the different evaluation methods is provided here, while details are described in the research chapters.

For evaluations of seed germination parameters, including rate, uniformity, area under the germination curve and total germination, we used a system based on the Jacobsen germination table, using filter papers that were connected to the water table with a paper wick. The main difference was that we applied different heights between the filter papers and water table by using Styrofoam plates to generate different moisture levels. This floating germination system was moveable and could fit in climate-controlled cabinets for evaluation at different temperatures, and in an air-tight box for evaluation at different oxygen levels (**Chapter 2**).

For the evaluation of damping-off tolerance (**Chapters 3 to 5**), we started performing trials in fields from which we also used soil samples to plant seeds of a susceptible spinach cultivar for extracting potential damping-off pathogens to choose our pathogen of interest. The variation in emergence results among field and greenhouse trials emphasised the necessity to minimise the environmental effects on emergence, other than pathogen infection. Therefore, a phenotyping method with standardised abiotic and biotic conditions was developed, using a standardised, nutrient poor, and relatively dry substrate that was inoculated with *Pythium ultimum* using a standardised protocol, and a control substrate that was not inoculated (**Chapter 3**). The use of a non-inoculated control substrate enabled us to correct for lack of emergence that was not a result of pathogen infection. The statistical analysis followed a logistic regression of emergence with the different independent variables. By dividing the odds of emergence in the inoculated substrate by the odds of emergence in the non-inoculated substrate (control treatment) we obtained an odds ratio that was referred to as the pre-emergence damping-off tolerance level.

In addition to the experiments with untreated seeds, a seed priming method was applied to increase the uniformity of germination and to test its effect on the rate of germination (**Chapter 2**), emergence and pre-emergence damping-off tolerance (**Chapter 4**). Seed dehulling was applied to test the effect of the seed pericarp on germination on filter paper in relation to high moisture levels (**Chapter 2**), on emergence in a substrate, and on pre-emergence damping-off tolerance in *P. ultimum*-inoculated versus non-inoculated (control) substrate (**Chapter 4**). By recording emergence daily, the rate of emergence could be determined and correlated with damping-off tolerance levels.

Various seed traits were measured with multispectral imaging of individual seeds of each seed lot and of a seed lot fractionated into seed sizes and based on high and low seed maturity levels (**Chapter 5**). Seeds were subsequently sown in *P. ultimum*-inoculated or control substrate. By sowing them in individual cells and scoring seedling emergence from individual seeds, we were able to relate those seed traits to emergence. The seed trait measurements were also used to check for variation among spinach seed lots.

Outline of chapters

This dissertation consists of six chapters, including this introduction (Chapter 1), four research chapters (Chapters 2 to 5) and a general discussion (Chapter 6). A summary is provided thereafter. The connections between the chapters are visualised in Figure 5.

The general introduction (**Chapter 1**) provides a background on the production of

spinach as a leafy vegetable and its seed production, with the most important challenges in both organic and conventional production, giving the most attention to damping-off disease and seed vigour.

In **Chapter 2,** the effects of seed size and the pericarp on seed germination at different moisture levels, temperatures, and at elevated and normal (air-based) oxygen levels are described. For these evaluations, a floating germination system was developed to assess seed germination at standardised moisture levels of commercially produced seed lots and of one seed lot fractionated into different seed sizes.

In **Chapter 3**, the same spinach cultivars and seed lots were evaluated for their tolerance towards damping off in the field, greenhouse, and in a climate-controlled cabinet. In field soils, incidences of emergence and non-emergence were scored and compared among cultivars and seed lots. Potential damping-off pathogens were isolated from the same field soils, tested for their pathogenicity on spinach, and characterized to species or genus. After the field and greenhouse trials, a standardised phenotyping assay was developed to assess pre-emergence damping-off tolerance to *P. ultimum*.

In **Chapter 4**, the same spinach seed lots were evaluated for the rate of emergence in relation to damping-off tolerance levels. The effects of priming and dehulling of seeds on rate and uniformity of germination and emergence were also related to the tolerance levels.

In **Chapter 5**, we zoomed in on individual seeds of the same spinach seed lots used in the previous chapters, and correlated those seed traits to *P. ultimum* tolerance. An experiment with a seed lot fractionated into different seed sizes and maturity levels was performed to validate the potential effects of seed size and seed maturity on emergence success in the absence and presence of *P. ultimum*.

Finally, the answers to the research questions are summarised and discussed in a broader context with recommendations for further research and development (**Chapter 6**).

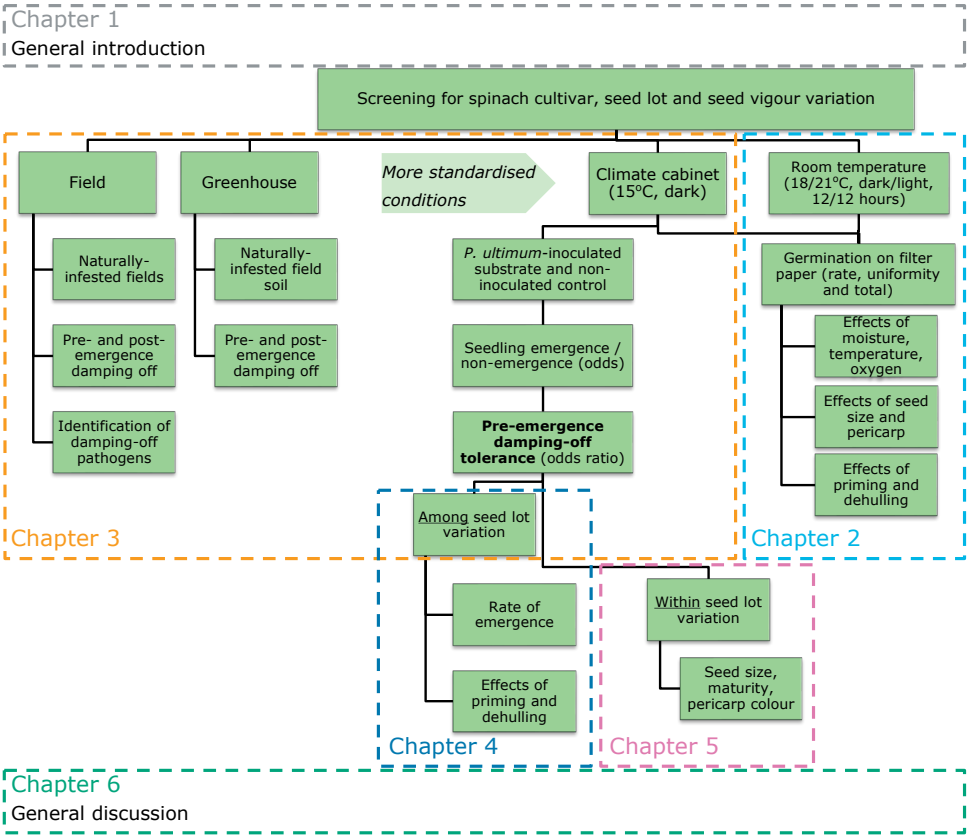


Figure 5. Diagram of the research steps and how they connect the different chapters.

Chapter 2

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

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

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 Science and Technology, 48(1), 117-131. https://doi.org/10.15258/sst.2020.48.1.13

Abstract

Uniform seedling emergence is crucial for economically viable spinach (*Spinacia oleracea* L.) production. However, non-uniform seed germination occurs due to variation in moisture sensitivity among and within spinach seed lots. To test moisture sensitivity, we developed a floating germination system with fixed distances between the water table and germination papers, so that moisture levels were standardised. We tested germination performance of different cultivar seed lots, with one seed lot fractionated into different seed sizes, and of seeds with an intact, open, or removed pericarp. At 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. The pericarp of smaller seeds was relatively thinner than the pericarp of larger seeds. A lower temperature or increased oxygen level resulted in improved germination of differently-sized seeds at a high moisture level. In conclusion, the sensitivity of spinach seed germination to moisture is influenced by seed size, hence pericarp thickness and intactness, and is driven by 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 to 20°C.

Keywords: germination, moisture, oxygen, pericarp, seed size, *Spinacia oleracea*, temperature

Introduction

Over about 50 years, the worldwide production of spinach (*Spinacia oleracea* L.) has increased tenfold, with a rapid increase of threefold in the past decade (FAO, 2019). The growing demands for fresh-market bagged spinach products has been the main reason for this rapid increase. Bagged spinach contains either very small young leaves (baby-leaf spinach) or slightly older, medium-sized leaves (teenage spinach). Depending on the season, baby-leaf 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. Therefore, non-uniform or poor seed germination resulting in non-uniform field emergence, is a major problem for spinach production (Simko et al., 2014).

Spinach seeds have a similar structure as sugar beet seeds (*Beta vulgaris* L.), both family of Amaranthaceae. What the industry calls the 'seed' is botanically an indehiscent fruit, consisting of a true seed loosely surrounded by a fruit wall, called 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 is called operculum. Due to the indeterminate flowering pattern and once-over seed harvesting of spinach, a seed lot varies in seed sizes and maturity levels (Deleuran et al., 2013). In general, less mature seeds have lower seed vigour (Jalink et al., 1998). Seed vigour is the sum of seed properties that determine the potential of a seed lot for fast and uniform seed germination and seedling establishment in optimal and suboptimal 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), may drop to 50% when it rises to 30°C (Atherton and Farooque, 1983), and germination does not occur at 35°C (Leskovar et al., 1999). 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 pre-chilling in addition to a low moisture level in the spinach germination test. In current ISTA rules (ISTA, 2020) pre-chilling 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 high moisture levels of germination papers. To determine the effects of various moisture levels on spinach seed germination, we developed a floating germination system with fixed distances between the water table and germination papers, so that the moisture content of the germination papers 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 to high moisture conditions 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 acts as a physical barrier to oxygen that 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 in case of true seeds (without pericarp) compared to intact seeds (fruits) at high moisture levels (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 showed that by increasing the oxygen level, spinach seed germination improved at room temperature (Sifton, 1927) and high moisture level (Heydecker and Orphanos, 1968). We performed germination studies with a floating germination system, 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 were used, including 'Carmel', 'Chevelle', 'Shelby', and 'Novico' (Table 1). No information was provided by the suppliers on the history or maturity of the seed lots. After arrival, 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 to 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 multiple sides than the operculum side only, or from another side than the operculum side, e.g., placenta side.

Table 1. Information on spinach seed lots 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. Seed lot D of cultivar Carmel was sorted into five seed size fractions (D.1 to D.5).

Cultivar	Seed lot	Seed size (mm)	TSW (g)	Providing company
Carmel	D.1	2.50–2.75	7.44	Pop Vriend Seeds, Andijk, the Netherlands
Carmel	D.2	2.75–3.50	12.60	
Carmel	D.3	3.50–4.25	19.99	
Carmel	D.4	4.25–4.50	25.17	
Carmel	D.5	4.50–5.00	29.24	
Chevelle	A	2.25–2.75	5.47	Enza Zaden/Vitalis, Enkhuizen/Voorst, the Netherlands
Chevelle	B	2.50–3.75	9.98	
Chevelle	C	2.50–3.75	10.81	
Novico	A	2.50–3.50	9.68	Nunhems/BASF, Nunhem, the Netherlands
Novico	B	2.75–4.25	12.85	
Novico	C	3.00–5.00	17.17	
Shelby	A	2.50–3.75	9.47	Enza Zaden/Vitalis, Enkhuizen/Voorst, the Netherlands
Shelby	B	2.50–3.75	11.01	
Shelby	C	3.30–4.50	16.13	

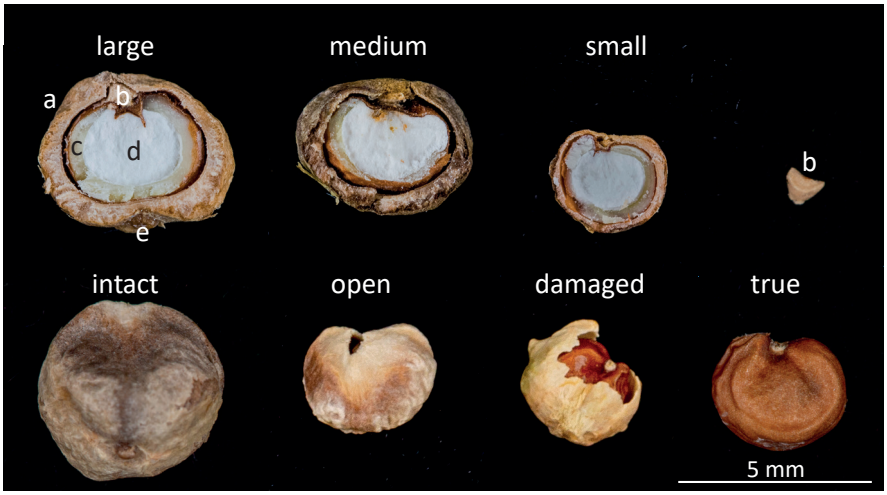


Figure 1. Cross-sections of spinach seeds (fruit) from seed lot D of cultivar Carmel with a large size (4.50–5.00 mm, Carmel-D.5), medium size (3.50–4.25 mm, Carmel-D.3), and small size (2.50–2.75 mm, Carmel-D.1) with pericarp **(a)**, operculum **(b)**, embryo **(c)**, perisperm **(d)**, and placenta **(e)**; and side views of an intact, open, damaged seed (fruit) and true seed (pericarp removed).

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}, Allpaper B.V., Zevenaar, NL) through holes in the Styrofoam with 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 are placed over the seeds to maintain a high air humidity level. The seeds have an equal distance to the light source. Two days after setting-up the floating germination system, the seeds were placed on the moist papers ('day 0').

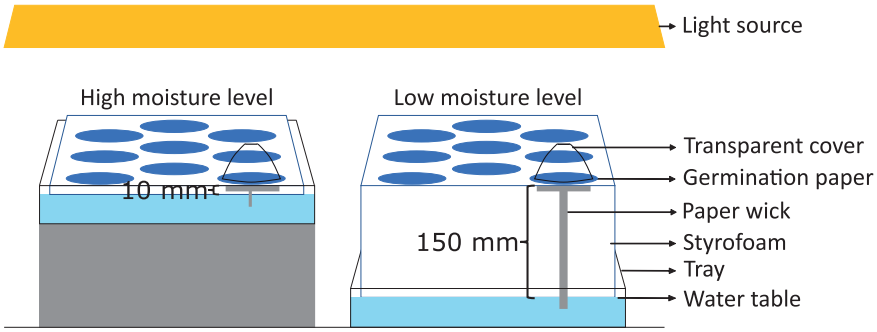


Figure 2. Illustration of germination system with germination papers on top of floating Styrofoam plates with fixed distances to the water table at 10 mm (left) and 150 mm (right) distance.

Experimental conditions

With the use of the described germination system, we tested germination performance of the seed size fractions of cultivar Carmel and seed lots of cultivars Chevelle, Novico, and Shelby at standardised moisture conditions and temperatures. 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 mm (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 facing up. Environmental conditions included 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

Germination performance was determined by several parameters: fitted maximum germination percentage (G_{max}), as the percentage of total germinated seeds at the end of the experiment; rate 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: $y_x = y_0 + \frac{(a \cdot x^b)}{(c^b + x^b)}$, 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 an Analysis of Variances (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 (α = 0.05). In case of significant interaction effects, *post hoc* multiple comparisons were carried out with the Fisher's Protected Least Significantly Difference (LSD) test (α=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 in grams water per gram germination paper decreased (Figure 3). Over time, there was an increase in moisture content. After seven days, there was no significant increase in moisture content, except for 10 and 50 mm Styrofoam height.

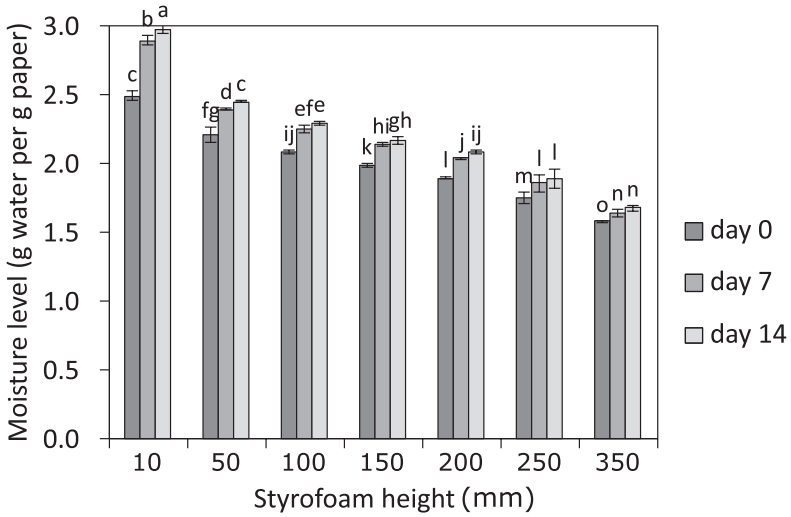


Figure 3. Moisture content of germination papers depending on Styrofoam height (mm) on the day of sowing (day 0), day 7, and on day 14 of the experiment shown in Figure 5. The same letter indicates homogeneous subsets according to Fisher's protected $LSD_{0.05}$. Error bars represent standard deviation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

Effect of moisture level on germination performance of differently-sized seeds

To test the effect of moisture level and seed size on germination performance, we conducted an experiment with five seed size fractions of the spinach cultivar 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 D.1 and D.2) germinated better than the larger seeds (seed size fractions D.3, D.4 and D.5), with a significantly

higher G_{max}, lower t_{50} and higher AUC (over 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.

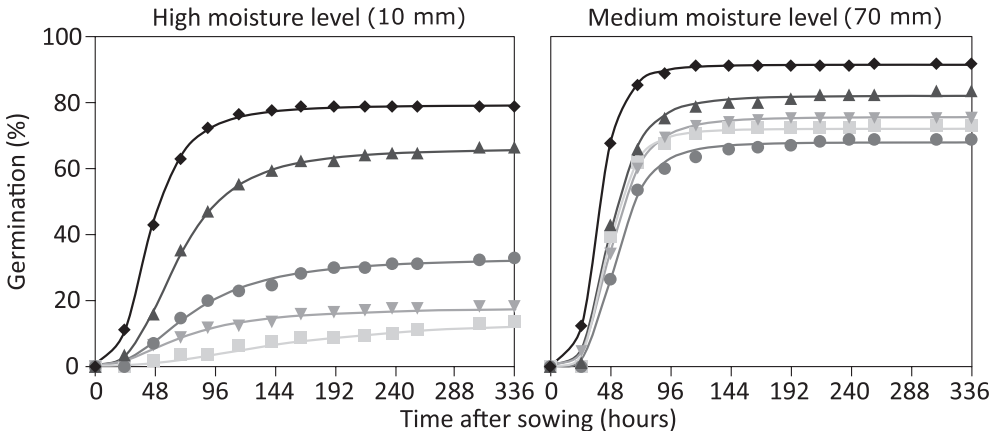


Figure 4. Germination over time of five seed size fractions of seed lot D of spinach cultivar Carmel: 2.50–2.75 mm (D.1, ◆), 2.75–3.50 mm (D.2, ▲), 3.50–4.25 mm (D.3, ●), 4.25–4.50 mm (D.4, ▼) and 4.50–5.00 mm (D.5, ■). The germination test was performed at high and medium moisture level (10 and 70 mm Styrofoam), with 12/12 hours light/darkness at 21/18°C. Percentages are averaged over four replicates of 50 randomly picked seeds.

Optimal moisture level for germination of differently-sized 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 on 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 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 seed lot D of spinach cultivar Carmel: 2.50–2.75 mm (D.1) and 4.50–5.00 mm (D.5). 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.

Carmel fraction	Height (mm)	G_{max} (%)	t_{50} (hours)	AUC (after 220 hours)
D.1	10	77.5 cd	72.1 a	109.9 f
	50	90.8 ab	50.5 a	148.5 abcd
	100	94.2 a	48.1 a	158.3 ab
	150	93.3 a	48.1 a	158.6 ab
	200	95.0 a	47.7 a	161.1 a
	250	94.2 a	51.8 a	154.4 abc
	350	95.0 a	57.3 a	148.2 abcd
D.5	10	6.7 e	91.8 a	7.4 g
	50	70.0 d	68.1 a	97.3 f
	100	80.8 bcd	52.9 a	130.4 de
	150	92.5 a	52.4 a	152.9 abc
	200	92.5 a	63.3 a	141.1 bcd
	250	95.0 a	68.8 a	138.6 cd
	350	84.2 abc	81.9 a	111.6 ef
SEM		0.04	9.04	6.51

Effect of moisture on germination performance of different seed lots

In addition to the previous results, seeds of different sizes from cultivars Chevelle, Novico and Shelby generally germinated better at low moisture level (Table 3). They had a significantly higher G_{max} and AUC (after 220 hours) at low moisture level (150 mm Styrofoam) than at high moisture level (10 mm). Remarkably, seed lot A of 'Chevelle', containing relatively smaller seeds (2.25–2.75 mm), germinated equally well at both moisture levels. At a high moisture level, Chevelle-A even germinated best compared with all other seed lots. We observed that this seed lot also contained more seeds with a damaged pericarp compared with the other seed lots (*data not shown*). At high moisture level, all three seed lots of cultivar Chevelle showed greater germination, with a significantly higher G_{max} and AUC, than the other cultivar seed lots. Even at low moisture level, the germination of seed lots B and C of 'Chevelle', containing seed sizes between 2.50 and 3.75 mm, was greater compared with '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).

Table 3. Germination performance of three seed lots of the spinach cultivars Chevelle, Novico and Shelby with different seed size ranges (Table 1). The germination test was performed at high and low moisture level (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.

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

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 (150 mm) moisture level and at constant 20, 15 or 10°C, without light. At high moisture level, small seeds (2.50–2.75 mm) generally germinated significantly better than medium-sized (3.50–4.25 mm) and large seeds (4.50–5.00 mm) (Table 4). At 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 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 moisture level (150 mm Styrofoam height) than at the high moisture level (10 mm Styrofoam height). At 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.

Table 4. Germination performance of three seed size fractions of seed lot D of spinach cultivar Carmel: 2.50–2.75 mm (D.1), 3.50–4.25 mm (D.3) and 4.50–5.00 mm (D.5). The germination test was performed at high and low moisture level (10 and 150 mm Styrofoam), at 10, 15, 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.

		G_{max} (%)				t_{50} (hrs)				AUC (after 220 hours)			
Carmel fraction	**°C**	**10 mm**		**150 mm**		**10 mm**		**150 mm**		**10 mm**		**150 mm**	
D.1	10	90.7	ab	94.0	a	82.1	de	89.0	d	246.1	de	248.6	de
	15	91.3	a	94.7	a	59.2	gh	54.6	h	269.0	bc	284.9	ab
	20	76.0	c	93.3	a	48.1	hi	37.9	i	230.9	ef	295.3	a
D.3	10	68.0	d	90.7	ab	121.6	b	111.6	bc	155.9	gh	217.7	f
	15	70.7	cd	90.0	ab	101.5	c	70.8	efg	173.3	g	257.2	cd
	20	18.7	f	84.0	b	70.1	fg	52.3	h	52.3	j	254.7	cd
D.5	10	72.7	cd	96.0	a	155.9	a	118.6	b	141.4	hi	225.9	f
	15	52.0	e	93.3	a	104.6	c	80.5	def	123.6	i	253.9	cd
	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			

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 the seed was 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 as quickly 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 (fruits).

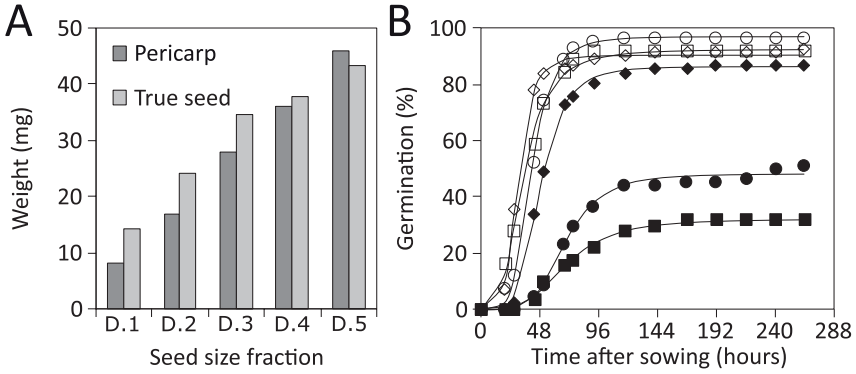


Figure 5. A) Pericarp (dark grey) and true seed (light grey) fresh weight in mg per 200 seeds of the five seed size fractions of seed lot D of spinach cultivar Carmel (no replicates): 2.50–2.75 mm (D.1), 2.75–3.50 mm (D.2), 3.50–4.25 mm (D.3), 4.25–5.00 mm (D.4) and 4.50–5.00 mm (D.5). **B)** Germination over time of seed size fractions D.1 (◆,◇), D.3 (●,○) and D.5 (■,□). Closed symbols display measurements of intact seeds (fruits) and open symbols display measurements of true seeds, averaged over three replicates of 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 a limiting factor for germination at high moisture levels, we applied normal or high oxygen levels to the floating germination system with high moisture level (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 (over 240 hours), and lower t_{50} at both oxygen levels, compared with medium-sized and large seeds. At normal oxygen level (19%), open seeds showed a significantly higher G_{max} and AUC than intact seeds, but at high oxygen level (89%), intact and open seeds performed equally well.

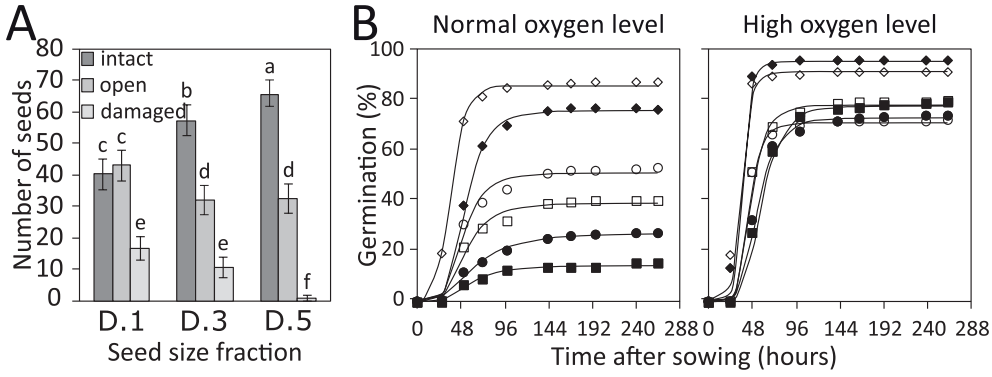


Figure 6. A) Number of intact, open, and damaged seeds of three seed size fractions of spinach cultivar Carmel: 2.50–2.75 mm (D.1), 3.50–4.25 mm (D.3) and 4.50–5.00 mm (D.5). Data are averaged over three replicates of 100 seeds. Error bars represent approximate standard deviation of the means and same letters indicate homogeneous subsets according to Fisher's protected $LSD_{0.05}$. **B)** Germination over time of seed size fractions D.1 (◆,◇), D.3 (●,○) and D.5 (■,□). Closed symbols display measurements of intact seeds and open symbols display measurements of open seeds, averaged over three replicates of 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 confirmed 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 fractionated into different seed sizes, we found that spinach seeds show considerable variation in sensitivity to high moisture levels. For instance, germination of large seeds varied from 90% at low moisture level to 10% at high moisture level, whereas small seeds still had 80% germination at 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 height) compared with total germination at a high moisture level (10 mm Styrofoam height). 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 gave the optimal moisture level for germination of spinach seeds of a diameter between 2.50 and 5.00 mm.

This study showed that variation in moisture sensitivity is related to variation in seed size that was observed among and within spinach seed lots, and that pericarp thickness and intactness plays a role. Larger seeds, which have a thicker pericarp, were more sensitive to high moisture levels, with a lower germination performance than smaller seeds, irrespective of cultivar or seed lot. Seed size ranges differed among the cultivars, which complicated interpretation of the results. However, the results of the 'Carmel' seed size fractions together with the results of the other seed lots confirmed 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 fewer 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 percentages of spinach seeds at a high moisture level (Heydecker and Orphanos, 1968). Neither study discriminated between seed sizes or pericarp intactness. With the use of 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 less 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 can hamper germination at high moisture levels.

Finally, this study led to the confirmation that oxygen shortage at high moisture levels is an important cause of spinach seed germination inhibition. Germination results using air-tight boxes with normal or high oxygen levels showed that total germination, especially of large intact seeds, at high moisture level improved with an increased oxygen level. This indicated that the pericarp of large seeds limits oxygen diffusion to the seed embryo to a larger extent 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 spinach seed germination percentages at high moisture levels (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 a better oxygen diffusion into the embryo and, therefore, smaller seeds, with 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 up than with it down in another study (Coumans et al., 1976). These results suggest that air supply through pericarp openings or a thin pericarp improves oxygen availability to the seed embryo and, therefore, stimulates germination.

At present it is not clear whether the physical limitations to oxygen diffusion by the pericarp are the only reason or if additional factors play a role. Heydecker and Orphanos (1968) 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 does not prevent germination as long as no mucilage is formed. They hypothesised that without mucilage formation, 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 an oxygen diffusion barrier at high moisture level. We cannot exclude that other pericarp factors

also play a role. For instance, the pericarp of sugar beet seeds contains abscisic acid, a germination inhibiting plant hormone, and phenolic compounds that bind oxygen (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-inhibitory effect.

Recommendations

With the knowledge gained from this study, we have some recommendations for spinach seed production and seed testing. When sown in the field or a substrate, measures should be taken to prevent high soil or substrate 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 even germinate 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 could be (partly) removed, if commercially feasible, for instance by a seed peeling apparatus (Toshiyuki, 1992). For breeding purposes, it may 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 high moisture levels. 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 recommend the ISTA to consider moisture level again in the spinach seed germination testing protocol, using standardised low moisture levels and higher temperatures of 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 of a diameter of 2.5 to 5.0 mm. When other germination papers are used, the optimal moisture content can be determined easily by using the floating system with different fixed heights. Our method has some similarity to the "Rodewald Method" (Willan, 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, 2020). We consider our system simpler and easier to use when it comes to determining the optimal distance between the water table and germination paper with 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.

Acknowledgements

We would like to thank our master students Nathalie Blom, Arjan Hendriksen and Jinwook Kim for their experimental contributions, Paul Keizer (WUR Biometris) for his statistical advice, and our technical assistant Jan Kodde (WUR Bioscience) for his practical support. The research was funded by the Ministry of Agriculture, Nature and Food Quality of the Netherlands under the Research Programme 'Groene Veredeling' (Green Breeding, KV 1409-031) and by the seed and breeding companies Pop Vriend Seeds B.V. (The Netherlands), Sakata Seeds (France, Japan, California). Pop Vriend Seeds, Enza Zaden/Vitalis Organic Seeds B.V. (The Netherlands), and Nunhems/BASF (The Netherlands) are also acknowledged for providing the spinach seed lots used in this study.

Chapter 3

Evaluation of damping-off tolerance in spinach cultivars in field soils and in a standardised lab assay with *Pythium ultimum*

Kim J.H. Magnée[1], Joeke Postma[2], Steven P.C. Groot[3], Gerrit Gort[4], Edith T. Lammerts van Bueren[1] and Olga E. Scholten[1]

[1] Plant Breeding, Wageningen University & Research, P.O. Box 386, 6700 AJ Wageningen, The Netherlands
[2] Biointeractions and Plant Health, Wageningen University & Research, The Netherlands
[3] Bioscience, Wageningen University & Research, The Netherlands
[4] Biometris, Wageningen University & Research, The Netherlands

Magnée, K. J. H., Postma, J., Groot, S. P. C., Gort, G., Lammerts van Bueren, E. T., & Scholten, O. E. (2021). Evaluation of damping-off tolerance in spinach cultivars in field soils and in a standardised lab assay with Pythium ultimum. Plant Health Progress, 23(2), in press. https://doi.org/10.1094/PHP-07-21-0107-RS

Abstract

Spinach growers face increasing problems of damping off in the production of fresh-market (baby-leaf) spinach due to increasing restrictions on chemical treatments. Damping-off tolerant cultivars are increasingly important, requiring effective evaluation methods. Potential damping-off pathogens were isolated from the soil of three locations in the Netherlands with a history of spinach cultivation and of one location in France. These were identified to species or genus, and tested for their pathogenicity on spinach. *Pythium ultimum* was the most prevalent pathogen in those fields, causing spinach pre- and post-emergence damping off. Eight spinach cultivars, with two or three seed lots of each, were evaluated at the same field locations and in a greenhouse with soil sampled from one of the Dutch field sites. Pre-emergence damping off was more discriminating for differences among cultivars than post-emergence damping off. Variation in levels of infection among trials, replicates within trials, and seed lots of the same cultivar, emphasised the need for a more standardised phenotyping assay. For such an assay, a cornmeal/sand-based inoculum of a pathogenic *P. ultimum* isolate was added to a substrate mixture of sand, perlite, and vermiculite, that was moistened to 50% water holding capacity. In this substrate and in a control substrate without *P. ultimum* inoculum, spinach seeds were incubated for 10 days in a climate-controlled cabinet at 15°C in the dark, and seedling emergence was assessed. The assay showed reproducible results for discriminating differences in pre-emergence damping-off tolerance levels among seed lots. However, cultivar differences in pre-emergence damping-off tolerance levels could not be confirmed due to a larger variation among seed lots, even of the same cultivar, which needs further investigation.

Keywords: damping off, phenotyping assay, *Pythium ultimum*, seed lots, *Spinacia oleracea*

Introduction

Global production of spinach (*Spinacia oleracea* L.) has increased fourfold during the past two decades (Food and Agriculture Organization (FAO), 2020). In the USA, spinach production has increased mainly due to the rising popularity of fresh-market leafy vegetables, particularly baby-leaf spinach (Simko et al., 2014). As consumers become more aware of food safety and environmental impacts of pesticides and fungicides, the demand for non-chemically treated seeds to produce leafy vegetables is increasing too. The reduction in fungicide seed treatments can increase problems of non-uniform seedling emergence and damping off in leafy vegetable production fields. For the production of baby-leaf spinach, in particular, damping off is a major concern because the short production cycle of three to six weeks requires uniform seedling establishment, resulting in uniform leaf sizes at the time of harvest (Correll et al., 1994; Koike et al., 2011). Damping off does not only occur in spinach, but in a wide variety of crops such as alfalfa (Berg et al., 2017), cucumber (Abbasi & Lazarovits, 2005), soybean (Ellis et al., 2013), and sugar beet (Bardin et al., 2003). Symptoms of damping off can be roughly divided into two types based on the seedling development: pre- and post-emergence damping off. Pre-emergence damping-off symptoms include seed decay before germination and seedling decay before emergence above the soil or substrate level. Post-emergence damping off includes seedling and plant wilt, and usually death, after emergence (Lamichhane et al., 2017).

Biotic and abiotic conditions for damping off

Several soilborne pathogens can cause damping-off symptoms, including species from the fungal genera *Fusarium* and *Rhizoctonia*, and the oomycete genera *Aphanomyces, Phytophthora*, and *Pythium* (Lamichhane et al., 2017). Most *Pythium* spp. have a broad host range and mainly infect juvenile tissue, including the seed and emerging root, causing pre-emergence damping off, or they infect emerging seedlings, causing post-emergence damping off (Hendrix & Campbell, 1973; Kamoun et al., 1999). At later plant growth stages, infection is usually restricted to the feeder roots, causing stunted growth, dark roots, and seedling chlorosis (Hendrix & Campbell, 1973; Kraft et al., 2000). As a result, most mature plants are partially resistant to *Pythium* spp. (Kamoun et al., 1999). *Pythium* species that can cause damping off of spinach include, for example, *Pythium aphanidermatum*, *P. paroecandrum* (Naiki et al., 1986), *P. ultimum*, *P. heterothallicum*, *P. sylvaticum* (Larsson, 1994), and *P. spinosum* (Hirayama & Tojo, 1999). Studies have characterised *P. ultimum* as the most prevalent damping-off pathogen in spinach fields in Sweden (Europe) (Larsson, 1994) and in California (USA) (Koike et al., 2011). In a study in Georgia (USA), other pathogens isolated in association with spinach damping off were *Fusarium oxysporum*, *F. solani*, *F. roseum*, and *Rhizoctonia solani*, in addition

to *Pythium* spp. (primarily *P. irregulare*) (Sumner et al., 1976). Since different species can cause spinach seeds or seedlings to dampen off, identification of the most prevalent damping-off pathogens is needed for targeted management or breeding purposes.

The severity of damping off is not only influenced by the pathogen(s), but also by the presence of other microorganisms, the host plant genotype, soil moisture, soil temperature, soil pH, light intensity, and cropping history (Hendrix & Campbell, 1973; Green et al., 2012). In general, greater severity of damping off occurs in wet and compact soils, associated with low oxygen levels (Lamichhane et al., 2017). More saturated soil moisture conditions are favourable for the saprophytic growth of *Pythium* spp., which tolerates conditions of poor gas exchange (Griffin, 1963). Severity is also greater when the temperature is unfavourable for the host plant and/or when it is favourable for the pathogen (Agrios, 2005c). In the case of spinach, a cool-season crop that grows best at soil temperatures of 15 to 18°C, more severe damping-off symptoms may occur at lower or higher temperatures, depending on the pathogen (Koike et al., 2011). *P. ultimum* has optimum growth at 25 to 30°C in culture, but below 20°C in soil. The germination of sporangia or oospores by germ tubes is optimal at temperatures above 18°C, whereas temperatures between 10 and 18°C induce the germination of zoospores (Agrios, 2005b).

Control of damping off

Damping off can be controlled through sanitation practices, ensuring good soil drainage and aeration, preventing excessively wet soils, using well-decomposed compost, planting at soil temperatures favourable for rapid seedling emergence, and rotation with non-host crops (Lamichhane et al., 2017). Direct control of *Pythium*-induced diseases on a field scale is difficult. Once established in the soil, the persistent resting structures of *Pythium* spp., including thick-walled oospores, are difficult to eliminate (Stanghellini & Hancock, 1971), except with soil fumigants such as methyl bromide and/or chloropicrin (Hendrix & Powell, 1970; Munnecke et al., 1971). On a greenhouse scale, *Pythium* spp. can be eliminated by steam treatment or pasteurization of planting media (Hendrix & Campbell, 1973). Also, seeds can be treated with fungicides like mefenoxam (or the isomer metalaxyl), but resistance to these fungicides in some *Pythium* spp. and *Phytophthora* spp. has been found (Taylor et al., 2002; Tekale et al., 2019). The increasing awareness of the environmental impacts of chemical treatments and of food safety has led to more and more restrictions on the use of chemical field and seed treatments to manage damping off (United Nations Environment Programme (UNEP), 2006; European Commission, 2021). Therefore, an integrated management of damping off is increasingly relevant. This can include preventive field measures, seed treatments to enhance seed germination and vigour, biocontrol products as a more environment-

friendly alternative to fungicides, in addition to using spinach cultivars more tolerant to damping off (Lamichhane et al., 2017), of which the latter was investigated in this study.

Search for damping-off resistance or tolerance

Resistance to *P. ultimum* has been identified in legume crops, including alfalfa (Altier & Thies, 1995), common bean (Namayanja et al., 2014), soybean (Klepadlo et al., 2019), pea (Stasz et al., 1980), and chickpea (Kumar et al., 1991). Resistance or tolerance to *P. ultimum* in spinach has not yet been described. For other plant species in the Amaranthaceae family, including sugar beet cultivars and accessions of *Amaranthus* weeds, resistance to *R. solani* (Scholten et al., 2001) and *P. myriotylum* (Sealy et al., 1988) has been reported. In accessions of wild species of the genus *Beta*, related to sugar beet, resistance was found against the damping-off pathogens *P. ultimum* and *Aphanomyces cochlioides* (Luterbacher et al., 2005). It is not clear whether the resistance involved true resistance or apparent resistance in the form of tolerance to or escape from pathogen infection (Agrios, 2005a). Generally, resistant plants limit the pathogen to infect and to develop disease symptoms, while tolerant plants do not limit infection, but are able to establish well, even when they become infected (Roy & Kirchner, 2000). A third mechanism could be an escape from severe infection, e.g., through fast germination and/or seedling development (Agrios, 2005a). As the defence mechanism of spinach against damping-off pathogens is not known, we chose to describe the disease severity in spinach by the level of damping-off tolerance, that could be split up into pre-emergence and post-emergence damping-off tolerance. Spinach growers worldwide experience variation in damping-off tolerance among spinach cultivars (Marcel van Diemen, Vitalis Organic Seeds, 2015, *personal communication*). However, they also experience differences among seed lots and among field locations, which complicates the search for genetic-based tolerance for breeding purposes. Hence, there is a need for a reliable phenotyping assay in which the environmental conditions are standardised to assess pre-emergence damping off as a result of pathogen infection rather than abiotic influences. In this study, we describe and discuss the development of a standardised phenotyping assay to screen spinach cultivars for tolerance to *P. ultimum*, including comparisons of multiple seed lots of each cultivar.

Materials and methods

Plant materials

Eight spinach cultivars, with two or three seed lots of each (coded 'A', 'B', 'C') from different seed productions and overlapping seed size ranges, were used in this study: 'Carmel', 'Chevelle', 'Cronos', 'Hudson', 'Mirage', 'Novico', 'Progress', and 'Shelby' (Table 1). The seed lots were stored at 30% relative humidity (RH) and 13°C after

receipt in 2015 until they were used from 2016 to 2020. For the readability when describing a seed lot of a cultivar, the cultivar name was hyphenated with the seed lot letter, e.g., Chevelle-B.

Table 1. Information on the seed lots of the spinach cultivars used in this study, including thousand seed weight (TSW) in grams (g), measured by the providing companies, and seed size ranges in mm covering 95% of the approximately 1200 measured seeds.

