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Spinach Downy Mildew: Advances in Our Understanding of the Disease Cycle and Prospects for Disease Management

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Fresh vegetable and fruit markets are rapidly expanding in the United States and Europe owing to increased awareness of the benefits of healthier diet, and the convenience of ready-to-eat salad packages or fruit platters (Rico et al. 2007; USDA-ERS 2017). Leafy greens such as lettuce (*Lactuca sativa* L., 2N=18), spinach (*Spinacia oleracea* L., 2N=12), kale (*Brassica oleracea* L., 2N=18), celery (*Apium graveolens* var. dulce, 2N=22), and others, compose the most consumed salad crops.

Spinach is typically grown for its edible dark green leaves and is cultivated in over 60 countries worldwide. China, the U.S.A., Iran, Japan, Turkey, and Indonesia are major spinach-producing countries. In the U.S.A., the majority of fresh market spinach production is located in the states of California and Arizona, followed by New Jersey and Texas (USDA-NASS 2016). The cool winters and mild summers make coastal California ideal for spinach production, where nearly 65% of the total U.S. spinach is produced (Koike et al. 2011; USDA-NASS 2016).

Spinach leaves have tender and soft textures; and contain high amounts of vitamin A and C, carotenoids, and antioxidant compounds (Cao et al. 1998; Eriksen et al. 2017; Tang et al. 2005). Spinach leaves are generally available at retail as fresh (bunched or bagged) or processed as frozen or canned spinach. Prewashed, ready-to-eat, young spinach leaves are consumed in salad, whereas bunched mature leaves are often used as an ingredient in cooked and steamed dishes. The recent greater demands for bagged salad industries have accelerated the production of baby leaf spinach in the U.S. and elsewhere. Likewise, the harvested acreage of spinach has also increased globally (FAOSTAT 2018). The per capita use of fresh spinach has increased from about 136 to 770 grams during the past 45 years (USDA-ERS 2017). However, like most crops, spinach production is hampered by biotic stresses from diseases, pests, and weed infestations, and abiotic stresses such as salinity, drought, and heat.

Downy mildew represents the major disease constraint on spinach production, decreasing both the quantity and quality of production worldwide (Correll et al. 2011; Klosterman 2016; Koike et al. 2011). Among the disease threats, downy mildew (*Peronospora effusa* (Grev.) Rabenh.), white rust (*Albugo occidentalis* G. W. Wilson), leaf spot diseases (*Colletotrichum dematium* (Pers.) Grove, *Cercospora beticola* Sacc., *Stemphyllium* sp.), seedling damping-off (*Pythium* spp.), and viral diseases are economically important (Correll et al. 2011).

In this article, we examine the disease cycle of *P. effusa* in the context of new research findings on oospore germination, seed infestation with oospores, and their role in initiating root infections, as well as recent highlights from our work examining windborne sporangia in coastal California using spore trap systems. This is particularly important as the type and role of primary inoculum has been an unresolved topic for many decades. These findings have shed additional light on the epidemiology of *P. effusa*, and have increased our knowledge base, allowing new and improved disease management approaches.

The Pathogen–Nomenclature and Host Range

The oomycete pathogen, *P. effusa*, has been observed in commercial spinach fields worldwide (Correll et al. 2011; Eriksson 1918; Feng et al. 2018b; Frinking and van der Stoel 1987; Inaba and Morinaka 1984; Klosterman et al. 2014; Nino et al. 2009; Richards 1939; Qian et al. 2016). *P. effusa* was first reported in 1824 (Greville 1824) and its morphology was described in 1885. The detailed life cycle of *P. effusa* was documented by Jakob Eriksson in 1918 (Eriksson 1918) and others (Richards 1939; Smith 1885). Smith (1885) suspected that *P. effusa* might infect multiple plant species belonging to family Chenopodiaceae and Polygonaceae. Subsequent attempts to cross-inoculate weedy plants and taxonomically related crops