Cultivar	Lot	TSW (g)	Seed size (mm)	Providing company (country)
Carmel	A	11.2	2.75–3.75	Pop Vriend Seeds
Carmel	B	12.6	2.75–3.75	(The Netherlands)
Carmel	C	12.7	2.50–3.75	
Chevelle	A	5.5	2.25–2.75	Enza Zaden/Vitalis
Chevelle	B	10.0	2.50–3.75	(The Netherlands)
Chevelle	C	10.8	2.50–3.75	
Cronos	A	11.0		Sakata Seeds
Cronos	B	11.1		(France)
Hudson	A	10.2		Pop Vriend Seeds
Hudson	B	10.9		(The Netherlands)
Hudson	C	11.2		
Mirage	A	9.8	2.75–3.75	Sakata Seeds
Mirage	B	9.9	2.50–3.75	(France)
Mirage	C	10.5	2.75–3.75	
Novico	A	9.7	2.50–3.50	Nunhems/BASF
Novico	B	12.6	2.75–4.25	(The Netherlands)
Novico	C	17.2	3.00–5.00	
Progress	A	9.7		Pop Vriend Seeds
Progress	B	10.2		(The Netherlands)
Progress	C	10.5		
Shelby	A	9.5	2.50–3.75	Enza Zaden/Vitalis
Shelby	B	11.0	2.50–3.75	(The Netherlands)
Shelby	C	16.1	3.30–4.50	

Field locations

For the field trials and for isolation of potential damping-off pathogens, three locations with a history of spinach cultivation were used. Three locations were in the Netherlands, one at Oosterdijk and two at Voorst. A fourth location, without a history of spinach cultivation, was in Uchaud, southern France. At Voorst, one field site was called 'de Bongerd' (Voorst B) and the other field site was called 'de Kamp' (Voorst K). The soils at Voorst were sandy and humus-rich (Marcel van Diemen, Vitalis Organic Seeds, 2021, *personal communication*), and the soils at Oosterdijk contained more marine clay, less organic matter and less sand than at Voorst (Panagos et al., 2012; European Soil Data Centre (ESDAC), 2021). The soil at Uchaud was a loam-sandy-clay soil (Sabrina Chandler, Sakata Seeds, 2021, *personal communication*). The fields at Voorst were used for organic crop production, whereas the fields at Oosterdijk and Uchaud were used for

conventional crop production.

Isolation of potential damping-off pathogens

Plastic trays (110 mm x 170 mm x 50 mm) were each filled with 300 g soil obtained from the four field sites (Voorst B, Voorst K, Oosterdijk, and Uchaud). A pathogen-free control soil consisted of a mixture of river sand from the IJssel in the Netherlands, and commercial peat-based potting soil (Lentse potting soil number 4 from Horticoop, Katwijk, the Netherlands, hereafter called 'peat') in a volume ratio of 3:1. In each tray, 20 seeds of Chevelle-B (with intermediate seed sizes from 2.50 to 3.75 mm, and assumed to be sensitive to damping off) were sown 5 mm deep. Trays were each covered with a transparent plastic lid to maintain high humidity, and were incubated for seven days at 15, 20, or 25°C. Potential damping-off pathogens were isolated from non-emerged seeds and from plants with post-emergence damping-off (wilting) symptoms by placing a small part of infected plant tissue (e.g., part of infected seed, stem base or root) on ¼ potato dextrose agar (10 g PDA (Thermo Scientific, CM139), 16 g agar (Thermo Scientific, LP0013) in 1 litre deionised water) with 100 mg/litre ampicillin to reduce bacterial growth. Plates were incubated at 20°C, and isolates were transferred to ¼ PDA medium for further use. Purified isolates were selected for genus or species identification, and for pathogenicity trials. From each soil-temperature combination, at least two isolates were selected. When isolates differed in colony morphology, one or two isolates from each different morphology type were selected, obtaining the highest possible variation in isolates.

Identification of damping-off pathogens

Mycelium was harvested from five-day-old isolates grown on PDA, placed in an Eppendorf tube with 50 µl TE buffer, boiled for five minutes, followed by a short spin. Then, 2 µl of the supernatant was used for DNA amplification with a PCR assay, using the polymerase GoTaq G2 from Promega (Catalog number M7841) in combination with the 5X colourless GoTaq reaction buffer. Primers for the Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990), and oomycete-specific cytochrome oxidase I (COI) primers (OomCoxI-Levup: 5'-TCAWCWMGATGGCTTTTTTCAAC-3'; and Fm85mod: 5'-RRHWACKTGACTDATRATACCAAA-3') were used for DNA sequencing (Robideau et al., 2011), performed by Macrogen Europe (Amsterdam, the Netherlands). The PCR procedure for the ITS rDNA was 2 min. at 95˚C, 1 min. at 95˚C, 30 min. at 55˚C, followed by 35 cycles of 1 min. at 72˚C, and 10 min. at 72˚C, followed by storage at 4˚C. For COI, the PCR procedure was 2 min. at 95˚C, 1 min. at 95˚C, 1 min. at 54˚C, followed by 35 cycles of 1 min. at 72˚C, and 10 min. at 72˚C, followed by storage

at 4˚C. Sequences were processed with CLC Genomics Workbench 8.0, after trimming the forward and reverse primers and removing low-quality sequences. Sequences were assembled into contigs, using standard assembly parameters from the CLC Genomics Workbench (Toolbox, Sequencing Data Analysis, Assemble sequences). The contig sequences were used in the Basic Local Alignment Search Tool (BLAST) on the website of the National Center for Biotechnology Information (NCBI) as a query against a nucleotide collection (nr/nt) database. In total, 43 isolates were identified based on the ITS rDNA sequence and/or COI sequence; two isolates could not be identified (Supplemental Table 1). For long-term storage, agar plugs (5 mm diameter) with mycelium of the isolate that grew on V8 agar medium (200 ml V8 juice, 5 g calcium carbonate, 800 ml deionised water) for 14 days at 25°C, were transferred into 2 ml Cryotubes with 15% DMSO (15 g dimethyl sulfoxide per litre water). The Cryotubes were slowly cooled to -80°C and placed in liquid nitrogen.

Pathogenicity trials

Two small-scale trials were used to evaluate the pathogenicity of the 43 isolates on spinach. First, pre-emergence damping off was tested in trays with pots, each 50 mm x 50 mm x 50 mm, filled with the 3:1 sand:peat mixture described above. A five-day-old PDA plug (5 mm diameter) with mycelium from the appropriate isolate was placed in the middle of the pot and simultaneously, four seeds of Chevelle-B were placed 5 mm deep at a 10 mm distance from the plug. With this short distance between the mycelial plug and the seed, the mycelium was already in contact with the seed before germination. Non-inoculated pots were included as a control treatment. Trays were incubated at 20°C with 16/8-hour light/dark cycle in plastic bags to create high humidity.

Second, post-emergence damping off was tested in trays with cylindrical pots (50 mm diameter x 50 mm tall) filled with the same 3:1 sand:peat mixture. One seed of Chevelle-B was sown 5 mm deep, and the pots were incubated at 15°C with 16/8-hour light/dark cycle in a plastic bag. After seven days, a five-day-old mycelial PDA plug was placed in contact with the stem of the emerged seedling. Non-inoculated pots were included as a control treatment. Pots were incubated further at 20°C with 16/8-hour light/dark cycle in plastic bags. Five and seven days after inoculation, the numbers of emerged and diseased seedlings were scored. Pathogenicity was determined for all identified isolates in three pots per isolate. Results were used to classify the isolates as non-pathogenic, weakly pathogenic or highly pathogenic, based on, respectively, 0 to 33%, 34 to 66%, and 67 to 100% non-emerged seedlings or emerged seedlings with post-emergence damping-off symptoms. To select a highly pathogenic isolate, and to check pathogenicity after storage in liquid nitrogen, 16 *Pythium* isolates were retested for pathogenicity on spinach using the pre- and post-emergence trials described before, with four blocks and three pots in each block.

Field trials

Five field trials were conducted in 2015, with two or three seed lots of each of eight spinach cultivars, including two trials at Oosterdijk, from 25 May to 15 June (A) and 22 June to 13 July (B); one trial at Voorst B, from 15 May to 2 June; one trial at Voorst K in August; and one trial at Uchaud, from 13 May to 3 June. The experimental design was the same for each trial, with a randomised complete block design with eight blocks. Each block consisted of 23 rows of spinach seeds, each 3 m in length with a 50 cm spacing between the rows. Within each row, 60 seeds (or 40 seeds in the Uchaud trial) of one seed lot were sown. Emergence was scored weekly after sowing. In the third week, total numbers of healthy seedlings and post-emergence damped-off seedlings were scored.

Greenhouse trial with naturally infested field soil

The same seed lots of the eight cultivars were also tested in a greenhouse in the Netherlands in 2016, using soil from the Voorst B field site. The soil was collected from the top 10 cm over a length of about 40 m. In total, 48 trays (600 mm x 400 mm x 80 mm, with a perforated bottom) were each filled with 10 kg of this soil and evenly spread in the tray. In each tray, four rows of 30 seeds were sown with a 1.8 cm distance between the seeds in a row, and a 7 cm distance between rows. The 23 seed lots were assigned randomly to the trays, with each seed lot sown eight times. The seeds were covered with 1 cm of the same soil, after the soil was sieved (<5 mm diameter particles). The soil moisture content was kept constant by weighing the trays three times a week and watering them when more than 150 ml water had evaporated. Numbers of emerged and diseased seedlings were scored daily for 11 days.

Development of a pre-emergence damping-off assay

For assessing pre-emergence damping-off tolerance in spinach, we developed a phenotyping assay with a cornmeal and sand-based inoculum containing *P. ultimum* mycelium, which was based on a protocol from Williams and Asher (1996). First, optimal emergence of spinach seedlings in the absence of *P. ultimum* was determined by testing different substrate mixtures and moisture levels as detailed below. Second, different *P. ultimum* inoculum doses were applied to the substrate in which seeds of the same seed lots of the eight spinach cultivars were sown to find an optimal dose for assessing pre-emergence damping-off tolerance.

Optimizing spinach emergence for the pre-emergence damping-off assay

Preliminary experiments were performed to find an optimal combination of substrate mixture and moisture level to obtain at least 85% spinach seedling emergence, of which the choices of substrate mixtures and moisture levels were based on in-house experience. The substrate mixtures contained autoclaved, dried, and sieved (<5 mm diameter

particles) river sand (IJssel, the Netherlands), peat (Lentse potting soil number 4 from Horticoop, Katwijk, the Netherlands), perlite and/or vermiculite (both from Agra Pull Rhenen, the Netherlands, no. 2: <3 mm diameter particles) at different volume-based ratios: sand:peat at a ratio of 3:1, sand:peat:perlite at ratios of 2:1:1 or 2:1:2, sand:perlite at a ratio of 1:1, and sand:perlite:vermiculite at a ratio of 1:1:1. One day before sowing seeds in the assay, the substrate mixtures were prepared in one bag per replicate, starting with the non-inoculated control treatment. Tap water was added in an amount based on the maximum water holding capacity (WHC) of the substrate mixture. The WHC was determined beforehand by placing 100 g of the dry substrate mixture into a funnel, lined with a mesh cloth on top of a 100 ml measuring cylinder. Then, 100 ml water was poured gently on the substrate mixture, and the amount of water collected in the cylinder was measured after two hours. A 100% WHC was obtained when the maximum amount of water remained in the 100 g substrate mixture. Based on preliminary experiments, each substrate mixture was moistened to 50 or 60% WHC. After the addition of tap water, the bags were closed tightly with some air enclosed, and stored overnight at room temperature.

Preparation of the pathogenic inoculum for the pre-emergence damping-off assay

A highly pathogenic *P. ultimum* isolate, 8b (Supplemental Table 2), was taken from the liquid nitrogen storage by thawing the Cryotube slowly and transferring the mycelial plugs to PDA for regeneration at 15°C. Five days before inoculation of the substrate mixtures, mycelial plugs of *P. ultimum* were transferred to new plates of PDA. The cornmeal/sand-based medium, as adapted from Williams and Asher (1996), was prepared in 500 ml Schott bottles and consisted of 247.5 g autoclaved, air-dried, and sieved river sand (<2 mm diameter particles), 2.5 g cornmeal (Sigma C6304), and 40 ml tap water. The bottles were shaken and autoclaved two times at 120°C for 20 minutes each time, with 24 hours rest in between. The bottles were cooled to room temperature before adding 15 mycelial plugs of *P. ultimum* from one PDA plate to the cornmeal/sand-based medium in each bottle. The bottles were closed tightly and incubated at 25°C for four weeks in darkness. Every week, the bottles were shaken and aerated in a biosafety cabinet. For the *P. ultimum* treatment, *P. ultimum* inoculum was added at a dose of 2.5, 5, 10, or 20%. The inoculum dose was based on the total substrate weight, including the dry weight of the substrate mixture and the fresh weight of the inoculum, before adding tap water to reach 50% WHC, resulting in approximately 27 to 28% water content of the total substrate weight.

Experimental conditions of the pre-emergence damping off assay

For the first experiments in which optimal substrate mixture and moisture level was determined, trays (380 mm x 240 mm x 55 mm) were each filled with 3000 g (excluding water) of one of the previously described substrate mixtures. The first experiment included

sand:peat (3:1), sand:peat:perlite (2:1:1, 2:1:2), and sand:perlite (1:1) with 50% and 60% WHC. The second experiment included sand:peat (3:1), sand:peat:perlite (2:1:2), and sand:perlite (1:1) with 50% WHC. In each tray, 40 seeds from Novico-B (2.75 to 4.25 mm seed size) and Novico-C (3 to 5 mm seed size) were sown in two rows with 45 mm space between rows and 15 mm between adjacent seeds, planted 10 mm deep, and covered with the same substrate mixture. There were no replicated trays within the two experiments. The trays were distributed randomly in a climate-controlled room set at 15°C with a 16/8-hour light/dark cycle (Philips Master TL-D 36W/840).

In follow-up experiments where a *P. ultimum* treatment was included, 100 g of the prepared substrate was evenly divided over the 25 cells of transparent polystyrene dishes (100 mm x 100 mm x 18 mm, Greiner-Bio One, Item No.: 638102). Two of these dishes were placed in a stackable tray (210 mm x 150 mm x 30 mm, DBP Plastics, Belgium), resulting in 50 cells per tray. One seed lot was sown per tray, with a single seed in each cell placed on the bottom of the tray, covered with the substrate, and pressed 3 mm deep. Trays were stacked with all seed lots in a stack placed in random order (15 trays each stack for 15 seed lots), with the control treatment and *P. ultimum* treatment separately. Each stack was wrapped in a plastic bag to prevent contamination between the control and *P. ultimum* treatment, and to limit dehydration. One replicate of control and one replicate of *P. ultimum* treatment together formed a time block, which was replicated three times. This resulted in a split-plot design with complete blocks 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. The three blocks were distributed randomly in a climate-controlled cabinet at 15°C in the dark, and the positions of the blocks and trays within each stack were switched daily. Spinach seedling emergence was assessed daily until the end of the experiment, 10 to 14 days after sowing (shown in figure caption). When scoring, we applied the rule "once emerged, always emerged", to assess primarily the pre-emergence damping off and not post-emergence damping off.

Data analysis

Statistical analyses were performed using R version 3.6.3 and RStudio version 1.2.5042 (RStudio Team, 2020). Count data of emerged, non-emerged and diseased seedlings were obtained at the end of the experiments. Mean percentages and standard deviations of these dependent variables were calculated for data visualizations. For statistically testing differences among cultivars or treatments, a generalised linear mixed model (glmm) was applied, assuming a binomial distribution of the count data. A logit link function was used to link the probability of emergence or diseased seedlings to the different independent variables, including fixed and random factors. Fixed factors

included the block, cultivar, treatment, and cultivar-by-treatment interaction. Random factors included the seed lot within cultivar, the seed lot-by-treatment interaction, and we included a random stack effect in case of a split-plot design, and a random tray effect to correct for potential overdispersion, as each tray within the developed assay contained a single seed lot. To compare individual seed lots, modified glmm's with seed lot and seed lot-by-treatment interaction as fixed factors, and necessary random factors, were applied. For each dataset, the fit of models was evaluated by residual plots with Pearson's residuals on the y-axis and fitted values on the x-axis. For data obtained from the field and greenhouse trials, binomial overdispersion was observed, so we replaced the binomial distribution with the betabinomial distribution using the glmmTMB package. Type II Wald Chi^2-tests were used to test effects of fixed factors. Using a logistic regression, the fixed and random effects were specified on a log odds scale. The odds were defined as the probability of successful emergence divided by the probability of non-emergence. Tukey's comparisons were performed to compare the log odds among the levels of fixed factors that showed significant differences (α=0.05).

To quantify the *P. ultimum* treatment effect in the pre-emergence damping-off assay, odds ratios (OR) were obtained from the logistic glmm output, showing the estimates for the *P. ultimum* treatment effect on emergence per seed lot on log scale, hence OR = $e^{estimate}$. This OR was used as a measure of pre-emergence damping-off tolerance level, defined as the odds of emergence in the substrate with *P. ultimum* versus the odds of emergence in the non-inoculated control substrate:

$$\text{Tolerance level=OR=}\frac{\text{emergence in } \textit{P. ultimum} \text{ substrate / non-emergence in } \textit{P. ultimum} \text{ substrate}}{\text{emergence in control substrate / non-emergence in control substrate}}$$

An OR smaller than 1 indicated sensitivity to *P. ultimum:* The closer the OR to 1, the greater the tolerance level. An OR of 1 or higher indicated complete tolerance to *P. ultimum*. For each OR estimate, the 95% confidence intervals were calculated ($e^{(estimate \pm 1.96 \cdot SE)}$). To test for significant differences (α=0.05) in tolerance levels among the seed lots, pairwise multiple comparisons with single-step adjusted p-values were performed on differences among estimates for the *P. ultimum* treatment effect on emergence per seed lot.

Results

Isolation and characterization of damping-off pathogens

When Chevelle-B was sown in the four field soils and in a pathogen-free control soil, incubated at three temperatures, 35% (±14%) of seeds did not emerge in the field soils compared to 10% (±5%) in the control soil. Post-emergence damping-off incidence was on average 21% (±17%) and was not observed in the control soil (*no replications*). From the non-emerged seeds and diseased seedlings sampled in the four field soils, 43 fungal and oomycete isolates were obtained, of which 25 were identified as *P. ultimum*, a species obtained from all four field soils at all three incubation temperatures (Supplemental Table 1). Of these 25 *P. ultimum* isolates, 23 were pathogenic on spinach, with 16 causing pre- and post-emergence damping off, two causing only pre-emergence damping off, and five causing only post-emergence damping off. Other pathogenic *Pythium* isolates included *P. terrestris* (five isolates) and *P. sylvaticum* (two isolates), of which *P. terrestris* was isolated from the Voorst B field soil, and *P. sylvaticum* from the Voorst K and Oosterdijk field soils. In addition, non-pathogenic *Pythium* isolates were obtained from the Oosterdijk field soil, including two *P. oligandrum* isolates and one *P. attrantheridium* isolate. A few fungal isolates were obtained from the Dutch soils, including *Rhizoctonia solani* and *Mortierella* spp. The *R. solani* isolate was highly pathogenic on spinach, inducing post-emergence symptoms. The *Mortierella* spp. isolates were not pathogenic or weakly pathogenic. From the Uchaud soil from France, only *P. ultimum* isolates were obtained. DNA sequences of all isolates are included in the NCBI database (MZ026493-MZ026534 for ITS and MZ151994-MZ152022 for COI) (https://www.ncbi.nlm.nih.gov/).

A selection of 14 *P. ultimum* and two *P. terrestris* isolates were retested for their pathogenicity on spinach after storage in liquid nitrogen (Supplemental Table 2). Twelve *P. ultimum* isolates were highly pathogenic, inducing pre-emergence and post-emergence damping off. Isolates of *P. terrestris* appeared to be less pathogenic than the *P. ultimum* isolates. Because of the abundance in all four field soils and the relatively high pathogenicity on spinach, *P. ultimum* isolate 8b (ITS: MZ026502; COI: MZ152003) was selected for the development of a pre-emergence damping-off phenotyping assay. In the pathogen-free control soils, a relatively high percentage (34%) of seeds did not emerge, illustrating the need for an optimised substrate when evaluating spinach seedling emergence.

Field trials

The overall incidence of emerged seedlings from the seed lots of eight spinach cultivars sown at the Voorst, Oosterdijk, and Uchaud field sites, was 58.3% (±13.6%) at Voorst B; 66.6% (±13.6%) at Voorst K; 83.9% (±11.4%) at Oosterdijk A; 82.8% (±9.2%) at Oosterdijk B; and 75.8 % (±24.5%) at Uchaud. The overall incidence of post-

emergence damped-off seedlings was 26.3% (±14.9%) at Voorst B; 7.0% (±6.4%) at Voorst K; 24.2% (±12.0%) at Oosterdijk A; 19.4% (±10.8%) at Oosterdijk B; and 13.0% (±19.7%) at Uchaud. At the Uchaud field site, the relatively low incidences of emerged seedlings and post-emergence damped-off seedlings did not show any significant differences among the cultivars or among the seed lots (*data not shown*).

At the four field sites in the Netherlands, variation was observed among cultivars and field trials, and among seed lots, even of the same cultivar (Figure 1A to 1D). A significant interaction effect was observed between the field trials and cultivars ($p<0.001$). For both field trials at Voorst, fewer seedlings of Chevelle emerged compared with Cronos (Voorst B: $p=0.006$; Voorst K: $p<0.001$), Mirage (Voorst B and K: $p=0.004$), and Shelby (Voorst B: $p=0.021$; Voorst K: $p=0.008$). At Voorst B, Chevelle also showed less emergence compared with Hudson ($p=0.004$). However, when comparing the seed lots (treating them as fixed instead of random factor), Chevelle-C did not significantly differ from any of the seed lots of those cultivars that showed greater emergence compared with the cultivar Chevelle. Only Chevelle-A and Chevelle-B showed fewer emerged seedlings compared with Cronos-B, Hudson-A, Mirage-A, and Mirage-C ($p<0.05$) at the Voorst B field site, and compared with Cronos-A, Cronos-B, Hudson-B, Mirage-A, Mirage-C, Novico-B, Progress-C, Shelby-A, and Shelby-C ($p<0.01$), at the Voorst K field site. Chevelle-C even showed more emerged seedlings than Chevelle-A ($p<0.001$) in both field trials at Voorst, and at Oosterdijk B. When comparing cultivar differences in both Oosterdijk field trials, Carmel showed less emergence compared with Cronos (Oosterdijk A and B: $p=0.005$), Hudson (Oosterdijk A and B: $p=0.002$), and Mirage (Oosterdijk A: $p=0.012$; Oosterdijk B: $p=0.003$). At Oosterdijk B, Carmel also emerged less than Novico ($p=0.009$) and Progress ($p=0.018$). When comparing the seed lots in the Oosterdijk A field trial, only Carmel-A showed fewer emerged seedlings compared with both seed lots of Cronos and seed lots B and C of Hudson ($p<0.01$), and only Carmel-C differed from Mirage-C ($p=0.047$). In the Oosterdijk B field trial, Carmel-A and Carmel-B only differed from Cronos-B ($p=0.03$), Hudson-B ($p=0.02$) and Novico-C ($p=0.01$), whereas Carmel-C differed from almost all other cultivar seed lots with a lower emergence ($p<0.01$).

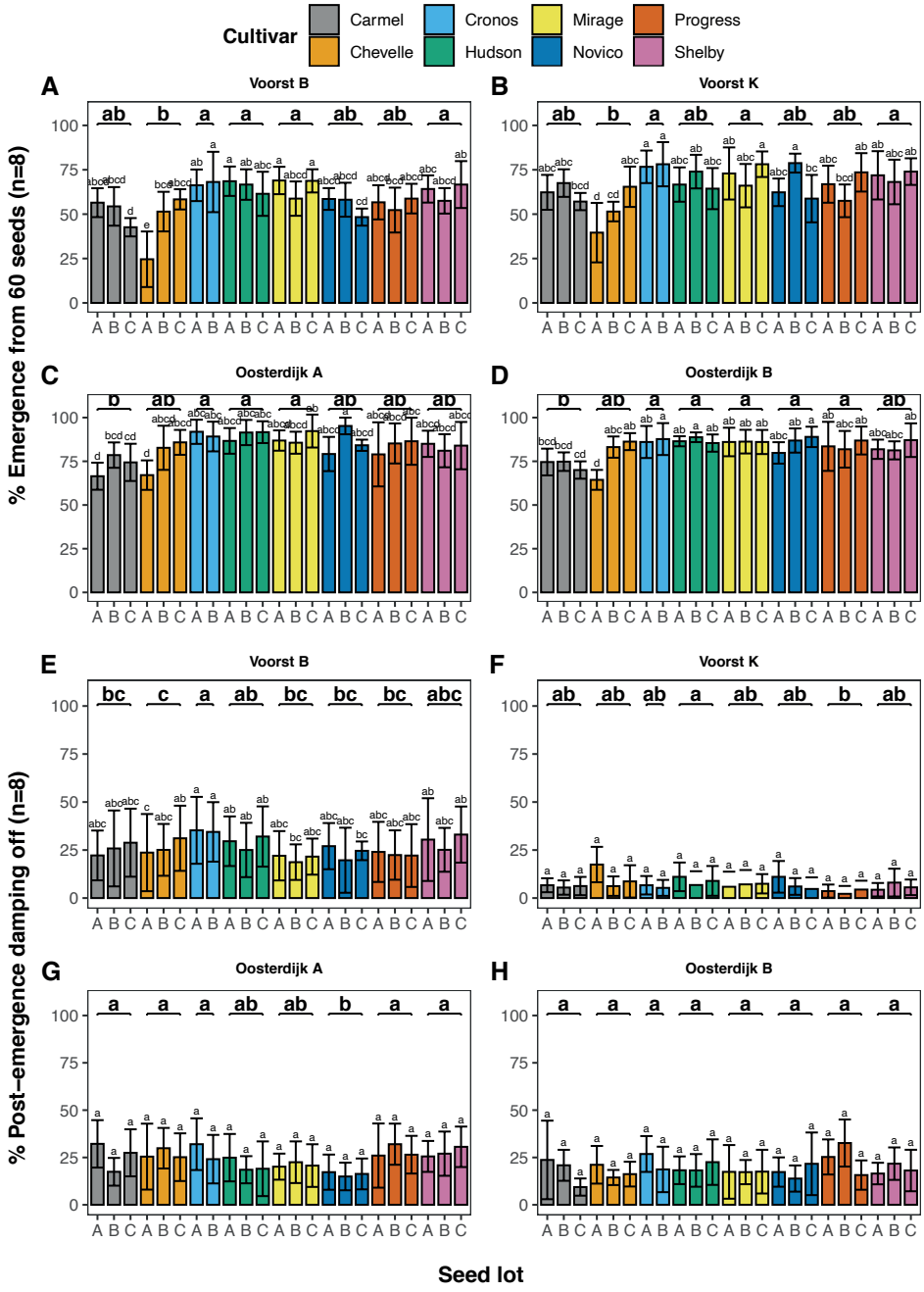


Figure 1. (A-D) Mean incidence (%) of emerged seedlings from two or three seed lots (coded 'A', 'B', 'C') of each of eight spinach cultivars three weeks after sowing in two field trials at Voorst ('Bongerd'=Voorst B and 'Kamp'=Voorst K) and Oosterdijk (A and B) (The Netherlands), and **(E-H)** mean incidence (%) of emerged seedlings with post-emergence damping-off symptoms. Error bars represent standard deviations (n=8, replicated plots with 60 seeds each plot). Same letters indicate no significant differences between means ($p>0.05$), based on Tukey's pairwise comparisons of the log of odds of emergence (emergence vs. non-emergence) or post-emergence damping off (healthy vs. diseased seedlings) between cultivars (with seed lots treated as random factor) or seed lots (treated as fixed factor).

When comparing the incidence of post-emergence damping off among cultivars, results were inconsistent (Figure 1E to 1H). At the Voorst B field site, Cronos showed more diseased seedlings than Carmel ($p=0.002$), Mirage ($p=0.005$), Novico ($p=0.001$), and Progress ($p<0.001$), which was not shown at Voorst K. When comparing individual seed lots, Cronos-A and Cronos-B differed from Chevelle-A ($p<0.001$), Mirage-B ($p<0.05$) and Novico-C ($p<0.05$). At Voorst K, the incidence of post-emergence damping off was less than at Voorst B ($p<0.001$), and there were no significant differences among seed lots. When comparing the cultivars, only Progress showed less post-emergence damping off than Hudson ($p=0.006$) at Voorst K. Also, within the field trials at Oosterdijk, there were no significant differences among cultivars or among seed lots. Only in field trial A at Oosterdijk, less post-emergence damping off was observed for Novico compared with Carmel ($p=0.040$), Chevelle ($p=0.013$), Cronos ($p=0.009$), Progress ($p=0.003$), and Shelby ($p=0.003$).

Greenhouse trial with field soil

The same cultivar seed lots were tested under more standardised conditions in a greenhouse with an overall emergence of 71.4% (±17.4%), and an overall incidence of 28.0% (±15.2%) post-emergence damping off. When comparing the means of the cultivars, emergence of Chevelle was less compared with the other cultivars ($p<0.05$), except Carmel (Figure 2A). Carmel emerged less than Mirage ($p=0.001$) and Shelby ($p=0.022$). However, when comparing the individual seed lots, Carmel-B and Carmel-C did not significantly differ from Shelby-A. Large variation also existed among the seed lots of Chevelle and Novico, with Chevelle-A that showed fewer emerged seedlings than Chevelle-C ($p<0.001$), and Novico-A that showed fewer emerged seedlings than Novico-B and Novico-C ($p<0.001$).

When evaluating post-emergence damping off, Chevelle showed a higher incidence of damped-off seedlings than Carmel ($p=0.034$). Shelby showed fewer damped-off seedlings compared with Chevelle ($p<0.001$), Cronos ($p=0.015$), Hudson ($p=0.003$), and Mirage ($p=0.018$) (Figure 2B). When evaluating individual seed lots, differences were confirmed statistically for Shelby-A and Shelby-C compared with the Chevelle seed lots ($p<0.02$), except for Shelby-A vs. Chevelle-C ($p=0.06$), for Shelby-A and Shelby-C compared with Cronos-A ($p=0.033$; $p=0.009$), Hudson-C ($p=0.002$; $p<0.001$), Novico-A ($p=0.023$; $p=0.006$), and Mirage-B ($p=0.020$; $p=0.005$). Large variation among seed lots and replicates ($p<0.001$) remained, as was also the case in the field trials.

Since the incidence of post-emergence damping off was relatively low, with less variation among seed lots in the field and greenhouse trials compared to the incidence of seedlings that did not emerge, pre-emergence damping off was expected to be more discriminating for differences among cultivars than post-emergence damping off.

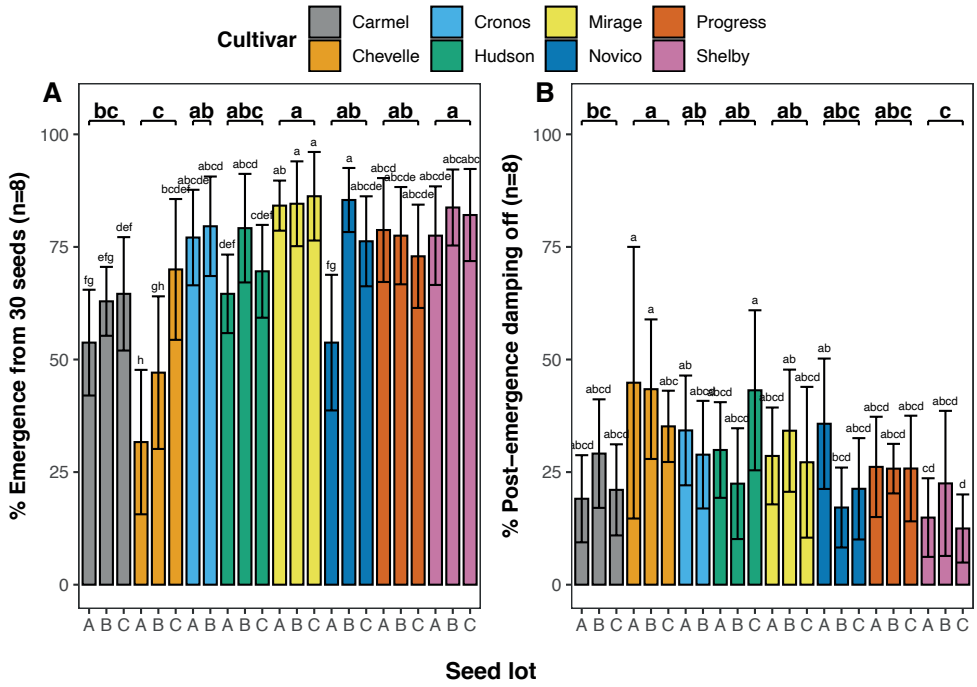


Figure 2. (A) Mean incidence (%) of emerged seedlings from two or three seed lots (coded 'A', 'B', 'C') of each of eight spinach cultivars, 11 days after sowing in soil sampled from Voorst B in the greenhouse, and **(B)** mean incidence (%) of emerged seedlings with post-emergence damping-off symptoms. Error bars represent standard deviations (n=8, replicated rows with 30 seeds each row). Same letters indicate no significant differences between means (p>0.05), based on Tukey's pairwise comparisons of the log of odds of emergence (emergence vs. non-emergence) or post-emergence damping off (healthy vs. diseased seedlings) between cultivars (with seed lots treated as random factor) or seed lots (treated as fixed factor).

Optimization of seedling emergence in a pre-emergence damping-off assay

Seedling emergence from the two Novico seed lots was, on average, greater ($p<0.001$) at 50% WHC (68.4% ±15.9% emergence) than at 60% WHC (55.9% ±14.0% emergence). In addition, average emergence from both Novico seed lots was greater ($p<0.001$) in the substrate mixtures with more perlite; 66.2% ±6.61% emergence in sand:peat:perlite (2:1:2), 80.6% ±10.1% emergence in sand:perlite (1:1), compared to 48.1% (±8.0%) emergence in sand:peat (3:1) mixture. A repeat of this experiment with three substrates maintained at 50% WHC also showed a greater emergence in the mixtures with perlite than in the sand:peat mixture ($p<0.001$) (Figure 3B), with an average emergence of 86.2% (±4.8%) in the sand:peat:perlite mixture, and 82.5% (±18.5%) in the sand:perlite mixture, compared to an average emergence of 38.8% (±11.1%) in the sand:peat mixture. In a later experiment, the addition of vermiculite to the sand:perlite mixture showed slightly greater emergence for some seed lots in the sand:perlite:vermiculite (1:1:1) mixture compared with the sand:perlite (1:1) mixture (*data not shown*). With the sand:perlite:vermiculite mixture maintained at 50% WHC,

the 15 tested seed lots showed an emergence of 88% to 98%, which was reproduced over the experiments (as example, see Figure 6A, 0% *P. ultimum* dose).

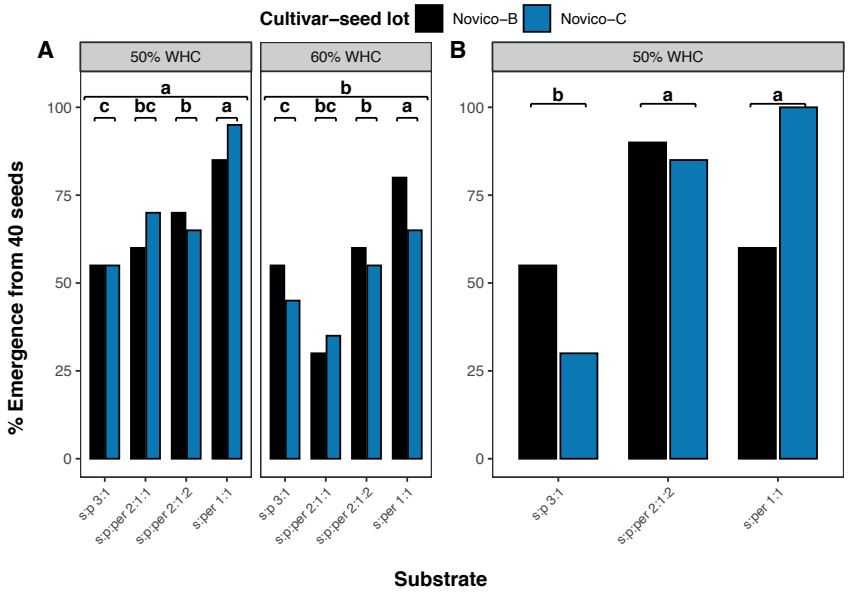


Figure 3. Incidence (%) of emerged seedlings 14 days after sowing 40 seeds of Novico seed lots B and C in **(A)** four different substrate mixtures (on volume basis): sand:peat (s:p) 3:1, sand:peat:perlite (s:p:per) 2:1:1 and 2:1:2, sand:perlite (s:per) 1:1, with different moisture levels: 50% and 60% WHC, and **(B)** a repeat with three substrate mixtures maintained at 50% WHC. Bars with same letters indicate no significant differences ($p>0.05$) with Tukey's pairwise comparisons between treatments (with seed lots treated as a random factor).

Dose effect of P. ultimum inoculum

When three concentrations of *P. ultimum* inoculum (5, 10, and 20%) were added to a sand:perlite:vermiculite mixture maintained at 50% WHC, the seedling emergence of two seed lots of cultivars Chevelle and Novico was less in the substrates with *P. ultimum* inoculum compared with the emergence in the control treatment without *P. ultimum* inoculum ($p<0.001$) (Figure 4A). In the control treatment, Novico showed fewer emerged seedlings than Chevelle ($p<0.001$), but their emergence did not differ in the three inoculated treatments. The repeat of this experiment with the same seed lots of cultivar Chevelle, but with seed lots B and C of cultivar Shelby, and a *P. inoculum* dose of 2.5 and 5%, resulted in fewer emerged seedlings in the 2.5% and 5% *P. ultimum* treatments than in the control treatment ($p<0.001$) (Figure 4B). The emergence of seedlings did not differ between the two cultivars in the control treatment or in the *P. ultimum* treatments.

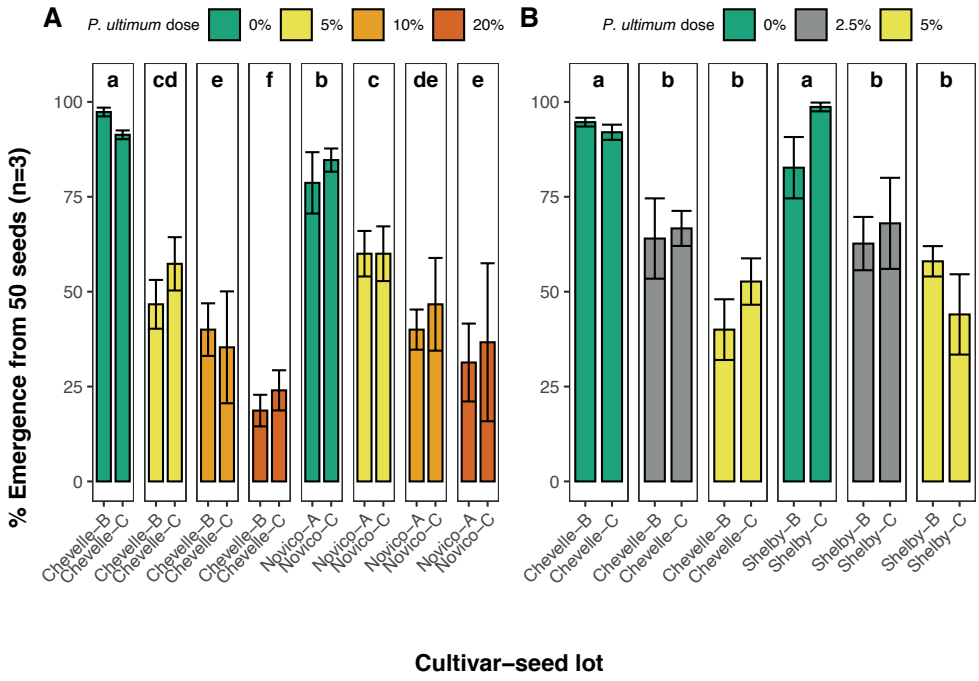


Figure 4. Mean incidence (%) of emerged seedlings 10 days after sowing 50 seeds of each of two seed lots of spinach cultivars Chevelle seed lots B and C and **(A)** Novico seed lots A and C or **(B)** Shelby seed lots B and C, in a substrate with sand:perlite:vermiculite mixture (1:1:1 volume basis) maintained at 50% WHC, with 0, 2.5, 5, 10, or 20% Pythium ultimum inoculum dose (% fresh weight inoculum / (dry weight mixture + fresh weight inoculum)). Error bars represent standard deviations (n=3, replicated trays with 50 seeds each tray). Bars with the same letter indicate no significant differences (p>0.05) among the cultivar and treatment means based on Tukey's pairwise comparisons between the log odds of emergence of cultivars within treatments (with seed lots treated as random factor).

Assessing pre-emergence damping-off tolerance using emergence odds ratios

The reproducibility of the phenotyping assay was assessed by evaluating two or three seed lots of spinach cultivars Chevelle and Shelby in four independent experiments (Figure 5). The overall emergence in the control treatment was 92.0% (±7.1%) in Exp. 1; 88.7% (±11.2%) in Exp. 2; 90.7% (±6.1%) in Exp. 3; and 93.0% (±4.5%) in Exp. 4, indicating good emergence conditions of the assay (>85% emergence). The overall emergence in the *P. ultimum* treatment was 48.7% (±9.8%) in Exp. 1; 26.3% (±11.3%) in Exp 2; 9.2% (±7.9%) in Exp 3; and 9.9% (±8.5%) in Exp 4, indicating sufficient infection levels but variation in effectiveness of the *P. ultimum* inoculum. The odds ratios between the emergence in the *P. ultimum* treatment and the emergence in the non-inoculated control treatment, calculated as a measure of pre-emergence damping-off tolerance level, showed similar results for the Chevelle and Shelby seed lots, indicating reproducibility of the developed assay. Shelby-B was more tolerant than Chevelle-B in all four experiments ($p<0.001$), and Shelby-B was more tolerant than Shelby-C

in experiments 1, 3 ($p<0.001$), and 4 ($p=0.005$), not in experiment 2 ($p=0.055$). In experiments 3 and 4, Shelby-A was more tolerant than Chevelle-B ($p<0.001$). Chevelle-A was also more tolerant than Chevelle-B in both 2020 experiments ($p<0.01$).