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including *Amaranthus* spp., *Artiplex* spp., *Allenrolfea occidentalis*, beets and swiss chard, *Bassia* spp., *Chenopodium* spp., *Salsola pestifer*, Epazote, Lambsquarters, and Nettleleaf goosefoot were unsuccessful (Byford 1967; Klosterman et al. 2014; Richards 1939). The available evidence therefore strongly suggests that the host range of *P. effusa* is limited to spinach. Such host specificity has also been observed in pathogens causing downy mildew on hop (*Pseudoperonospora humuli*), basil (*P. belbahrii*), sunflower (*Plasmopara halstedi*), and soybean (*P. manshurica*) (Gascuel et al. 2015; Gent et al. 2017; Homa et al. 2016; Roongruangsree et al. 1988). Host range limitations have historically guided the naming conventions of plant pathogenic *Peronospora* species. In older literature, and even in some of the extant literature, *P. farinosa* f. sp. *spinaciae* continues to be used as the causal agent of downy mildew of spinach. But the species name "farinosa" had been ascribed to an unrelated genus originally (*Atriplex*) and is no longer valid as a species name for *Peronospora* (Choi and Thines 2014; Thines and Choi 2016). Recent phylogenetic and morphological analyses further suggest that *P. effusa* is indeed a distinct species (Choi and Thines 2014; Choi et al. 2007) that is restricted to spinach.

Disease Cycle of *Peronospora effusa*

The disease cycle of *P. effusa* involves both asexual and sexual reproductive phases (Fig. 1). During asexual reproduction, sporangia are formed on branched sporangiophores emerging from stomata primarily on the abaxial surface of leaves, dislodged by wind or rain splash, and released into the environment. Sporangia land on receptive foliar spinach tissue to cause infection. The symptoms may begin at the cotyledon stage and progress rapidly in true leaves when nighttime temperature and humidity favor for the excessive dew formation on leaves. Moisture on leaf surfaces, cool temperature, and high relative humidity favor sporangial germination and infection (Choudhury et al. 2016; Klosterman 2016). Gray to brown sporangial masses are produced during the night, which become airborne during the day and disperse. Each sporangium has the potential to initiate new leaf infections on plants after short- or long-distance dispersal. Symptoms of irregular chlorotic spots (Fig. 2e), along with signs of the masses of sporangia and sporangiophores on the underside of the leaf are visible 7 to 10 days following inoculation. Symptomatic plants are not suitable for fresh market and asymptomatic plants in which the pathogen remains in the latent period (Subbarao et al. 2018) may show sporulation or tissue damage during postharvest storage.

Sexual reproduction. Two different mating types (P1 and P2) are required for sexual reproduction in *P. effusa* (Inaba and Morinaka 1984; Van Asch and Frinking 1988); the mating of the two results in nuclear fusion and genetic recombination. The outcome of the sexual union is the formation of thick-walled oospores, which have been observed within infected leaves and seeds (Figs. 3 and 4). Oospores are formed by fertilizing oogonia by antheridia; the female and male gametangia respectively (Figs. 4a and 4b). Eriksson (1918) observed both gametangia and documented them through detailed camera lucida drawings. An intrinsic hormonal system influencing the formation of oogonia and antheridia and their union has been described

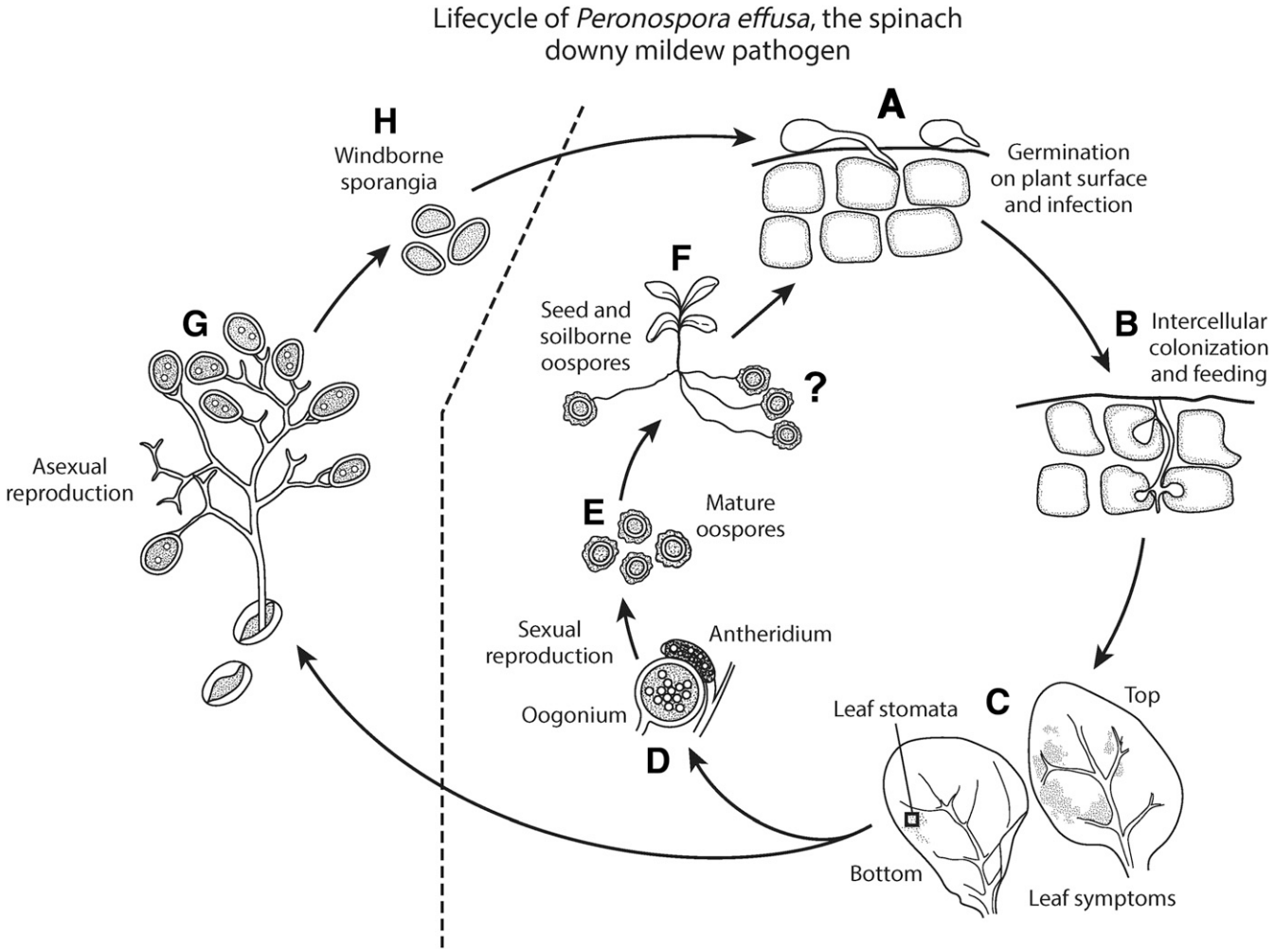

Fig. 1. Disease cycle of *Peronospora effusa* on spinach. (**A**) Germination and direct infection by sporangia and/or oospores; (**B**) intercellular colonization and haustorium formation; (**C**) symptoms on leaves occur 7 to 10 days after infection and include chlorotic areas on upper side of leaf and downy light gray to brownish sporulation on the underside of leaves; (**D**) oogonium coming in contact with antheridium resulting in sexual reproduction; (**E**) mature oospores in leaves and seeds; (**F**) root infection by germinating oospores (the question mark denotes whether infection from germinating oospores can occur as it has not been experimentally demonstrated); (**G**) branched sporangiophores, bearing oval-shaped sporangia, emerge from stomata in leaves; (**H**) sporangia become wind-borne and are disseminated.

in *Phytophthora* pathogens of crop plants (Judelson 2009, 2017). The host preference according to mating type identity of *Pseudoperonospora cubensis* was reported earlier, but the mechanisms regulating this phenomenon remain undiscovered (Cohen et al. 2013; Thomas et al. 2017b). It is not clear whether signaling determines the sexual orientation of gametangia or the host preference by different mating type isolates of *P. effusa*. The oospores of *P. effusa* are spherical with an outer casing of extramatrical material beyond the oogonial wall that sometimes appears wrinkled. The mature oospores are chocolate brown with an average diameter of nearly 37 μm (Choi et al. 2007; Kunjeti et al. 2016b; Richards 1939; Yerkes and Shaw 1959). As opposed to asexually produced spore masses observed on leaf surfaces, oospores are not visible to the naked eye, but can be observed under a microscope, where they are clearly seen below the epidermal layer of the infected leaf (Fig. 4c).