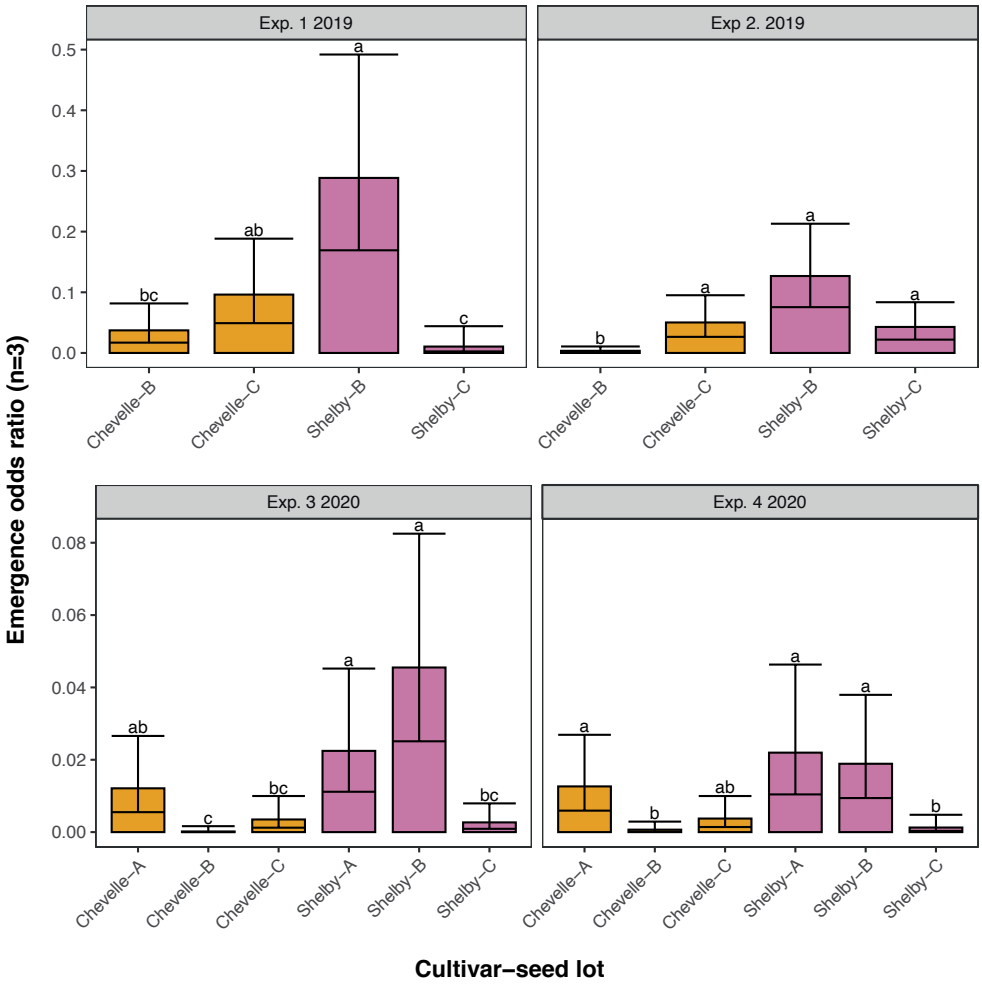


Figure 5. Pre-emergence damping-off tolerance levels of two or three seed lots of spinach cultivars Chevelle and Shelby, displayed as emergence odds ratios between seedling emergence in substrate with sand:vermiculite:perlite mixture (1:1:1 volume basis) maintained at 50% WHC and 5% Pythium ultimum dose, and emergence in the non-inoculated control substrate (n=3, replicated trays with 50 seeds each tray), in each of four experiments conducted in 2019 (Exp. 1 and 2) and 2020 (Exp. 3 and 4). Exp. 4 was part of a larger experiment with the rest of the data shown in Figure 6. Error bars show the upper and lower limits of the 95% confidence interval. Bars with the same letter indicate no significant differences ($p>0.05$) in tolerance levels among the seed lots based on pairwise multiple comparisons (with seed lots treated as a fixed factor).

Assessing seed lot variation in pre-emergence damping-off tolerance

Based on the reproducibility of the assay with sufficient infection in a substrate with 5% *P. ultimum* dose, the experiment was repeated with three seed lots of each of five spinach cultivars, sown in the developed assay with 0% (control) and 5% *P. ultimum* dose. Overall, seedling emergence in the control treatment was 92.2% (±4.6%). With a 5% *P. ultimum* dose, the seedling emergence of all five cultivars was reduced to an average emergence of 11.7% (±9.2%) (Figure 6A). When the seed lots were treated as a random factor in the analyses, there was no significant variation in pre-emergence damping-off tolerance levels among the five cultivars (Figure 6B). When the seed lots were treated as a fixed factor, they showed variation in tolerance levels ($p<0.001$), even within the same cultivar. Carmel-C was more tolerant compared with the three seed lots of Chevelle (A: $p=0.042$, B: $p<0.001$, C: $p<0.001$), the three seed lots of Mirage (A: $p<0.001$, B: $p=0.002$, C: $p=0.005$), Novico-B ($p=0.005$), and Shelby-C ($p<0.001$). However, Carmel-A and Carmel-B did not show greater tolerance levels compared with the other cultivar seed lots, and Carmel-C was more tolerant than Carmel-A ($p=0.002$). Chevelle-A was also more tolerant than Chevelle-B ($p=0.030$), and Shelby-C was less tolerant than Shelby-A ($p=0.018$) and Shelby-B ($p=0.028$). Tolerance levels did not significantly differ among the seed lots of Mirage and Novico.

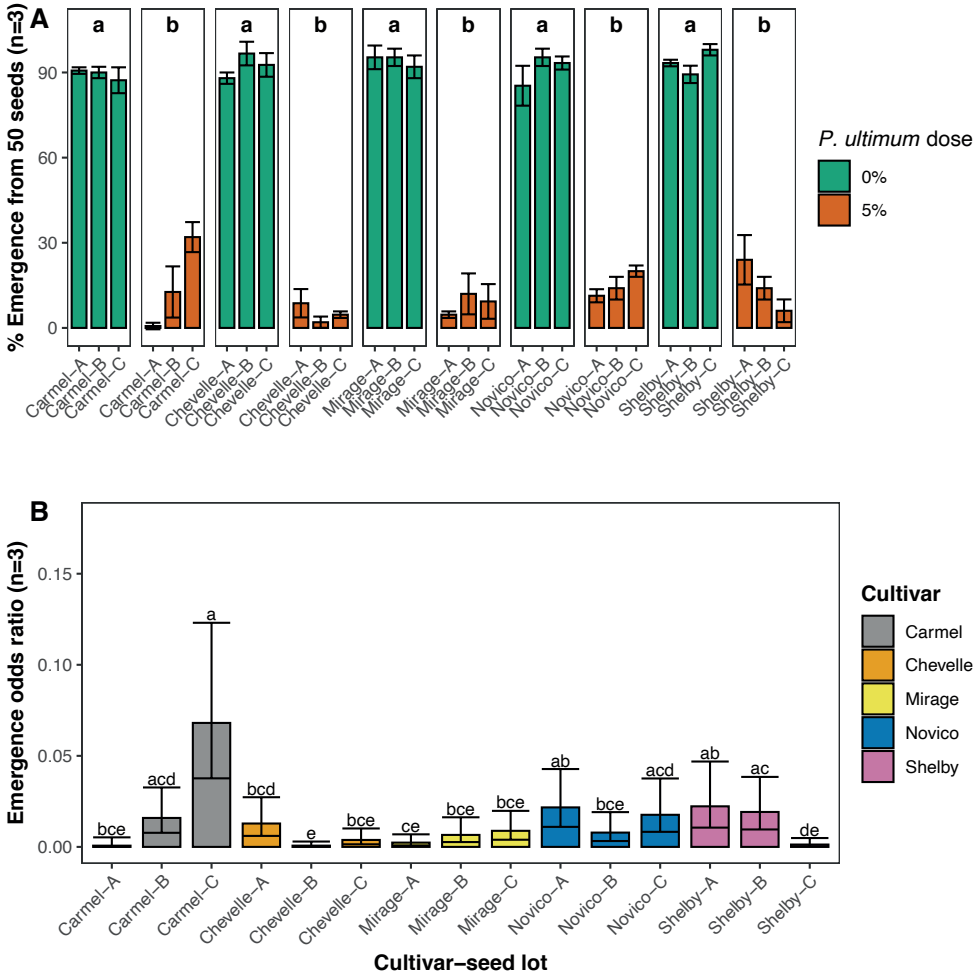


Figure 6. (A) Mean incidence (%) of emerged seedlings (n=3) 10 days after sowing 50 seeds of each of three seed lots of five cultivars in a substrate with sand:perlite:vermiculite mixture (1:1:1 volume basis) with 0% (control treatment) or 5% Pythium ultimum inoculum dose (P. ultimum treatment). Error bars represent standard deviations (n=3, replicated trays with 50 seeds each tray). Means with same letters indicate no significant differences (p>0.05) based on Tukey's pairwise comparisons among the cultivars (with seed lots treated as a random factor); and **(B)** pre-emergence damping-off tolerance levels for the same seed lots displayed as the emergence odds ratio, between emergence in P. ultimum treatment and emergence in control treatment. Error bars show the upper and lower limits of the 95% confidence interval. Bars with the same letter indicate no significant differences (p>0.05) in tolerance levels among the seed lots based on pairwise multiple comparisons (with seed lots treated as a fixed factor).

Discussion

Identification of isolates from field soils

From three field locations in the Netherlands with a history of spinach cultivation, and one field location in France, several *Pythium* spp., *Mortierella* spp., and *Rhizoctonia solani*

were isolated. We did not identify any species of the genus *Fusarium*, *Phytophthora* or *Aphanomyces*, whereas these pathogens have previously been isolated from spinach fields with damping off in other locations (Green et al., 2012). For instance, *F. oxysporum* has been isolated from wilting spinach seedlings in Sweden (Larsson & Olofsson, 1994), Japan (Naiki & Kanoh, 1978), Georgia in the USA (Sumner et al., 1976), Canada (Reyes, 1977), and Australia (Trimboli, 1977). *P. ultimum* was most abundant in evaluated field soils in the present study, and showed to be highly pathogenic on spinach, inducing both pre- and post-emergence damping off, similar to what was reported in Sweden (Larsson, 1994). Therefore, we chose *P. ultimum* as the pathogen to use in a phenotyping assay to screen for damping-off tolerance in spinach cultivars. According to the NCBI, *Pythium ultimum* has also been named *Globisporangium ultimum* (Uzuhashi et al., 2010).

Another species that we isolated was *P. sylvaticum*, which was also the second most abundant pathogenic *Pythium* species detected in Sweden (Larsson, 1994), followed by *P. ultimum* var. *ultimum* and *P. heterothallicum*. Two other pathogenic *Pythium* species isolated in this study were *P. terrestris,* described as a new species by Paul (2002), which showed various levels of pathogenicity on spinach in our pathogenicity trials, and *Pythium* sp. CAL-2011f, which was weakly pathogenic on spinach. To our knowledge, both species have not previously been reported as spinach damping-off pathogens. *P. terrestris* has recently been isolated from soybean and wheat seedlings, and the soils in which the plants were growing, and were highly pathogenic on wheat (Feng et al., 2020). Also, *Pythium* sp. CAL-2011f has been isolated recently from rhizosphere soils of soybean seedlings (Navarro-Acevedo et al., 2021). In addition, we isolated some *Pythium* strains that were non-pathogenic on spinach, including *P. oligandrum*, of which strains have been found to be a biocontrol agent against *P. ultimum* (Martin & Hancock, 1987), and *P. attrantheridium*, which induces cavity spot in carrots, and cavity lesions on apple and cherry seedlings (Allain-Boulé et al., 2004). Another group of interesting isolates that we identified, was the *Mortierella* spp., zygomycetes commonly isolated along with *Pythium* spp. in soybean fields with damping off (Zitnick-Anderson & Nelson Jr, 2015). Apparently, *Mortierella* species are the most abundant filamentous fungi in soils around the world, of which some have plant-growth-promoting abilities, and may even provide protection of agricultural plants from pathogens, such as *Fusarium* spp. (Ozimek & Hanaka, 2020).

Damping-off evaluations in the field and greenhouse trials

Among the five field trials, we observed large variation in non-emergence and post-emergence damping off. At the Uchaud field site in France, without a history of spinach cultivation, no differences among cultivars or seed lots were found in the relatively low incidences of emergence and post-emergence damping off. At the Dutch field sites at

Voorst and Oosterdijk, cultivar differences were found that were relatively consistent among the trials. At the two Voorst sites, seedlings of cultivar Chevelle emerged less compared with the other cultivars, whereas Carmel had the poorest emergence at Oosterdijk. However, there was large variation among seed lots of Chevelle, with Chevelle-A showing the least emergence at all field sites, even below 40% in both field trials at Voorst. The poorer emergence of cultivars Chevelle and Carmel compared with the other cultivars could be a result of greater pre-emergence infection by damping-off pathogens, but other environmental conditions may also have played a role (e.g., high soil moisture levels). When comparing post-emergence damping off, no differences among cultivars were found or results were inconsistent among seed lots or among field trials. At the Voorst K and Uchaud field sites, the infection levels were relatively low compared with other sites. Inconsistency among field sites could be related to various biotic (e.g., microbial species composition, abundance, and diversity in the soil, including pathogen species) and abiotic conditions (e.g., moisture, temperature, organic matter content, and soil texture). Variable abiotic conditions among field sites (e.g., soil properties), among field trials (e.g., weather conditions), and even among rows within fields (e.g., soil moisture level and soil compaction) complicated assessment of cultivar effects on seedling emergence and post-emergence damping off. Therefore, the same seed lots were evaluated under more standardised conditions in the greenhouse with field soil collected from the Voorst B field site, where the incidence of non-emergence was highest and differences among seed lots most visible.

Similar patterns in emergence were found among the cultivars and seed lots in the greenhouse and field trials. For instance, seed lots of the cultivar Chevelle showed less emergence compared with seed lots of other cultivars, except Carmel. These observations indicated that cultivars Carmel and Chevelle were most sensitive to pre-emergence damping-off infection and/or other factors influencing their germination and emergence. When comparing post-emergence damping off among cultivars in the greenhouse trial, only cultivar Shelby showed fewer damped-off seedlings compared with cultivars Chevelle, Cronos, Hudson, and Mirage. This indicated a potentially greater post-emergence damping-off tolerance of the cultivar Shelby. However, when comparing individual seed lots, we could not confirm these differences for all Shelby seed lots compared with the seed lots of other cultivars due to large variation among seed lots of the same cultivar. Variation in levels of infection among trials, among seed lots of the same cultivar, and among replicates, made clear that the development of a more standardised phenotyping assay was necessary. Therefore, we continued developing a more standardised assay that could be conducted in a climate-controlled cabinet. The results from the naturally-infested soils in the field and greenhouse indicated that measuring pre-emergence damping off of spinach was more discriminatory among

cultivars than post-emergence damping off. Therefore, the requirements were set for a more standardised pre-emergence damping-off assay and included: good and uniform germination and emergence in the absence of damping-off pathogen(s), the use of a pathogenic inoculum with reliable effectiveness, and minimal environmental variation to give reproducible results.

Optimizing a phenotyping assay for spinach pre-emergence damping off

To phenotype pre-emergence damping-off tolerance in spinach, it was crucial to optimise seedling emergence so that the incidence of non-emergence could be ascribed (mostly) to pre-emergence infection. With the sand:perlite:vermiculite (1:1:1 volume) substrate mixture maintained at 50% WHC, emergence of spinach seedlings by at least 85% was achieved in the pathogen-free control treatment. Since variation in emergence still existed among seed lots, a control treatment was necessary to correct for this variation and to assess the responses of cultivars towards *P. ultimum*. The choice of substrate was partly based on a study of Kear et al. (2005), who used a mixture of sieved peat moss, dolomitic limestones and vermiculite (called 'peatlite'). We excluded peat from the substrate in this study because emergence of spinach seedlings was greater with perlite instead. Also, peat can be highly variable among batches and can have *P. ultimum*-suppressive properties (Bongiorno et al., 2019). In addition, a low moisture level was preferred since spinach seed germination can be sensitive to high moisture levels, especially for relatively larger seeds (Chapter 2). The choice of a low substrate moisture level and a loosely structured substrate was based primarily on the optimization of spinach seed germination and seedling emergence rather than on *P. ultimum* development and infection. *P. ultimum* prefers high soil moisture conditions, e.g., in compacted soils, for infection of seeds, young roots, or seedlings (Hendrix & Campbell, 1973). Spinach seed germination does not tolerate low oxygen levels, whereas *P. ultimum* is less affected by poor gas exchange under high moisture levels (Griffin, 1963). The temperature of 15°C used in the assay was potentially good for both spinach seedling emergence and *P. ultimum* infection.

Reproducibility of the phenotyping assay

When comparing emergence results of the lab assay with emergence results in the field and greenhouse assays, seed lots were discriminated more clearly in the lab assay, especially for the Carmel and Shelby seed lots. The lab assay also showed a more reproducible pattern in emergence across the seed lots. For instance, comparable results for pre-emergence damping-off tolerance levels of seed lots of Chevelle and Shelby were obtained from the four experiments with the lab assay. However, the effect of the same *P. ultimum* inoculum dose on pre-emergence damping off in the 2020 experiments was greater than in 2019 and earlier experiments, despite using the same *P. ultimum* isolate, emphasising the importance of evaluating at least two doses when phenotyping spinach

pre-emergence damping off caused by *P. ultimum*. One potential reason for variable effectiveness of *P. ultimum* inoculum on spinach pre-emergence damping off could be the seed quality. Seeds used in the later experiments might have had slightly lower quality despite storage at 30% relative humidity and 13°C. There are several advantages of the developed phenotyping assay compared to the field and greenhouse trials. These include the capacity for detailed evaluations of pre-emergence damping-off tolerance due to the correction for non-emergence in a control treatment. The assay was optimised in small trays that could be stacked, each with 50 cells, so that less space was needed. The standardised environmental conditions also enabled evaluation of pre-emergence damping off in a relatively short time (10 days). Moreover, the set-up with a single seed per cell enabled the assessment of emergence from individual seeds over time, which created the possibility of relating seedling emergence to other seed characteristics that are measured individually. This system was less suitable for assessing post-emergence damping off because no light was provided, and no nutrients were added to the medium for seedling development. Our recommendations on the primary conditions of a pre-emergence damping-off assay for screening spinach cultivars are listed in Table 2.

Table 2. Recommended conditions for a pre-emergence damping-off assay for spinach cultivars, based on existing literature and the results described in this study.

Assay component	Condition
Substrate	sand:perlite:vermiculite 1:1:1 (volume basis)
Moisture	50% WHC (27-28% water of total substrate weight)
Temperature and light	15°C and dark (constant)
Pathogen isolate	*Pythium ultimum* (highly pathogenic)
Inoculum	1% cornmeal in sand (Williams & Asher, 1996) + 15 mycelial plugs of five-day-old *P. ultimum*, incubated for four weeks at 25°C in the dark (shaken and aerated weekly)
Inoculum dose	5% fresh weight inoculum out of dry weight substrate plus fresh weight inoculum Two doses (e.g., 2.5 and 5.0%) recommended as effectiveness of inoculum may vary

Relevance of phenotyping multiple seed lots with a standardised lab assay

With the phenotyping assay developed in this study, seed lots differed significantly in pre-emergence damping-off tolerance levels. If the seed lots were treated as random samples of each cultivar, there was no significant variation in tolerance levels among cultivars. Different seed lots of the same cultivar responded differently towards *P. ultimum*, which confounded the search for genotypic-based pre-emergence damping-off tolerance in spinach that is necessary for breeding purposes. Cultivar variation may be masked by large variation among seed lots due to varying environmental conditions during seed production, post-harvest treatments, or seed storage conditions, that can all contribute to the variation in seed quality among those seed lots. Additionally, the

indeterminate growth pattern of spinach can cause a high degree of variation in seed sizes, seed maturity, and seed quality within seed lots (Deleuran et al., 2013). Although quality differences can be expected within and among seed lots, in most studies just one seed lot per cultivar was used. Only Green et al. (2012) tested two seed lots of each of two spinach cultivars, and they indicated that the differences observed may have been the result of seed lot differences rather than cultivar differences. Therefore, we included three seed lots of each of five spinach cultivars. The seed lots that we used in the development of the phenotyping assay had similar seed size ranges, e.g., Chevelle-B, Chevelle-C, Novico-A, Shelby-A and Shelby-B with a seed size range of 2.50 to 3.75 mm diameter. This enabled us to check for cultivar and seed lot variation without a potential seed size effect. Shelby-A and Shelby-B showed a greater tolerance than Chevelle-B and Chevelle-C, indicating a potential cultivar effect. We also tested seeds with a diameter of 3.0 to 5.0 mm, such as Novico-C and Shelby-C. Shelby-C showed less tolerance compared with the other Shelby seed lots, suggesting that seed size may play a role. Also, Chevelle-A, with the smallest seed size (2.25 to 2.75 mm diameter), showed greater tolerance than Chevelle-B. However, an effect of seed size on pre-emergence damping-off tolerance levels was not observed for the Novico and Carmel seed lots. The Novico seed lots differed in seed size, but not in tolerance levels and the Carmel seed lots differed in tolerance levels even though their seed size did not differ. This implies that other seed lot differences may contribute to the large variation among seed lots in pre-emergence tolerance to *P. ultimum*. More research is needed to evaluate seed quality aspects or seed traits that contribute to pre-emergence damping-off tolerance in spinach, and whether genetic variation for certain traits exist. This is necessary to develop spinach cultivars with improved damping-off tolerance. For this research purpose, we recommend using the phenotyping assay that was developed in this study.

Acknowledgements

We gratefully acknowledge the financial and practical support from the seed and breeding companies Pop Vriend Seeds B.V. (The Netherlands), Sakata Seeds (France, Japan, California), and Enza Zaden/Vitalis Organic Seeds B.V. (The Netherlands). These companies and Nunhems/BASF B.V. (The Netherlands) are also acknowledged for providing the spinach seed lots. We thank Marieke Förch, Betty Henken, Marc Hendriks, and Mirjam Schilder for the isolation, identification, and storage of the damping-off pathogens. We thank Arjan Hendriksen and Jan Kodde for their experimental contributions. This research was funded by the Top Sector Horticulture & Propagation Materials as part of the research program 'Green Breeding' (KV 1409-031).

Supplemental

Supplemental Table 1. Characteristics of 43 isolates obtained from diseased spinach seed or seedling material of cultivar Chevelle seed lot B, grown in soil sampled from four field sites: Voorst 'de Bongerd' (B), Voorst 'de Kamp' (K), Oosterdijk (O) in the Netherlands; and Uchaud (U) in southern France. Species names show closest hit obtained from the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI), with accession numbers based on the ITS- and COI-primers-based sequences. Isolates were tested for pathogenicity in pre- and post-emergence stage of spinach seedlings, defined as non-pathogenic, weakly, or highly pathogenic in the pre- or post-emergence pathogenicity trial at different soil incubation temperatures (°C).

Species (closest hit BLAST tool)	ITS accession no. at NCBI [a]	COI accession no. at NCBI [a]	#	Field site	°C	Pathogenicity [b]
Pythium ultimum	MZ026494 MZ026495 MZ026496 MZ026497 MZ026498 MZ026499 MZ026500 MZ026502 MZ026503 MZ026508 MZ026510 MZ026511 MZ026512 MZ026513 MZ026515 MZ026521 MZ026522 MZ026526 - MZ026527 MZ026528 MZ026529 MZ026530 MZ026534 MZ026493	MZ151995 MZ151996 MZ151997 MZ151998 MZ151999 MZ152000 MZ152001 MZ152003 MZ152004 MZ152009 - MZ152011 MZ152012 - - MZ152014 MZ152015 MZ152016 MZ152017 - - MZ152018 MZ152019 MZ152022 MZ151994	25	B, K, O, U	15, 20, 25	2 non-pathogenic 23 weakly to highly pathogenic: 16 in pre- and post-emergence stage, 2 in only pre- and 5 in only post-emergence stage
P. terrestris	MZ026505 MZ026509 MZ026516 MZ026514 MZ026520	MZ152006 MZ152010 MZ152013 - -	5	B	15, 25	Weakly to highly
P. sylvaticum	MZ026504 MZ026533	MZ152005 MZ152021	2	K, O	15	Non-pathogenic to weakly
Pythium sp. CAL-2011f	MZ026519 MZ026531	- MZ152020	2	O	15, 20	Weakly
P. oligandrum	MZ026506 MZ026507	MZ152007 MZ152008	2	O	20	Non-pathogenic
P. attrantheridium	MZ026501	MZ152002	1	O	15	Non-pathogenic
Mortierella spp.	MZ026517 MZ026523 MZ026524 MZ026525 MZ026532	-	5	B, K, O	15, 20, 25	Non-pathogenic to weakly
Rhizoctonia solani	MZ026518	-	1	O	15	Highly: only post-emergence

[a] https://www.ncbi.nlm.nih.gov/

[b] Non-pathogenic means 0 to 33%, weakly pathogenic means 34 to 66%, and highly pathogenic means 67 to 100% non-emerged spinach seedlings in pre-emergence pathogenicity trial or seedlings with post-emergence damping-off symptoms in post-emergence pathogenicity trial.

Supplemental Table 2. Identity of 16 *Pythium* isolates obtained from diseased spinach seed or seedling material of cultivar Chevelle seed lot B, grown in soil sampled from four field sites: Voorst 'de Bongerd' (B), Voorst 'de Kamp' (K), Oosterdijk (O) in the Netherlands, and Uchaud (U) in southern France, with isolate number in this study and species names showing closest hit with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI), with accession numbers based on the ITS- and COI-primers-based sequences. Results of pre- and post-emergence pathogenicity trials with, respectively, incidences of non-emerged seedlings (% non-emerged) and emerged seedlings with wilting symptoms (% diseased).

Species (closest hit BLAST tool)	Isolate no.	ITS accession no. at NCBI [a]	COI accession no. at NCBI [a]	Field site	% non-emerged	% diseased
P. ultimum var. ultimum	1	MZ026493	MZ151994	K	85	92
P. ultimum	2b	MZ026495	MZ151996	K	85	92
P. ultimum	3a	MZ026496	MZ151997	K	63	83
P. ultimum	3b	MZ026497	MZ151998	K	58	42
P. ultimum	4	MZ026498	MZ151999	K	88	75
P. ultimum	5	MZ026499	MZ152000	B	81	92
P. ultimum	8b	MZ026502	MZ152003	O	88	100
P. ultimum	8c	MZ026503	MZ152004	O	94	92
P. ultimum	15	MZ026510	-	B	81	92
P. ultimum	20a	MZ026515	-	B	79	83
P. ultimum	36b	MZ026521	MZ152014	B	88	83
P. ultimum	41	MZ026522	MZ152015	K	48	92
P. ultimum	53	MZ026526	MZ152016	U	65	83
P. ultimum	68	MZ026530	MZ152019	U	31	8
P. terrestris	20c	MZ026516	MZ152013	B	54	58
P. terrestris (P. sylvaticum)	34	MZ026520	-	B	35	25
Control		-	-		34	0
LSD α=0.05					18	34

[a] https://www.ncbi.nlm.nih.gov/

Chapter 4

Rate of seedling emergence is not the only driver of pre-emergence damping-off tolerance in spinach

Kim J.H. Magnée[1], Steven P.C. Groot[2], Jan Kodde[2], Joeke Postma[3], Gerrit Gort[4], Edith T. Lammerts van Bueren[1] and Olga E. Scholten[1]

[1] Plant Breeding, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[2] Bioscience, Wageningen University & Research, The Netherlands
[3] Biointeractions and Plant Health, Wageningen University & Research, The Netherlands
[4] Biometris, Wageningen University & Research, The Netherlands

Submitted for publication in European Journal of Plant Pathology, under review

Abstract

Previous studies showed a large variation in pre-emergence damping-off tolerance levels among seed lots of the same spinach cultivars when evaluated in *Pythium ultimum*-inoculated versus non-inoculated substrates. Based on another study with mainly single seed lots per spinach cultivar, the hypothesis was that faster germinating and emerging seed lots show greater pre-emergence damping-off tolerance than slower seed lots. To test this hypothesis, seed priming or pericarp removal (dehulling) was applied prior to sowing, and the effects on germination, emergence, and pre-emergence damping-off tolerance were evaluated for three seed lots per cultivar. With both seed treatments, the rate and uniformity of seed germination improved. After priming, rate of seedling emergence and pre-emergence damping-off tolerance levels improved. Priming also reduced the variation in emergence rate and tolerance levels among the 15 seed lots tested. However, when pre-emergence damping-off tolerance levels of these seed lots was correlated with their rate of emergence in non-inoculated control substrates, a positive correlation could not be confirmed. After dehulling, some seed lots showed an increased rate of seedling emergence as well as an improved tolerance, while other seed lots of the same cultivars showed only one of the two effects. These results indicated that, besides rate of germination and seedling emergence, and the genotype (cultivar), other mechanisms or seed traits may be involved in pre-emergence damping-off tolerance in spinach.

Keywords: damping off, pericarp, *Pythium ultimum*, seedling emergence, seed vigour, *Spinacia oleracea*

Introduction

Global spinach (*Spinacia oleracea* L.) production faces increasing problems with non-uniform seedling establishment due to damping-off diseases. This is especially the case in the production of baby-leaf spinach, which requires dense and uniform stands (Correll et al., 1994; Koike et al., 2011). The increasing restrictions of using synthetic, chemical treatments, also in conventional production, emphasises the need for other methods to improve damping-off tolerance (United Nations Environment Programme (UNEP), 2006; European Commission, 2021).

Damping-off symptoms include seed decay and root rot before emergence, resulting in pre-emergence damping off, and root rot or seedling wilt after emergence, resulting in post-emergence damping off (Lamichhane et al., 2017). These symptoms can be caused by several soilborne pathogens, including species from the fungal genera *Fusarium* and *Rhizoctonia*, and oomycete genera *Aphanomyces, Phytophthora* and *Pythium* (Lamichhane et al., 2017). Studies have characterised *Pythium ultimum* as the most prevalent damping-off causing pathogen in spinach fields in Sweden (Larsson, 1994), California (Koike et al., 2011), and the Netherlands (Chapter 3). Generally, the severity of damping off is not only influenced by the pathogen, but also by the presence and absence of other microorganisms, the host plant genotype (or cultivar), soil moisture, soil temperature, soil pH, light intensity, and cropping history (Hendrix & Campbell, 1973; Green et al., 2012).

Our previous studies on pre-emergence damping-off tolerance in spinach cultivars showed larger variation among seed lots than among cultivars, confounding the expected differences in tolerance levels among cultivars (Chapter 3). Large variation in tolerance levels among seed lots may be a result of variation in seed lot quality. Varying environmental conditions during seed production, post-harvest treatments, or seed storage conditions can contribute to that variation. Also variation in seed quality within seed lots occurs due to the indeterminate flowering pattern of spinach, resulting in various seed sizes and maturity levels within a seed lot (Deleuran et al., 2013). Seed quality is intertwined with seed vigour, which comprises those seed properties that determine the potential for rapid and uniform germination and emergence of healthy seedlings under a wide range of environmental conditions (International Seed Testing Association (ISTA), 2021). Seed vigour mainly affects the early stages of vegetative growth and is, therefore, especially important for baby-leaf spinach production, where the crop is harvested early (TeKrony & Egli, 1991). The effects of seed vigour on seedling establishment may be direct, through physical properties (e.g., seed size and pericarp intactness) and seed health, or indirect, through rate and uniformity of seedling emergence. Seed vigour may also play a role in

biotic stress tolerance, especially with diseases that affect the seed or young seedling, e.g., pre-emergence damping off. Seeds with lower vigour showed an increased sensitivity to *Pythium* spp., for example in wheat (Das Gupta & Austenson, 1973), soybean (Schlub & Schmitthenner, 1978; Hamman et al., 2002), pea (Stasz & Harman, 1980), and lucerne (Hawthorne, 1988). A previous study with spinach indicated that slower germinating seed lots were more prone to damping off than faster germinating seed lots (Green et al., 2012). However, this was only confirmed for two seed lots of two cultivars, and other cultivars were represented by a single seed lot only. Based on this study, the hypothesis arose that faster germination and faster seedling emergence results in greater pre-emergence damping-off tolerance, which was tested by using multiple seed lots of five cultivars and by treating the seeds to deliberately improve the rate of germination and emergence.

Spinach seed priming with hydrogen peroxide increased the rate and uniformity of seed germination in a hydroponic system at 18°C (Katzman et al., 2001). In general, priming methods are based on the process of hydrating seeds in a controlled manner to initiate germination related processes, while preventing root protrusion by limiting the extent of water uptake, which would otherwise result in a loss of desiccation tolerance. A limited water uptake can result from soaking seeds in a low-water-potential solution (osmopriming), by restricting the duration of soaking seeds in water (hydropriming) (Heydecker et al., 1975), or by applying a limited amount of water to the seeds (in this study). After priming, seeds are dried and seeds within a seed lot that would otherwise germinate relatively slow will germinate faster, thereby increasing the uniformity of germination. Repair of damage, restoration of seed vigour, or a faster germination, resulting in a potential escape of pathogen attack, or a combination of those priming effects, may result in an increased tolerance towards pre-emergence infection.

In addition to the pathogen and plant genotype, abiotic conditions in the field play a role in damping-off severity (Lamichhane et al., 2017) and germination capacity (Chapter 2). For instance, infection by *P. ultimum* is worse at high soil moisture levels and temperatures that are optimal for its growth and development, but less optimal for the host plant (Agrios, 2005c). Spinach seed germination is hampered by high moisture levels at relatively warm temperatures (>15°C), interfering with oxygen availability to the seed embryo (Chapter 2), whereas *P. ultimum* tolerates low oxygen conditions (Schmitthenner, 1970). The spinach seed is botanically an indehiscent fruit with a true seed surrounded by dry perianth tissue, the fruit coat, or so-called pericarp (Sifton, 1927). Previous studies indicated that after pericarp removal (also called dehulling), spinach seed germination becomes less sensitive to low oxygen levels and dehulled seeds germinated better than untreated seeds under high moisture conditions (Heydecker & Orphanos, 1968; Chapter 2) and at relatively warm temperatures (Sifton, 1927;

Atherton & Farooque, 1983; Suganuma & Ohno, 1984; Leskovar et al., 1999). Hence, we hypothesised that with dehulling of spinach seeds, rate of germination and seedling emergence, and pre-emergence damping-off tolerance levels will increase.

To test the relation between pre-emergence damping-off tolerance and rate of seedling emergence, we evaluated the effects of seed priming and dehulling on seed germination, seedling emergence and pre-emergence damping-off tolerance. The use of three seed lots of each of five spinach cultivars enabled us to correlate rate of seedling emergence with pre-emergence damping-off tolerance levels of those seed lots.

Materials & Methods

Plant materials

Five spinach cultivars, with three independent seed lots of each (coded 'A', 'B', 'C'), were used in this study: 'Carmel', 'Chevelle', 'Mirage', 'Novico', and 'Shelby' (Table 1). For the readability when describing a seed lot of a cultivar, the cultivar name was hyphenated with the seed lot letter, e.g., Carmel-A. Seeds were stored at 30% relative humidity (RH) and 13°C after they were received in 2015 until they were used from 2017 to 2020. Seeds were randomly picked from the bags for the experiments, except for the 'intact' seeds that were obtained by selecting against seeds with a damaged pericarp, detected by using a binocular microscope. To obtain seeds without a pericarp ('dehulled' seeds), the pericarp was manually removed using liquid nitrogen when gently crushing the seeds with a mortar in a pestle, and undamaged dehulled seeds were selected.

Table 1. Information on the spinach seed lots used in this study. Seed size is the range in millimetres (mm) covering 95% of the approximately 1200 measured seeds and thousand seed weight (TSW) in grams (g), measured by the providing companies.

Cultivar	Seed lot	Seed size (mm)	TSW (g)	Providing company (country)
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	
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	
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	
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	
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	

Seed priming method

To obtain primed seeds, 5.5 g of seeds were moistened in 194 ml glass jars, each closed with a metal lid containing a 1 mm hole to provide sufficient oxygen for the seeds. Tap water was added to obtain a seed moisture content between 35 and 36%. This seed moisture content was chosen based on a pilot experiment with a seed lot of cultivar Carmel fractionated into five seed sizes, resulting in a more uniform seed germination on paper as compared to untreated seeds (*data not shown*). The jars were placed in a climate-controlled cabinet at 20°C in the dark and shaken daily. After seven days, the few seeds (0.4%) that had already germinated (protruded radicle) or that became mouldy (1.1%) were discarded. The remaining seeds were dried back to the initial seed moisture content of 8 to 9% water content versus seed fresh weight, by spreading the seeds in trays with filter paper for three days in a climate-controlled cabinet set at 20°C and 30% relative humidity.

Seed germination test

Seed germination was tested using a floating germination system described earlier (Chapter 2). Seeds were sown on germination papers (ø 100 mm, 300 g m^{-2}, Allpaper B.V., Zevenaar, the Netherlands). Standardised moisture conditions were obtained by placing moistened germination papers on top of 150 mm thick polystyrene foam that floated on the water in a plastic tray (600 mm x 400 mm x 80 mm). The germination papers were connected to the water with long folded paper wicks (460 mm by 30 mm, 235 g m^{-2}). On each germination paper, 25 or 50 seeds of a seed lot were sown (Table 2), with nine germination papers randomly distributed over each tray. This resulted in a randomised complete block design with trays as blocks and germination papers within trays as experimental units, with seed lot as the experimental factor. Tests were performed in triplicate on a Jacobsen germination table set at 20°C with 16/8-hour light/dark cycle (Exp. 1 and 2), or in a climate-controlled cabinet at 15°C in the dark (Exp. 3). Spinach seed germination was assessed daily until the end of the experiment (Table 2). On day 1 to 3, germination was assessed at least at two different times per day, with 8 hours difference to generate a more accurate estimation of the germination rate.

Pre-emergence damping-off assay

Pre-emergence damping-off tolerance was tested as described previously (Chapter 3). One day before each experiment, the substrates were prepared, consisting of autoclaved, dried, and sieved (< 5 mm diameter particles) river sand (IJssel, the Netherlands), perlite and vermiculite (both nr. 2, ≤ 3 mm, Pull B.V., Rhenen, the Netherlands) at a volume-based ratio of 1:1:1 (sand:perlite:vermiculite), and moistened with tap water to reach 50% of the maximum water holding capacity of the substrate. The cornmeal/sand-based inoculum of *P. ultimum* isolate 8b (see Chapter 3) was added to the substrate at a dose

of 0, 5, 10 or 20% (Table 2). Each treatment was replicated three times. For each experiment, a new batch of inoculum was prepared using the same *P. ultimum* isolate (8b) that was stored in liquid nitrogen in the form of mycelium PDA plugs in Cryotubes with 15% DMSO. The mycelium plugs were always regenerated on PDA.

The experiment started when the seeds were sown in the substrate. 200 g of the prepared substrate was divided evenly over the 50 cells of two transparent polystyrene dishes (100 mm x 100 mm x 18 mm, Item No.: 638102, Greiner-Bio One B.V., Alphen aan den Rijn, the Netherlands) that were placed in a stackable tray (210 mm x 150 mm x 30 mm, DBP Plastics N.V., Antwerpen, Belgium). One replicate of the control treatment and one replicate of the *P. ultimum* treatment together formed a block, resulting in a split-plot design with complete blocks including stacks as main plot units and *P. ultimum* treatment as the main plot factor, and trays (within stack) as subplot units with seed lot as the subplot factor. The three blocks were distributed randomly in a climate-controlled cabinet at 15°C in the dark and the positions of the blocks and trays within each stack were switched daily. Spinach seedling emergence was assessed daily until the end of the experiment (Table 2), and rotten seeds or seedlings were identified in compartments of the *P. ultimum*-inoculated substrate without emerged seedlings.

Table 2. Overview of the nine experiments (Exp. 1-9) used in this study with information on tested spinach seed lots, applied seed treatment (untreated, selected intact, primed or dehulled), number of seeds per replicate (n=3), substrate on which the seeds were sown (filter paper in floating germination system or substrate in damping-off assay containing moistened (50% WHC) sand:perlite:vermiculite mixture, 1:1:1 volume ratio), incubation temperature (°C), *Pythium ultimum* inoculum dose (Dose), and final day of the experiment.