Sexual recombination, as evidenced by the production of oospores in a heterothallic organism such as *P. effusa*, can generate extensive genetic variation in a pathogen population. Thus, the progeny of oospores may acquire the new virulence and can trigger new epidemics in unaffected spinach plantations. For this reason, it remains critical to understand the role of the oospores in the disease cycle. Oospores potentially play a critical role in the disease cycle of *P. effusa* in two major ways: i) by serving as the primary inoculum to initiate disease in the field, and ii) by fostering genetic diversity within pathogen populations. Correll et al. (2011) reported an exponential appearance of new races of *P. effusa* over the past 20 years, a condition that is likely exacerbated by sexual recombination that has taken place in *P. effusa* populations. Also, the rapidly evolving populations of *P. effusa* have been recently documented in California and Arizona, the two major spinach production sites in the U.S. (Lyon et al. 2016). Therefore, our recent efforts have focused on understanding the oospore viability as well as the optimum conditions for their production, survival, and germination to guide effective long-term strategies for disease management.

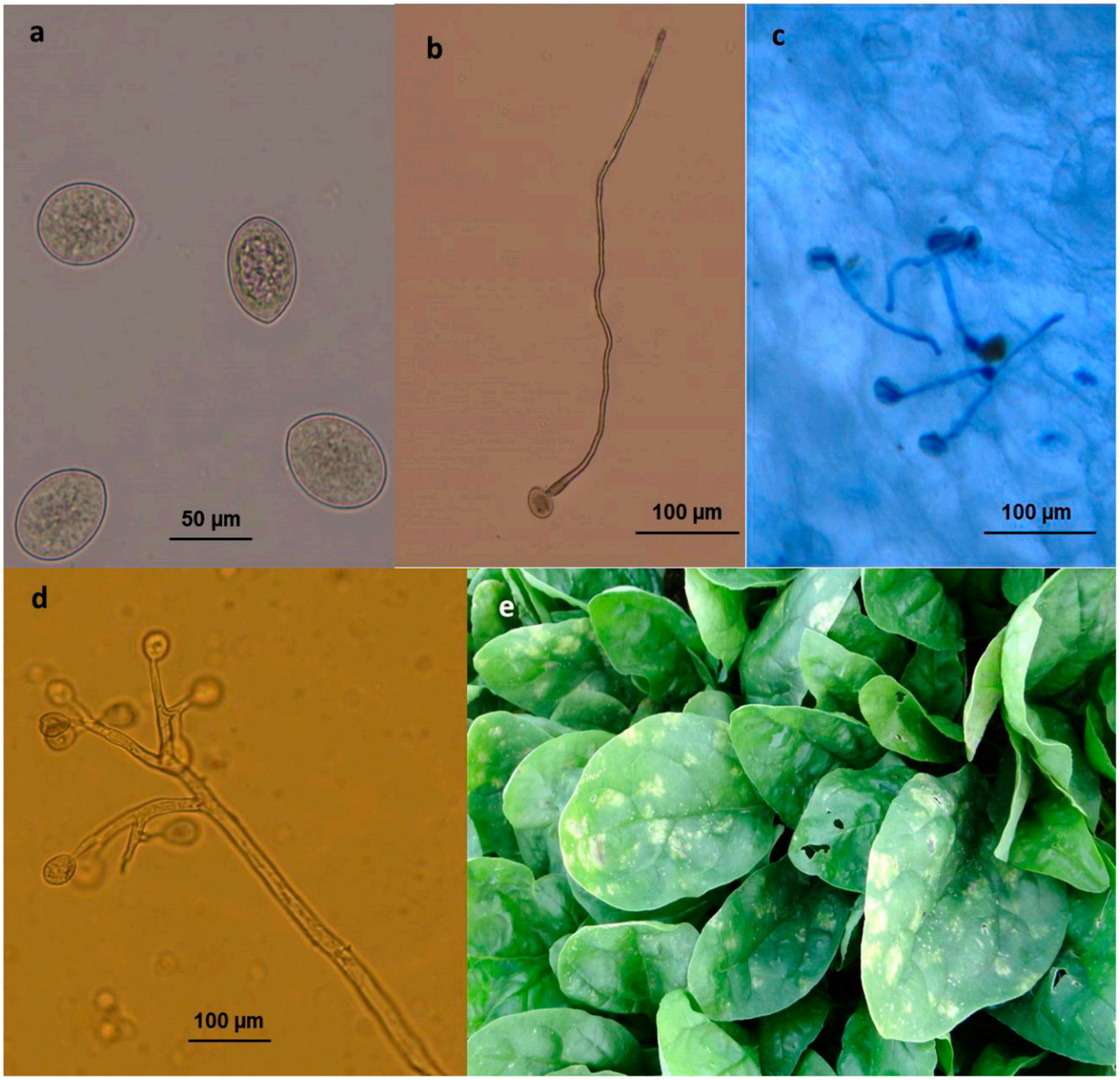

Fig. 2. The asexual phase of *Peronospora effusa*. (**a**) Sporangia are round to oval (21 to 33 µm length and 18 to 23 µm width), and brown (Choi et al. 2007); (**b**) germinated sporangium and a single unbranched coenocytic germ tube from a sporangium on water agar (1.5%); (**c**) germinated sporangia on spinach (cultivar Viroflay) leaf stained with 0.01% trypan blue (Gibco by Life Technologies); (**d**) young sporangiophore with terminal sporangia, and (**e**) spinach (cultivar Viroflay) plants with downy mildew symptoms in an experimental field plot in Salinas, CA.

Production and dispersal of oospores. The presence of oospores in spinach seeds and leaves has been reported in different spinach production areas around the world (Inaba et al. 1983; Smith 1885; Van Asch and Frinking 1988), and thus has likely been a contributing factor in the observed genetic changes in different populations of *P. effusa*. Frinking et al. (1985) determined the weather variables and growth conditions that influence oospore production in spinach using detached leaf assays and seedling inoculation assays under growth chamber and field conditions.

Sporangia collected from different isolates belonging to race 3 were inoculated on detached leaves and seedlings. In detached cotyledons, oospores were produced in nearly 40% of observed cotyledons in 3-week-old seedlings, and 35 and 17% in undetached cotyledons of 4- and 3-week-old growth chamber and field grown seedlings, respectively. Moderate humidity (about 70 to 80%), cool night temperature (15 ± 5°C), and sunny days favored oospore formation in spinach. In some instances, oospores may not form due to exclusion of one or the other mating type or failure to provide suitable conditions for gametogenesis (Richards 1939). The extent to which oospores of *P. effusa* form in spinach leaves in commercial production fields requires further scrutiny. One or both mating types may be excluded in resistant cultivars, as it is established that the status of host resistance influences oospore formation in *Phytophthora infestans* (Turkensteen et al. 2000). Higher numbers of oospores were reported in potato cultivars with moderate levels of late blight resistance in comparison of resistant and susceptible cultivars (Hanson and Shattock 1998; Hermansena et al. 2000). In spinach, no significant influence of cultivars on oospore formation was reported (Frinking et al. 1985), but this information for modern spinach cultivars is unavailable.

Dispersal of plant pathogens through infested/infected seed is a major concern in agriculture owing to the prospect of introducing exotic pathogens into new areas (Choudhury et al. 2017; Fry and Goodwin 1997; Gascuel et al. 2015; Maciel et al. 2014). Due to the specific photoperiod requirement for bolting and seed maturation, spinach seed production is concentrated in western Washington, Denmark, The Netherlands, New Zealand, and more recently in Chile. Seed is then distributed to different fresh market spinach production regions worldwide. It is therefore important to document the environmental variables and the mating type identity of *P. effusa* isolates in seed production regions. In a recent evaluation of 82 commercial spinach seed lots, oospores were recovered from 16% of the lots (Kunjeti et al. 2016b). The presence of *P. effusa* in these commercial seed lots was also confirmed through PCR or qPCR assays, demonstrating that 95% of seed lots evaluated were contaminated with DNA of *P. effusa* (Kunjeti et al. 2016b). Because 95% of the seed lots tested were PCR-positive, this finding indicates further investigations may be warranted to assess the numbers of oospores further.