Exp.	Seed lots	Seed treatment	Seeds (n=3)	Sowing substrate	°C	Dose	Final day
1	Chevelle-A/B/C Novico-A/B/C Shelby-A/B/C	untreated	50	filter paper	20	-	9
2	All 15 seed lots	primed	25	filter paper	20	-	8
3	Chevelle-C Shelby-B	selected intact, dehulled	50	filter paper	15	-	11
4	Chevelle-C Shelby-B	untreated, primed	50	substrate	15	0, 20%	10
5	All 15 seed lots	untreated	50	substrate	15	0, 5%	10
6	All 15 seed lots	primed	50	substrate	15	0, 10%	11
7	All 15 seed lots	primed	50	substrate	15	0, 20%	11
8	Chevelle-C Mirage-B Novico-A Shelby-B	selected intact, dehulled	50	substrate	15	0, 5%	14
9	Chevelle-A/B/C Shelby-A/B/C	untreated, dehulled	50	substrate	15	0, 5%	11

Data analysis

Count data of germinated seeds on filter paper and emerged seedlings in substrate were obtained over time. With the GERMINATOR curve fitting tool (Joosen et al., 2010), parameters for seed germination and seedling emergence were calculated: 1) total germination or emergence as fitted maximum percentage; 2) rate of germination or emergence as the time point (t50) when 50% of the fitted maximum percentage was reached; and 3) uniformity of germination or emergence as time between 16 and 84% germination or emergence (u8416). Further statistical analyses were performed using R version 4.0.4 and RStudio version 1.2.5042 (RStudio Team, 2020). A log transformation on the u8416 data was necessary to improve the homogeneity and normality of residuals. On the t50 data and log transformed u8416 data, an ANOVA was performed to check for effects (α=0.05) of fixed factors: replicate, seed lot by cultivar or seed treatment by seed lot by cultivar combination when different seed treatments were included. In case of significant effects, *post hoc* multiple comparisons among seed lots and treatments were carried out based on Tukey's pairwise comparisons (α=0.05).

For statistically testing differences in total germination or total emergence at the end of the experiments, a generalised linear mixed model (glmm) was applied, assuming a binomial distribution of the count data. A logit link function was used to link the probability of germination or emergence to the different independent variables as a linear predictor, including fixed and random factors. The fixed factors included block (three blocks consisting of one stack of trays with control treatment and one stack of trays with *P. ultimum* treatment), cultivar seed lot, substrate treatment (control and *P. ultimum* treatment), seed treatment (untreated vs. primed, intact vs. dehulled, or untreated vs. dehulled), and treatment by cultivar seed lot (by seed) interaction. The random factors included stack (for the split-plot design) and tray (to correct for potential binomial overdispersion). Type II Wald Chi^2-tests were used to test the effect of fixed factors. Tukey's pairwise comparisons were performed on the estimated means of significant fixed effects. For the total germination of primed seeds, 100% germination of Chevelle-B limited the use of the Wald test. For that case, pairwise likelihood ratio tests were performed manually between seed lots, and multiple comparisons were performed with Bonferroni-corrected p-values.

To quantify the *P. ultimum* treatment effect in the pre-emergence damping-off assays, odds ratios (OR) were obtained from the logistic glmm output, showing the estimates for the *P. ultimum* treatment effect on emergence per seed lot on a log scale, hence $OR = e^{estimate}$. This OR was used as a measure of damping-off tolerance level, defined as the odds of emergence in substrate with *P. ultimum* versus the odds of emergence in control substrate:

$$\text{Tolerance level=OR=}\frac{\text{emergence in } \textit{P. ultimum} \text{ substrate / non-emergence in } \textit{P. ultimum} \text{ substrate}}{\text{emergence in control substrate / non-emergence in control substrate}}$$

An OR smaller than 1 indicated sensitivity to *P. ultimum:* The closer the OR to 1, the greater the tolerance level. An OR of 1 or higher indicated complete tolerance to *P. ultimum*. For each OR estimate, the 95% confidence intervals were calculated ($e^{(estimate \pm 1.96 \cdot SE)}$). To test for significant differences (α=0.05) in tolerance levels, pairwise multiple comparisons with single-step adjusted p-values were performed on the differences among the estimates for the *P. ultimum* treatment effect on emergence. Pearson's correlations were calculated to correlate rate of emergence (t50) with the tolerance level (OR) of the spinach seed lots tested.

Results

Germination of untreated, primed and dehulled seeds

With untreated seeds, the rate and uniformity of germination on paper showed large variation among seed lots of cultivars Chevelle, Novico and Shelby (Table 3, Exp. 1). The Chevelle seed lots germinated faster (smaller t50) and more uniform (smaller u8416) than most of the other cultivar seed lots ($p<0.05$). Shelby-C germinated slowest and least uniform (longest duration between 16 and 84% total germination). Total germination of Chevelle-B and Chevelle-C was greater than total germination of Novico-B ($p<0.001$), Novico-C ($p<0.001$), and Shelby-C ($p=0.018$). Novico-C germinated less than Chevelle-A ($p<0.001$), Chevelle-B ($p<0.001$), Chevelle-C ($p<0.001$), Shelby-A ($p<0.001$), Shelby-B ($p=0.003$), and Novico-A ($p=0.003$).

Primed seed lots showed more uniform germination (shorter duration between 16% and 84% total germination) with less significant differences among seed lots than among untreated seeds. Primed seeds of Carmel-A germinated slower than primed seeds of all other seed lots ($p<0.01$), and primed Mirage-B seeds germinated faster compared to Mirage-C ($p=0.02$), Shelby-B ($p<0.001$) and Shelby-C ($p<0.001$). Total germination still showed variation among seed lots, with the greatest germination for Chevelle-B and least germination of Shelby-A (Table 3, Exp. 2). The effect of dehulling (pericarp removal) on germination of Chevelle-C and Shelby-B, which had similar seed sizes, showed that dehulled seeds germinated faster and more uniform than the selected seeds with an intact pericarp ($p=0.003$ and $p<0.001$ respectively) (Table 3, Exp. 3). Dehulling did not result in greater total germination of Chevelle-C or Shelby-B, as under the applied germination conditions the germination was already high for untreated seeds of these seed lots. Important to note is that the rate of seedling emergence of the selected intact seeds was relatively slow due to the lower temperature of 15°C used in experiment 3 compared to 20°C in experiment 1.

Table 3. Germination results of untreated (**Exp. 1**) and primed (**Exp. 2**) seeds of three seed lots of each spinach cultivar, and of selected intact and dehulled seeds of Chevelle-C and Shelby-B (**Exp. 3**) on germination paper. Rate of germination as time point when 50% of seeds had germinated (t50) and uniformity of germination as duration of time between 16 and 84% germination (u8416), obtained from the curve fitting tool, were analysed by ANOVA on t50 data and log-transformed u8416 data. Total germination at the end of the experiments, shown as mean percentages of three replicates of 50 seeds (Exp. 1 and 3) or 25 seeds (Exp. 2), was analysed by a generalised linear model with binomial distribution and logit link function. Results are on response scale with standard error of means (SEM) based on the model. Same letters indicate homogeneous subsets according to Tukey's pairwise comparisons within Exp. 1 and within Exp. 3, and according to Bonferroni-corrected p-values from Likelihood Ratio tests within Exp. 2.

Exp.	Seed treatment	Cultivar – seed lot	t50 ± SEM (hours)			u8416 ± SEM (hours)			Total germination ± SEM (%)		
1[a]	untreated	Chevelle-A	34.3	±2.6	d	24.6	±3.2	de	93.0	±2.0	ab
		Chevelle-B	35.5	±2.6	d	12.9	±1.7	e	98.7	±0.9	a
		Chevelle-C	44.1	±2.6	cd	19.7	±2.6	de	98.7	±0.9	a
		Novico-A	54.2	±2.6	bc	52.0	±6.8	abc	91.7	±2.2	ab
		Novico-B	61.3	±2.6	b	58.4	±7.6	ab	83.9	±3.0	bc
		Novico-C	62.1	±2.6	b	52.1	±6.8	abc	73.9	±3.6	c
		Shelby-A	57.0	±2.6	bc	33.2	±4.3	bcd	94.9	±1.8	ab
		Shelby-B	51.3	±2.6	bc	29.4	±3.8	cd	91.7	±2.2	ab
		Shelby-C	81.4	±2.6	a	79.4	±10.4	a	85.9	±2.8	bc
		average		53.3			40.2			90.3	
2	primed	Carmel-A	34.0	±1.0	a	18.9	±3.0	a	89.3	±3.6	ac
		Carmel-B	20.3	±1.0	cde	17.1	±2.7	a	89.3	±3.6	ac
		Carmel-C	20.4	±1.0	cde	16.9	±2.7	a	88.0	±3.8	bc
		Chevelle-A	18.7	±1.0	de	9.7	±1.5	ab	90.7	±3.4	ac
		Chevelle-B	17.5	±1.0	de	10.4	±1.7	ab	100.0	±0.0	a
		Chevelle-C	20.2	±1.0	cde	10.9	±1.7	ab	92.0	±3.1	ac
		Mirage-A	18.5	±1.0	de	10.7	±1.7	ab	96.0	±2.3	ab
		Mirage-B	15.0	±1.0	e	6.5	±1.0	b	97.3	±1.9	ab
		Mirage-C	20.8	±1.0	cd	13.2	±2.1	ab	90.7	±3.4	ac
		Novico-A	19.8	±1.0	cde	9.6	±1.5	ab	86.7	±3.9	bc
		Novico-B	18.7	±1.0	de	7.3	±1.2	b	98.7	±1.3	ab
		Novico-C	16.8	±1.0	de	14.2	±2.3	ab	98.7	±1.3	ab
		Shelby-A	19.3	±1.0	de	7.1	±1.1	b	76.0	±4.9	c
		Shelby-B	27.8	±1.0	b	17.3	±2.7	a	94.7	±2.6	ac
		Shelby-C	24.7	±1.0	bc	14.4	±2.3	ab	85.3	±4.1	bc
		average		20.8			12.3			91.6	
3	selected intact	Chevelle-C	62.5	±2.1	b	21.4	±5.3	ab	98.7	±0.9	a
		Shelby-B	95.6	±2.1	a	60.5	±14.9	a	94.1	±1.9	a
	dehulled	Chevelle-C	44.0	±2.1	c	13.2	±3.3	b	98.7	±0.9	a
		Shelby-B	47.0	±2.1	c	28.3	±6.9	ab	100.0	±0.0	a

[a] Data on t50 and total germination of untreated seeds (Exp. 1) were also used in Chapter 2 (Table 3) but are re-analysed.

Priming effects on emergence and pre-emergence damping-off tolerance

Testing the effect of priming on emergence and pre-emergence damping-off tolerance with two untreated and two primed seed lots, Chevelle-C and Shelby-B (with similar seed sizes), showed that the primed seed lots emerged faster ($p<0.001$) and had greater pre-emergence damping-off tolerance ($p<0.001$ for Chevelle-C and $p=0.018$ for Shelby-B) (Table 4, Exp. 4). Tolerance levels of primed seed lots even exceeded 1 due to a slightly but not significantly greater emergence in *P. ultimum* treatment compared to

the control treatment. Untreated seeds of Chevelle-C emerged faster but with less pre-emergence damping-off tolerance than untreated Shelby-B seeds. After priming, these differences in emergence rate and tolerance disappeared.

To correlate the emergence rate with pre-emergence damping-off tolerance levels, all 15 seed lots were evaluated in an assay with either untreated or primed seeds. For the 15 untreated seed lots, 50% total emergence was reached around six days after sowing in the control substrate (Table 4, Exp. 5). Primed seeds emerged faster, with 50% total emergence around day four, and more uniform, without differences in uniformity among seed lots (Table 4, Exp. 7). The total emergence from untreated seeds sown in the substrate inoculated with 5% *P. ultimum* inoculum dose was less than the total emergence in the non-inoculated control substrate, and this difference was significant ($p<0.001$) (Table 4, Exp. 5). With primed seeds, a higher inoculum dose was necessary to obtain a significant emergence reduction. The total emergence of primed seeds in the substrate inoculated with 10% *P. ultimum* inoculum dose did not significantly differ from the emergence in the control substrate (Figure 1A, Exp. 6). In the substrate with 20% inoculum dose, the total emergence of primed seed lots was reduced, and the differences were significant ($p<0.007$), except for Carmel-C ($p=0.95$), Shelby-A ($p=0.56$), Shelby-B ($p=0.057$), and Shelby-C ($p=0.057$) (Figure 1B; Table 4, Exp. 7). The variation in total emergence in the non-inoculated substrate was larger among the primed seed lots than among the untreated seed lots, whereas the variation in emergence rate in the control substrate and the variation in tolerance levels among the primed seed lots was smaller. The correlation between emergence rate and pre-emergence damping-off tolerance among the untreated seed lots was not significant (Pearson's $r=-0.11$, $p=0.70$), and similarly for the primed seed lots (Pearson's $r=0.087$, $p=0.76$).

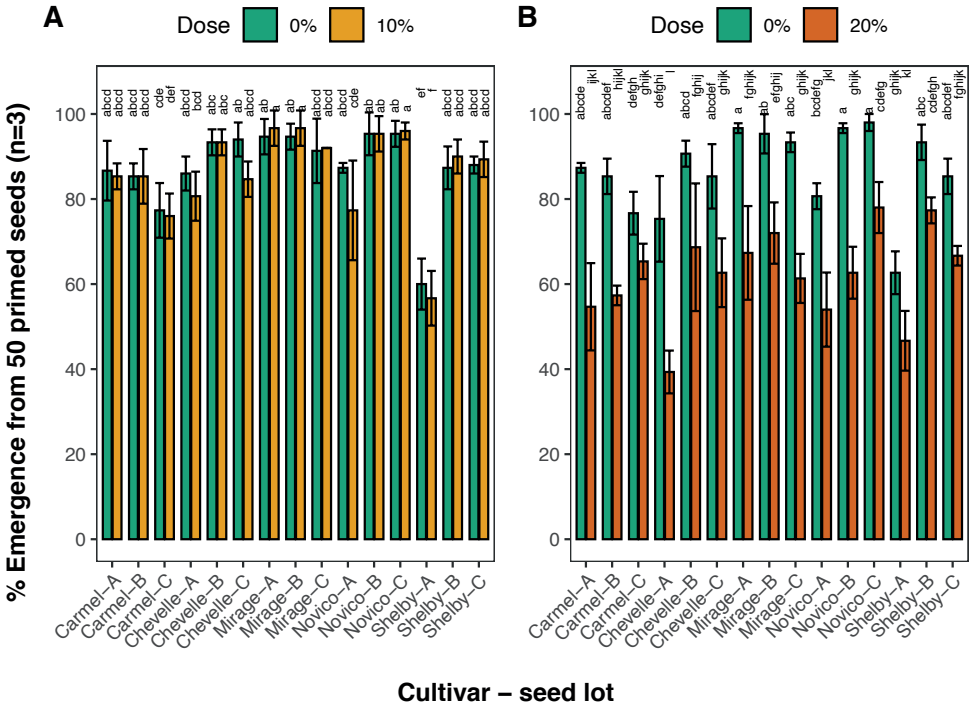


Figure 1. Mean incidence (%) of emerged seedlings 10 days after sowing 50 primed seeds of each of three seed lots of five spinach cultivars in a substrate with sand:perlite:vermiculite mixture (1:1:1 volume basis) maintained at 50% WHC, with 0% (control) and 10% **(A, Exp. 6)** or 0% and 20% **(B, Exp. 7)** *Pythium ultimum* inoculum dose. Error bars represent standard deviations (n=3, replicated trays with 50 seeds each tray). Bars with the same letter indicate no significant differences among seed lots and treatments based on Tukey's pairwise comparisons (α=0.05).

Table 4. Emergence results and pre-emergence damping-off tolerance levels for untreated and primed seeds of Chevelle-C and Shelby-B (**Exp. 4**), for 15 untreated seed lots (**Exp. 5**), and for 15 primed seed lots (**Exp. 7**), including three seed lots of spinach cultivars Carmel, Chevelle, Mirage, Novico and Shelby. Rate (t50) and uniformity (u8416) of seedling emergence were analysed by a linear model with normal distribution. Data of total emergence in non-inoculated control and *P. ultimum*-inoculated ("Pythium") substrates with a 5% inoculum dose (Exp. 5) or 20% inoculum dose (Exp. 4 and 6), shown as mean percentages of three replicates of 50 seeds, were analysed by a generalised linear mixed model with binomial distribution and logit link function. Same letters indicate homogeneous subsets according to Tukey's pairwise comparisons within experiments. Emergence data are on a response scale with standard errors of the means (SEM), and tolerance levels as odds ratios (OR) with 95% confidence intervals (CI), and significance letters based on the model (α=0.05).

Exp.	Seed treatment	Cultivar -seed lot	t50 ±SEM in days			u8416 ±SEM in days			Total emergence in control ±SEM (%)			Total emergence in Pythium ±SEM (%)			Tolerance level (OR Pythium vs. control with CI)		
4	untreated	Chevelle-C	6.7	±0.1	b	1.3	±0.2	ab	92.7	±2.1	a	59.4	±4.0	b	0.11	(0.06-0.23)	c
		Shelby-B	7.4	±0.1	a	1.9	±0.2	a	82.7	±3.1	a	64.7	±3.9	b	0.38	(0.22-0.66)	b
	primed	Chevelle-C	4.9	±0.1	c	1.0	±0.2	ab	91.4	±2.3	a	93.4	±2.0	a	1.33	(0.56-3.13)	ab
		Shelby-B	4.8	±0.1	c	0.6	±0.2	b	89.4	±2.5	a	93.4	±2.0	a	1.67	(0.73-3.82)	a
5[a]	untreated	Carmel-A	6.1	±0.1	bcde	1.9	±0.2	abc	90.7	±2.4	a	0.7	±0.7	de	0.001	(0.000-0.005)	bce
		Carmel-B	5.7	±0.1	def	1.2	±0.2	cde	90.1	±2.4	a	12.6	±2.7	cde	0.016	(0.008-0.033)	acd
		Carmel-C	5.8	±0.1	cdef	1.5	±0.2	bcde	87.3	±2.7	a	31.9	±3.8	b	0.068	(0.038-0.123)	a
		Chevelle-A	5.5	±0.1	ef	1.5	±0.2	bcde	88.1	±2.6	a	8.7	±2.3	cde	0.013	(0.006-0.027)	bcd
		Chevelle-B	5.5	±0.1	f	1.0	±0.2	e	96.7	±1.5	a	2.0	±1.1	e	0.001	(0.000-0.003)	e
		Chevelle-C	5.8	±0.1	cdef	1.4	±0.2	bcde	92.7	±2.1	a	4.6	±1.7	de	0.004	(0.001-0.010)	bce
		Mirage-A	6.3	±0.1	bc	1.9	±0.2	abcd	95.4	±1.7	a	4.6	±1.7	de	0.002	(0.001-0.007)	ce
		Mirage-B	6.5	±0.1	b	1.8	±0.2	abcde	95.4	±1.7	a	11.9	±2.6	cde	0.007	(0.003-0.016)	bce
		Mirage-C	6.2	±0.1	bcd	2.1	±0.2	abc	92.1	±2.2	a	9.3	±2.4	cde	0.009	(0.004-0.020)	bce
		Novico-A	5.8	±0.1	cdef	1.5	±0.2	bcde	85.4	±2.9	a	11.3	±2.6	cde	0.022	(0.011-0.043)	ab
		Novico-B	6.0	±0.1	bcdef	2.2	±0.2	ab	95.4	±1.7	a	13.9	±2.8	bcde	0.008	(0.003-0.019)	bce
		Novico-C	5.9	±0.1	cdef	1.5	±0.2	bcde	93.4	±2.0	a	19.9	±3.3	bcd	0.018	(0.008-0.038)	acd
		Shelby-A	6.1	±0.1	bcde	1.1	±0.2	de	93.4	±2.0	a	23.9	±3.5	bc	0.022	(0.011-0.047)	ab
		Shelby-B	7.2	±0.1	a	2.4	±0.2	a	89.4	±2.5	a	13.9	±2.8	bcde	0.019	(0.010-0.038)	ac
		Shelby-C	6.5	±0.1	b	2.0	±0.2	abc	98.0	±1.1	a	6.0	±1.9	de	0.001	(0.000-0.005)	de
		average	6.1			1.7			92.2			11.7					

Table 4. Continued.

Exp.	Seed treatment	Cultivar -seed lot	t50 ±SEM in days			u8416 ±SEM in days			Total emergence in control ±SEM (%)			Total emergence in Pythium ±SEM (%)			Tolerance level (OR Pythium vs. control with CI)		
7	primed	Carmel-A	4.7	±0.1	a	1.2	±0.2	a	87.4	±2.7	abcde	54.7	±4.1	ijkl	0.17	(0.10-0.31)	ab
		Carmel-B	4.0	±0.1	ab	1.1	±0.2	a	85.4	±2.9	bcde	57.4	±4.0	hijkl	0.23	(0.13-0.40)	ab
		Carmel-C	3.8	±0.1	b	0.8	±0.2	a	76.8	±3.4	def	65.4	±3.9	ghijk	0.57	(0.34-0.95)	a
		Chevelle-A	3.6	±0.1	b	1.9	±0.2	a	75.5	±3.5	ef	39.3	±4.0	l	0.21	(0.13-0.35)	ab
		Chevelle-B	3.8	±0.1	b	1.0	±0.2	a	90.7	±2.4	abcd	68.8	±3.8	fghij	0.22	(0.12-0.43)	ab
		Chevelle-C	3.9	±0.1	b	1.1	±0.2	a	85.4	±2.9	bcde	62.7	±4.0	ghijk	0.29	(0.16-0.50)	ab
		Mirage-A	3.8	±0.1	b	0.9	±0.2	a	96.7	±1.5	ab	67.4	±3.8	fghijk	0.07	(0.03-0.18)	b
		Mirage-B	3.6	±0.1	b	0.8	±0.2	a	95.4	±1.7	ab	72.1	±3.7	efghij	0.13	(0.05-0.29)	ab
		Mirage-C	4.1	±0.1	ab	1.1	±0.2	a	93.4	±2.0	abc	61.4	±4.0	ghijk	0.11	(0.05-0.23)	b
		Novico-A	3.9	±0.1	b	1.8	±0.2	a	80.8	±3.2	cde	54.0	±4.1	jkl	0.28	(0.17-0.47)	ab
		Novico-B	3.6	±0.1	b	1.1	±0.2	a	96.7	±1.5	ab	62.7	±4.0	ghijk	0.06	(0.02-0.15)	b
		Novico-C	3.5	±0.1	b	0.9	±0.2	a	98.0	±1.1	a	78.1	±3.4	cdefg	0.07	(0.02-0.24)	ab
		Shelby-A	3.7	±0.1	b	0.8	±0.2	a	62.7	±4.0	f	46.7	±4.1	kl	0.52	(0.33-0.82)	a
		Shelby-B	4.1	±0.1	ab	1.7	±0.2	a	93.4	±2.0	abc	77.5	±3.4	cdefgh	0.24	(0.11-0.51)	ab
		Shelby-C	4.0	±0.1	ab	0.9	±0.2	a	85.4	±2.9	bcde	66.8	±3.9	fghijk	0.34	(0.19-0.60)	ab
		average	3.9			1.1			86.9			62.3					

[a] Data on total emergence and tolerance levels of untreated seeds (Exp. 5) were visualised in Chapter 3 (Figure 6) but are now related to rate and uniformity of emergence.

Seed dehulling effect on emergence and pre-emergence damping off tolerance

Dehulled seeds of seed lots Chevelle-C, Mirage-B, Novico-A, and Shelby-B (with similar seed sizes) differed significantly from the selected intact seeds of the same seed lots, with a greater pre-emergence damping-off tolerance of the dehulled seeds of Chevelle-C ($p<0.001$), Mirage-B ($p<0.001$), and Novico-A ($p=0.006$), but there was no significant difference for Shelby-B ($p=0.078$) (Table 5, Exp. 8). Dehulled seeds of Mirage-B and Shelby-B also emerged faster than the intact seeds of Mirage-B ($p=0.004$) and Shelby-B ($p<0.001$), respectively. Shelby-B was the slowest emerging seed lot and showed least effect of dehulling on damping-off tolerance compared to the other three seed lots. A replicate experiment (Exp. 9) with dehulled and (non-selected) untreated seeds from three seed lots of Chevelle and Shelby showed no or hardly any influence of dehulling on the rate of emergence and on the total emergence of seedlings in the non-inoculated substrate (Table 5). In the *P. ultimum*-inoculated substrate, emergence was greater for the dehulled seed lots than for the untreated seed lots, but the difference was only significant for Chevelle-B, Chevelle-C, and Shelby-C ($p<0.001$). Similarly, the pre-emergence damping-off tolerance was improved for all seed lots after dehulling, but the difference was only significant for Chevelle-B and Chevelle-C ($p<0.001$). Dehulled seeds of Shelby-B emerged faster than untreated seeds of Shelby-B ($p=0.005$), but the tolerance of the Shelby seed lots did not improve after dehulling.

Table 5. Emergence incidence (%) and pre-emergence damping-off tolerance levels of (**Exp. 8**) selected intact and dehulled seeds of Chevelle-C, Mirage-B, Novico-A and Shelby-B; and (**Exp. 9**) untreated and dehulled seeds of three seed lots of Chevelle and Shelby. Rate (t50) and uniformity (u8416) of seedling emergence were analysed by a linear model with normal distribution. Data of total emergence in non-inoculated control and *P. ultimum*-inoculated substrates with 5% inoculum dose ("Pythium"), shown as mean percentages of three replicates of 50 seeds, were analysed by a generalised linear mixed model with binomial distribution and logit link function. Same letters indicate homogeneous subsets according to Tukey's pairwise comparisons within experiments. Emergence data are on response scale with standard errors of means (SEM), and tolerance levels are odds ratios (OR) with 95% confidence intervals (CI), and significance letters based on the model (α=0.05).

Exp.	Seed treatment	Cultivar - seed lot	t50 ±SEM (days)			u8416 ±SEM (days)			Emergence in control ±SEM (%)			Emergence in Pythium ±SEM (%)			Tolerance level (OR Pythium vs. control with CI)		
8	intact	Chevelle-C	6.9	±0.1	c	1.2	±0.2	b	94.8	±1.8	ab	5.7	±1.9	d	0.003	(0.001-0.009)	c
	intact	Mirage-B	7.6	±0.1	b	1.8	±0.2	b	97.4	±1.3	a	7.6	±2.2	d	0.002	(0.001-0.007)	c
	intact	Novico-A	7.1	±0.1	bc	1.8	±0.2	b	84.8	±3.1	ab	5.1	±1.8	d	0.010	(0.004-0.023)	c
	intact	Shelby-B	8.4	±0.1	a	3.3	±0.2	a	94.8	±1.8	ab	17.4	±3.3	cd	0.012	(0.005-0.027)	bc
	dehulled	Chevelle-C	6.7	±0.1	c	1.7	±0.2	b	84.2	±3.1	b	24.8	±3.8	c	0.062	(0.034-0.114)	a
	dehulled	Mirage-B	6.7	±0.1	c	1.1	±0.2	b	83.5	±3.2	b	28.1	±4.0	c	0.077	(0.043-0.140)	a
	dehulled	Novico-A	6.6	±0.1	c	1.2	±0.2	b	85.5	±3.0	ab	27.5	±4.0	c	0.064	(0.035-0.119)	a
	dehulled	Shelby-B	7.2	±0.1	bc	1.8	±0.2	b	86.8	±2.9	ab	25.4	±3.8	c	0.052	(0.027-0.097)	ab
9[a]	untreated	Chevelle-A	5.7	±0.2	bc	1.6	±0.2	ab	85.5	±3.4	ab	6.4	±2.2	f	0.011	(0.004-0.027)	ab
	untreated	Chevelle-B	5.5	±0.2	c	1.3	±0.2	b	97.4	±1.3	a	0.6	±0.6	f	0.000	(0.000-0.001)	c
	untreated	Chevelle-C	5.9	±0.2	bc	1.5	±0.2	ab	90.9	±2.7	ab	3.2	±1.5	f	0.003	(0.001-0.010)	bc
	untreated	Shelby-A	6.3	±0.2	bc	1.3	±0.2	b	91.6	±2.5	ab	19.1	±4.0	def	0.021	(0.009-0.049)	ab
	untreated	Shelby-B	7.1	±0.2	a	2.2	±0.2	a	82.9	±3.8	ab	17.8	±3.8	def	0.034	(0.016-0.073)	a
	untreated	Shelby-C	6.4	±0.2	ab	1.8	±0.2	ab	96.8	±1.5	a	7.1	±2.3	f	0.003	(0.001-0.008)	bc
	dehulled	Chevelle-A	5.8	±0.2	bc	1.4	±0.2	b	77.7	±4.3	b	15.0	±3.5	ef	0.047	(0.022-0.098)	a
	dehulled	Chevelle-B	5.6	±0.2	bc	1.5	±0.2	ab	93.5	±2.2	ab	48.7	±5.7	c	0.065	(0.028-0.152)	a
	dehulled	Chevelle-C	5.6	±0.2	bc	1.6	±0.2	ab	92.2	±2.4	ab	41.5	±5.5	cd	0.060	(0.027-0.134)	a
	dehulled	Shelby-A	6.1	±0.2	bc	1.7	±0.2	ab	89.5	±2.9	ab	36.1	±5.4	cde	0.065	(0.030-0.139)	a
	dehulled	Shelby-B	6.0	±0.2	bc	1.7	±0.2	ab	94.1	±2.1	ab	35.3	±5.3	cde	0.034	(0.014-0.081)	a
	dehulled	Shelby-C	5.7	±0.2	bc	1.5	±0.2	ab	98.1	±1.1	a	40.5	±5.5	cd	0.013	(0.004-0.047)	ab

[a] Data on tolerance levels of untreated seeds (Exp. 9) were visualised in Chapter 3 (3rd plot in Figure 5) but are now related to other parameters.

Discussion

Priming of spinach seeds resulted in an increased uniformity of seed germination and seedling emergence compared to untreated seeds. Primed seed lots emerged faster and showed increased pre-emergence damping-off tolerance levels compared to untreated seed lots, which was in accordance with our hypothesis. Priming can induce seed metabolic activities that prepare the seed for radicle protrusion, but can also induce repair and protection mechanisms (Rajjou et al., 2012; Sano et al., 2015). Those mechanisms include abiotic stress tolerance during germination, such as drought and salinity (Chen & Arora, 2013). Also, biotic stress tolerance may be induced by priming. For instance, priming of taxonomically and morphologically-related sugar beet seeds resulted in reduced exudation of soluble carbohydrates during seed germination, which resulted in reduced seed colonization by *P. ultimum* (Osburn & Schroth, 1988). In addition, the seed microbiome may be activated by priming, like with seed imbibition, activating dormant endophytic (in the seed) or epiphytic (on the pericarp) microorganisms (Nelson, 2018). These microorganisms could be antagonists of *P. ultimum* and thereby enhance tolerance of spinach seeds towards infection (Osburn & Schroth, 1988). The pericarp surrounding the spinach seed also contains phenolic compounds, such as water-soluble polyphenols (e.g., tannins) with antimicrobial activity (Scalbert, 1991). Since our priming method only moistened the spinach seeds to a limited extent and the seeds were redried, it is not likely that the priming removed these water-soluble compounds.

Although priming reduced the variation in pre-emergence damping-off tolerance levels among seed lots, variation in emergence in the absence of *P. ultimum* still existed among primed seed lots. This emphasised the need for a control treatment without *P. ultimum* when predicting pre-emergence damping-off tolerance levels of seed lots. The reduced variation in tolerance levels among cultivars limits the usefulness of priming as a tool in the search for cultivar variation in pre-emergence damping-off tolerance in spinach.

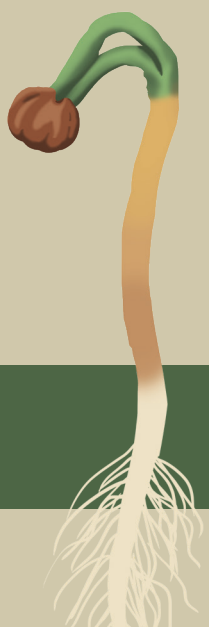
When seeds were dehulled prior to sowing, positive effects were found on pre-emergence damping-off tolerance levels for four out of eight spinach seed lots tested. For only one seed lot, positive effects of seed dehulling were found on both the rate of seedling emergence and the pre-emergence damping-off tolerance. For the other seed lots, only one of the two effects occurred. In these cases, other mechanisms than rate of seedling emergence alone must have played a role. For instance, genotypic effects could play a role in the case of Shelby-B that showed greater damping-off tolerance than Chevelle-B, despite faster emergence of Chevelle-B. Also, other seed lot effects than seed vigour alone might be involved for Shelby-C, which showed less damping-off tolerance than Shelby-B, despite the faster emergence of Shelby-C.

The effects of seed dehulling on emergence and pre-emergence damping-off tolerance may be related to the removal of germination inhibitors, such as abscisic acid (ABA) and phenolic compounds, that are present in the pericarp. Phenolic compounds bind oxygen and, when present in large quantities, may hamper seed germination, as shown for birch (*Betula* spp. L.) and sugar beet (*Beta vulgaris* L.) seeds (Black & Wareing, 1959; Heydecker et al., 1971). For sugar beet seeds, polishing and washing improved the germination rate as well as total germination by reducing the physical and chemical barriers for oxygen diffusion (Ignatz et al., 2019). With these techniques, especially when used in combination, the concentration of germination inhibitors was reduced, increasing the rate of germination and emergence. Instead of complete removal of the pericarp, removal of the operculum (the ovary cap of the fruit) already promoted radicle emergence of sugar beet seeds (Coumans et al., 1976). For spinach seeds, an open or thinner pericarp also promoted germination through increased oxygen availability to the seed embryo (Chapter 2). Similar to sugar beet seeds, the pericarp of spinach seeds contains biologically active compounds that regulate seed germination, or have antibacterial or antifungal effects that may improve biotic stress tolerance during the early stages of seedling development (Oksana & Artur, 2019). Therefore, removing the pericarp may, on one hand, increase the ability of seeds to germinate and emerge faster, and provide potential escape from *P. ultimum* infection, but on the other hand, it can also reduce antimicrobial capacity, which was not expected with the priming method used in this study.

In conclusion, spinach seeds that were primed were more tolerant to pre-emergence damping off, with less variation among the primed seed lots than among the untreated seed lots. Dehulling of seeds resulted in improved tolerance for some seed lots and was not always accompanied by an improved rate of seedling emergence. Although we generally found a positive relationship between emergence rate and damping-off tolerance when seed lots were treated compared to untreated, we did not find a positive correlation between emergence rate and damping-off tolerance for untreated seed lots. This was not in accordance with our hypothesis that faster emerging seed lots will have greater pre-emergence damping-off tolerance. For the seed lots with faster emergence and less tolerance to *P. ultimum* than slower emerging seed lots, other mechanisms must be involved. So, we presume that rate of seedling emergence is not the only driver for spinach pre-emergence damping-off tolerance. Further studies on the effects of other vigour-related seed traits may aid in the development of more damping-off tolerant spinach cultivars.

Acknowledgements

We gratefully acknowledge the financial and practical support from the seed and breeding companies Pop Vriend Seeds B.V. (The Netherlands), 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. We thank Nathalie Blom and Ranthi Whesi Umbarani for their experimental contributions. This research was funded by the Top Sector Horticulture & Propagation Materials as part of the research program 'Green Breeding' (KV 1409-031).



Chapter 5

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

Kim J.H. Magnée[1], Olga E. Scholten[1], Jan Kodde[2], Joeke Postma[3], Gerrit Gort[4], Edith T. Lammerts van Bueren[1], and Steven P.C. Groot2

[1] Plant Breeding, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[2] Bioscience, Wageningen University & Research, The Netherlands
[3] Biointeractions and Plant Health, Wageningen University & Research, The Netherlands
[4] Biometris, Wageningen University & Research, The Netherlands

To be submitted

With the growing demand for baby-leaf spinach (*Spinacia oleracea* L.) and 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 five spinach cultivars that did not correlate with their variation in rate of seedling emergence. We hypothesised that other seed vigour-related traits, such as seed size and maturity, can influence tolerance towards pre-emergence infection by *P. ultimum*. To study this, individual seeds of the same seed lots were measured for their morphological and multispectral properties. The seedling emergence from those individual seeds was assessed at different *P. ultimum* doses. The results showed that chlorophyll fluorescence (a reliable predictor for seed maturity according to previous studies) had a negative relationship 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 emergence in the presence or absence of *P. ultimum*, and that seed size had a negative association with 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. Further research is needed to understand the negative effects of violet-blue light reflectance and associated secondary metabolites. Selecting the most mature seeds with relatively less light reflectance (darker pericarp) or smaller seed size, is recommended to obtain seed lots with improved pre-emergence damping-off tolerance levels.

Keywords: chlorophyll fluorescence, damping off, multispectral imaging, light reflectance, *Spinacia oleracea*

Abbreviations: AIC, Aikaike's information criterion; AU, arbitrary units; Blue, reflectance at 420-480 nm; CF, chlorophyll fluorescence; Dose, *P. ultimum* dose; glmm, generalised linear mixed model; Green, reflectance at 535-585 nm; Hue, colour reflectance; Int, light reflection intensity; Red, reflectance at 602.5-677.5 nm; 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.

Abstract

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 losses of spinach yields. Damping off symptoms can occur in the seed germination or seedling stages, either causing seed or root rot before the seedling emerges above soil level (pre-emergence damping off), or seedling root rot or 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 at causing spinach pre-emergence damping off (Larsson, 1994; Koike et al., 2011; Chapter 3). Management of damping off includes seed treatments with synthetic fungicides, many of which are increasingly banned worldwide (United Nations Environment Programme (UNEP), 2006; European Commission, 2021) and not allowed in organic production. As the demand for non-chemically treated seeds increases, issues with pre-emergence damping off and the need for alternative solutions, e.g., cultivars with improved tolerance levels, are increasing too. In this study, the aim was 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 variation among seed lots, confounding the search for potential genotypic variation (Chapter 3). The standardised lab assay resulted in 92.2% (±4.6%) emergence in the control treatment, without significant differences among the 15 tested seed lots, and 11.7% (±9.2%) emergence in the treatment with a 5% *P. ultimum* dose, with significant differences among the seed lots that could not be explained by their variation in rate of emergence (Chapter 4). Therefore, we hypothesised that other 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 growth habit of spinach and 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 some seed characteristics.

The general observation is that larger seeds result in greater germination and emergence capacity, more vigorous seedlings, higher survival rates, and greater crop yields (i.e., as shown for wheat (Das Gupta & Austenson, 1973), broccoli (Heather & Sieczka, 1991), olive (Rey et al., 2004), legumes (Arellano & Peco, 2012), soybean (Vange et al., 2016), and ginseng (Zhang et al., 2018)). Seed size may relate to biotic stress tolerance, as a study on lucerne seeds showed that seedlings that developed from heavier seeds grew

more vigorously in *Pythium*-infected soils than seedlings from lighter seeds (Hawthorne, 1988). Also for spinach, a positive correlation between seed size and germination has been reported, although other 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 as 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 or seed production environments. Within a seed lot, however, seed size is expected to correlate positively with maturity. Hence, seed size and maturity levels 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.

During seed maturation, the amount of chlorophyll content in the seed coat of many seed species decreases (Steckel et al., 1989; Ward et al., 1995). The chlorophyll content of a seed can be determined by its fluorescent properties, and for cabbage, pepper, and tomato seeds, 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 instead of the seed coat, 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 colour and seed quality, including abiotic stress tolerance, e.g, salt tolerance in red clover (Atis et al., 2011), and biotic stress tolerance, e.g., damping-off tolerance in pine seeds (Grzywacz & Rosochacka, 1980). Pericarp colour, measured by light reflectance, was also a good marker for the presence of fungal infection of spinach seeds (Olesen et al., 2011) and barley seeds (Boelt et al., 2018). This has triggered our interest in studying pericarp colour in relation to pre-emergence damping-off tolerance.

In this study, we investigated the potential effects of seed size, seed maturity, and pericarp colour on pre-emergence damping-off tolerance by measuring the seed area, length and width, roundness, CF levels, and light reflectance along the visible and near-infrared light spectrum of individual seeds, prior to sowing them in a *P. ultimum*-inoculated or non-inoculated (control) substrate. Seedling emergence from individual seeds was recorded and related to the different seed characteristics. Individual seeds of three

commercially produced seed lots of each of five spinach cultivars were used to determine which seed traits correlate significantly and in which direction 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 fractions and maturity fractions of a single seed lot were screened to validate a potential seed size and/or maturity effect without the influence of a potential genotype-by-environment effect. Seed characteristics, such as seed size, rate of ripening and pericarp colour, are determined by both the genotype and the environment of the mother plant. Therefore, 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.

Materials & Methods

Plant materials

Untreated seeds of three independent seed lots (coded 'A', 'B', 'C') of five spinach cultivars, including 'Carmel', 'Chevelle', 'Mirage', 'Novico', 'Shelby', were used for experiments A and B (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 seed lot (coded 'D') of the cultivar Carmel were obtained, fractionated by the supplier into five different seed size ranges (1-5), from which three fractions (1, 3 and 5) were used in experiment C. All seeds were stored at 30% relative humidity (RH) and 13°C between receipt in 2015 and use in 2020 (experiments A and B) and in 2021 (experiment C). A week before the experiment, 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 6000 CF and high CF seeds had a level above 12000 CF (see seed trait measurements).

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)
A and B	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	
C	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	
A and B	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	
A and B	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	
A and B	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	
A and B	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	

Seed trait measurements

Before sowing, seeds were individually measured for several seed traits, including area, roundness, and colour, 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. Colour 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 colour 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 colour space defined by the International Commission on Illumination (Commission Internationale de l'Eclairage (CIE), www.cie.co.at). RefL stands for luminescence, 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 colour 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, 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 6000 CF in AU. High CF seeds were selected after setting the CF mask at 12000 CF in AU.

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 A, 1.25 and 2.50% (Experiment B), and 0, 1.9 and 2.5% (Experiment C) 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 (Chapter 3), using *P. ultimum* isolate 8b (NCBI accession no. MZ026502 for ITS and MZ152003 for COI). For experiments A and B, 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, 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. For experiment C, all trays were randomly stacked, including the three *P. ultimum* treatments (0, 1.875%, and 2.5% *P. ultimum* dose), resulting in a randomised complete block design with five blocks. For each experiment, the blocks 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 was assessed daily until 10 days after sowing.

Statistical analysis

The combined binary data of emergence (1) versus non-emergence (0), and continuous data of the individual seed traits, including 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 the Pearson's correlations among the seed traits, measured from a total of 8950 seeds (50 seed of a single tray were not measured for RGB). For the development of a model 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 independent variables, including fixed and random factors. The fixed factors included block, cultivar, and treatment. The random factors included

seed lot, stack (only for the split-plot designs of experiments A and B) and tray. With Type II Wald Chi^2-tests, we tested for significant effects of the fixed factors.

To determine which seed traits contributed most to the emergence success, 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 general 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 Aikaike's information criterion (AIC) value, which is an indicator of the model fit: The lower the AIC, the better the fit of the model. The model improved when the AIC was reduced by at least 2 units ($\Delta AIC \geq 2$). 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 and inserted as a fixed main effect, and in interaction with the selected seed traits. Again, the model fit was evaluated by the AIC, following a forward regression approach with the "dredge" function. The most contributing traits with their interactions with *P. ultimum* were selected and an all-possible subset approach was used 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 C. The contributions of these two quantitative traits as main effects and their interaction with *P. ultimum* treatment were evaluated again using the AIC and their 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.

Results

Correlations among seed trait variables measured from individual seeds

From all individual seeds used in Experiments A and B (n=8950), the morphological

trait variables Area, Length, and Width showed a large collinearity, with correlations close to 1 (Figure 1). Roundness (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. RefB and CF showed a negative correlation (-0.2) with Area. CF was correlated positively with all other spectral measurements (0.4 to 0.7).

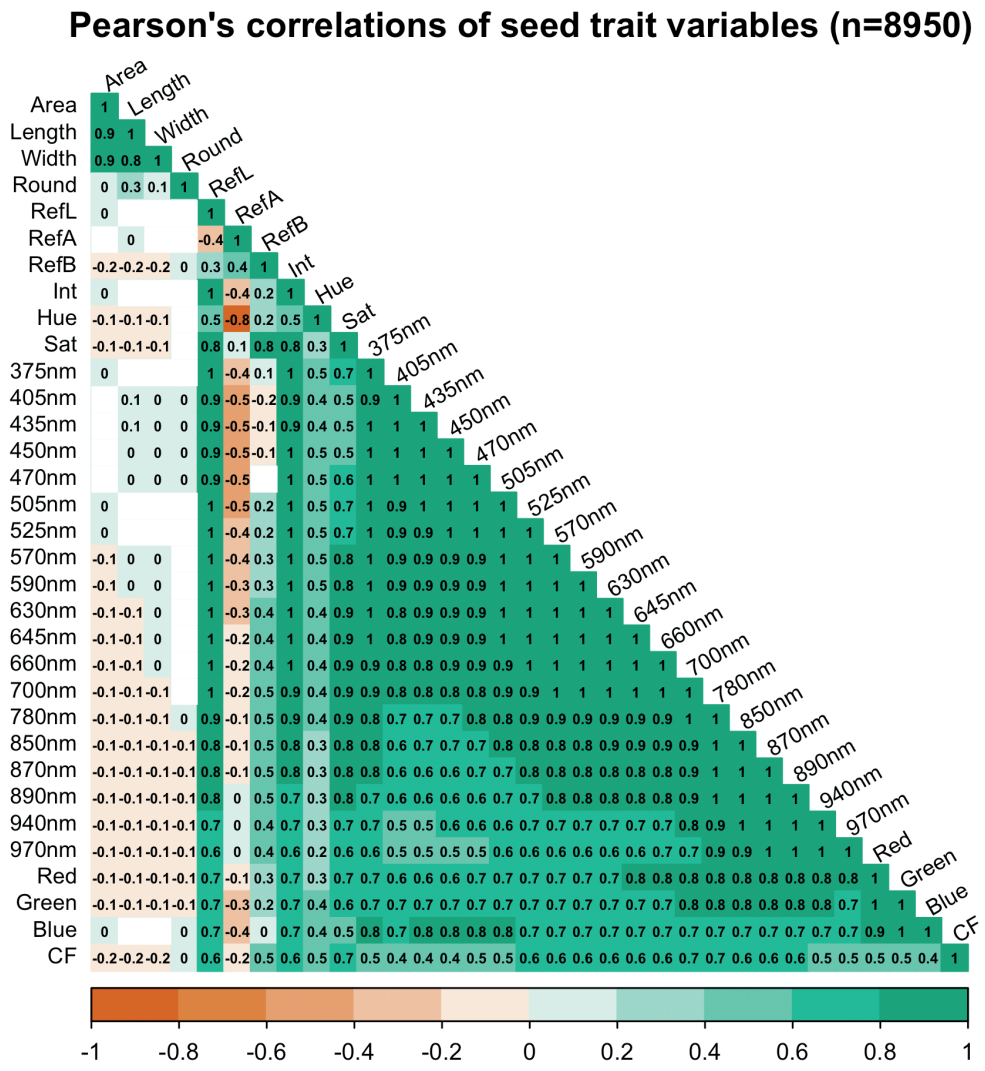


Figure 1. Visualization of Pearson's correlation coefficients among seed trait variables of individual seeds of 15 spinach seed lots tested (n=8950). All variables were standardised by subtracting the overall mean and dividing by the standard deviation, and CF was log-transformed before standardization. Colour and colour intensity are proportional to the correlation coefficients with positive correlations displayed in green and negative correlations displayed in orange. Insignificant correlations ($p<0.01$) were left blank.

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

For the development of a model for emergence dependent on *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 were first split into the four separate *P. ultimum* doses. For emergence in the substrate without *P. ultimum*, the addition of Area (seed size) to the general model resulted in the greatest model fit, with 7.5 AIC units difference, compared to the other models with a single trait variable (Supplemental Table 1B). 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 general model, with positive regression coefficients (Supplemental Table 1A). 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* (Table 2).

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 Aikaike's information criterion (AIC) of the general 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.

***P. ultimum* dose**	**model**	**model fit (AIC)**	**coefficient of additional trait variable**	
0.00%	general	1204.5		
	general + **Area**	1197.0	+0.34	
	general + Area + **405nm**	1194.5	+0.19	
1.25%	general	2453.1		
	general + **^{c}log(CF)**	2442.7	-0.22*	-0.25**
	general + ^{c}log(CF) + **RefA**	2440.5	-0.11	
	general + ^{c}log(CF) + **Hue**	2440.6	+0.12	
2.50%	general	2654.4		
	general + **^{c}log(CF)**	2639.4	-0.22	
5.00%	general	1538.7		
	general + **Blue**	1520.8	-0.37	

*coefficient for ^{c}log(CF) in model with RefA
**coefficient for ^{c}log(CF) in model with Hue

When *P. ultimum* was added at a 1.25% inoculum dose, the addition of CF to the general model resulted in the best model fit with more than 10 AIC units difference compared to the other models (Supplemental Table 1B). In addition, reflection measurements ranging from 700 nm (red) to 970 (near-infrared) improved the model fit by at least 2 AIC units compared to the general 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 general model (ΔAIC≥2) (Supplemental Table 1B). The addition of CF had the greatest contribution to the model fit with at least 3.9 AIC units difference compared to the other models. Compared to the general 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 general 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) resulted in the greatest model fit (ΔAIC=17.8) compared to the general model (Supplemental Table 1B). However, the addition of 470 nm (blue) and 450 nm (blue) fell within the 2-unit range, 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.

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, grey-shaded area). 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, grey-shaded area).

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 reflectance measurements, chlorophyll fluorescence (CF), colour (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 Aikaike'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 Chi^2 test. **B**) Second step: evaluating the model fit by AIC of the best possible combinations of the most significantly contributing traits in addition to CF. Models were sorted by AIC from high to low. The grey-shaded areas highlight 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**
General	7863.9		
General + Area + Area:Dose	7863.6	0.696	0.040
General + Hue + Hue:Dose	7859.5	0.023	0.088
General + RefA + RefA:Dose	7852.8	0.070	0.001
General + 405nm + 405nm:Dose	7843.0	0.011	<0.001
General + 435nm + 435nm:Dose	7834.2	<0.001	<0.001
General + 450nm + 450nm:Dose	7832.1	<0.001	<0.001
General + Blue+ Blue:Dose	7831.9	<0.001	<0.001
General + 470nm + 470nm:Dose	7831.8	<0.001	<0.001
General + ^{e}log(CF) + ^{e}log(CF):Dose	7828.8	<0.001	0.049
B) Second step full model selection	**model fit (AIC)**		
General + ^{e}log(CF) + CF:Dose + Area + Area:Dose	7827.7		
General + ^{e}log(CF) + CF:Dose + Blue + Blue:Dose + Area	7815.5		
General + ^{e}log(CF) + Area + Blue + Blue:Dose	7813.9		
General + ^{e}log(CF) + CF:Dose + Blue + Blue:Dose	7813.6		
General + ^{e}log(CF) + CF:Dose + Area + Area:Dose + Blue + Blue:Dose	7813.5		
General + ^{e}log(CF) + Blue + Blue:Dose	7811.9		
General + ^{e}log(CF) + Area + Area:Dose + Blue + Blue:Dose	7811.6		

The coefficients for the main and interaction effects of the selected model parameters were determined (Table 4). CF had a significantly negative correlation with emergence. The positive correlation between Area and emergence, independent of *P. ultimum* dose, 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 correlated positively with emergence, independent of *P. ultimum* dose. The effect of *P. ultimum* dose increased with increasing seed size and with increasing reflectance at 420-480 nm.

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 (^{e}log(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

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

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

with:

Area: seed size in standard deviations, with 0 equals mean=9.59 mm^2 and 1 SD=2.57 mm^2,

^{e}log(CF): natural logarithm of chlorophyll fluorescence, with 0 equals mean=9.05 ^{e}log(CF) in AU and 1 SD=0.27 ^{e}log(CF) in AU,

Blue: reflectance at 420-480 nm in standard deviations, with 0 equals mean=6230.1 reflectance in AU and 1 SD=1831.7 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 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 ($=e^{2.43-1.05x3} \times (1+e^{2.43-1.05x3})^{-1}$). 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 ($=e^{2.43+0.11-(1.05+0.05)x3} \times (1+e^{2.43+0.11-(1.05+0.05)x3})^{-1}$, and to 0.29 with the higher reflectance at violet-blue light ($=e^{2.43+0.23-(1.05+0.13)x3} \times (1+e^{2.43+0.23-(1.05+0.13)x3})^{-1}$).

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

Since seed size correlated negatively with CF (Figure 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 most. The three seed size fractions from seed lot Carmel-D showed little overlap in seed size (Area) (Figure 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 (Figure 2B). Beside seed size and CF, individual seeds showed considerable overlap among the size fractions in the distributions of RefLAB, and RGB levels (Figure 2C-H). The two CF-sorted subfractions of the smallest seeds (fraction D.1) showed clear morphological distinctions in pericarp intactness and colour (Figure 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 (Figure 3A) and had a lower light reflectance (RefL) (Figure 2D), including Red, Green, and Blue light reflectances (Figure 2C, 2E, 2G).

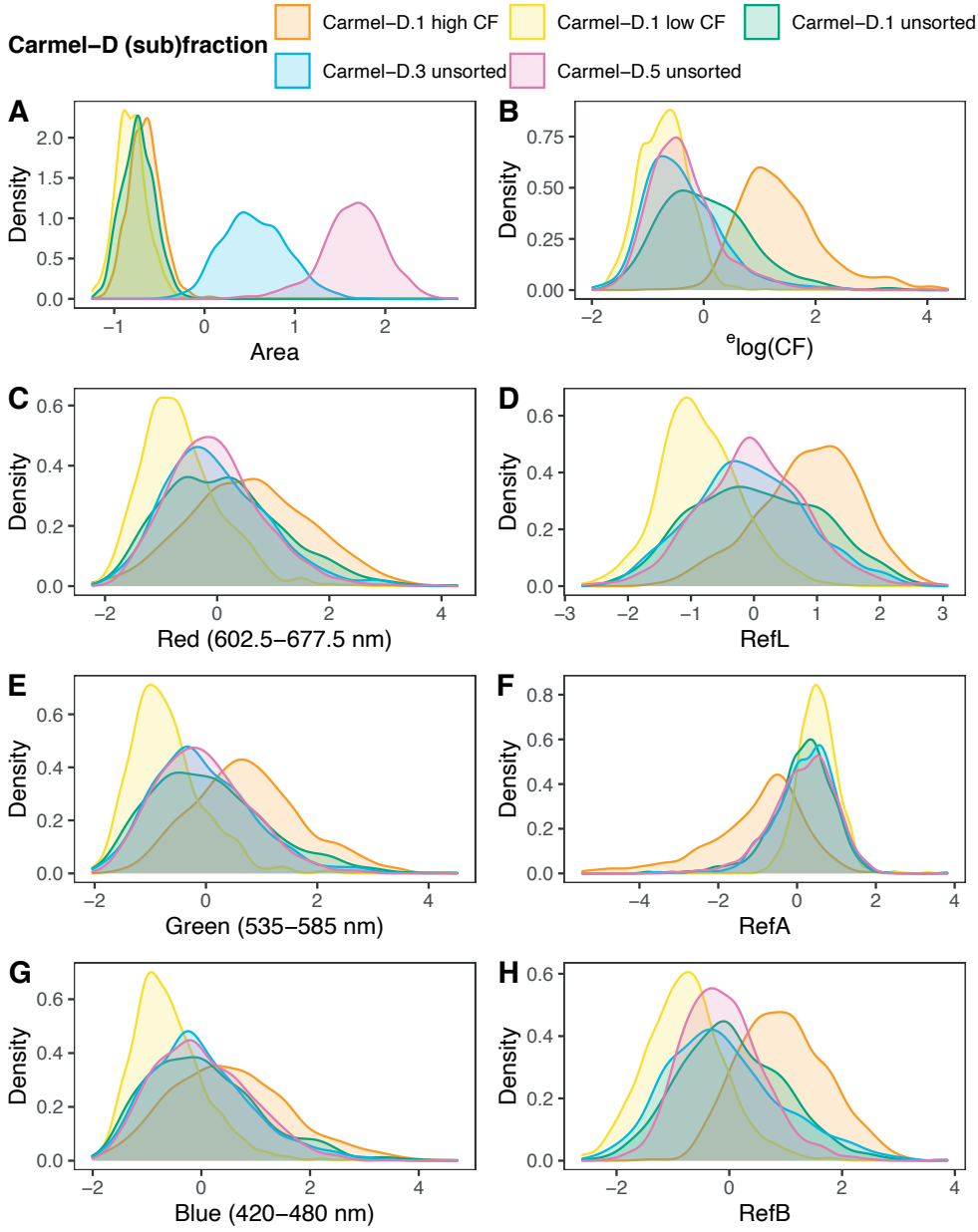


Figure 2. Distributions of standardised continuous data of **(A)** seed size (Area), **(B)** log-transformed chlorophyll fluorescence (^{e}log(CF)), **(C)** reflectance at 602.5-677.5 nm (Red), **(D)** lightness (RefL), **(E)** reflectance at 535-585 nm (Green), **(F)** reflectance from red to green (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 C: 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; D.1 high = high CF level (low maturity); and D.1 low = low CF level (high maturity). The area under each curve is 1.

Figure 3. Seeds from Carmel fraction D.1 sorted by chlorophyll fluorescence (CF) with **(A)** low CF seeds (<6000 CF arbitrary units), and **(B)** high CF seeds (>12.000 CF arbitrary units).

The Carmel-D (sub)fractions were tested simultaneously in an assay with 0, 1.9, and 2.5% *P. ultimum* doses. The statistical analysis confirmed significant main effects of the CF fractions, seed size fractions, and *P. ultimum* dose ($p<0.05$). On average, emergence was 85.2% (±7.7%) in the control substrate, 31.3% (±12.1%) in the substrate with a 1.9% *P. ultimum* dose, and 19.4% (±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). Seed size had no effect on emergence in the control substrate without *P. ultimum*, but 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 small mature seeds emerged comparable to the fractions D.3 and D.5 with larger seeds. In the presence of *P. ultimum*, the smallest seeds even showed a greater emergence than the largest seeds.

Table 5. Mean ± 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.

***P. ultimum* dose (%)**	**Size fraction**	**CF level**	**Mean (±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

Instead of looking at seed size and CF fractions as 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 main 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.

Seed lot variation in emergence and other seed traits

With *P. ultimum* doses of 1.25% and 2.5% (Experiment B), the average emergence over the 15 seed lots was 76.1% (±8.6%) and 29.5% (±12.6%), respectively. These averages

fell between those of the previous experiment with 0% and 5% *P. ultimum* doses (Experiment A), 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, large variation among the seed lots and cultivars was possibly 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 (Figure 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.

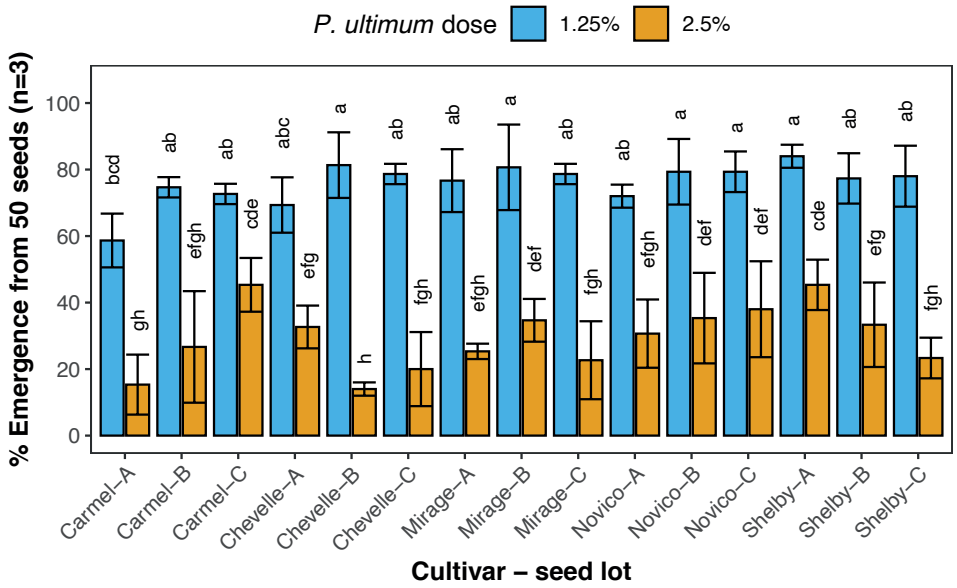


Figure 4. Mean incidence (%) of emerged seedlings 10 days after sowing three seed lots of five cultivars in a substrate with sand:perlite:vermiculite mixture (1:1:1 volume basis) with 1.25% or 2.5% *Pythium ultimum* dose. Error bars represent standard deviations (n=3, replicated trays with 50 seeds each 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.

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 model 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 general 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.

Table 6. Variance components of spinach seed lot effects in the general 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). Grey-shaded seed traits caused a reduction in seed lot variance compared to the general model.

Model	Seed lot variance
General model	0.1253
+ Blue	0.1084
+ Int	0.1086
+ RefL	0.1118
+ Green	0.1143
+ Red	0.1149
+ Area	0.1185
+ Round	0.1246
+ Sat	0.1253
+ ^{c}log(CF)	0.1271
+ RefB	0.1306
+ RefA	0.1337
+ Hue	0.1470

The box-and-whiskers plots of the continuous data for seed measurements showed considerable overlap among the 15 seed lots tested (Figure 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 (Figure 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 (Figure 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 (Figure 5F). Shelby-C also showed less emergence in the presence of 2.5% *P. ultimum* dose than Shelby-A (Figure 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 (Figure 4).

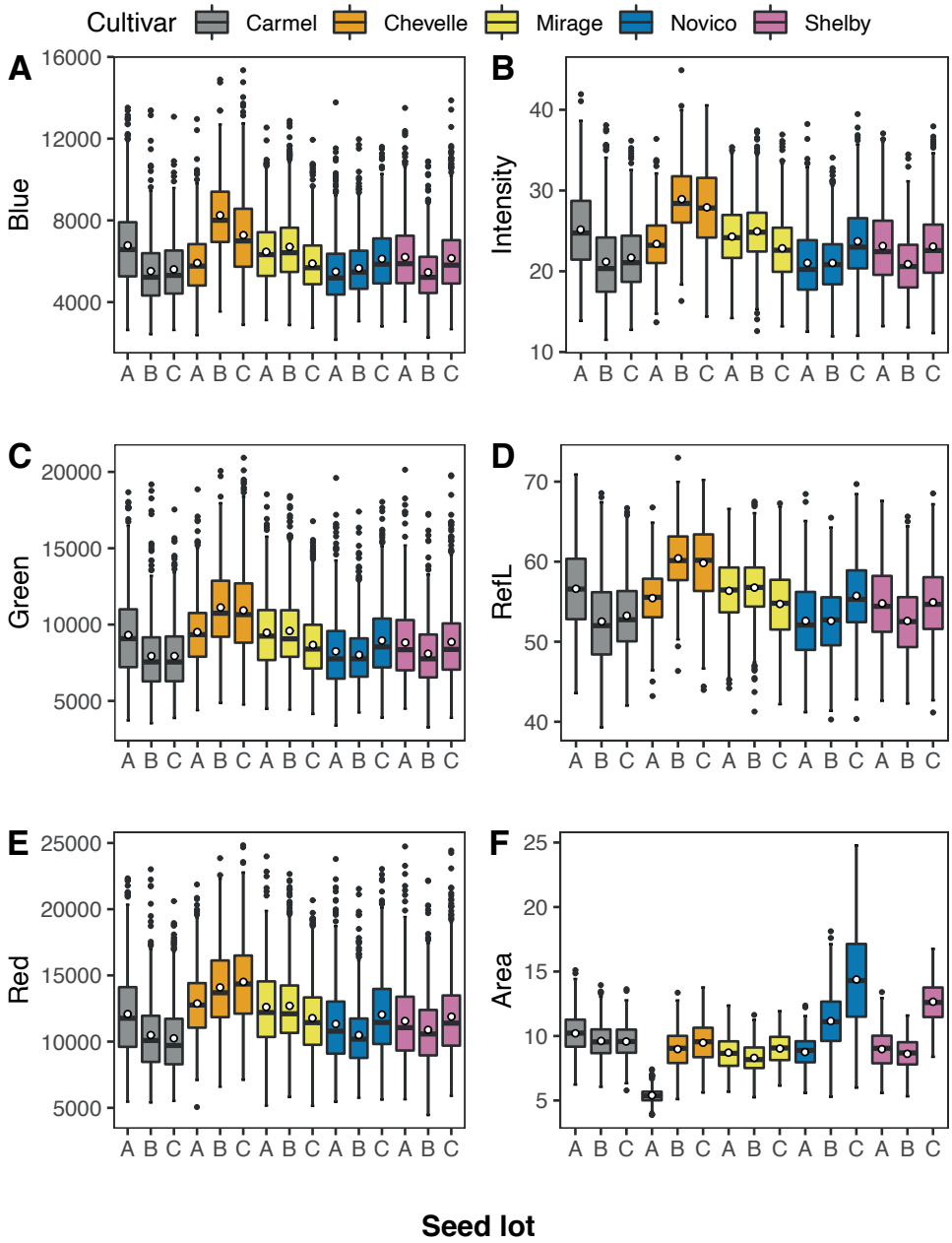


Figure 5. Box-and-whiskers plots of non-scaled data of **(A)** light reflectance at 420-480 nm (Blue), **(B)** light reflectance intensity (Intensity), **(C)** reflectance at 535-585 nm (Green), **(D)** lightness (RefL), **(E)** reflectance at 602.5-677.5 nm (Red), and **(F)** seed size (Area) of individual seeds (n=600) from each seed lot, with same colours for seed lots of the same spinach cultivar. 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 minimum and maximum values within the range of -1.5·IQR to +1.5·IQR.

Discussion

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 for the pre-emergence damping-off tolerance levels, as calculated for the same 15 seed lots before (Chapter 3). 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 reflected by 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. However, 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 within seed lots triggered our interest to zoom in on the individual seed level.

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 fluorescence (CF) of 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 (Jalink et al., 1998; Deleuran et al., 2013; 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 small and mature Carmel-D seeds, emergence was comparable with or even higher than emergence from the unsorted, larger seeds of Carmel, indicating 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 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 of an effect of the seed maturity than of seed size.

Seed traits in relation to P. ultimum tolerance

Overall, a high CF level for a seed had a negative relationship with seedling emergence in the presence and the absence of *P. ultimum*. The relationship of seedling emergence with 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 (Chapter 2). Such fast germination may enable an 'escape' of the seedling from *P. ultimum* infection (Agrios, 2005a). 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 vigour. Although studies on seed vigour in relation to biotic stress tolerance are limited, in some studies seeds with lower vigour were observed to have increased sensitivity towards soilborne pathogens, including *P. ultimum*, for example, seeds of wheat (Das Gupta & Austenson, 1973), soybean (Schlub & Schmitthenner, 1978; Hamman et al., 2002), pea (Perry, 1973; Stasz & Harman, 1980), and lucerne (Hawthorne, 1988).

Light reflectance over a broad range of wavelengths, from 375 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 colour is associated with higher seed maturity. With 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). In another study, the maturity of sugar

beet seeds was associated with reduced lightness and increased germination (Mirzaei & Rajabi, 2021). The colour of the pericarp may also be associated with the presence and concentration of secondary metabolites. With sugar beet seeds, the pericarp contains various secondary metabolites with different biological activities, e.g., regulation of seed germination and seedling emergence, and antimicrobial activity (Oksana & 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 (Junttila, 1976; Chiji et al., 1980; Morris et al., 1984). When sugar beet seeds were polished and washed, the removal of water-soluble germination inhibitors resulted in an increased germination (Ignatz et al., 2019; Salimi & Boelt, 2019a). The pericarp can also contain phenolic compounds that have 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 8950 seeds measured in this study, we found no correlation between light reflectance and seed size, indicating that the light reflectance effect was independent of 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.

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 colour-grading (Salimi & Boelt, 2019a) 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 favour of less light-reflecting (darker) seeds and smaller seeds.

Acknowledgements

We gratefully acknowledge the financial and practical support from the seed and breeding companies Pop Vriend Seeds B.V. (The Netherlands), 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' (KV 1409-031).

Supplemental

Supplemental Table 1. **A)** The regression coefficients (RC) of single seed trait variables, and **B)** Aikaike's information criterion (AIC) of the general and extended models for each dataset with emergence in substrate with 0, 1.25, 2.5, or 5% *Pythium ultimum* dose. ΔAIC displays the difference in AIC between the extended model with a single seed trait variable and the general model. The colour scale of the RC goes from orange (negative) to green (positive) over all *P. ultimum* doses. The colour scale of ΔAIC goes from orange (no improvement of model fit) to green (highest improvement of model fit) for each *P. ultimum* dose separately.

A) Regression coefficients				
General model +	***Pythium ultimum* dose**			
	0%	**1.25%**	**2.5%**	**5%**
Intercept	2.53	1.19	-0.94	-2.30
Area	0.37			
Round				0.11
375nm			-0.19	-0.33
405nm	0.20		-0.15	-0.29
435nm	0.18		-0.19	-0.34
450nm	0.17		-0.20	-0.35
470nm	0.16		-0.20	-0.35
505nm			-0.20	-0.32
525nm			-0.20	-0.31
570nm		-0.08	-0.19	-0.30
590nm		-0.08	-0.19	-0.28
630nm		-0.09	-0.19	-0.27
645nm		-0.09	-0.19	-0.27
660nm		-0.09	-0.17	-0.26
700nm		-0.11	-0.19	-0.26
780nm		-0.14	-0.19	-0.24
850nm		-0.15	-0.18	-0.22
870nm		-0.15	-0.18	-0.21
890nm		-0.15	-0.17	-0.20
940nm		-0.14	-0.17	-0.18
970nm		-0.14	-0.16	-0.17
Red		-0.09	-0.15	-0.28
Green			-0.17	-0.31
Blue			-0.17	-0.37
RefL			-0.19	-0.29
RefA	-0.13		0.12	0.22
RefB	-0.17	-0.12		
Hue			-0.14	-0.14
Int			-0.20	-0.31
Sat	-0.04	-0.12	-0.14	-0.15
CF		-0.19	-0.22	-0.28

Supplemental Table 1. Continued.

B) Aikaike's information criterion (AIC)								
General model +	***Pythium ultimum* dose**							
	0%		**1.25%**		**2.5%**		**5%**	
	AIC	**ΔAIC**	**AIC**	**ΔAIC**	**AIC**	**ΔAIC**	**AIC**	**ΔAIC**
	1204.51	**0.00**	**2453.14**	**0.00**	**2654.37**	**0.00**	**1538.67**	**0.00**
Area	1196.97	7.54						
Round							1538.22	0.45
375nm					2644.82	9.55	1524.63	14.03
405nm	1202.09	2.42			2648.95	5.42	1528.95	9.72
435nm	1203.04	1.47			2645.30	9.07	1523.81	14.86
450nm	1203.46	1.04			2644.06	10.31	1522.73	15.93
470nm	1203.76	0.75			2643.61	10.76	1522.70	15.97
505nm					2643.35	11.02	1524.75	13.91
525nm					2643.39	10.99	1525.80	12.86
570nm			2453.08	0.05	2643.85	10.53	1526.81	11.86
590nm			2452.78	0.36	2644.08	10.29	1527.83	10.84
630nm			2452.42	0.72	2644.83	9.55	1528.72	9.95
645nm			2452.38	0.75	2644.94	9.43	1528.68	9.99
660nm			2452.48	0.66	2646.32	8.05	1529.65	9.01
700nm			2450.98	2.16	2644.83	9.54	1529.76	8.91
780nm			2448.90	4.24	2644.34	10.03	1530.78	7.89
850nm			2447.94	5.19	2644.93	9.44	1531.93	6.73
870nm			2447.90	5.24	2645.46	8.91	1532.51	6.15
890nm			2447.97	5.17	2646.00	8.37	1532.93	5.73
940nm			2448.41	4.73	2646.65	7.72	1534.14	4.52
970nm			2448.92	4.21	2646.86	7.51	1535.07	3.59
Red			2452.51	0.62	2649.50	4.88	1528.96	9.71
Green					2648.24	6.13	1525.74	12.93
Blue					2647.86	6.52	1520.86	17.80
RefL					2643.99	10.38	1526.10	12.57
RefA	1204.14	0.37			2650.56	3.82	1532.55	6.11
RefB	1202.87	1.64	2449.94	3.19				
Hue					2648.95	5.42	1537.32	1.35
Int					2643.76	10.61	1525.89	12.77
Sat	1206.32	-1.81	2450.27	2.86	2650.03	4.35	1537.17	1.50
CF			2442.71	10.43	2639.42	14.95	1528.20	10.47

Supplemental Table 2. The regression coefficients of each seed trait variable of spinach seeds in relation to the *Pythium ultimum* dose of the substrate in which they were sown, when this variable was added to the model in addition to the trait variable selected from Supplemental Table 1. Models were sorted by Aikaike's information criterion (AIC), which corresponds to the model fit. ΔAIC is the difference between the model with the first selected seed trait and the model with a second seed trait. The grey-shaded models were selected for further analysis.

***P. ultimum* dose**	**Seed trait variables**										**Model fit**	
0%	Intercept	Area	405nm	435nm	450nm	470nm	RefA	RefB			AIC	ΔAIC
	2.54	0.34	0.19								1194.49	2.48
	2.54	0.34						-0.16			1195.29	1.68
	2.54	0.34		0.17							1195.40	1.57
	2.54	0.34			0.16						1195.77	1.20
	2.54	0.35				0.15					1196.04	0.93
	2.53	0.36					-0.13				1196.22	0.75
	2.53	0.37									1196.97	0.00
1.25%	Intercept	CF	RefA	Hue							AIC	ΔAIC
	1.20	-0.22	-0.11								2440.50	2.21
	1.20	-0.25		0.12							2440.62	2.09
	1.19	-0.19									2442.71	0.00
2.50%	Intercept	CF	435nm	450nm	470nm	505nm	525nm	Int	RefA	RefB	AIC	ΔAIC
	-0.94	-0.17			-0.12						2638.22	1.21
	-0.94	-0.17		-0.11							2638.27	1.15
	-0.94	-0.18	-0.11								2638.73	0.69
	-0.94	-0.21							0.09		2638.83	0.59
	-0.94	-0.16				-0.11					2638.93	0.50
	-0.94	-0.16					-0.10				2639.20	0.22
	-0.94	-0.27								0.10	2639.29	0.13
	-0.94	-0.17						-0.10			2639.35	0.07
	-0.94	-0.22									2639.42	0.00
5%	Intercept	Blue									AIC	ΔAIC
	-2.31	-0.37									1520.86	0.00

Chapter 6

General discussion

Introduction

This chapter provides an overview of the main findings of the present study on spinach damping-off tolerance in relation to seed vigour and underlying traits of the spinach seed. First, a brief introduction is provided about the background and main results of this study, as covered in previous chapters. In the following paragraphs, the results are discussed in more detail in relation to each other and to existing literature, which is also visualised in a concept map (Figure 1). In the final paragraphs, recommendations for further research and development are provided based on the knowledge gained on specific seed traits of spinach seeds that related to a greater tolerance to stressful conditions (low oxygen levels or *Pythium ultimum* presence) during spinach seed germination and seedling emergence. The possibilities for integrated damping-off management with an emphasis on increasing seed vigour through seed selection, non-chemical seed treatments, and breeding are discussed, ending with some concluding remarks.

Background and main results of this study

Both organic and conventional cultivation face increasing challenges to meet the growing demand for leafy vegetables, such as spinach (*Spinacia oleracea* L., Amaranthaceae family). These challenges include crop diseases, an increasing ban on chemical disease treatments, and more extreme weather events due to climate change (e.g., longer periods of drought and/or excessive rainfalls), leading to severe losses in field crop production. For instance, spinach production can suffer serious losses due to damping-off diseases, which include seed or seedling rot before emergence, called pre-emergence damping off, and seedling rot at the root or stem base resulting in wilting symptoms or death after emergence, called post-emergence damping off. Infection can be caused by soilborne pathogens from the fungal genera *Fusarium* and *Rhizoctonia*, or oomycete genera *Aphanomyces, Phytophthora* and *Pythium*. High soil moisture conditions and high temperatures can stimulate spinach damping off. Damping off can be particularly devastating with baby-leaf spinach, because the production requires uniform stand establishment in a short period of time, and frequent and dense sowing can promote the spread of infection. In conventional spinach production, damping-off diseases are usually controlled by chemical seed and field treatments, as genetic-based resistance has not yet been found in spinach. However, synthetic fungicide treatments are not allowed in organic spinach production and there is an increasing ban of such treatments in conventional production as well, due to potential risks of phytotoxicity, pathogen resistance to fungicides, and potential negative impacts on human and environmental health. Therefore, alternative solutions, including the use of damping-off tolerant spinach cultivars, are becoming more urgent. Since damping off mainly occurs in the early stages of seed germination and seedling emergence, seed vigour and underlying seed traits were hypothesised to play an important role in pre-emergence damping-off tolerance. Seed vigour can be defined as the sum of seed properties that determine the ability of viable seeds to germinate fast and uniformly, and to produce healthy seedlings with rapid and uniform emergence under both optimal and suboptimal environmental conditions.

The main research question of this study was whether improvements in damping-off tolerance in spinach are possible through plant breeding or through seed vigour enhancement by selecting for specific seed traits. For breeding, the existence of genotypic variation for the desired characteristic, e.g., damping-off tolerance or specific seed traits, is required. Under field and greenhouse conditions, pre-emergence damping off appeared to be more discriminatory among spinach cultivars than post-emergence damping off, but consistent genotypic variation could not be confirmed (**Chapter 3**). The large variation among fields and replicates emphasised the need for more standardised phenotyping assays, which I developed for assessing pre-emergence damping off, using a substrate

inoculated with a pathogenic *P. ultimum* isolate. This isolate was chosen based on its occurrence in all studied field soils and its high pathogenicity on spinach. Despite the more reproducible results for pre-emergence damping-off tolerance levels within seed lots, the variation among seed lots remained larger than the variation among cultivars (**Chapter 3**). This research further focused on pre-emergence damping-off tolerance, as a considerable variation in sensitivity occurred in the pre-emergence stage, which should be overcome first, and a relationship with seed vigour was hypothesised. Using in-house developed methods of spinach seed priming and pericarp removal (dehulling), more uniform performance of seed lots was found, but genotypic variation for the evaluated cultivars, still could not be confirmed. However, positive effects of priming and dehulling on the rate of germination and emergence, and on pre-emergence damping-off tolerance levels were promising (**Chapter 4**). These results indicated that seed vigour enhancement by priming or dehulling can improve pre-emergence damping-off tolerance levels and that greater seed vigour, e.g., a faster emergence, underlies this improvement. However, rate of emergence was not the only driver of increased damping-off tolerance levels, as priming and dehulling also improved tolerance levels without causing increased rates of emergence. Also, the rate of emergence of the tested seed lots did not correlate with the tolerance levels. Other seed vigour related traits, such as seed maturity, seed size, and pericarp colour, also contributed to pre-emergence damping-off tolerance in spinach (**Chapter 5**). Seeds with a relative higher maturity (measured by a lower chlorophyll fluorescence), smaller size and darker pericarp (measured by less light reflectance), were generally more tolerant. These results are discussed in relation to oxygen stress tolerance, that was already considered in **Chapter 2**. Larger seeds, which have a thicker and more intact pericarp, were more sensitive to high moisture in terms of less germination than smaller seeds. Seed dehulling and elevated oxygen levels improved seed germination at high moisture levels (**Chapter 2**). Smaller seeds and dehulled seeds were also more tolerant to pre-emergence damping off (**Chapters 4 and 5**). Hence, seed vigour enhancements through seed sorting after seed harvest could also improve pre-emergence damping-off tolerance levels in spinach seed lots. In the next paragraphs, these results will be discussed in more detail in relation to each other and in relation to existing literature, with connections visualised in a concept map (Figure 1).

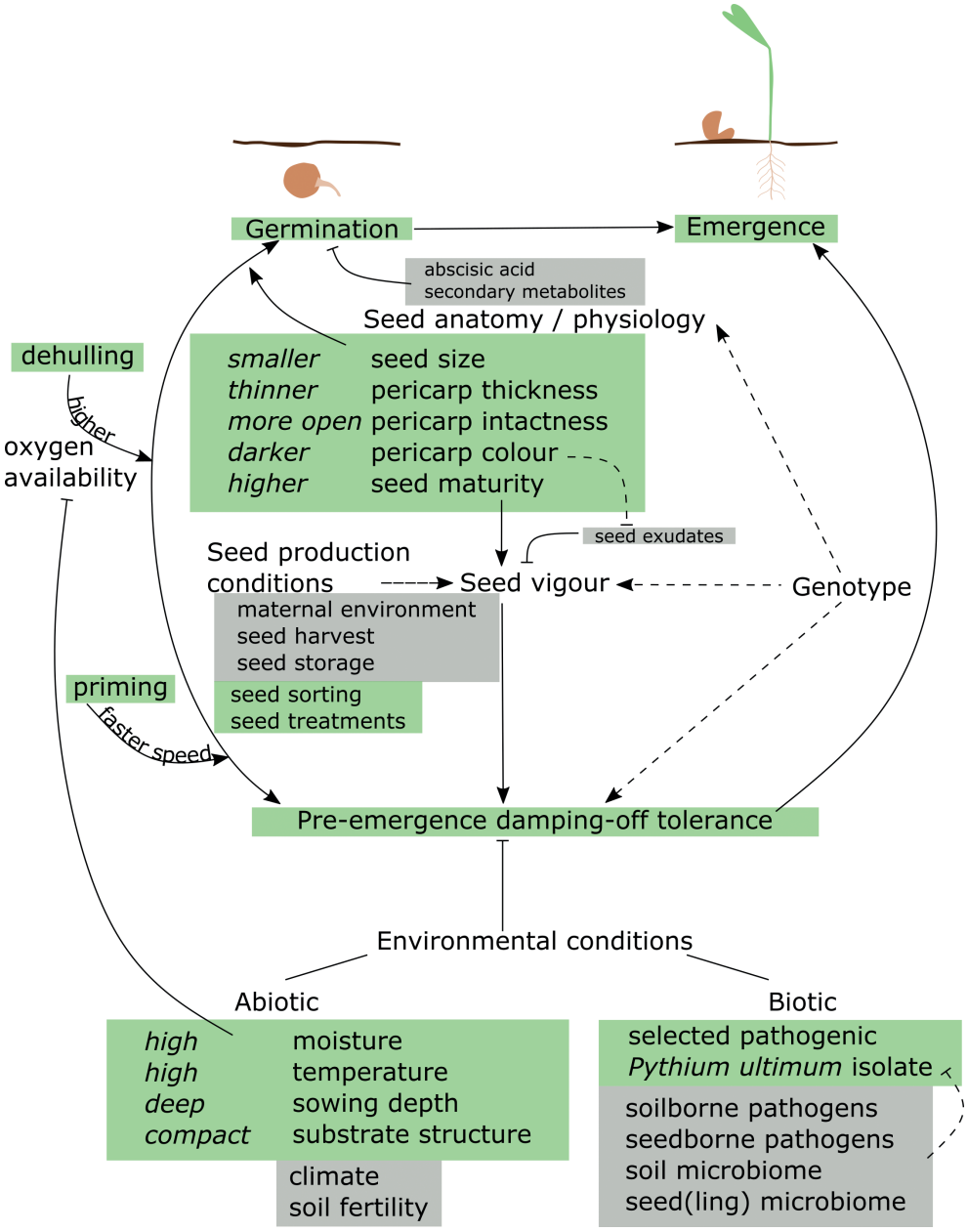


Figure 1. Concept map of factors with confirmed (solid lines) and hypothetical (dashed lines) interactions between those factors, with arrows indicating stimulative effects and flat ends indicating inhibitory effects. Green marked factors are studied in this research, grey marked factors are suggested based on published literature.