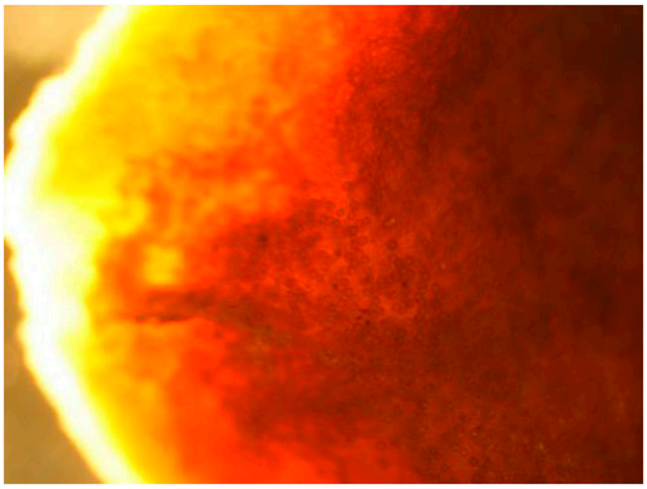

Fig. 3. Hundreds of circular oospores of *Peronospora effusa* shown on the inner layer of spinach seed pericarp section.

In the U.S., it is estimated that approximately 200 commercial seed lots are produced for planting each year. During last five years, examination of 168 commercial seed lots revealed that nearly 20% of the seed lots were infested with oospores (Klosterman et al., *unpublished data*). Together, this indicates that only about 15% of total seed lots produced per year were examined for oospore infestation. Further seed examination is likely to provide better estimates of the extent of infested seed that is distributed for spinach production. Figure 3 illustrates the numbers of oospores found on the interior surface of a spinach seed pericarp from a contemporary seed lot sample. Additionally, since late 2016, oospore formation in the susceptible cultivar Viroflay leaf tissue has been routinely recorded at the USDA ARS station located in Salinas, CA (Fig. 4), indicating that both mating types of *P. effusa* are also routinely present in the Salinas Valley.

We also detected oospores in spinach seedlings at 2 weeks after inoculation with a suspension of *P. effusa* sporangia (Fig. 4), revealing that oospore formation may occur not only in seed crops, but also in spinach production for fresh market. Moreover, oospores may be routinely introduced in fields where the infested seed has been planted. For baby leaf production, the crop is densely planted at about 10 million seeds per hectare on 2-m wide beds (Koike et al. 2011), and thus millions of oospores are potentially introduced into spinach producing regions on seeds (Kunjeti et al. 2016b).

Production of oospores in spinach roots. The occurrence of oospores in roots has been observed in a few oomycete-host interactions (Heyman et al. 2013; Pratt 1978; Shishkoff 2018; Widmer 2010). Our analyses indicate that *P. effusa* can also produce oospores in roots under field conditions (Kandel et al., *unpublished data*). Six weeks after planting, spinach plants with yellowing leaves and poor growth were randomly collected from an experimental field plot near Salinas, CA, in 2017 to detect oospores. Oospores characteristic of *P. effusa* were observed in the root tissues of all 10 plants examined and were consistent with the dimensions of *P. effusa* oospores previously reported (Choi et al. 2007; Kunjeti et al. 2016b). Additionally, DNA was extracted from spinach roots containing oospores and an amplicon was obtained using primer sequences specific for *P. effusa* (Klosterman et al. 2014). Thus, the available evidence indicates that the oospores observed in roots are those of *P. effusa*, but this requires further verification. The duration of survival of the oospores in soil or seed is not yet established for *P. effusa*, but in other *Peronospora* species, this can range from 3 to 25 years (Gaag and Frinking 1997; McKay 1957; Montes-Borrego et al. 2009).

Oospore germination. The obligate nature of the pathogen and specific conditions required for germination of oospores has undoubtedly been challenging to the epidemiological studies of the role of oospores. Historically, demonstration of oospore germination has proven difficult among oomycete pathogens (Gent et al. 2017; McMeekin 1960; Michelmore and Ingram 1981). A variety of techniques have been tried to stimulate germination including incubation with root exudates, soil leachates, $KMnO_4$, passage through snails, and others (Gent et al. 2017; McMeekin 1960; Michelmore and Ingram 1981). However, these treatments have only yielded limited success.