Genotypic variation in damping-off tolerance

The general observation from spinach growers is that spinach cultivars can differ in their tolerance to damping-off diseases, and that there is also variation among years, fields, and seed lots. To explore the potential of genotypic variation in damping-off tolerance, several cultivars that were expected to differ in their tolerance levels based on the experience of growers, were evaluated in this study. Multiple seed lots were used that were obtained from various seed productions. It was hypothesised that when the seed lots of each genotype perform similarly, the performance is likely to be genotypic-based. Despite the variation in damping-off tolerance among cultivars, observed in field and greenhouse trials, more variation was observed among seed lots, even among seed lots of the same cultivars, as demonstrated in Chapter 3. Also, large variation was observed in the incidence of non-emergence and post-emergence damping off among field trials. The use of a more standardised assay in a climate-controlled cabinet made it possible to assess pre-emergence damping-off tolerance with more reproducible results. Still, large variation was observed among the spinach seed lots, even among seed lots of the same cultivar. Another study on spinach damping off also found large variation among seed lots and the researchers suggested that the differences they found among cultivars could be due to variation among the seed lots rather than genotypic variation (Green et al., 2012). Until now, genotypic variation for spinach damping-off tolerance has not been demonstrated.

In other plant species, genotypic variation for resistance to specific damping-off pathogens has been confirmed, often with the use of single seed batches of different genotypes. For family-related sugar beet (*Beta vulgaris* L.) cultivars and *Amaranthus* accessions, resistance to *Rhizoctonia solani* (Scholten et al., 2001) and *Pythium myriotylum* (Sealy et al., 1988) was reported. Resistance towards pre-emergence infection by *P. ultimum* and *Aphanomyces cochlioides* was also found in *Beta* accessions (Luterbacher et al., 2005). Studies have been performed on resistance to *Pythium* spp. in legume crops, including alfalfa (Altier & Thies, 1995), chickpea (Kumar et al., 1991), common bean (Namayanja et al., 2014), pea (Stasz et al., 1980), and soybean (Urrea Romero, 2015; Klepadlo et al., 2019). For instance, resistance genes have been characterised for common bean (Namayanja et al., 2014) and soybean (Klepadlo et al., 2019). Some studies stated that the *P. ultimum* resistance in legumes is a polygenic, quantitative trait (Dickson & Abawi, 1974; Kumar et al., 1991), while others stated that the inheritance of *P. ultimum* resistance could also be due to a single dominant gene (Rosso et al., 2008; Namayanja et al., 2014), or the inheritance could be both quantitative and qualitative (Campa et al., 2010). Even for the more extensively studied legume crops, the genetic background of damping-off resistance is still uncertain, as is the defence mechanism against *Pythium*

spp., i.e., monogenic (complete) resistance, polygenic (partial) resistance, tolerance towards infection, or escape from infection (Agrios, 2005a). The present study explored the existence of genotypic-based resistance or tolerance towards *P. ultimum* infection of spinach. Based on the research results (Chapter 3 and 4) and on published literature, I hypothesised that the time between sowing and emergence is the most crucial phase for infection, and that an escape from infection is a possible strategy for rapidly emerging seedlings to tolerate certain levels of *P. ultimum* and other damping-off pathogens (Agrios, 2005a). If *P. ultimum* resistance exists in spinach, I expect the resistance to be partial, polygenic, and influenced by multiple environmental factors. Therefore, I preferred the term spinach damping-off tolerance rather than resistance.

Seed lot variation: a burden and opportunity

Despite the burden of large seed lot variation confounding the search for genotypic variation in spinach damping-off tolerance, the seed lot variation also gave an opportunity to zoom in on the seed lot level and to study different seed characteristics in relation to damping-off tolerance levels. Based on the results of seed priming, the hypothesis was that a higher rate of germination resulted in a higher tolerance level, suggesting that faster germinating seed lots are more tolerant than slower germinating seed lots, as suggested from an earlier spinach damping-off study (Green et al., 2012). Despite the variation in rate of emergence and tolerance levels among the studied spinach seed lots, a positive correlation between these traits could not be confirmed. Also, in a study with *Beta* accessions, no correlations were found between the rate of seedling emergence and *P. ultimum* tolerance (Luterbacher et al., 2005). This suggested that rate of emergence may be a driver but is not the only driver of *P. ultimum* tolerance. Other seed traits could also be involved that may relate to seed vigour.

Some of the spinach seed lots tested in this study differed in specific seed traits, e.g., seed size, seed maturity, and pericarp colour, that could have an influence on *P. ultimum* tolerance, as was observed with the phenotyping assay developed for use in a climate-controlled cabinet (Chapter 5), and there may be a relationship with seedling emergence rate (Chapter 4). For instance, the smallest seeds of the spinach cultivar Chevelle had a greater *P. ultimum* tolerance and germinated faster than larger seeds of Chevelle. The largest seeds of cultivar Shelby also showed less tolerance and slower germination than the other two Shelby seed lots that had relatively smaller seeds. In contrast, the Novico seed lots differed in seed size distributions, but not in tolerance levels, possibly due to the large variation in seed sizes among the seeds within these seed lots or strong genotypic effects. Also, the variation among the three Carmel seed lots could not be associated with differences in seed size or seed maturity, and only partly by pericarp light

reflectance. The Carmel seed lot with the highest tolerance level contained relatively more seeds with less light reflectance compared to the other two seed lots. Also, seeds of the cultivar Chevelle, particularly for two of the three seed lots, showed relatively more light reflectance (lighter-coloured pericarp) and were relatively less tolerant to *P. ultimum* compared to the other seed lot and seed lots of the other cultivars. However, most of the seed lot variation could not be explained by any of the measured seed characteristics. Differences in seed characteristics among seed lots could also be due to differences in seed lot production conditions. For instance, in dry environments seeds, may become larger as the plant develops fewer branches and allocates resources to fewer seeds (Baker, 1972). In nutrient-deficient environments, seeds may remain smaller, as the resources for seed filling become limited (Fenner & Thompson, 2005). The variation in seed lot production conditions may also have caused some variation in seed vigour among the studied seed lots, whereas seed vigour might have partly been genotypic as well. To unravel genotypic-based seed vigour effects, seed lots should have been produced under the same environmental, harvesting, and storage conditions. Also, large variation was observed among seeds within the seed lots. This could be the result of the indeterminate seed maturation pattern on spinach plants and once-over seed harvest. For other crops that show an indeterminate seed maturation pattern, the large variation among harvested seeds should, therefore, be considered when studying diseases that occur in the early stages of seed germination until emergence. This is, for instance, the case with *Beta vulgaris* (including sugar beet, beet root, and chard), and for many species within the Fabaceae family (e.g., alfalfa, soybean, and pea), for which damping off is a common disease as well. To our best knowledge, studies on these crops in relation to damping-off tolerance have not included the interactions with seed morphological traits, as was done in this study.

Seed traits in relation to abiotic stress and pre-emergence damping off

The large variation in damping-off tolerance levels among seed lots and the variation among and within seed lots for seed maturity, size and colour urged to zoom in on the individual seed level. I hypothesised that some seed characteristics influence the sensitivity of spinach seeds to abiotic stress and, directly or indirectly, their pre-emergence damping-off tolerance. The developed phenotyping assay with seeds sown in individual cells made it possible to relate seed trait measurements from individual seeds to emergence in substrate with or without the pathogen. A seed lot fractionated into seed sizes and maturity levels excluded the potential genotype-by-seed lot interaction effect. The effects of seed size, pericarp structure, and pericarp colour on pre-emergence damping-off tolerance are discussed below and in relation to oxygen sensitivity and seed vigour.

Seed size in relation to stress tolerance

The studied seed lots had large variation in seed sizes and maturity levels, probably due to the non-uniform seed development on spinach plants and once-over seed harvest. This may result in large variation in seed quality among seeds within spinach seed lots, as was demonstrated before (Deleuran et al., 2013). In the present study, a large variation was found in seed germination under abiotic stress (high moisture levels during germination, Chapter 2) and seedling emergence under biotic stress (presence of *P. ultimum*, Chapter 5), even for seed lots that contained similar seed sizes and maturity levels. Seed size is also influenced by the maternal genotype and environment. Therefore, the seed size effect was also analysed from seed size fractions that were obtained from a single seed lot. Results showed that smaller seeds were less sensitive to high moisture levels than larger seeds (Chapter 2). When temperatures were lowered, the rate of germination was reduced but the total germination of the seeds, particularly of larger seeds, increased. Total germination of differently-sized spinach seeds also improved when atmospheric oxygen levels were increased, indicating that oxygen availability to the seed embryo was the main limiting factor at high moisture levels. Apparently, seed embryos of smaller seeds are less sensitive to lower oxygen levels surrounding the seed. This finding is in line with previous studies that indicated that the availability of oxygen to spinach or sugar beet seeds with a pericarp is a limiting factor for germination at high moisture levels (Heydecker & Orphanos, 1968; Coumans et al., 1976). The smaller, mature seeds were also more tolerant towards pre-emergence infection by *P. ultimum* than the larger, mature seeds (Chapter 5). Stressful conditions, in the presence of high moisture levels or *P. ultimum*, seem to give smaller (mature) seeds an advantage over larger (mature) seeds. The faster germination of smaller spinach seeds, as was observed on filter paper, may have resulted in an 'escape' from *P. ultimum* infection when they were sown in substrate.

Pericarp structure and composition in relation to stress tolerance

Seed size was mostly determined by the pericarp thickness. When comparing the weight of the pericarp and true seeds of the seed size fractions of one seed lot of cultivar Carmel, the pericarp thickness increased relatively more than the true seed size with increasing whole seed size (Chapter 2). Smaller spinach seeds also contained more frequently openings in the pericarp compared with larger seeds. These two factors may have resulted in better oxygen diffusion to the embryo, which is necessary for germination, resulting in a greater tolerance to low oxygen levels by the smaller seeds. After removal of the pericarp, both small and large seeds showed improved germination on filter paper, and the sensitivity to low oxygen was reduced, particularly for the larger seeds. Moreover, pericarp removal reduced the sensitivity to *P. ultimum* in substrate (Chapter 4). The thicker, more intact pericarp of the larger seeds may have had a stronger germination-inhibitory effect due to a potential physical and/or chemical barrier of the pericarp.

Removal of the operculum of the pericarp or placing the basal pore facing up improved the germination of sugar beet seeds due to less physical constraint as well as increased water and oxygen uptake (Coumans et al., 1976). Also with spinach seeds, removal of the entire pericarp improved the germination at warmer temperatures, confirming the physical barrier of the pericarp to germination (Leskovar et al., 1999).

The presence of the plant hormone ABA in the pericarp has been shown to inhibit germination of spinach seeds (Leskovar et al., 1999) as well as sugar beet seeds (Hermann et al., 2007; Ignatz et al., 2019). There is also evidence that other chemical germination-inhibitors are present in the sugar beet seed pericarp, such as phenolic compounds and inorganic salts (Ignatz et al., 2019; Oksana & Artur, 2019). The pericarp also consists of an inner layer of thick-walled sclerenchyma cells containing lignin, and a more porous outer layer of thin-walled parenchyma cells containing chlorophyll (Ignatz et al., 2019). Lignin is a water-insoluble polyphenolic compound that is synthesised during seed ripening through enzymatic dehydrogenation and oxidation of phenylpropanoids (Katahira et al., 2018). Lignin plays a role in seed hardness and resistance to mechanical seed damage, reducing water and oxygen permeability and, thereby, inhibiting germination (Mohamed-Yasseen et al., 1994; Debeaujon et al., 2007). In addition to lignin, other germination-inhibiting phenolic compounds are formed via the phenylpropanoid pathway, such as flavonoids (Dardick & Callahan, 2014). Flavonoids are a large group of polyphenolic compounds that can protect seeds from pathogen attack (e.g., condensed tannins), UV-B radiation by scavenging free radicals, imbibitional damage by decreasing the permeability for water uptake, and by inhibiting germination through oxygen-binding (Debeaujon et al., 2007). The outer-layer of the pericarp contains chlorophyll, which is a water-insoluble photosynthetic pigment that degrades during late seed maturation. Therefore, the level of chlorophyll remaining in the pericarp can be used as a marker for seed maturity, which can be linked directly to germination performance and abiotic stress tolerance of seeds (Jalink et al., 1998; Smolikova et al., 2011). Polishing of sugar beet seeds removed the outer layer of the pericarp, while the inner layer remained unaffected and germination improved, especially after washing, due to the reduced content of ABA, secondary metabolites, and inorganic salts (Ignatz et al., 2019). There may also be compounds in the pericarp that have an anti-pathogenic function, as was demonstrated with water-soluble pericarp extracts from *Acer saccharum* that inhibited germination of sporangia of *P. irregulare* (Webb & Agnihotri, 1970). For instance, condensed tannins are described as anti-pathogenic phenolic compounds (Scalbert, 1991).

In summary, the pericarp of achenes like spinach and sugar beet seeds seems to regulate seed germination in multiple ways: by creating a mechanical barrier for radicle

protrusion; a permeability barrier for water uptake and oxygen diffusion to the seed embryo; and a chemical barrier with hormones and secondary metabolites that regulate germination directly (ABA) or indirectly through oxygen binding. For larger spinach seeds, germination may be inhibited by the thicker pericarp and a higher content of germination-inhibiting compounds. This inhibitory effect was observed only under stressful conditions of high moisture levels or in the presence of *P. ultimum*, possibly due to interference with low oxygen levels, which is explained later.

Pericarp colour in relation to maturity and stress tolerance

In addition to pericarp thickness and intactness, pericarp colour was associated with *P. ultimum* tolerance of spinach seed lots (Chapter 5). Studies on spinach or beet pericarp colour have mainly focused on the association with seed health or pericarp damage and germination potential under optimal conditions. For instance, a reduced intensity of light reflectance in the visible and near-infrared light spectrum indicated the presence of seedborne pathogens on spinach seeds (Olesen et al., 2011), and different levels of pericarp damage of sugar beet seeds could be discriminated by multispectral imaging (Salimi & Boelt, 2019b). In the present study, light reflectance of the spinach seed pericarp was examined in relation to seedling emergence in the presence of soilborne *P. ultimum*, with less light reflectance indicating darker seeds were more tolerant to the pathogen. Light reflectance was also associated with the seed maturity level. The more mature spinach seeds reflected less light over the whole visible light spectrum, as they were darker brown compared with less mature seeds (Chapter 5). A similar observation was reported with different maturity classes of sugar beet seeds (Boelt et al., 2018). Mature sugar beet seeds reflected relatively less light in the visible to NIR light spectrum. The wash-water of mature sugar beet seeds, however, had higher reflectance of light compared to the seed wash solutions of the lower maturity classes, with the largest difference in the ultraviolet (UV) to blue spectrum, between 260 and 420 nm (Boelt et al., 2018; Salimi & Boelt, 2019a). This indicates that, during seed maturation, the concentration of water-soluble phenolic compounds that absorb UV to blue light declines. Based on the measured reflectance (375 to 970 nm) of the pericarp of spinach seeds, reflectance in the violet-blue range showed the strongest negative association with seedling emergence in the presence of *P. ultimum* (Chapter 5). Less violet-blue reflectance, hence higher violet-blue absorbance, related positively to *P. ultimum* tolerance of spinach seeds. Higher violet-blue light absorbance (at 375 to 480 nm) may be associated with the presence of flavonoids or other secondary metabolites. In other studies, flavonoid content was associated with light absorbance at various wavelengths within that range and below, between 250 and 430 nm (Tsimogiannis et al., 2007; da Silva et al., 2015; Oksana & Artur, 2019). Therefore, to draw conclusions, more research is needed on the presence and composition of specific secondary metabolites in the spinach seed pericarp, their

association with light absorbance, and their specific biological activities.

More studies have been conducted on the association between seed coat (instead of seed pericarp) colour and seed vigour. For instance, white-coloured varieties of legumes (e.g., chickpea, cowpea) had lower total germination, faster water uptake and higher electrical conductivity than coloured varieties (Peksen et al., 2004; Lamichaney et al., 2016). Generally, a higher leakage of compounds from seeds has been associated with a lower seed vigour as a result of cell membrane damage, which is measured by a higher electrical conductivity (ISTA, 2021). Another example is that brown seeds of watermelon were relatively larger and showed lower electrical conductivity and water uptake ratios, and greater germination and emergence, resulting in greater seedling weights compared to yellow seeds (Mavi, 2010). Possibly, darker melon seeds were more mature, similar to the darker-coloured cabbage seeds with lower chlorophyll fluorescence levels and greater seed vigour (Jalink et al., 1998). With rapeseed, a darker seed coat colour was associated with a slower water uptake and greater tolerance to excessive water (Zhang et al., 2008). The darker seeds also suffered less from mechanical seed coat injury during harvest than yellow seeds of rapeseed. Although the darker seeds germinated slower, they had a higher seedling emergence than the yellow seeds (Neubert et al., 2003). Also, brown seeds of flax had higher seed vigour than yellow seeds, probably due to a higher content of tannins (Saeidi & Rowland, 1999). Few studies have investigated the relationship of seed coat colour with susceptibility to *P. ultimum* infection. For instance, darker seeds of pine (Grzywacz & Rosochacka, 1980) and soybean (Campa et al., 2010) had higher resistance to *P. ultimum* infection than lighter-coloured seeds. Higher seed exudation levels of, e.g., carbohydrates and amino acids, stimulated *P. ultimum* mycelium growth and infection, as demonstrated for pea (Matthews, 1971), soybean (Schlub & Schmitthenner, 1978), and cotton (Nelson & Craft, 1989). With less mature spinach seeds that showed less *P. ultimum* tolerance, higher exudation levels can be expected due to less vigour and more openings in the pericarp, resulting in greater leakage of sugars that may have stimulated *P. ultimum* infection.

Oxygen sensitivity as a potential driver of stress tolerance

The sensitivity of larger and more intact seeds and seeds with a thicker pericarp towards high moisture levels was thought to be related to reduced oxygen availability to the embryo, which inhibits germination. In experiments with germination on filter papers, higher moisture levels of the filter papers may have resulted in a more saturated pericarp, restricting oxygen diffusion through the pericarp to the embryo, particularly for seeds with a thicker and more intact pericarp. The air surrounding seeds on the filter papers was assumed to be at normal atmospheric oxygen levels due to the opening in the cover.

Another possibility is that compounds in the pericarp form a mucilage layer around the embryo at excessive moisture levels, which restricts oxygen diffusion to the embryo, as was described previously for spinach seeds (Heydecker & Orphanos, 1968). However, with the tested seed lots I did not observe the formation of a mucilage layer. At high moisture level, the germination of seeds increased when atmospheric oxygen levels were elevated or when temperatures were lowered. At lower temperatures, the seed metabolic activity and, hence, oxygen consumption is lower, while the solubility of oxygen in water is higher. This, or the combination of both, may explain the higher total germination observed at high moisture levels with lower temperatures, despite the slower germination (Chapter 2).

In the field, high soil moisture conditions and soil compaction result in reduced oxygen levels and increased severity of damping off (Agrios, 2005c; Lamichhane et al., 2018). Reduced oxygen levels are harmful for spinach seed germination and seedling emergence, whereas *P. ultimum* can withstand low oxygen levels and favours high soil moisture for production and motility of zoospores (Griffin, 1963; Agrios, 2005c). When spinach seeds were sown in the field trials in the present study, I could not exclude potential moisture (hence oxygen) stress levels that interfered with emergence success and damping-off severity. The same seeds were also sown in samples of these field soils in the greenhouse where the soil moisture content was kept relatively constant. Comparable results from the field and greenhouse trials suggest that there was no substantial oxygen stress. In the lab assay, the substrate in which the seeds were sown was relatively dry (50% water-holding capacity) compared to the field. In addition, the substrate had a loose structure, enabling good aeration and drainage with sufficient water and oxygen supply to the seeds to obtain at least 85% germination. The trays were not closed tightly, and were opened daily to take photos, which allowed fresh air to enter. In presence of *P. ultimum* inoculum, there may be reduced oxygen levels in the substrate due to oxygen consumption by the mycelium. Therefore, it may be possible that there was, temporarily, a lower oxygen level in the substrate with *P. ultimum*, especially during the first three or four days after sowing when the trays were not photographed. This may have affected germination of seeds, increasing the sensitivity towards pre-emergence infection by *P. ultimum*. At the end of the experiments, it was hard to determine whether the seeds rotted before or after germination, or before seedling emergence above substrate level. Reduced oxygen levels alone may limit germination rate and capacity of seeds (Yasin & Andreasen, 2016). Platenius (1943) already determined that an oxygen level below 20.9% is limiting for respiration of spinach seeds, as well as for asparagus, bean, pea and carrot. Yasin and Andreasen (2016) confirmed that the germination of Swiss chard, also member of the Amaranthaceae, declined with reduced oxygen levels from 20.9% to 2.5%. The seeds from crops of the family Apiaceae (carrot, celeriac, and parsley)

were also sensitive to low oxygen levels, with reduced seed germination rate and total germination, whereas members of the Asteraceae (e.g., lettuce) and Brassicaceae (e.g., broccoli and white cabbage) still showed considerable germination at low oxygen levels. Some researchers have suggested that oxygen sensitivity can depend on the cultivar and on seed size, as was demonstrated with carrot (Corbineau et al., 1995).

Seed vigour as an overarching trait involved in stress tolerance

Seed vigour is affected by seed maturity, genotype, and seed production conditions, including the maternal environment, seed harvesting methods, and seed storage conditions (temperature, humidity, oxygen) (TeKrony & Hunter, 1995; Hatzig et al., 2015). Of the seed traits that we measured, seed maturity seemed to be the most important trait associated with emergence success. The results indicated that seed maturity influences the vigour of the seed, with a positive relationship between the maturity level and emergence success in the absence and presence of *P. ultimum* (Chapter 5). In accordance with previous studies on sugar beet seeds, a correlation between the darkness of pericarp colour and seed maturity of spinach seeds was confirmed. In particular, a less violet-blue pericarp colour was associated with a higher *P. ultimum* tolerance. Based on previous studies that associated seed coat colour with seed vigour, pericarp colour may also be associated with spinach seed vigour. Seed vigour may not be related to the lightness of seed pericarp colour alone, but with specific colours associated with secondary metabolites that have various biological functions, such as the regulation of germination and seedling emergence, and antimicrobial or pathogen-stimulative activities, as described above.

Based on the research and literature findings, I hypothesise that pericarp colour-associated compounds affect spinach seed vigour and indirectly relate to *P. ultimum* tolerance of spinach seeds. A lower reflectance of light from the spinach seed pericarp (i.e., darker pericarp) may be associated with a higher concentration of phenolic compounds that have antimicrobial capacity, which may have reduced *P. ultimum* infection of the more mature seeds and darker seeds that showed a higher *P. ultimum* tolerance. At the same time, other phenolic compounds may inhibit germination, but since the substrate conditions were optimal for germination in this study, this negative effect was not observed with mature seeds. Less mature seeds may have lower seed vigour due to greater leakage of carbohydrates or water-soluble metabolites from the less ripened pericarp that also contained more openings. These factors may have stimulated *P. ultimum* infection, resulting in less *P. ultimum* tolerance of the less mature seeds. Smaller, mature seeds were less sensitive to pre-emergence damping off than larger, mature seeds, which is hypothesised to be related to a lower content of ABA and less physical constraint by the thinner pericarp of smaller

seeds. Further studies are needed to measure the presence and concentration of phenolic compounds and carbohydrates in the spinach pericarp, their water-solubility, and their association with seed germination and *P. ultimum* growth and infection.

Recommendations for seed production, breeding, and further research

In seed production, post-harvest seed grading is usually done, resulting in discarding of small, immature seeds. Subsequently, seed treatments can be applied, if economically feasible, to increase seed quality. Such treatments include polishing (in case of seeds with a thick pericarp); priming to increase the uniformity and rate of germination; seed sterilization by steam or chlorine and/or coating with pesticides, biological solutions, or nutrients to enhance stress tolerance. Breeding for specific seed traits should be considered based on the results of this research. Opportunities for product development and breeding are discussed and suggestions for further research are provided.

Seed selection to enhance seed vigour and damping-off tolerance

In nature, indeterminate seed development on a plant can be advantageous. Due to indeterminate seed ripening, seeds are released from the plant at different times, which is also affected by environmental conditions (Rubatzky & Yamaguchi, 1997). Variation in seed size, pericarp thickness and, potentially, other traits, broadens the germination range of the produced seeds, thereby increasing the survival chances of the genotype. However, for commercial seed production, uniformity of maturity levels is preferred, and seeds with a thin or open pericarp or even no pericarp may provide an advantage over seeds with a thick pericarp when sown under stressful conditions (low oxygen levels or *P. ultimum* presence), as shown by this research. Post-harvest seed selection for uniformity of maturity level, and a thinner or darker pericarp could be relevant strategies in seed production of indeterminately growing crops like spinach and sugar beet.

More research is needed to validate the effect of pericarp colour and associated compounds on seed vigour and *P. ultimum* tolerance. If so, it could be useful to include measurements of pericarp light reflectance at wavelengths lower than 375 nm since various secondary metabolites are also represented by absorbance in the ultra-violet light spectrum. In addition, the presence and concentration of specific polyphenolic compounds could be measured in the pericarp using high performance liquid chromatography, as was performed with sugar beet seeds (Oksana). Discrimination between water-soluble and water-insoluble compounds was not made in the present study, except for the specific measurement of chlorophyll fluorescence, which is associated with the presence of the insoluble chlorophyll pigment. In further studies, water-soluble and insoluble compounds could be distinguished by washing and re-drying seeds prior to reflectance

measurements of both, the seeds and the wash-water. In addition, the inhibitory effect of the water-soluble compounds on *P. ultimum* growth could be evaluated with the wash-water. It should also be considered that spinach seed reflectance of light may be associated with various secondary metabolites with potentially contrasting biological functions. If a negative effect of compounds on *P. ultimum* tolerance is associated with violet-blue light reflectance of the spinach seeds, sorting in favour of seeds with less violet-blue reflectance could be valuable to improve *P. ultimum* tolerance of spinach seed lots.

Chemical and non-chemical seed treatments to improve damping-off tolerance

Chemical seed treatments are still an important application to manage damping-off diseases in conventional agriculture. Currently, pesticides applied for damping-off management may contain active substances like cyazofamid, propamocarb hydrochloride, aluminum tris phosphonate, etridiazole, and metalaxyl, that are active against *Phytophtora* and *Pythium* (Meadows et al., 2020). There are also combinations of fungicides available and broad-spectrum fungicides like captan and ferbam, that are used particularly when the damping-off pathogen is not known. However, the efficacy of fungicides can be reduced over time due to fungicide resistance, as was shown with mefenoxam (based on metalaxyl) resistance in *Pythium* spp. and *Phytophthora* spp. in some regions (Taylor et al., 2002; Tekale et al., 2019). Fungicides can also negatively affect seed germination and seedling growth, due to phytotoxicity besides potential negative impacts on environmental and human health. This has led to an increasing ban on the use of such chemical treatments in many countries (United Nations Environment Programme (UNEP), 2006; European Commission, 2021). The increased pressure on the application of chemical seed treatments has forced the development of alternative solutions for damping-off management (Lamichhane et al., 2017).

Biocontrol agents can be an important substitute to conventional fungicides with lower potential negative impacts. To control damping-off diseases, there are promising results on priming with antagonistic fungi, including *Gliocladium* spp. and *Trichoderma* spp., or bacteria such as *Bacillus* spp., *Pseudomonas* spp., and *Streptomyces* spp. (Lamichhane et al., 2017). For instance, organic seed treatments with *Trichoderma harzianum*, *Streptomycete* products or *Bacillus subtilis* could suppress pre- and/or post-emergence damping off of spinach caused by *P. ultimum*, with similar levels of control to that of the conventional fungicide mefenoxam (Cummings et al., 2009). Also, antagonistic microbes can already be present in the spinach seed microbiome (Lopez-Velasco et al., 2013). Most biocontrol products are not yet registered or marketed for use, despite knowledge of their effectivity for disease control (Paulitz & Bélanger, 2001). Due to the increasing demand for chemical-free seeds, the industry for crop protection products

is increasingly using the available scientific knowledge to develop and commercialise biocontrol agents (Lamichhane et al., 2017).

Seed-surface sterilization with chlorine or hot water can be used to remove some seedborne pathogens, such as *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* on spinach seeds (du Toit & Hernandez-Perez, 2005). However, these methods could potentially also remove beneficial microbes that might have anti-pathogenic functions (Nelson, 2018). Also, the seed microbiome is determined by the plant genotype, environment, and management practices (Berg & Raaijmakers, 2018). In the present study, seeds were not sterilised due to the possibility of a genotypic basis for the seed microbiome and potential interactions with *P. ultimum*.

Other non-chemical treatments of seeds include those that change the seed physically or physiologically, or both. For instance, seed dehulling increased the germination potential of spinach seeds when sown at high moisture conditions (Chapter 2) or in the presence of *P. ultimum* (Chapter 4). For some seed lots, removal of the pericarp of seeds resulted in higher tolerance levels, whereas the rate of emergence did not increase. This indicated that seed dehulling could improve tolerance levels not only as a result of an increased rate of germination. Removal of the physical and chemical barrier for oxygen diffusion may have improved the vigour of the seed, not in terms of rate but in terms of total germination under stressful conditions. Seed vigour may have also improved by a reduction in leakage of *P. ultimum* stimulating compounds that were removed by dehulling and dehulling may have also reduced the presence of seedborne pathogens.

After hydropriming spinach seeds, the rate of germination and emergence increased, and tolerance towards *P. ultimum* infection increased (Chapter 4). Still, some variation was observed among the seed lots, but they performed more uniform in terms of germination rate and damping-off tolerance levels. The positive effects of priming could be a direct result of the pre-germination of seeds or an indirect result of stimulating the activity of microbes present in the spinach seed microbiome. Like with seed imbibition, priming with a limited amount of water can activate dormant endophytic (in the embryo) or epiphytic (in or on the pericarp) microorganisms (Nelson, 2018). Many microorganisms have been identified in the area surrounding the germinating seed that are known to be antagonistic to *P. ultimum* (Lopez-Velasco et al., 2013; Centre for Agriculture and Bioscience International (CABI), 2021).

Spinach seed priming and dehulling have not yet been applied commercially because the seeds are relatively inexpensive, whereas these treatments are still rather expensive (Johan Rijk, Pop Vriend Seeds, 2021, *personal communication*). For instance, drying

large amounts of seeds after priming is a challenge logistically and the shelf-life of the seeds is likely to be reduced. Only in the breeding process, priming is used commercially for synchronization of flowering time of male and female plants (Germains, 2017). A seed dehulling technique has been developed and used commercially for spinach seed production in Japan (Toshiyuki, 1992). For sugar beet seeds, related techniques of washing and polishing have already been introduced for commercial seed production (Ignatz et al., 2019). Techniques for priming and dehulling spinach seeds may become economically feasible as the demand increases for organic spinach seeds with uniform seedling emergence.

Field measures and treatments to control damping off

In addition to seed selection and seed treatments, field measures to prevent damping off are still needed. Those measures include sanitation practices, enabling good soil drainage and aeration, preventing excessively wet soils, using well-decomposed compost, and planting at soil temperatures favourable for rapid seedling emergence (Lamichhane et al., 2017). Rotation with non-host crops is probably impossible with *P. ultimum*-infested soils due to its wide host range and the persistence of its resting structures (oospores and sporangia) in the soil (Stanghellini & Hancock, 1971). If *Pythium* spp. are present in the soil, there are limited management strategies, including chemical and non-chemical field treatments. For a long time, soil fumigants, such as methyl bromide and chloropicrin, were used to eliminate *Pythium* spp. (Hendrix & Powell, 1970; Munnecke et al., 1971). However, the use of methyl bromide has been banned in most countries since the Montreal protocol in 1987 due to the large-scale negative effects on human health and the environment, including depletion of the ozone layer (United States Environmental Protection Agency (EPA), 2021). Also, pesticides like chloropicrin are being banned increasingly, but are not yet eradicated from agricultural use in the USA (Donley, 2019). A traditional alternative to chemical field treatments is soil solarization, including covering the moistened soil with transparent polyethylene sheets to be heated by the sun to increase the soil temperature, which is proven to be effective against *Pythium* spp. (Christensen & Thinggaard, 1999). However, soil solarization can also have great impacts on other soil microbial life (Yokoe et al., 2015). A more recently developed method is anaerobic soil disinfestation, which includes the addition of organic nutrients to the soil prior to covering the soil completely to generate anaerobic conditions. This method has proven to be effective in suppressing soilborne diseases, including some caused by *P. ultimum* (Mazzola et al., 2012). Addition of organic compost to the soil without soil coverage can also reduce damping-off occurrence by increasing soil suppressiveness against *P. ultimum* (Pane et al., 2011; Meghvansi & Varma, 2015). The addition of compost tea or *Gliocladium* spp. suppressed post-emergence wilt caused by *Fusarium oxysporum* in spinach in greenhouse trials (Cummings et al., 2009). Soil suppressiveness in the field may be associated with several biotic and abiotic conditions,

such as soil pH, micronutrients (e.g, Cu, Fe, Zn) and macronutrients (e.g., N, K, Mg), and microbial biomass, activity, and diversity (Bünemann et al., 2018), which needs further validation for assessing the efficacy of biocontrol products.

Breeding for enhanced seed vigour and damping-off tolerance

Breeding usually focuses on the development of resistant cultivars with genetic uniformity that are cultivated in a monoculture to generate high yields. However, with the ever-changing environment (e.g., climate change and evolving pathogens) it may be relevant to breed for crop diversity to obtain site-specific crops and to sustain genetic resources. In case of polygenic resistance or tolerance that is largely influenced by the environment, it may also be necessary to integrate multiple traits in a cultivar to obtain crops that are resilient towards multiple biotic and abiotic stressors. Based on the current research, it may be relevant to study the genetic base for seed vigour or seed characteristics that relate to higher pre-emergence damping-off tolerance levels, such as a thinner pericarp, a darker (or less violet-blue reflecting) pericarp, greater uniformity in seed maturity, and improved capacity for germination under low oxygen levels. A genetic base for some of these traits has been found in other crops.

For pericarp thickness, a genetic base was found in several crops, including maize (Helm & Zuber, 1972) and sorghum (Blakely et al., 1979). Recently, a method has been published to obtain spinach plants through breeding that produces seeds that are substantially free from the pericarp (Schrijver et al., 2019). In buckwheat, progress has been made to unravel the genetics behind the determinate and indeterminate growth patterns that were attributed to a single recessive gene (Luthar, 2018). Plants with determinate growth contained thinner seeds and were adapted to lowland soil and climatic conditions, whereas plants with indeterminate growth contained slightly thicker, dark seeds, adapted for elevated locations and a shorter growing season. This knowledge may be useful for the development of determinately growing spinach plants that may also produce seeds with a thinner pericarp. Also, an increased rate of maturation of seeds, resulting in more synchronised flowering and more uniform seed maturity by the time of harvest, could be an interesting selection trait for breeders. In tomato, genes that underlie rate of maturation have been identified, and selection led to more uniform flowering and seed development (Powell et al., 2012).

For sugar beet, it has been demonstrated that the pericarp colour is influenced by both genotype and environment and is related to seed vigour (Mirzaei & Rajabi, 2021). Differences in seed vigour of crosses between female lines and a single male line indicated that the differences were due to differences among female lines, which is also probable

since the pericarp is derived from the ripened ovary wall of the female plants. Mirzaei and Rajabi (2021) also suggested that the traits RefA (red/green colour), RefB (blue/yellow colour), Blue, Hue and Saturation can be selection criteria in breeding programs to enhance seed vigour. As discussed above, light reflectance from the spinach pericarp may be associated with phenolic compounds that inhibit seed germination, but also that inhibit *P. ultimum* infection. With sorghum, the presence of phenolic compounds, such as anti-pathogenic tannins, in a mature seed is regulated by two genes, whereas the pigmentation is regulated by a different gene (Blakely et al., 1979). For *Arabidopsis thaliana*, mutants have been identified that have a transparent seed coat phenotype associated with yellow seed colour due to the lack of anthocyanin (Nesi et al., 2001). Candidate genes underlying seed coat colour have also been identified in crops like chickpea (Bajaj et al., 2015), Chinese cabbage (Ren et al., 2017) and rapeseed (Somers et al., 2001). Seed colour reflectance has been demonstrated to be a variety distinguishing factor in buckwheat, winter and spring barley, and pea (Klepacka et al., 2011). Based on the germination results that indicated differences in moisture sensitivity among spinach cultivars, I would also suggest researching whether there is a genetic base for higher total germination under reduced oxygen conditions. For instance, with carrot, high levels of seed germination under low oxygen levels (10% O_2) were found with certain cultivars, which depended partly on the cultivar in addition to seed size (Corbineau et al., 1995).

Moreover, varietal tolerance to other root rot pathogens could be considered when searching for varieties with *P. ultimum* tolerance. For instance, spinach varieties with resistance to *Fusarium* spp., a major focus in spinach breeding, may be linked to tolerance to *Pythium* spp. and *Rhizoctonia* spp. (Grigg, 2015). For breeders it would be ideal if resistance to these three pathogenic species could be obtained in one breeding programme and if the same genes are involved in the resistance. Previous research indicated the presence of combined resistance in some *Beta* accessions towards *P. ultimum* as well as *Rhizoctonia* spp. or *Aphanomyces* spp., or all three soilborne pathogens (Luterbacher et al., 2005).

Further research on genotyping variation in damping-off tolerance

The large variation among the studied seed lots emphasises the relevance of including multiple seed lots of each genotype in genotypic studies on damping-off tolerance. However, in the studies that were cited in this research, single seed lots of each genotype were mostly compared. Also, large variation in seed characteristics existed among seed lots of the same cultivars, and among seeds within the same seed lots. Based on this variability in damping-off tolerance levels and seed traits among and within the spinach seed lots studied, I make some recommendations on how to evaluate genotypic-based damping-off tolerance.

With seed priming, I intended to increase the uniformity of emergence among the seed lots to reduce the variation in seed vigour among seed lots and to emphasise differences in damping-off tolerance levels among the cultivars. However, it turned out that, due to seed priming, the tolerance levels of all seed lots improved compared to the untreated seed lots, and the differences in tolerance levels among the cultivars were reduced. Instead of enhancing seed vigour through seed priming, further research should consider a reduction of seed vigour, for instance by applying low oxygen conditions in the phenotyping assay. This may increase the variation in pre-emergence damping-off tolerance detected among cultivars. A control treatment without the pathogen should always be included to correct for differences in emergence that are not due to pathogen infection.

In further evaluations, I suggest evaluating cultivars (genotypes) that are developed under the same environmental conditions and whose seeds were threshed and sorted in the same way. In addition, multiple seed lots of each genotype should be obtained from different seed productions to evaluate the potential genotype-by-environment interactions. The environmental conditions during seed production should be monitored. If differences in damping-off tolerance levels among genotypes are independent of seed lot and environmental background, then a genetic base for damping-off tolerance is likely.

Concluding remarks

For further research on spinach damping off, I recommend focusing on spinach breeding for improved damping-off tolerance with emphasis on enhanced seed vigour and related seed traits. Such traits include a thin pericarp that is still protective against mechanical injury, leaking less pathogen-stimulating compounds and which contains low levels of germination-inhibitors and/or high levels of anti-pathogenic phenolic compounds. Further studies on the associations of pericarp colour and secondary metabolites are needed for a better understanding of their role in damping-off tolerance.

Until breeding for improved damping-off tolerance in spinach cultivars is possible, seed sorting techniques and seed treatments remain highly valuable, especially when the tolerance turns out to be polygenic and highly influenced by the abiotic environment. Seed sorting should integrate seed maturity selection by low chlorophyll fluorescence, removal of seeds with an underdeveloped pericarp, seed size grading in favour of smaller seeds, and selection of seeds with a darker pericarp colour (e.g., less violet-blue light reflectance). Moreover, dehulling or polishing techniques should be taken into consideration, which may become more economically feasible than priming, even though priming showed the most promising results for improved seed vigour and *P. ultimum* tolerance. Since the development of biocontrol products is continuing, this

may be a good option for damping-off management as well. Biocontrol products should contain an effective cocktail of disease suppressive microorganisms and/or plant growth promoting microorganisms or (micro-)nutrients that can be applied to the seed.

In addition to the use of disease tolerant cultivars, seed sorting techniques, (non-chemical) seed treatments, or combinations of these three, and the integration of site-specific (non-chemical) field measures (e.g., good soil drainage and using well-decomposed compost) will remain necessary to provide sustainable control of damping off in both organic and conventional spinach production.

Bibliography

Abbasi, P. A., & Lazarovits, G. (2005). Effects of AG3 phosphonate formulations on incidence and severity of *Pythium* damping-off of cucumber seedlings under growth room, microplot, and field conditions. *Canadian Journal of Plant Pathology, 27*(3), 420-429. https://doi.org/10.1080/07060660509507241

Abd-Elsalam, K. A., & Amal-Asran. (2020). Chapter 10: Host plants and specificity of the genus *Pythium*. In *Pythium: Diagnosis, DIseases and Management* (pp. 162-175). M. Rai, K. A. Abd-Elsalam, & A. P. Ingle (Eds.). *CRC Press*.

Agrios, G. N. (2005a). Chapter 4: Genetics of plant disease. In *Plant Pathology, 5th Edition*. *Elsevier Academic Press*. Burlington.

Agrios, G. N. (2005b). Chapter 11: Plant diseases caused by fungi. In *Plant Pathology, 5th Edition*. *Elsevier Academic Press*. Burlington.

Agrios, G. N. (2005c). Chapter 7: Environmental effects on the development of infectious plant disease. In *Plant Pathology, 5th Edition*. *Elsevier Academic Press*. Burlington.

Allain-Boulé, N., Tweddell, R., Mazzola, M., Bélanger, R., & Lévesque, C. A. (2004). *Pythium attrantheridium* sp. nov.: taxonomy and comparison with related species. *Mycological Research, 108*(7), 795-805. https://doi.org/10.1017/s095375620400053x

Altier, N. A., & Thies, J. A. (1995). Identification of resistance to *Pythium* seedling diseases in alfalfa using a culture plate method. *Plant Disease, 79*(4), 341-346.

Andersen, S. B., & Torp, A. M. (2011). *Spinacia*. In *Wild Crop Relatives: Genomic and Breeding Resources: Vegetables* (pp. 273-276). C. Kole (Ed.). *Springer*. Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-20450-0_13

Arellano, G., & Peco, B. (2012). Testing the role of seed size in annual legume seedling performance under experimental autumn moisture conditions. *Journal of Vegetation Science, 23*(4), 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. *The Journal of Wildlife Management, 74*(6), 1175-1178. https://doi.org/10.1111/j.1937-2817.2010.tb01236.x

Association of Official Seed Analysts (AOSA). (1983). *Seed Vigor Testing Handbook. Contribution No. 32 to the AOSA Handbook on Seed Testing*. Lincoln, NE.

Atherton, J. G., & Farooque, A. M. (1983). High temperature and germination in spinach. I. The role of the pericarp. *Scientia Horticulturae, 19*(1-2), 25-32. https://doi.org/10.1016/0304-4238(83)90040-7

Atis, I., Atak, M., Can, E., & Mavi, K. (2011). Seed coat color effects on seed quality and salt tolerance of red clover (*Trifolium pratense*). *International Journal of Agriculture & Biology, 13*(3).

Bajaj, D., Das, S., Upadhyaya, H. D., Ranjan, R., Badoni, S., Kumar, V., Tripathi, S., Gowda, C. L., Sharma, S., & Singh, S. (2015). A genome-wide combinatorial

strategy dissects complex genetic architecture of seed coat colour in chickpea. *Frontiers in Plant Science, 6*, 979-996. https://doi.org/10.3389/fpls.2015.00979

Baker, H. G. (1972). Seed weight in relation to environmental conditions in California. *Ecology, 53*(6), 997-1010. https://doi.org/10.2307/1935413

Bardin, S. D., Huang, H. C., Liu, L., & Yanke, L. J. (2003). Control, by microbial seed treatment, of dampingoff caused by *Pythium* sp. on canola, safflower, dry pea, and sugar beet. *Canadian Journal of Plant Pathology, 25*(3), 268-275. https://doi.org/10.1080/07060660309507079

Beakes, G. W., Honda, D., & Thines, M. (2014). Systematics of the Straminipila: Labyrinthulomycota, Hyphochytriomycota, and Oomycota. In *Systematics and Evolution: Part A* (pp. 39-97). D. J. McLaughlin & J. W. Spatafora (Eds.). *Springer*. Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-55318-9_3

Bemis, W. P., & Wilson, G. B. (1953). A new hypothesis explaining the genetics of sex determination: In *Spinacia oleracea* L. *Journal of Heredity, 44*(3), 91-95. https://doi.org/10.1093/oxfordjournals.jhered.a106370

Berg, G., & Raaijmakers, J. M. (2018). Saving seed microbiomes. *The ISME journal, 12*(5), 1167-1170.

Berg, L. E., Miller, S. S., Dornbusch, M. R., & Samac, D. A. (2017). Seed rot and damping-off of alfalfa in Minnesota caused by *Pythium* and *Fusarium* species. *Plant Disease, 101*(11), 1860-1867. https://doi.org/10.1094/PDIS-02-17-0185-RE

Bionext. (2020). *Trendrapport: Ontwikkelingen in de biologische sector*. https://bionext.nl/application/files/5916/3333/2378/BIONEXT_TRENDRAPPORT_2020.pdf

Black, M., & Wareing, P. F. (1959). The role of germination inhibitors and oxygen in the dormancy of the light-densitive seed of *Betula* spp. *Journal of Experimental Botany, 10*(1), 134-145. https://doi.org/10.1093/jxb/10.1.134

Blakely, M. E., Rooney, L. W., Sullins, R. D., & Miller, F. R. (1979). Microscopy of the pericarp and the testa of different genotypes of Sorghum. *Crop Science, 19*(6), 837-842.

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 Science Research, 28*(3), 222-228. https://doi.org/10.1017/S0960258518000235

Bongiorno, G., Postma, J., Bünemann, E. K., Brussaard, L., de Goede, R. G. M., Mäder, P., Tamm, L., & Thuerig, B. (2019). Soil suppressiveness to *Pythium ultimum* in ten European long-term field experiments and its relation with soil parameters. *Soil Biology and Biochemistry, 133*, 174-187. https://doi.org/10.1016/j.soilbio.2019.03.012

Bünemann, E. K., Bongiorno, G., Bai, Z., Creamer, R. E., De Deyn, G., de Goede, R., Fleskens, L., Geissen, V., Kuyper, T. W., Mäder, P., Pulleman, M., Sukkel, W., van Groenigen, J. W., & Brussaard, L. (2018). Soil quality – A critical review. *Soil Biology*

and Biochemistry, 120, 105-125. https://doi.org/10.1016/j.soilbio.2018.01.030

Campa, A., Pérez-Vega, E., Pascual, A., & Ferreira, J. J. (2010). Genetic analysis and molecular mapping of quantitative trait loci in common bean against *Pythium ultimum*. *Phytopathology, 100*(12), 1315-1320.

CBS StatLine. (2021). *Landbouw; gewassen, dieren en grondgebruik naar regio. Onderwerp: spinazie*. https://opendata.cbs.nl/#/CBS/nl/dataset/80780ned/table?ts=1641566503006.

Centre for Agriculture and Bioscience International (CABI). (2021). *Pythium ultimum (black-leg of seedlings)*. In: *Invasive Species Compendium*. from https://www.cabi.org/isc/datasheet/46172

Chen, K., Arora, R., & Arora, U. (2010). Osmopriming of spinach (*Spinacia oleracea* L. cv. Bloomsdale) seeds and germination performance under temperature and water stress. *Seed Science and Technology, 38*(1), 36-48. https://doi.org/10.15258/sst.2010.38.1.04

Chen, K., & Arora, R. (2013). Priming memory invokes seed stress-tolerance. *Environmental and Experimental Botany, 94*, 33-45. https://doi.org/10.1016/j.envexpbot.2012.03.005

Chiji, H., Tanaka, S., & Izawa, M. (1980). Phenolic germination inhibitors in the seed balls of red beet (*Beta vulgaris* L. var. *rubra*). *Agricultural and Biological Chemistry, 44*(1), 205-207. https://doi.org/10.1080/00021369.1980.10863924

Christensen, L., & Thinggaard, K. (1999). Solarization of greenhouse soil for prevention of *Pythium* root rot in organically grown cucumber. *Journal of Plant Pathology*, 137-144.

Collins, K., Zhao, K., Jiao, C., Xu, C., Cai, X., Wang, X., Ge, C., Dai, S., Wang, Q., Wang, Q., Fei, Z., & Zheng, Y. (2019). SpinachBase: a central portal for spinach genomics. *Database, 2019*. https://doi.org/10.1093/database/baz072

Corbineau, F., Picard, M. A., Bonnet, A., & Côme, D. (1995). Effects of production factors on germination responses of carrot seeds to temperature and oxygen. *Seed Science Research, 5*(3), 129-135. https://doi.org/10.1017/S0960258500002749

Correll, J. C., Morelock, T. E., Black, M. C., Koike, S. T., Brandenberger, L. P., & Dainello, F. J. (1994). Economically important diseases of spinach. *Plant Disease, 78*(7), 653-660. https://doi.org/10.1094/PD-78-0653

Coumans, M., Come, D., & Gaspar, T. (1976). Stabilized dormancy in sugarbeet fruits. I. Seed coats as a physicochemical barrier to oxygen. *Botanical Gazette, 137*(3), 274-278. https://doi.org/10.1086/336870

Cummings, J. A., Miles, C. A., & du Toit, L. J. (2009). Greenhouse evaluation of seed and drench treatments for organic management of soilborne pathogens of spinach. *Plant Disease, 93*(12), 1281-1292. https://doi.org/10.1094/PDIS-93-12-1281

da Silva, L. A. L., Pezzini, B. R., & Soares, L. (2015). Spectrophotometric

determination of the total flavonoid content in *Ocimum basilicum* L. (Lamiaceae) leaves. *Pharmacognosy Magazine, 11*(41), 96-101. http://doi.org/10.4103/0973-1296.149721

Dardick, C., & Callahan, A. M. (2014). Evolution of the fruit endocarp: molecular mechanisms underlying adaptations in seed protection and dispersal strategies. *Frontiers in Plant Science, 5*, 284-293. https://doi.org/10.3389/fpls.2014.00284

Das Gupta, P. R., & Austenson, H. M. (1973). Analysis of Interrelationships among seedling vigor, field emergence, and yield in wheat. *Agronomy Journal, 65*(3), 417-422. https://doi.org/10.2134/agronj1973.00021962006500030019x

Debeaujon, I., Lepiniec, L., Pourcel, L., & Routaboul, J.-M. (2007). Chapter 3: Seed coat development and dormancy. In *Seed development, Dormancy and Germination* (Vol. 27, pp. 392-436). K. Bradford & H. Nonogaki (Eds.). *Wiley-Blackwell, Annual Plant Reviews*. https://hal.archives-ouvertes.fr/hal-01203973

Deleuran, L. C., Olesen, M. H., & Boelt, B. (2013). Spinach seed quality: Potential for combining seed size grading and chlorophyll fluorescence sorting. *Seed Science Research, 23*(4), 271-278. https://doi.org/10.1017/S0960258513000202

Dickson, M., & Abawi, G. (1974). Resistance to *Pythium ultimum* in white-seeded beans (*Phaseolus vulgaris*). *Plant Disease Reporter, 58*(9), 774-776.

Donley, N. (2019). The USA lags behind other agricultural nations in banning harmful pesticides. *Environmental Health, 18*(1), 44. http://doi.org/10.1186/s12940-019-0488-0

du Toit, L. J., & Hernandez-Perez, P. (2005). Efficacy of hot water and chlorine for eradication of *Cladosporium variabile*, *Stemphylium botryosum*, and *Verticillium dahliae* from spinach seed. *Plant Disease, 89*(12), 1305-1312. https://doi.org/10.1094/PD-89-1305

El-Kassaby, Y. A., Moss, I., Kolotelo, D., & Stoehr, M. (2008). Seed germination: mathematical representation and parameters extraction. *Forest Science, 54*(2), 220-227. https://doi.org/10.1093/forestscience/54.2.220

Ellis, M. L., Arias, M. M. D., Jimenez, D. R. C., Munkvold, G. P., & Leandro, L. F. (2013). First report of *Fusarium commune* causing damping-off, seed rot, and seedling root rot on soybean (*Glycine max*) in the United States. *Plant Disease, 97*(2), 284-284. https://doi.org/10.1094/PDIS-07-12-0644-PDN

ElMasry, G., Mandour, N., Al-Rejaie, S., Belin, E., & Rousseau, D. (2019). Recent Applications of Multispectral Imaging in Seed Phenotyping and Quality Monitoring—An Overview. *Sensors, 19*(5), 1090(1091-1032). https://www.mdpi.com/1424-8220/19/5/1090

European Commission. (2021). *European Green Deal, Strategy priorities 2019-2024*. Retrieved 18/02/2021 from https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal_en

European Soil Data Centre (ESDAC). (2021). *European Soil Database & Soil Properties*. Retrieved 12/03/21 from https://esdac.jrc.ec.europa.eu

Feng, H., Chen, J., Yu, Z., Li, K., Li, Z., Li, Y., Sun, Z., Wang, Y., Ye, W., & Zheng, X. (2020). Pathogenicity and fungicide sensitivity of *Pythium* and *Phytopythium* spp. associated with soybean in the Huang-Huai region of China. *Plant Pathology, 69*(6), 1083-1092. https://doi.org/10.1111/ppa.13187

Fenner, M. (1992). Environmental influences on seed size and composition. *Horticultural Reviews, 13*, 183-213.

Fenner, M., & Thompson, K. (2005). *The Ecology of Seeds*. Cambridge University Press. Cambridge, UK. https://genderi.org/pars_docs/refs/62/61494/61494.pdf

Finch-Savage, W. E., & Bassel, G. W. (2015). Seed vigour and crop establishment: extending performance beyond adaptation. *Journal of Experimental Botany, 67*(3), 567-591. https://doi.org/10.1093/jxb/erv490

Food and Agriculture Organization (FAO). (2020, 20/12/2020). *Production/Yield quantities of Spinach in the World 1994 - 2019*. Retrieved 18/02/2021 from http://www.fao.org/faostat/en/#data/QC

Fuentes-Bazan, S., Mansion, G., & Borsch, T. (2012). Towards a species level tree of the globally diverse genus Chenopodium (Chenopodiaceae). *Molecular Phylogenetics and Evolution, 62*(1), 359-374. https://doi.org/10.1016/j.ympev.2011.10.006

Germains. (2017). *Spinach seed priming boosts Danish seed production*. from https://germains.com/spinach-seed-priming-denmark/

Green, K., Gladders, P., Ritchie, F., Williams, S., Caspell, N., & Pettitt, T. (2012). *Spinach: biology and management of damping-off disease* (Project Number FV 367 (Final Report)). Pages 1-51. ADAS UK Ltd. https://ahdb.org.uk/fv-367-spinach-biology-and-management-of-damping-off-disease

Griffin, D. M. (1963). Soil physical factors and the ecology of fungi: II. Behaviour of *Pythium ultimum* at small soil water suctions. *Transactions of the British Mycological Society, 46*(3), 368-372. https://doi.org/10.1016/S0007-1536(63)80029-7

Grigg, S. (2015). *Spinach–A global expedition to understand the prevalence of damping off and practical management techniques*. M. International Specialised Skills Institute. https://www.issinstitute.org.au/wp-content/uploads/2015/04/Report-Grigg-FINAL-LowRes.pdf

Grzywacz, A. P., & Rosochacka, J. (1980). The colour of *Pinus silvestris* L. seed and their susceptibility to damping-off: I. The colour and quality of seeds and fatty acids content of the seed coat. *European Journal of Forest Pathology, 10*(2-3), 138-144. https://doi.org/10.1111/j.1439-0329.1980.tb00019.x

Hallavant, C., & Ruas, M.-P. (2014). The first archaeobotanical evidence of *Spinacia oleracea* L.(spinach) in late 12th–mid 13th century AD France. *Vegetation History and Archaeobotany, 23*(2), 153-165. https://doi.org/10.1007/s00334-013-0400-8

Hamman, B., Egli, D. B., & Koning, G. (2002). Seed vigor, soilborne pathogens, preemergent growth, and soybean seedling emergence. *Crop Science, 42*(2), 451-457. https://doi.org/10.2135/cropsci2002.4510

Hatzig, S. V., Frisch, M., Breuer, F., Nesi, N., Ducournau, S., Wagner, M.-H., Leckband, G., Abbadi, A., & Snowdon, R. J. (2015). Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. *Frontiers in Plant Science, 6*, 221(221-213). https://doi.org/10.3389/fpls.2015.00221

Hawthorne, B. T. (1988). Pathogenicity of *Pythium* spp. to lucerne seedlings and relationships between seed weight, seedling vigour, and infection by *Pythium*. *New Zealand Journal of Agricultural Research, 31*(3), 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. *Journal of the American Society for Horticultural Science, 116*(6), 946-949. https://doi.org/10.21273/JASHS.116.6.946

Helm, J. L., & Zuber, M. S. (1972). Inheritance of pericarp thickness in corn belt maize. *Crop Science, 12*(4), 428-430.

Hendrix, F. F., & Powell, W. M. (1970). Control of root pathogens in peach decline sites. *Phytopathology, 60*, 16-19.

Hendrix, F. F., & Campbell, W. A. (1973). Pythiums as plant pathogens. *Annual Review of Phytopathology, 11*(1), 77-98. https://doi.org/10.1146/annurev.py.11.090173.000453

Hermann, K., Meinhard, J., Dobrev, P., Linkies, A., Pesek, B., Heß, B., Macháčková, I., Fischer, U., & Leubner-Metzger, G. (2007). 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): A comparative study of fruits and seeds. *Journal of Experimental Botany, 58*(11), 3047-3060. https://doi.org/10.1093/jxb/erm162

Heydecker, W., & Orphanos, P. I. (1968). The effect of excess moisture on the germination of *Spinacia oleracea* L. *Planta, 83*(3), 237-247. https://doi.org/10.1007/BF00385333

Heydecker, W., Chetram, R. S., & Heydecker, J. C. (1971). Water relations of beetroot seed germination II. Effects of the ovary cap and of the endogenous inhibitors. *Annals of Botany, 35*(1), 31-42. https://doi.org/10.1093/oxfordjournals.aob.a084465

Heydecker, W., Higgins, J., & Turner, Y. J. (1975). Invigoration of seeds. *Seed Science and Technology, 3*(3/4), 881-888.

Hirayama, Y., & Tojo, M. (1999). Threshold level of oospore density of *Pythium aphanidermatum* and *P. spinosum* causing damping-off in spinach seedlings. *Soil Microorganisms, 53*(1), 53-56. https://doi.org/10.18946/jssm.53.1_53

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*(5), 1717-1729. https://doi.org/10.1007/s00425-019-03257-5

International Seed Testing Association (ISTA). (2021). International Rules for Seed Testing. *International Seed Testing Association, Bassersdorf, Switzerland*(1), i-19-18. https://doi.org/10.15258/istarules.2021.F

Jalink, 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 Science Research, 8*(4), 437-443. https://doi.org/10.1017/S0960258500004402

Joosen, R. V. L., Kodde, J., Willems, L. A. J., Ligterink, W., Van Der Plas, L. H. W., & Hilhorst, H. W. M. (2010). Germinator: A software package for high-throughput scoring and curve fitting of *Arabidopsis* seed germination. *Plant Journal, 62*(1), 148-159. https://doi.org/10.1111/j.1365-313X.2009.04116.x

Junttila, O. (1976). Germination Inhibitors in Fruit Extracts of Red Beet (*Beta vulgaris* cv. *rubra*. *Journal of Experimental Botany, 27*(4), 827-836. https://doi.org/10.1093/jxb/27.4.827

Kadereit, G., Mavrodiev, E. V., Zacharias, E. H., & Sukhorukov, A. P. (2010). Molecular phylogeny of Atripliceae (Chenopodioideae, Chenopodiaceae): implications for systematics, biogeography, flower and fruit evolution, and the origin of C4 photosynthesis. *American Journal of Botany, 97*(10), 1664-1687. https://doi.org/10.3732/ajb.1000169

Kamoun, S., Huitema, E., & Vleeshouwers, V. G. A. A. (1999). Resistance to oomycetes: a general role for the hypersensitive response? *Trends in Plant Science, 4*(5), 196-200. https://doi.org/10.1016/s1360-1385(99)01404-1

Katahira, R., Elder, T. J., & Beckham, G. T. (2018). Chapter 1: A brief introduction to lignin structure. In *Lignin Valorization: Emerging Approaches* (pp. 1-20). https://doi.org/10.1039/9781788010351-00001

Katzman, L. S., Taylor, A. G., & Langhans, R. W. (2001). Seed enhancements to improve spinach germination. *HortScience, 36*(5), 979-981. https://doi.org/10.21273/HORTSCI.36.5.979

Kear, E., Albright, L. D., de Villiers, D. S., Langhans, R. W., Katzman, L. S., Johnson, C., & Uchigasalci, M. (2005). Seed germination and seedling production. In *A Commercially Viable Controlled Environment Agriculture (CEA) Spinach Production System* (pp. 1-55). *Cornell University, Department of Biological and Environmental Engineering*. Ithaca.

Klepacka, J., Gujska, E., & Michalak, J. (2011). Phenolic compounds as cultivar- and variety-distinguishing factors in some plant products. *Plant Foods for Human Nutrition, 66*(1), 64-69. https://doi.org/10.1007/s11130-010-0205-1

Klepadlo, M., Balk, C. S., Vuong, T. D., Dorrance, A. E., & Nguyen, H. T. (2019).

Molecular characterization of genomic regions for resistance to *Pythium ultimum* var. *ultimum* in the soybean cultivar Magellan. *Theoretical and Applied Genetics, 132*(2), 405-417. https://doi.org/10.1007/s00122-018-3228-x

Klisiewicz, J. M. (1968). Relation of *Pythium* spp. to root rot and damping-off of safflower. *Phytopathology, 58*(10), 1384-1386.

Koike, S. T., Cahn, M., Cantwell, M., Fennimore, S., Lestrange, M., Natwick, E., Smith, R. F., & Takele, E. (2011). *Spinach production in California* (Publication Number 7212).

Kraft, J. M., Haware, M. P., Halila, H., Sweetingham, M., & Bayaa, B. (2000). Soilborne diseases and their control. In *Linking Research and Marketing Opportunities for Pulses in the 21st Century* (pp. 457-466). *Springer*. https://doi.org/10.1007/978-94-011-4385-1_42

Kumar, J., Kaiser, W. J., & Hannan, R. M. (1991). Damping-off resistance in chickpeas. *Plant Disease, 75*(12), 1244-1245.

Lamichaney, A., Katiyar, P. K., Natarajan, S., & Sripathy, K. V. (2016). Relationship among some seed characters, laboratory germination and field emergence in chickpea (*Cicer arietinum* L.) genotypes differing in testa colour. *Journal of Food Legumes, 29*(1), 29-32.

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. *Agronomy for Sustainable Development, 37*(10), 1-25. https://doi.org/10.1007/s13593-017-0417-y

Lamichhane, J. R., Debaeke, P., Steinberg, C., You, M. P., Barbetti, M. J., & Aubertot, J.-N. (2018). Abiotic and biotic factors affecting crop seed germination and seedling emergence: a conceptual framework. *Plant and Soil, 432*(1), 1-28. https://doi.org/10.1007/s11104-018-3780-9

Lammerts van Bueren, E., Struik, P., & Jacobsen, E. (2003). Organic propagation of seed and planting material: an overview of problems and challenges for research. *NJAS: Wageningen Journal of Life Sciences, 51*(3), 263-277. https://doi.org/10.1016/S1573-5214(03)80019-2

Lammerts van Bueren, E. T., Struik, P. C., & Jacobsen, E. (2002). Ecological concepts in organic farming and their consequences for an organic crop ideotype. *NJAS - Wageningen Journal of Life Sciences, 50*(1), 1-26. https://doi.org/10.1016/S1573-5214(02)80001-X

Larsson, M. (1994). Prevalence and pathogenicity of spinach root pathogens of the genus *Pythium* in Sweden. *Plant Pathology, 43*(2), 261-268. https://doi.org/10.1111/j.1365-3059.1994.tb02684.x

Larsson, M., & Olofsson, J. (1994). Prevalence and pathogenicity of spinach root pathogens of the genera *Aphanomyces*, *Phytophthora*, *Fusarium*, *Cylindrocarpon*,

and *Rhizoctonia* in Sweden. *Plant Pathology, 43*(2), 251-260. https://doi.org/10.1111/j.1365-3059.1994.tb02683.x

Laufer, B. (1919). *Sino-Iranica: Chinese contributions to the history of civilization in ancient Iran: with special reference to the history of cultivated plants and products*. Field Museum of Natural History. Chicago, IL.

Leach, L. D. (1947). Growth rates of host and pathogen as factors determining the severity of preemergence damping-off. *Journal of Agricultural Research, 75*(5, 6), 161-179. https://naldc-legacy.nal.usda.gov/catalog/IND43970169

Leskovar, D. I., Esensee, V., & Belefant-Miller, H. (1999). Pericarp, leachate, and carbohydrate involvement in thermoinhibition of germinating spinach seeds. *Journal of the American Society for Horticultural Science, 124*(3), 301-306. https://doi.org/10.21273/JASHS.124.3.301

Lopez-Velasco, G., Carder, P. A., Welbaum, G. E., & Ponder, M. A. (2013). Diversity of the spinach (*Spinacia oleracea*) spermosphere and phyllosphere bacterial communities. *FEMS Microbiology Letters, 346*(2), 146-154. https://doi.org/10.1111/1574-6968.12216

Lucier, G., Allshouse, J. E., & Lin, B.-H. (2004). *Factors affecting spinach consumption in the United States*. US Department of Agriculture, Economic Research Service.

Luterbacher, M. C., Asher, M. J. C., Beyer, W., Mandolino, G., Scholten, O. E., Frese, L., Biancardi, E., Stevanato, P., Mechelke, W., & Slyvchenko, O. (2005). Sources of resistance to diseases of sugar beet in related *Beta* germplasm: II. Soil-borne diseases. *Euphytica, 141*(1), 49-63. https://doi.org/10.1007/s10681-005-5231-y

Luthar, Z. (2018). Chapter 14: Buckwheat genetic resources in Central Europe. In *Buckwheat Germplasm in the World* (pp. 127-143). *Elsevier*. https://doi.org/10.1016/B978-0-12-811006-5.00014-8

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 Science and Technology, 48*(1), 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. (2021). Evaluation of damping-off tolerance in spinach cultivars in field soils and in a standardized lab assay with *Pythium ultimum*. *Plant Health Progress, 23*(2), *in press*. https://doi.org/10.1094/PHP-07-21-0107-RS

Martin, F. N., & Hancock, J. G. (1987). The use of *Pythium oligandrum* for biological control of preemergence damping-off caused by *P. ultimum*. *Phytopathology, 77*(7), 1013-1020.

Matthews, S. (1971). A study of seed lots of peas (*Pisum sativum* L.) differing in predisposition to pre-emergence mortality in soil. *Annals of Applied Biology, 68*(2), 177-183. https://doi.org/10.1111/j.1744-7348.1971.tb06454.x

Mavi, K. (2010). The relationship between seed coat color and seed quality in watermelon Crimson sweet. *Horticultural Science, 37*(2), 62-69.

Mazzola, M., Hewavitharana, S., Muramoto, J., & Shennan, C. (2012). Application sequence and soil biology influence anaerobic soil disinfestation induced disease suppression. Proceedings of International Research Conference on Methyl Bromide Alternatives, Vol. 57, 1-3.

Meadows, I., Sharpe, S., & Henson, M. (2020). *Damping-off in Flower and Vegetable Seedlings*. Retrieved in January 2022 from https://content.ces.ncsu.edu/damping-off-in-flower-and-vegetable-seedlings

Meemken, E.-M., & Qaim, M. (2018). Organic agriculture, food security, and the environment. *Annual Review of Resource Economics, 10*, 39-63. https://doi.org/10.1146/annurev-resource-100517-023252

Meghvansi, M. K., & Varma, A. (2015). *Organic Amendments and Soil Suppressiveness in Plant Disease Management* (Vol. 46). Springer. Switzerland.

Mirzaei, M. R., & Rajabi, A. (2021). Relationship of seed pericarp color with seed quality in sugar beet (*Beta vulgaris* L. var. *altissima* Döll). *Genetic Resources and Crop Evolution, 68*(5), 2093-2105. https://doi.org/10.1007/s10722-021-01120-9

Mohamed-Yasseen, Y., Barringer, S. A., Splittstoesser, W. E., & Costanza, S. (1994). The role of seed coats in seed viability. *Botanical Review, 60*(4), 426-439. https://doi.org/10.1007/Bf02857926

Morelock, T. E., & Correll, J. C. (2008). Spinach. In *Vegetables I: Asteraceae, Brassicaceae, Chenopodicaceae, and Cucurbitaceae* (pp. 189-218). J. Prohens & F. Nuez (Eds.). *Springer New York*. New York, NY. https://doi.org/10.1007/978-0-387-30443-4_6

Morris, P. C., Grierson, D., & Whittington, W. J. (1984). Endogenous inhibitors and germination of *Beta vulgaris*. *Journal of Experimental Botany, 35*(7), 994-1002. https://doi.org/10.1093/jxb/35.7.994

Munnecke, D. E., Moore, B. J., & Abu-El-Haj, F. (1971). Soil moisture effects on control of *Pythium ultimum* or *Rhizoctonia solani* with methyl bromide. *Phytopathology, 61*, 194-197.

Naiki, T., & Kanoh, M. (1978). Root diseases of spinach in plastic house cropping and their pathogens. *Japanese Journal of Phytopathology, 44*(5), 543-553. https://doi.org/10.3186/jjphytopath.44.543

Naiki, T., Gonda, Y., & Kageyama, K. (1986). *Pythium* species causing damping-off of spinach seedlings under plastic-house cropping. *Japanese Journal of Phytopathology, 52*(5), 772-778. https://doi.org/10.3186/jjphytopath.52.772

Namayanja, A., Msolla, S. N., Buruchara, R., & Namusoke, A. (2014). Genetic analysis of resistance to *Pythium* root rot disease in common bean (*Phaseolus vulgaris* L.) genotypes. *Journal of Crop Improvement, 28*(2), 184-202. https://doi.org/10.1080/15427528.2013.863815

Navarro-Acevedo, K. A., Wijeratne, S., Culman, S. W., Benitez, M.-S., & Dorrance, A. E. (2021). The effect of incubation temperature on the species composition of *Phytophthora*, *Phytopythium*, and *Pythium* communities associated with soybean. *Phytobiomes Journal*, PBIOMES-01. https://doi.org/10.1094/PBIOMES-01-20-0016-R

Nelson, E. B., & Craft, C. M. (1989). Comparative germination of culture-produced and plant-produced sporangia of *Pythium ultimum* in response to soluble seed exudates and exudate components. *Phytopathology, 79*(10), 1009-1013.

Nelson, E. B. (2018). The seed microbiome: Origins, interactions, and impacts. *Plant and Soil, 422*(1), 7-34. https://doi.org/10.1007/s11104-017-3289-7

Nesi, N., Jond, C., Debeaujon, I., Caboche, M., & Lepiniec, L. (2001). The Arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *The Plant Cell, 13*(9), 2099-2114.

Neubert, A. B., Lühs, W., Baetzel, R., & Friedt, W. (2003). Influence of seed colour on seed vigour in *Brassica napus*. Proceedings of the 11th International Rapeseed Congress, Vol., 253-256.

Oksana, K., & Artur, L. (2019). Biological activity of endometabolites from sugar beet (*Beta vulgaris* L.) pericarps. *Journal of Microbiology, Biotechnology and Food Sciences, 8*(6), 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 Science and Technology, 39*(1), 140-150. https://doi.org/10.15258/sst.2011.39.1.12

Onodera, Y., Yonaha, I., Masumo, H., Tanaka, A., Niikura, S., Yamazaki, S., & Mikami, T. (2011). Mapping of the genes for dioecism and monoecism in *Spinacia oleracea* L.: evidence that both genes are closely linked. *Plant Cell Reports, 30*(6), 965-971. https://doi.org/10.1007/s00299-010-0998-2

Osburn, R. M., & Schroth, M. N. (1988). Effect of osmopriming sugar beet seed on exudation and subsequent damping-off caused by *Pythium ultimum*. *Phytopathology, 78*(9), 1246-1250.

Ozimek, E., & Hanaka, A. (2020). *Mortierella* species as the plant growth-promoting fungi present in the agricultural soils. *Agriculture, 11*(1), 1-18.

Panagos, P., Van Liedekerke, M., Jones, A., & Montanarella, L. (2012). European Soil Data Centre: Response to European policy support and public data requirements. *Land Use Policy, 29*(2), 329-338. https://doi.org/10.1016/j.landusepol.2011.07.003

Pane, C., Spaccini, R., Piccolo, A., Scala, F., & Bonanomi, G. (2011). Compost amendments enhance peat suppressiveness to *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia minor*. *Biological Control, 56*(2), 115-124.

Parlevliet, J. E. (1967). The influence of external factors on the growth and development

of spinach cultivars (*Spinacia oleracea* L.). https://edepot.wur.nl/284960

Paul, B. (2002). *Pythium terrestris*, a new species isolated from France, its ITS region, taxonomy and its comparison with related species. *FEMS Microbiology Letters, 212*(2), 255-260. https://doi.org/10.1111/j.1574-6968.2002.tb11275.x

Paulitz, T. C., & Baker, R. (1987). Biological control of *Pythium* damping-off of cucumbers with *Pythium nunn*: Population dynamics and disease suppression. *Phytopathology, 77*(2), 335-340.

Paulitz, T. C., & Bélanger, R. R. (2001). Biological control in greenhouse systems. *Annual Review of Phytopathology, 39*(1), 103-133. https://doi.org/10.1146/annurev.phyto.39.1.103

Peksen, A., Peksen, E., & Bozoglu, H. (2004). Relationships among some seed traits, laboratory germination and field emergence in cowpea (*Vigna unguiculata* (L.) Walp.) genotypes. *Pakistan Journal of Botany, 36*(2), 311-320.

Perry, D. A. (1973). Infection of seeds of *Pisum sativum* by *Pythium ultimum*. *Transactions of the British Mycological Society, 61*(1), 135-144. https://doi.org/10.1016/S0007-1536(73)80095-6

Plaats-Niterink, A. J. (1981). Monograph of the genus *Pythium*. *Studies in Mycology, 21*.

Platenius, H. (1943). Effect of oxygen concentration on the respiration of some vegetables. *Plant Physiology, 18*(4), 671–684. https://doi.org/10.1104/pp.18.4.671

Powell, A. L. T., Nguyen, C. V., Hill, T., Cheng, K. L., Figueroa-Balderas, R., Aktas, H., Ashrafi, H., Pons, C., Fernández-Muñoz, R., & Vicente, A. (2012). Uniform ripening encodes a Golden 2-like transcription factor regulating tomato fruit chloroplast development. *Science, 336*(6089), 1711-1715. https://doi.org/10.1126/science.1222218

Prior, R. L., & Cao, G. (2000). Antioxidant phytochemicals in fruits and vegetables: diet and health implications. *HortScience, 35*(4), 588-592.

Raaijmakers, M., Bruszik, A., & Sommer, M. (2021). *Policy Brief: A national roadmap towards 100% organic seed by 2036*. LIVESEED, Bionext Netherlands, & IFOAM Organics Europe. https://www.liveseed.eu/wp-content/uploads/2021/09/Policy-brief-RoadMap-National-Authorities_final_compressed-4.pdf

Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C., & Job, D. (2012). Seed germination and vigor. *Annual Review of Plant Biology, 63*, 507-533. https://doi.org/10.1146/annurev-arplant-042811-105550

Ren, Y., He, Q., Ma, X., & Zhang, L. (2017). Characteristics of color development in seeds of brown-and yellow-seeded heading Chinese cabbage and molecular analysis of Brsc, the candidate gene controlling seed coat color. *Frontiers in Plant Science, 8*, 1-9. https://doi.org/10.3389/fpls.2017.01410

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*(3), 310-320. https://doi.org/10.1080/11956860.2004.11682838

Reyes, A. A. (1977). Spinach wilt in Ontario. *Plant Disease Reporter, 61*(12), 1067-1070.

Ribera, A., van Treuren, R., Kik, C., Bai, Y., & Wolters, A.-M. A. (2021). On the origin and dispersal of cultivated spinach (*Spinacia oleracea* L.). *Genetic Resources and Crop Evolution, 68*(3), 1023-1032. https://doi.org/10.1007/s10722-020-01042-y

Robideau, G. P., De Cock, A. W. A. M., Coffey, M. D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D. W., Désaulniers, N., Eggertson, Q. A., & Gachon, C. M. M. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular Ecology Resources, 11*(6), 1002-1011. https://doi.org/10.1111/j.1755-0998.2011.03041.x

Røeggen, O. (1984). Effect of temperature and moisture on germination in spinach (*Spinacia oleracea* L.). *Scientia Horticulturae, 24*(3), 221-229. https://doi.org/10.1016/0304-4238(84)90105-5

Rosso, M. L., Rupe, J. C., Chen, P., & Mozzoni, L. A. (2008). Inheritance and genetic mapping of resistance to *Pythium* damping-off caused by *Pythium aphanidermatum* in 'Archer' soybean. *Crop Science, 48*(6), 2215-2222. https://doi.org/10.2135/cropsci2008.01.0035

Roy, B. A., & Kirchner, J. W. (2000). Evolutionary dynamics of pathogen resistance and tolerance. *Evolution, 54*(1), 51-63. https://doi.org/10.1111/j.0014-3820.2000.tb00007.x

RStudio Team. (2020). *RStudio: Integrated Development Environment for R*. from http://www.rstudio.com/

Rubarth Apparate GmbH. (2020). *The Seed-Line – ideally applicable for systematic ISTA compliant seed tests: The Rodewald Method*. Rubarth Apparate GmbH. from https://www.rumed.de/en/saatgut-linie/

Rubatzky, V. E., & Yamaguchi, M. (1997). Chapter 21: Spinach, Table Beets, and Other Vegetable Chenopods. In *World Vegetables: Principles, Production, and Nutritive Values* (pp. 457-473). *Springer US*. Boston, MA. https://doi.org/10.1007/978-1-4615-6015-9_21

Saeidi, G., & Rowland, G. G. (1999). The effect of temperature, seed colour and linolenic acid concentration on germination and seed vigour in flax. *Canadian Journal of Plant Science, 79*(3), 315-319. https://doi.org/10.4141/P98-100

Salimi, Z., & Boelt, B. (2019a). Optimization of germination inhibitors elimination from sugar beet (*Beta vulgaris* L.) seeds of different maturity classes. *Agronomy, 9*(11), 763-773. https://doi.org/10.3390/agronomy9110763

Salimi, Z., & Boelt, B. (2019b). Classification of processing damage in sugar beet (*Beta vulgaris*) seeds by multispectral image analysis. *Sensors, 19*(10), 2360-2368. https://doi.org/10.3390/s19102360

Sano, N., Rajjou, L., North, H. M., Debeaujon, I., Marion-Poll, A., & Seo, M. (2015). Staying alive: molecular aspects of seed longevity. *Plant and Cell Physiology, 57*(4), 660-674. https://doi.org/10.1093/pcp/pcv186

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry, 30*(12), 3875-3883.

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*(8), 1186-1191.

Schmitthenner, A. F. (1970). Significance of populations of *Pythium* and *Phytophthora* in soil. In *Root Diseases and Soil-borne Pathogens* (pp. 25-27). *University of California Press*. https://doi.org/10.1525/9780520339941-008

Scholten, O. E., Panella, L. W., De Bock, T. S. M., & Lange, W. (2001). A greenhouse test for screening sugar beet (*Beta vulgaris*) for resistance to *Rhizoctonia solani*. *European Journal of Plant Pathology, 107*(2), 161-166. https://doi.org/10.1023/A:1011208903344

Schrijver, A. J. M., Stam, E. L. M., van Dorp, J., & Zutt, N. A. (2019). *Pericarp free Spinacia oleracea seeds.* Patent number: EP3462845A1. from https://worldwide.espacenet.com/patent/search/family/056121043/publication/EP3462845A1?q=pn%3DEP3462845A1

Sealy, R. L., Kenerley, C. M., & McWilliams, E. L. (1988). Evaluation of Amaranthus accessions for resistance to damping-off by *Pythium myriotylum*. *Plant Disease, 72*, 985-989.

Sherry, R. A., Eckard, K. J., & Lord, E. M. (1993). Flower development in dioecious *Spinacia oleracea* (Chenopodiaceae). *American Journal of Botany, 80*(3), 283-291. https://doi.org/10.1002/j.1537-2197.1993.tb13801.x

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. *Journal of Chemometrics, 26*(8-9), 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. *Annals of Botany, 41*(163), 557-568. https://www.jstor.org/stable/43237080

Simko, I., Hayes, R. J., Mou, B., & McCreight, J. D. (2014). Chapter 4: Lettuce and Spinach. In *Yield Gains in Major U.S. Field Crops* (pp. 53-86). *American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc.* Madison, WI. https://doi.org/10.2135/cssaspecpub33.c4

Smolikova, G. N., Laman, N. A., & Boriskevich, O. V. (2011). Role of chlorophylls and carotenoids in seed tolerance to abiotic stressors. *Russian Journal of Plant Physiology, 58*(6), 965-973.

Sneep, J. (1958). The present position of spinach breeding. *Euphytica, 7*(1), 1-8.

Somers, D. J., Rakow, G., Prabhu, V. K., & Friesen, K. R. D. (2001). Identification of a major gene and RAPD markers for yellow seed coat colour in *Brassica napus*. *Genome, 44*(6), 1077-1082. https://doi.org/10.1139/g01-097

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. *Journal of Agronomy and Crop Science, 207*(6), 1024-1040. https://doi.org/10.1111/jac.12559

Stanghellini, M. E., & Hancock, J. G. (1971). The sporangium of *Pythium ultimum* Trow as a survival structure in soil. *Phytopathology, 61*, 157-164.

Stasz, T. E., & Harman, G. E. (1980). Interactions of *Pythium ultimum* with germinating resistant or susceptible pea seeds. *Phytopathology, 70*(1), 27-31. https://doi.org/10.1094/Phyto-70-27

Stasz, T. E., Harman, G. E., & Marx, G. A. (1980). Time and site of infection of resistant and susceptible germinating pea seeds by *Pythium ultimum*. *Phytopathology, 70*(8), 730-733.

Steckel, J. R., Gray, D., & Rowse, H. R. (1989). Relationships between indices of seed maturity and carrot seed quality. *Annals of Applied Biology, 114*(1), 177-183. https://doi.org/10.1111/j.1744-7348.1989.tb06798.x

Suganuma, N., & Ohno, H. (1984). Role of pericarp in reducing spinach (*Spinacia oleracea* L.) seed germination at supra-optimal temperatures. *Journal of the Japanese Society for Horticultural Science, 53*(1), 38-44. https://doi.org/10.2503/jjshs.53.38

Sumner, D. R., Kays, S. J., & Johnson, A. W. (1976). Etiology and control of root diseases of spinach. *Phytopathology, 66*(11), 1267-1273.

Taylor, R. J., Salas, B., Secor, G. A., Rivera, V., & Gudmestad, N. C. (2002). Sensitivity of North American isolates of *Phytophthora erythroseptica* and *Pythium ultimum* to mefenoxam (metalaxyl). *Plant Disease, 86*(7), 797-802. https://doi.org/10.1094/PDIS.2002.86.7.797

Tekale, A. G., Guldekar, D. D., Kendre, V. P., Deshmukh, A. P., & Potdukhe, S. R. (2019). Efficacy of fungicides and bioagents against damping off in chilli caused by *Pythium aphanidermatum*. *International Journal of Current Microbiology and Applied Sciences, 8*(6), 637-648.