Looking back at the last 100 years of research literature on the *P. effusa* pathosystem, oospore germination has only been reported once (Eriksson 1918) and the lack of clarity of the photography in that publication had even left some doubt as to whether images truly showed oospore germination. We have pursued the analysis of oospore germination of *P. effusa* using spinach (cultivar Viroflay) leaves collected from the field. Leaf tissues with high numbers of oospores (as in Fig. 4c) were macerated in water and stored in the water suspension at 4°C for about 4 weeks. Under these conditions, the oospores germinated and produced a coenocytic mycelium (Fig. 5). Since this initial finding, we also demonstrated that *P. effusa* oospores from seed also germinate under the same conditions. Given the additional evidence that oospores of *P. effusa* are capable of germination, it lends support to our hypothesis that oospores, including those that are imported into California on seed (Kunjeti et al. 2016b), potentially serve as primary inoculum sources in spinach crops. The finding of oospores in spinach, and their germination, prompted Eriksson (1918) to recommend the use healthy seeds to minimize

the downy mildew damage in spinach. Unfortunately, this finding did not find much currency until recently.

Potential role of oospores in the disease cycle. In several downy mildew pathosystems, seedborne oospores have been described as a source of primary inoculum (Cohen et al. 2017; Danielsen et al. 2004; Inaba et al. 1983; Montes-Borrego et al. 2009; Ojiambo et al. 2015). Inaba et al. (1983) observed oospores in seed wash-offs from commercial spinach seed lots. They further demonstrated sporulation and appearance of downy mildew in spinach seedlings grown from oospore-infested seeds. Routine observations of leaf and root inhabiting oospores in the Salinas Valley, CA, and their germination suggest an important role for oospores in the *P. effusa* disease cycle, and efforts are underway to reconfirm the results of Inaba et al. (1983) in our laboratories.