TeKrony, D. M., & Egli, D. B. (1991). Relationship of seed vigor to crop yield: a review. *Crop Science, 31*(3), 816-822. https://doi.org/10.2135/cropsci1991.0011183X003100030054x

TeKrony, D. M., & Hunter, J. L. (1995). Effect of seed maturation and genotype on seed vigor in maize. *Crop Science, 35*(3), 857-862. https://doi.org/10.2135/cropsci1995.0011183X003500030037x

Toshiyuki, I. (1992). Seed Peeling Apparatus. European Patent Application, Patent

No. 0478181A1. https://worldwide.espacenet.com/publicationDetails/biblio?CC=EP&NR=0478181A1&KC=A1&FT=D&ND=4&date=19920401&DB=&locale=en_EP#

Trimboli, D. S. (1977). *Fusarium* wilt of spinach. *Australian Plant Pathology Society Newsletter, 6*(3), 44-44.

Tsimogiannis, D., Samiotaki, M., Panayotou, G., & Oreopoulou, V. (2007). Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS. *Molecules, 12*(3), 593-606. https://doi.org/10.3390/12030593

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. https://www.unep.org/resources/report/handbook-montreal-protocol-substances-deplete-ozone-layer-seventh-edition-2006

United States Department of Agriculture (USDA). (2019). *2017 Census of Agriculture. 2019 Organic Survey.* (AC-17-SS-4). (Certified Organic Farm Data, table 2: Certified organic vegetables grown in the open harvested and value of sales: 2019 Issue October 2020). Volume 3. Special Studies. Part 4. Pages 27-28. *N. A. S. Service*. https://www.nass.usda.gov/Publications/AgCensus/2017/Online_Resources/Organics/ORGANICS.pdf

United States Department of Agriculture (USDA). (2020). *Vegetables 2019 Summary* (ISSN: 0884-6413). February 2020). Pages 72-73. *N. A. S. Service*. https://www.nass.usda.gov/Publications/Todays_Reports/reports/vegean20.pdf

United States Department of Agriculture (USDA). (2021). *FoodData Central*. Retrieved in November 2021 from https://fdc.nal.usda.gov/fdc-app.html#/?query=spinach

United States Environmental Protection Agency (EPA). (2021). *Methyl Bromide*. Retrieved in January 2022 from https://www.epa.gov/ods-phaseout/methyl-bromide

Urrea Romero, K. E. (2015). *Pythium*: Characterization of Resistance in Soybean and Population Diversity. *Theses and Dissertations, 1272*. http://scholarworks.uark.edu/etd/1272

Uzuhashi, S., Tojo, M., & Kakishima, M. (2010). Phylogeny of the genus *Pythium* and description of new genera. *Mycoscience, 51*(5), 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*(1), 81-87. https://www.jstor.org/stable/26625375

Wadlington, W. H., & Ming, R. (2018). Development of an X-specific marker and identification of YY individuals in spinach. *Theoretical and Applied Genetics, 131*(9), 1987-1994. https://doi.org/10.1007/s00122-018-3127-1

Ward, K., Scarth, R., Vessey, J. K., & Daun, J. K. (1995). Chlorophyll degradation in summer oilseed rape and summer turnip rape during seed ripening. *Canadian Journal of Plant Science, 75*(2), 413-420. https://doi.org/10.4141/cjps95-069

Webb, D. P., & Agnihotri, V. P. (1970). Presence of a fungal inhibitor in the pericarps of *Acer saccharum* fruits. *Canadian Journal of Botany, 48*(12), 2109-2116. https://doi.org/10.1139/b70-305

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Chapter 38: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: A Guide to Methods and Applications* (pp. 315-322). *Academic Press, Inc.*

Willan, R. L. (1985). Chapter 9: Seed testing. In *A guide to forest seed handling*. *Food and Agriculture Organization (FAO) of the United Nations*. Rome. http://www.fao.org/3/ad232e/AD232E09.htm#ch9

Williams, G. E., & Asher, M. J. C. (1996). Selection of rhizobacteria for the control of *Pythium ultimum* and *Aphanomyces cochlioides* on sugar-beet seedlings. *Crop Protection, 15*(5), 479-486. https://doi.org/10.1016/0261-2194(96)00014-2

Yamamoto, K., Oda, Y., Haseda, A., Fujito, S., Mikami, T., & Onodera, Y. (2014). Molecular evidence that the genes for dioecism and monoecism in *Spinacia oleracea* L. are located at different loci in a chromosomal region. *Heredity, 112*(3), 317-324. https://doi.org/10.1038/hdy.2013.112

Yasin, M., & Andreasen, C. (2016). Effect of reduced oxygen concentration on the germination behavior of vegetable seeds. *Horticulture, Environment, and Biotechnology, 57*(5), 453-461. https://doi.org/10.1007/s13580-016-0170-1

Yokoe, K., Maesaka, M., Murase, J., & Asakawa, S. (2015). Solarization makes a great impact on the abundance and composition of microbial communities in soil. *Soil Science and Plant Nutrition, 61*(4), 641-652. https://doi.org/10.1080/00380768.2015.1048183

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 Science and Technology, 46*(2), 407-417. https://doi.org/10.15258/sst.2018.46.2.22

Zhang, X. K., Chen, J., Chen, L., Wang, H. Z., & Li, J. N. (2008). Imbibition behavior and flooding tolerance of rapeseed seed (*Brassica napus* L.) with different testa color. *Genetic Resources and Crop Evolution, 55*, 1175-1184. https://doi.org/10.1007/s10722-008-9318-x

Zitnick-Anderson, K. K., & Nelson Jr, B. D. (2015). Identification and pathogenicity of *Pythium* on soybean in North Dakota. *Plant Disease, 99*(1), 31-38. https://doi.org/10.1094/PDIS-02-14-0161-RE

Summary

Spinacia oleracea L. (spinach) is an economically important, highly nutritious leafy vegetable. During the past twenty years, global spinach production has increased fourfold, mainly due to the growing demand for the freshly consumed young leaves, also known as baby-leaf spinach. At the same time, there is a growing demand for organically produced crops, and, in general, chemical treatments are increasingly banned due to public opposition and potential negative effects on environmental and human health. These developments have revealed weak points in management of diseases, such as damping-off diseases in spinach production. Damping off includes non-emergence of seedlings (pre-emergence damping off) and wilting of seedlings or plants after emergence (post-emergence damping off). Damping off is an even greater problem in the production of baby-leaf spinach, which requires uniform stands within a very short production cycle of a maximum of six weeks, and the frequent and dense sowing increases disease pressure. Damping off can be caused by multiple soilborne pathogens, including the fungi *Fusarium* spp. and *Rhizoctonia* spp., and oomycetes *Aphanomyces* spp., *Phytophthora* spp. and *Pythium* spp. Generally, the severity of damping off depends on the host plant, the pathogen, and abiotic field conditions. Field measures, such as good soil drainage and sowing at optimal temperatures to promote rapid seedling emergence, can partly limit damping-off occurrence. However, once the pathogen has been established in the soil, these measures are not sufficient and, since chemical treatments are increasingly banned, alternative solutions are urgently needed. This dissertation focuses on solutions at the plant level, exploring the potential of plant breeding and seed vigour enhancement to improve pre-emergence damping-off tolerance levels in spinach. More background on spinach and damping off, the knowledge gaps and studied research questions are provided in **Chapter 1**.

In **Chapter 2**, the moisture sensitivity of spinach seeds in the absence of the pathogen was described. The spinach seed is botanically an indehiscent fruit with a 'true seed' surrounded by a fruit wall, called the pericarp. Based on literature, the hypothesis was that seed size, pericarp thickness and pericarp intactness play a role in spinach seed germination in relation to high moisture conditions. Seeds from multiple seed lots of commercial cultivars and from one seed lot fractionated into different seed sizes were sown in a floating germination system that was developed to create different standardised moisture levels. At a high moisture level, the total germination and germination rate differed among cultivars and seed lots of the same cultivars, and between larger and smaller seeds of the same seed lot. Larger seeds (>3.50 mm), having a thicker pericarp, germinated less at high moisture level compared to smaller seeds, which had a thinner pericarp and, more frequently, openings in the pericarp. The sensitivity to high moisture

conditions was reduced by germinating spinach seeds under lower temperatures or elevated oxygen levels, or by removing the pericarp from the seed, also called dehulling. These results indicate that the pericarp interferes with oxygen availability to the embryo. Based on this study, a germination system with a standardised low moisture level and a temperature of 15°C to 20°C is recommended to determine the germination potential of differently-sized spinach seeds.

In **Chapter 3**, the aim is to elucidate whether genotypic variation in damping-off tolerance exists in spinach, which is necessary for breeding purposes. Eight commercial spinach cultivars, with two or three seed lots of each, were screened for seedling emergence and for post-emergence damping off in fields with a history of spinach cultivation. Large variation was found among field locations, replicates, and among seed lots of the same cultivars. The same seed lots were screened in the greenhouse with the use of field soil obtained from one of the fields with severe damping-off occurrence. Large variation was still observed among replicates and seed lots of the same cultivars. These results emphasised the need for a more standardised phenotyping assay. The pathogen of interest, *Pythium ultimum*, was chosen based on its abundance in spinach fields with damping off and its pathogenicity on spinach. The development eventually resulted in an assay with stackable trays and individual cells, suitable for individual seed sowing (50 seeds per tray), placed in a climate-controlled cabinet at 15°C and in the dark. The cells were filled with a relatively dry substrate that consisted of sand, perlite, and vermiculite in equal volume ratios, moistened until 50% water holding capacity. Seeds were sown in this substrate as a control treatment and in the same substrate inoculated with a cornmeal/sand-based inoculum of a pathogenic *P. ultimum* isolate. This enabled reliable evaluation of *P. ultimum* tolerance levels of seed lots by correcting for non-emergence in the control treatment that was not due to infection. The assay was developed specifically to assess pre-emergence damping-off tolerance for two reasons. Pre-emergence damping off seemed to be more discriminating for differences among cultivars than post-emergence damping off in field and greenhouse trials, and it was hypothesised that there is a relationship with seed vigour, e.g., the ability of seeds to germinate fast and uniformly under optimal and stressful conditions. The assay showed reproducible results for pre-emergence damping-off tolerance levels, but variation in tolerance levels among cultivars could not be confirmed due to larger variation among seed lots, even among seed lots of the same cultivars.

In **Chapter 4**, the relationship between emergence rate and pre-emergence damping-off tolerance is evaluated. The hypothesis was that enhanced rates of germination and emergence would improve tolerance levels. By pre-treating seeds using priming and dehulling methods, the rate and uniformity of seed germination and seedling emergence

increased. Primed seed lots showed an increased rate of seedling emergence and improved pre-emergence damping-off tolerance compared to untreated seed lots. The variation among seed lots and cultivars was reduced and damping-off tolerance levels increased. After dehulling, some seed lots had an increased rate of seedling emergence and improved damping-off tolerance, while other seed lots from the same cultivars showed only one of the two effects. The results indicated that rate of germination and emergence is not the only driver of pre-emergence damping-off tolerance. Priming and dehulling may also result in improved damping-off tolerance through other mechanisms, such as enhanced anti-pathogenic activity or increased oxygen availability to the seed embryo, which needs further investigation. A positive correlation between emergence rate and damping-off tolerance of seed lots could not be confirmed, possibly due to other seed lot differences, such as vigour-related seed traits.

In **Chapter 5**, spinach seed lots are characterised for several seed traits, including seed size, maturity, and pericarp colour. Only a small part of the variation in pre-emergence damping-off tolerance levels among seeds lots could be attributed to differences in seed size and pericarp colour. Large variation in damping-off tolerance levels could not be explained, possibly due to seed lot production differences. The large variation among and within seed lots urged to zoom in on the individual seed level. Prior to sowing, untreated seeds were measured individually for morphological and spectral seed traits, including seed size and roundness, chlorophyll fluorescence level (which is correlated negatively with seed maturity), and light reflectance at various wavelengths, ranging from visible to near-infrared light. By sowing the seeds in individual cells of the developed damping-off assay, it was possible to relate the measured seed traits to seedling emergence from each individual seed. Generally, more mature seeds showed higher *P. ultimum* tolerance than less mature seeds. Of those seeds, the smaller seeds and the seeds with a darker pericarp were more tolerant than the larger seeds and lighter-coloured seeds. The light reflectance in the violet to blue light spectrum showed the strongest negative association with *P. ultimum* tolerance. According to the literature, seed pericarp colour may be associated with the presence of phenolic compounds that can have multiple biological activities, including inhibition of seed germination and anti-pathogenic effects. Further studies are required to investigate the association between pericarp colour and specific secondary metabolites, and to understand their role in damping-off tolerance of spinach seeds.

In **Chapter 6**, the main findings of the four research chapters are discussed in relation to each other and to existing literature. Based on the knowledge that was gained on specific seed traits and treatments of spinach seeds that related to improved damping-off tolerance levels, recommendations for further research and development are provided. In conclusion, to develop spinach cultivars with improved tolerance to *P. ultimum*, seed

vigour of spinach seeds should be enhanced. According to this study, preferable traits are a smaller seed size, thinner pericarp, and darker pericarp in addition to high levels of seed maturity. Further research is needed to confirm a genetic base for one or more of these traits, which is necessary for breeding purposes. In the meantime, seeds can be pre-selected for these traits, or they can be pre-treated by dehulling (removal of the pericarp) or priming (increasing their germination rate). A combination of damping-off tolerant cultivars, post-harvest seed selection or seed treatments, and site-specific field measures will provide more sustainable control of damping off in both organic and conventional spinach production.

Samenvatting

Spinacia oleracea L. (spinazie) is een economisch belangrijk en zeer voedzaam bladgewas. De afgelopen twintig jaar is de wereldwijde spinazieproductie verviervoudigd, vooral door de groeiende vraag naar de vers geconsumeerde jonge bladeren, ook wel 'baby-leaf' spinazie genoemd. Tegelijkertijd is er een groeiende vraag naar biologisch geproduceerde gewassen dat het gebruik van synthetisch chemische middelen verbiedt, terwijl deze middelen worden gebruikt in de conventionele productie, o.a. om ziektes te onderdrukken. In het algemeen worden chemische behandelingen van zaden en velden in toenemende mate verboden vanwege de mogelijk negatieve effecten op het milieu en de menselijke gezondheid. Deze ontwikkelingen hebben ertoe geleid dat 'damping off' een toenemend probleem is in de spinazieteelt. Damping off, ook wel 'wegval ziekte', omvat het niet opkomen van zaailingen (pre-opkomst damping off) en de verwelking van zaailingen of planten na opkomst (post-opkomst damping off). Damping off is een nog groter probleem bij de productie van baby-leaf spinazie vanwege de vereisten van een uniforme opkomst binnen een zeer korte productiecyclus van maximaal zes weken na zaaien. Daarnaast zorgt het frequente en dichte zaaien voor een verhoogde ziektedruk. Damping off kan worden veroorzaakt door meerdere bodempathogenen, waaronder de schimmels *Fusarium* spp. en *Rhizoctonia* spp., en schimmelachtigen (ook wel 'oomyceten') *Aphanomyces* spp., *Phytophthora* spp. en *Pythium* spp. Over het algemeen hangt de ernst van de ziekte af van de waardplant, de ziekteverwekker en abiotische veldomstandigheden. Veldmaatregelen, zoals een goede bodemdrainage en zaaien bij optimale temperaturen om een snelle opkomst van zaailingen te bevorderen, kunnen het optreden van damping off deels beperken. Als de ziekteverwekker zich eenmaal in de bodem heeft gevestigd, zijn deze maatregelen echter niet voldoende en omdat chemische behandelingen steeds vaker worden verboden, zijn alternatieve oplossingen dringend nodig. Dit proefschrift richt zich op oplossingen op plantniveau, waarbij mogelijkheden van plantenveredeling en verbeterde zaadkracht (ook wel 'seed vigour') wordt onderzocht om met name pre-opkomst damping-off tolerantieniveaus in spinazie te verbeteren. Meer achtergrondinformatie over spinazie en damping-off ziekte, de kennishiaten en bestudeerde onderzoeksvragen vindt u in **Hoofdstuk 1**.

In **Hoofdstuk 2** wordt beschreven welke zaadeigenschappen betrokken zijn bij de vochtgevoeligheid van spinaziezaden in afwezigheid van de ziekteverwekker. Het spinaziezaad is botanisch gezien een niet-splijtende vrucht met een 'echt zaadje' omgeven door een vruchtwand, het 'pericarp'. Gebaseerd op de literatuur was de hypothese dat zaadgrootte, pericarpdikte en pericarpintactheid een rol spelen bij het ontkiemen van spinaziezaden in interactie met het vochtgehalte. Zaden van meerdere zaadpartijen van commerciële rassen ('cultivars') en van één zaadpartij gefractioneerd in verschillende

zaadgroottes, werden gezaaid op een drijvend kiemsysteem dat we hadden ontwikkeld om verschillende gestandaardiseerde vochtgehaltes te creëren. Bij een hoog vochtgehalte verschilde de totale kieming en kiemsnelheid tussen rassen en tussen zaadpartijen van dezelfde rassen, en tussen de grotere en kleinere zaden van dezelfde zaadpartij. Grotere zaden (>3.50 mm), die tevens een dikker pericarp bevatte, vertoonden een lagere kiemkracht bij een hoog vochtgehalte in vergelijking met kleinere zaden, die tevens een dunner pericarp met meer openingen bevatte. De gevoeligheid voor hoge vochtgehaltes werd verminderd door de spinaziezaden te laten ontkiemen bij lagere temperaturen of bij verhoogde zuurstofgehaltes, of door het pericarp van het echte zaadje te verwijderen, ook wel pellen ('dehulling') genoemd. Deze resultaten geven aan dat het pericarp de beschikbaarheid van zuurstof voor het zaadembryo kan belemmeren. Op basis van dit onderzoek wordt aanbevolen om een kiemsysteem te gebruiken met een gestandaardiseerd laag vochtgehalte en een temperatuur van 15°C tot 20°C om het kiempotentieel van spinaziezaden van verschillende groottes te bepalen.

In **Hoofdstuk 3** wordt bekeken of er genotypische variatie in damping-off tolerantie bestaat bij spinazie dat noodzakelijk is voor veredeling. Acht commerciële spinazierassen, met elk twee of drie zaadpartijen, werden getest op opkomst van zaailingen en op damping off na opkomst in velden met een geschiedenis van spinazieteelt. Er werd een grote variatie gevonden tussen veldlocaties, herhalingen en tussen zaadpartijen van dezelfde rassen. Dezelfde zaadpartijen werden getoetst in de kas met behulp van natuurlijke grond van een van de velden met ernstige ziektedruk. Ook hier werd een grote variatie waargenomen tussen herhalingen en zaadpartijen van dezelfde rassen. Deze resultaten benadrukten de noodzaak van een meer gestandaardiseerde toets. De pathogeen, *Pythium ultimum*, werd gekozen op basis van zijn hoge aanwezigheid in spinazievelden en zijn ziekteverwekkend vermogen in spinazie. De ontwikkeling van deze toets resulteerde in een opstelling met stapelbare bakjes met individuele cellen, geschikt voor individueel zaaien (50 zaden per bakje), geplaatst in een kweekkast bij 15°C en in het donker. De cellen werden gevuld met een relatief droog substraat dat bestond uit zand, perliet en vermiculiet in gelijke volumeverhoudingen, bevochtigd tot 50% watervasthoudend vermogen. Zaden werden gezaaid in dit substraat als controlebehandeling en in hetzelfde substraat dat was geïnoculeerd met een op maïsmeel en zand gebaseerd inoculum met het gekozen *P. ultimum* isolaat. Deze opstelling maakte een betrouwbare evaluatie mogelijk van *P. ultimum* tolerantie in zaadpartijen van spinazie doordat er gecorrigeerd kon worden voor het niet opkomen van zaailingen in de controlebehandeling dat niet het gevolg was van infectie. De toets was speciaal ontwikkeld om de tolerantie voor pre-opkomst damping off te beoordelen vanwege twee redenen. Pre-opkomst damping off leek meer onderscheidend te zijn voor verschillen tussen rassen dan post-opkomst damping off in veld- en kasproeven, en de verwachting was dat er een verband is met zaadkracht,

zoals het vermogen van zaden om snel en uniform te ontkiemen onder optimale en stressvolle omstandigheden. De toetsingsmethode vertoonde herhaalbare resultaten in tolerantieniveaus, maar een variatie in tolerantieniveaus tussen rassen kon niet worden bevestigd vanwege een grotere variatie tussen zaadpartijen, zelfs tussen zaadpartijen van dezelfde rassen.

In **Hoofdstuk 4** wordt de relatie tussen opkomstsnelheid en pre-opkomst damping-off tolerantie onderzocht. De hypothese was dat een verhoogde snelheid van kieming en opkomst de tolerantie zal verbeteren. Door de zaden voor te behandelen met behulp van ontwikkelde methoden van priming (voorkiemen) of dehulling (pellen), nam de snelheid en uniformiteit van zaadkieming en opkomst van zaailingen toe. Geprimede zaadpartijen vertoonden een verhoogde snelheid van opkomst van zaailingen en verhoogde tolerantieniveaus voor pre-opkomst damping off vergeleken met onbehandelde zaadpartijen. De variatie tussen zaadpartijen, maar ook tussen rassen nam af bij de toegenomen tolerantieniveaus, waardoor de verschillen tussen rassen niet duidelijker werden. Na het pellen vertoonden sommige zaadpartijen een verhoogde snelheid van opkomst en een verbeterd tolerantieniveau, terwijl andere zaadpartijen van dezelfde rassen slechts één van de twee effecten vertoonden. De resultaten geven aan dat de snelheid van kieming en opkomst niet de enige oorzaak is van pre-opkomst damping-off tolerantie. Priming en dehulling geven mogelijk ook verbeterde tolerantieniveaus via andere mechanismen, zoals een verhoogde anti-pathogene werking of verhoogde zuurstofbeschikbaarheid voor het zaadembryo, wat verder onderzoek behoeft. Een positieve correlatie tussen opkomstsnelheid en tolerantieniveaus van zaadpartijen kon niet worden bevestigd, mogelijk vanwege andere verschillen tussen zaadpartijen, zoals zaadkracht-gerelateerde kenmerken.

In **Hoofdstuk 5** worden de verschillende zaadpartijen gekarakteriseerd op basis van verschillende zaadkenmerken, waaronder zaadgrootte, rijpheidsniveau en vruchtwandkleur. Slechts een klein deel van de variatie in pre-opkomst damping-off tolerantie tussen zaadpartijen kon worden toegeschreven aan verschillen in zaadgrootte en vruchtwandkleur. Een grote variatie in tolerantieniveaus kon niet worden verklaard, mogelijk als gevolg van verschillen in de productie van zaadpartijen. De grote variatie tussen en binnen zaadpartijen noopte tot inzoomen op individueel zaadniveau. Voorafgaand aan het zaaien werden onbehandelde zaden afzonderlijk gemeten op morfologische en spectrale zaadkenmerken, waaronder zaadgrootte en rondheid, chlorofylfluorescentieniveau (wat negatief correleert met zaadrijpheid) en lichtreflectie bij verschillende golflengten, variërend van zichtbaar tot bijna-infrarood licht. Door de zaden in individuele cellen van de ontwikkelde toets te zaaien, was het mogelijk om de gemeten zaadeigenschappen te relateren aan de zaailingopkomst van elk afzonderlijk

zaadje. Over het algemeen vertoonden rijpere zaden een hogere *P. ultimum* tolerantie dan minder rijpe zaden. Van deze zaden waren de kleinere zaden en de zaden met een donkerdere vruchtwand toleranter dan de grotere en lichter gekleurde zaden. De lichtreflectie in het violet tot blauwe lichtspectrum had de meest negatieve associatie met *P. ultimum* tolerantie. Volgens de literatuur kan de kleur van het pericarp worden geassocieerd met de aanwezigheid van fenolische verbindingen die meerdere biologische activiteiten kunnen hebben, waaronder remming van zaadkieming en anti-pathogene effecten. Verdere studies zijn nodig om het verband tussen pericarpkleur en specifieke secundaire metabolieten te onderzoeken, en om hun rol bij damping-off tolerantie van spinaziezaden te begrijpen.

In **Hoofdstuk 6** worden de belangrijkste bevindingen van de vier onderzoekshoofdstukken in relatie tot elkaar en met behulp van bestaande literatuur besproken. Op basis van de kennis die is opgedaan, over specifieke zaadeigenschappen en behandelingen van spinaziezaden die relateren aan verbeterde ziektetolerantieniveaus, worden aanbevelingen gedaan voor verder onderzoek en productie van spinaziezaden. Concluderend, om spinazierassen te verkrijgen met een verbeterde tolerantie voor *P. ultimum* kan de zaadkracht van spinaziezaden worden verbeterd. Volgens deze studie zijn eigenschappen die de voorkeur hebben, naast zaadrijpheid, een kleinere zaadgrootte, dunnere vruchtwand en donkerdere vruchtwand. Verder onderzoek is nodig om een genetische basis voor een of meer van deze eigenschappen te bevestigen voor veredelingsdoeleinden. In de tussentijd kunnen zaden worden voorgeselecteerd op basis van deze eigenschappen of ze kunnen worden voorbehandeld door pellen (verwijderen van de vruchtwand) of primen (verhogen van kiemsnelheid). Een combinatie van ziektetolerante rassen, zaadselectie of zaadbehandelingen na zaadoogst, en locatie-specifieke veldmaatregelen zal zorgen voor een duurzame beheersing van damping-off ziekten in zowel de biologische als conventionele spinazieteelt.

Acknowledgements

For a long time, I have been looking forward to the moment I could write these words of thanks. Now that the time has come, it appears to be more difficult than expected to write them. Since I had troubles expressing my feelings in English, I wrote the majority in Dutch and for the non-Dutch, I wrote the English words of thanks in italics. Due to Corona in the past two years, working almost entirely from home, I am also afraid I forget to mention a lot of nice people that I met and that supported me during the first years. *Therefore, I would like to thank everyone who supported me or contributed directly or indirectly to this dissertation in advance, including those I have not mentioned explicitly by name.*

Op 16 april 2016 begon ik mijn PhD carrière met veel enthousiasme in een team van toegewijde begeleiders die mij vanaf de eerste ontmoeting een vertrouwd gevoel gaven. Zeer dankbaar ben ik voor de kans die zij mij gegeven hebben om mij te ontplooien tot wie ik nu ben. Mijn promotor Edith wil ik bedanken voor haar onvoorwaardelijke support en het delen van haar ervaringen als onderzoekster en als persoon. Zoals zij dat mooi verwoordde, heeft het traject mijn schil dikker gemaakt. Ik ben trots dat ik een van haar laatste PhD kandidaten mocht zijn. Olga wil ik bedanken voor haar deur die altijd open stond om te sparren over het onderzoek, maar ook over persoonlijke zaken. Heel fijn om af en toe een spiegel voor te krijgen. Joeke wil ik bedanken voor haar enorme kennis van biologische interacties tussen planten en micro-organismen en haar creatieve ideeën. Ook bij haar kon ik altijd terecht. Steven wil ik bedanken voor zijn onuitputtelijke bron van adviezen omtrent het onderzoek aan zaden, zijn snelle redeneren en altijd kritische blik. Van hem leerde ik hoe waardevol de meest kritische opmerkingen zijn en hoe je perfectionisme het beste inzet. *Besides my supervisors from WUR, I would like to gratefully acknowledge the people from Pop Vriend and Sakata that contributed to my project financially and in-kind with lab and greenhouse work. Thank you very much, Jan de Visser, Johan Rijk, Anita van Nieuwenhoven, Suxian Zhu, Noriyuki Onozuka, Bill Johnson, Ryo Kimura, and Sabrina Chandler, for all your input and support despite the complexity of the research.*

Zoals bij elk PhD traject in meer of mindere mate het geval is, heb ik zowel hoogte- als dieptepunten ervaren. Een van de hoogtepunten was het spinaziecongres in Murcia in februari 2018 waar Olga en ik samen naartoe gingen. Daar heb ik met trots ons project gepresenteerd aan een grote groep spinazietelers en -onderzoekers en zijn we naar proefvelden geweest om verschillende variëteiten en valse meeldauw van spinazie te zien. Het was een erg leuke ervaring, behalve dan die ochtend dat we heel vroeg de taxi moesten hebben naar het vliegveld. Andere hoogtepunten waren uiteraard de

publicaties, al werd ons geduld hiervoor ook veel op de proef gesteld. In een tijdsbestek van anderhalf jaar waren er helaas ook een aantal dieptepunten. De complexiteit van het onderzoek maakte deze tijd zeker niet gemakkelijker. Ik ben mijn teamgenoten enorm dankbaar dat zij mij de tijd en ruimte hebben gegeven voor de verwerking hiervan om daarna met voldoende energie mijn PhD mooi af te kunnen ronden.

Tijdens het onderzoek zijn er een aantal mensen geweest die mij erg hebben geholpen bij de praktische uitvoering. Jan, heel erg bedankt voor jouw praktische hulp, ideeën en gezelligheid in het lab. Betty, Mirjam, Trudy, Gerrie, hartelijk dank voor jullie hulp tijdens het inzetten van proeven, bij het zoeken naar materialen (zelfs op plekken waar ik het laatste jaar het bestaan nog niet van wist), en uiteraard ook voor de gezelligheid en fijne gesprekken. Gerrit en Jan, enorm bedankt voor jullie oneindige hulp bij de statistische analyses en de leerzame discussies hieromtrent. Marc, bedankt voor jouw hulp met de sequentiedata van de isolaten. José, bedankt dat ik zomaar af en toe een stapel PDA platen mocht halen. Zo fijn dat er zulke mensen zijn die het leven van een PhD student gemakkelijker maken, waaronder ook Gerrit, Johan, Taede en Wim van Unifarm, bedankt. *I would like to sincerely thank my students, Jinwook, Nathalie and Ranthi for their experimental help, support, and for increasing the fun of my job. My sincere thanks also go to the former students, Arjan and Yuji, for their studies that gave me already some useful directions at the start of my PhD.*

I would like to gratefully thank all other colleagues from Plant Breeding, including my office mates and lunch buddies, colleagues from Bio-Interactions and Plant Health, colleagues from BioScience, and my PhD fellows and coordinators from the PE&RC graduate school for supporting me during this PhD trajectory. Micaela, as Edith's other last PhD candidate, it was a pleasure meeting you and I appreciate a lot your support and hospitality. Thank you so much for sharing our experiences in research and life, and for your help with some spinach facts from the USA for my introduction. Lorena, Viviana, and Ying, thank you for sharing our ups and downs and for having nice times at Doppio, although we still have a few cups of tea to catch up. Makrina, thank you for being such an inspiring and positive person who gave me a lot of support in the final stretch of my PhD, and thank you for being my paranymph. Jasper, jij was vanaf dag 1 mijn maatje waarmee ik alles kon delen onder het genot van een koffie of thee, taartje (met twee vorkjes) of tijdens een wandelingetje over de campus. Ik ben trots dat we elkaars paranimfen konden zijn.

Muziek was de beste afleiding voor mij tijdens het gehele traject. Ik vond het dan ook erg leuk dat we met een aantal collega's van verschillende afdelingen binnen de Plant Sciences Group een band hadden opgericht, "The Evergreens". Iedereen die hierbij zat enorm bedankt voor de gezellige tijd en gave optredens die we hebben gedaan. Daarnaast

ben ik lid van OBK Bennekom en heb ik nog een tijdje djembé gespeeld. Alle mensen die ik hier heb leren kennen, wil ik graag bedanken voor de afleiding en oneindige support. Ook heb ik tijdens mij PhD ontdekt dat yoga goed voor mij is. Herma, Jolanda, Isabelle, en Eva, jullie hebben mij op een fijne manier kennis laten maken met zowel actieve als rustige vormen van yoga waar ik de rest van mijn leven profijt van zal hebben, waarvoor mijn grote dank. Verschillende mensen hebben mij geholpen om door de moeilijkere tijden te komen. Petra, bedankt voor je inzichten en handvatten om mijn gedachtes en lijf beter te begrijpen. Ton, bedankt dat je mij hebt laten inzien wat ik kan bereiken op een manier die gezonder is voor mijzelf en mijn omgeving. Ik adviseer alle PhD studenten regelmatig met professionele mensen te praten die helemaal los staan van het onderzoek. Een PhD traject omvat namelijk niet alleen een wetenschappelijk onderzoek, maar ook een onderzoek naar jezelf.

Dankbaar ben ik voor al mijn vrienden die mij de tijd en steun hebben gegeven om dit proefschrift tot een goed einde te brengen. Ondanks de nodige lange-afstandsrelaties weet ik dat we regelmatig aan elkaar denken en als we elkaar dan treffen, dan is dat altijd als vanouds. Lieve vriendinnen uit Zuid-Limburg, bedankt voor jullie steun, de nodige afleiding, spontane berichtjes of bezoekjes, bloemetje of knuffel wanneer ik dat nodig had. Anke, bedankt voor onze onvoorwaardelijke vriendschap, jouw interesse in alles wat mij bezighoudt en eerlijke adviezen. Leonie, bedankt voor jouw interesse en steun op afstand, al hadden we elkaar liever wat vaker omhelsd. Mijn vrienden uit Nijmegen die ik heb leren kennen bij het studentenorkest QHarmony, waar ik ook Timo heb ontmoet, zijn tevens onze vrienden voor het leven. De gespreksonderwerpen zijn geleidelijk veranderd van orkest naar verhuizen naar baby's. De groepsuitbreiding maakt onze banden alleen maar sterker en de weekendjesweg nog gezelliger, alhoewel we wellicht een extra weekend zullen moeten inplannen om aan de spellen toe te komen. Lieve vrienden, enorm bedankt voor jullie liefdevolle steun en welgemeende interesse in mijn onderzoek.

Mijn familie en schoonfamilie wil ik van harte bedanken voor alle liefdevolle steun en gezellige afleiding als we samen waren, en voor jullie begrip dat ik niet zo vaak kon langskomen als ik zou willen. Lieve Petra en Frank, vanaf de allereerste ontmoeting voelde ik de warmte van jullie gezin en ik ben blij daar deel van uit te mogen maken. Jasper & Carmen en Iris, ik ben trots en dankbaar jullie schoonzus te mogen zijn. Ook ben ik dankbaar jullie opa en oma te hebben leren kennen. Lieve (schoon)oma, ik ben enorm trots op uw kracht en dankbaar voor de fijne momenten samen die we hopelijk nog veel vaker samen zullen beleven.

Lieve pap en mam, woorden kunnen niet omschrijven hoe groot mijn dank is naar jullie toe. Jullie hebben mij laten leren, studeren, geleidelijk aan een beetje los moeten laten en mijzelf laten ontdekken wat ik kan. Veel woorden hebben wij ook niet nodig. Ik ben trots op ons alle drie, wat we hebben bereikt en hoe wij nu in het leven staan, en ik weet dat we nog een hele mooie tijd samen tegemoet gaan. Jullie vaders en moeders, mijn opa's en oma's, zullen ook super trots op ons zijn. Ook ben ik hen zeer dankbaar voor alle liefde, de groene vingers en muzikale vaardigheden, die zij mij gegeven hebben.

Lieve Timo, jij wist al vanaf het begin dat het mij zou lukken, terwijl ik dat zelf vaak niet geloofde. Ik had bewijs, dit proefschrift, nodig voordat ik het kon geloven. Ik ben jou enorm dankbaar voor jouw liefde, zorgzaamheid en oneindige behulpzaamheid, zeker in de laatste fase, en voor het schrijven van mijn biografie. Ik ben trots op ons en zie erg uit naar ons volgende hoofdstuk als getrouwd PhD stel.

Biography

Kim Jacqueline Hubertine Magnée was born in Rothem (Meerssen, the Netherlands), on the 18th of December 1990. She obtained the VWO diploma at Stella Maris College in Nature & Health, after which she started her Bachelor of Science in Biology at Radboud University Nijmegen in 2009. During the subsequent Master of Science in Biology at the same university, she wrote a thesis on (a)biotic stress in *Solanum dulcamara* at the department of Ecogenomics, under the supervision of Prof. Dr Nicole van Dam and Dr Duy Nguyen. Additionally, she did a six-month internship at Bejo Zaden B.V. about seed treatments with micronutrients to enhance damping-off tolerance in carrot and onion, under supervision of Dr Liesbeth van der Heijden and Dr Ivo Rieu.

After graduating bene meritum, Kim pursued a second master's degree in Plant Sciences at Wageningen University, with specialisation Phytopathology & Entomology and minor Plant Breeding. She graduated in 2016 on a study at the Netherlands Institute for Ecology (NIOO-KNAW) on the effects of soil and caterpillar herbivory on native and invasive *Centaurea* plants, under supervision of Prof. Dr Wim van der Putten and Dr Olga Kostenko.

In April 2016, Kim was employed at the Plant Breeding group at Wageningen University. She started to work as a PhD candidate on plant-environment interactions for spinach seeds and *Pythium*, under supervision of an interdisciplinary team consisting of Dr Olga Scholten, Dr Steven Groot, Dr Joeke Postma and Prof. Dr Edith Lammerts van Bueren. This study was performed in collaboration with the seed and breeding companies Pop Vriend Seeds and Sakata. She enjoyed the teamwork and supervision of three master students.

Since February 2022, Kim has been working as a product development specialist in plant protection at Certis Europe. She lives in Ede, together with her fiancé Timo. In her free time, she plays the French horn and the piano, practices yoga, and loves to work in the garden.

Training and education statement

With the training and education activities listed below, resulting in a total of 44 ECTS, the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum of 32 ECTS (= 22 weeks of activities).

Review of literature (4.5 ECTS)

- Introduction of dissertation

Writing of project proposal (3 ECTS)

- A multidisciplinary approach to improve damping-off tolerance in spinach, *Spinacia oleracea* L.

Post-graduate courses (6.3 ECTS)

- Course JoinMap; WUR Plant Breeding (2016)
- Introduction to R for statistical analysis; PE&RC/SENSE (2016)
- Basic statistics; PE&RC/SENSE (2016)
- Advanced statistics course Design of experiments; PE&RC/WIAS (2016)
- 6th Workshop on the molecular aspects of seed dormancy and germination; International Society for Seed Science, Seed Lab Wageningen, WUR, Seed Valley (2019)
- Tidy data transformation and visualization with R; online; PE&RC (2020)

Laboratory training and working visits (0.2 ECTS)

- Sharing knowledge on spinach seed production and breeding; Pop Vriend Seeds (2017)
- Sharing knowledge on spinach crop production and breeding; Seminis/Bayer (2019)

Invited review of journal manuscripts (1 ECTS)

- HortTech: the physiological potential of spinach seeds in response to hydropriming (2016)
- APS Compendium on Spinach Diseases and Pests: spinach seed germination, quality and technology (2020)

Deficiency, refresh, brush-up courses (4 ECTS)

- Markers in quantitative genetics and plant breeding; WUR Plant Breeding (2020)

Competence strengthening / skills courses (4.7 ECTS)

- Competence assessment; Wageningen Graduate Schools (WGS) (2016)
- Scientific writing; WGS (2017)
- PhD workshop carousel; WGS (2017):
  - Supervising MSc students during their thesis
  - Brain efficiency
  - Essentials in time management
  - Jump-in theatre 'Dealing with stress'
- The choice; WGS (2020)
- Career orientation; WGS (2020)
- Brain-friendly working & writing; WGS (2020)

Scientific integrity / ethics in science activity (0.3 ECTS)

- Ethics in plant and environmental sciences; WGS (2018)

PE&RC Annual meetings, seminars or weekends (1.95 ECTS)

- PE&RC First-years weekend (2016)
- PE&RC Midterm weekend (2017)
- PE&RC Day 'Preventing the end of the world - How science can save the planet' (2017)
- PE&RC Last-years afternoon online (2020)

Discussion groups / local seminars or scientific meetings (4.1 ECTS)

- 'Studiekring plantenveredeling'; WUR Plant Breeding (2016)
- Plant-soil interaction discussion group; PE&RC (2016-2017)
- PhD Discussion club; WUR Plant Breeding (2017-2018)
- EPS Conference; poster presentation; Lunteren; graduate school Experimental Plant Sciences (EPS) (2017, 2019)
- Seminar at Impulse: 'Uncovering the hidden half of plants: revealing how roots adapt to water availability'; Ritzema Bos Lectures (2019)
- Plant microbiomes discussion group; PE&RC (2020-2021)

International symposia, workshops and conferences (5.4 ECTS)

- International spinach conference; oral presentation; Murcia, Spain; University of Arkansas (2018)
- Plant breeding and biotechnology symposium; oral and poster presentation; Wageningen, the Netherlands; Swedish University of Agricultural Sciences (SLU), WUR, PE&RC, EPS (2019)
- 21st EUCARPIA General congress; online; oral presentation; Rotterdam, the Netherlands; European Association for Research on Plant Breeding (2021)

Lecturing / supervision of practicals / tutorials (1.5 ECTS)

- Hosting the insect and nonhost resistance group meetings; WUR Plant Breeding (2019-2021)

BSc / MSc thesis supervision (7 ECTS)

- Jinwook Kim: testing moisture sensitivity of spinach seed germination
- Nathalie Blom: improving screening for damping-off tolerance in spinach
- Ranthi Whesi Umbarani: unravelling traits of spinach seeds that improve damping-off tolerance

The research described in this dissertation was funded through the Dutch Top consortium for Knowledge and Innovation (TKI) in horticulture and starting materials ("Tuinbouw en Uitgangsmaterialen", T&U), as part of the research program 'Green Breeding' with project number KV 1409-031. As partners in this research, the seed and breeding companies Pop Vriend Seeds and Sakata co-financed the research. Financial support from Wageningen University for the printing of the dissertation is gratefully acknowledged.

Artwork of cover and title pages:	Stijn Schreven
Layout:	Dennis Hendriks || ProefschriftMaken.nl
Printing:	ProefschriftMaken.nl

Printed on FSC certified and recycled paper.