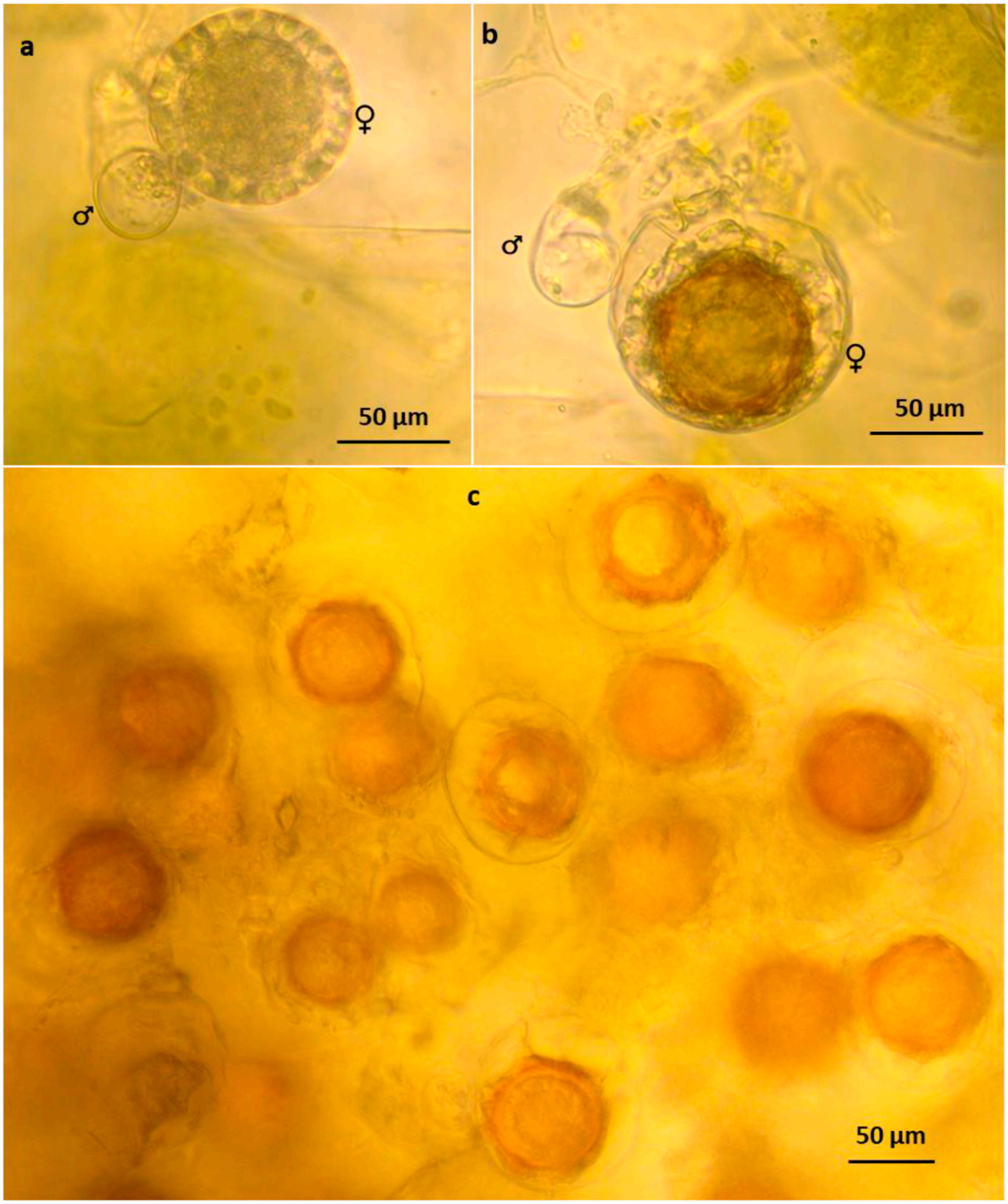

Fig. 4. Stages of oospore maturation in *Peronospora effusa* within the leaf tissue of naturally infected spinach cultivar Viroflay. (**a**) Pairing of antheridium (♂) and oogonium (♀); (**b**) newly formed oospores; (**c**) mature oospores embedded in leaf tissues.

Downy mildew (*Peronospora variabilis* Gaum (formerly *Peronospora farinosa* f. sp. *chenopodii* Byford) of quinoa was regarded as endemic in the Andes for many years. The detection of *P. variabilis* oospores was first reported in quinoa seeds in 1979 (Alandia et al. 1979). Seed transmission of the disease was confirmed from symptomatic seedlings produced from oospore-infested quinoa seeds (Danielsen et al. 2004). Quinoa production has now expanded from its native range to different parts of the world, and downy mildew has been reported in Canada, Denmark, India, the U.S., and the Republic of Korea (Choi et al. 2014; Danielsen et al. 2002; Kumar et al. 2006; Testen et al. 2012; Tewari and Boyetchko 1990). Thus, seedborne oospores may have facilitated the distribution of downy mildew beyond the Andean territory.

There are numerous other examples in the literature that documented the role of seedborne oospores in downy mildew transmission. The transmission of *Peronospora belbahrii* thorough infested seeds (Cohen et al. 2017; Garibaldi et al. 2004) has been viewed as the main source for the rapid global spread of the downy mildew pathogen in sweet basil (*Ocimum basilicum*). However, limited information is available about oospore production and its role in initiating infection (Cohen et al. 2017; Wyenandt et al. 2015). Downy mildew (*P. manshurica*) in soybean produces oospores in leaves but occasionally on seeds. The oospores overwinter in crop residues or infested seeds and may cause seedling infections in the following crop (Hildebrand and Koch 1951; Roongruangsree et al. 1988). Oospores of *Peronosclerospora sorghi*, the causal agent of maize downy mildew, were detected in 21% of the 14 seed lots examined. Also, 12.3 and 10% seedling infection was reported when untreated and treated with metalaxyl seeds were used, respectively (Adenle and Cardwell 2000). In poppy downy mildew (*Peronospora somniferi* and *P. meconopsidis*), oospores were observed in 19 of 20 seed lots examined. The majority of seedborne oospores were found viable through staining and seedling infection, suggesting pathogen transmission through seeds (Thangavel et al. 2018). Additionally, oospore

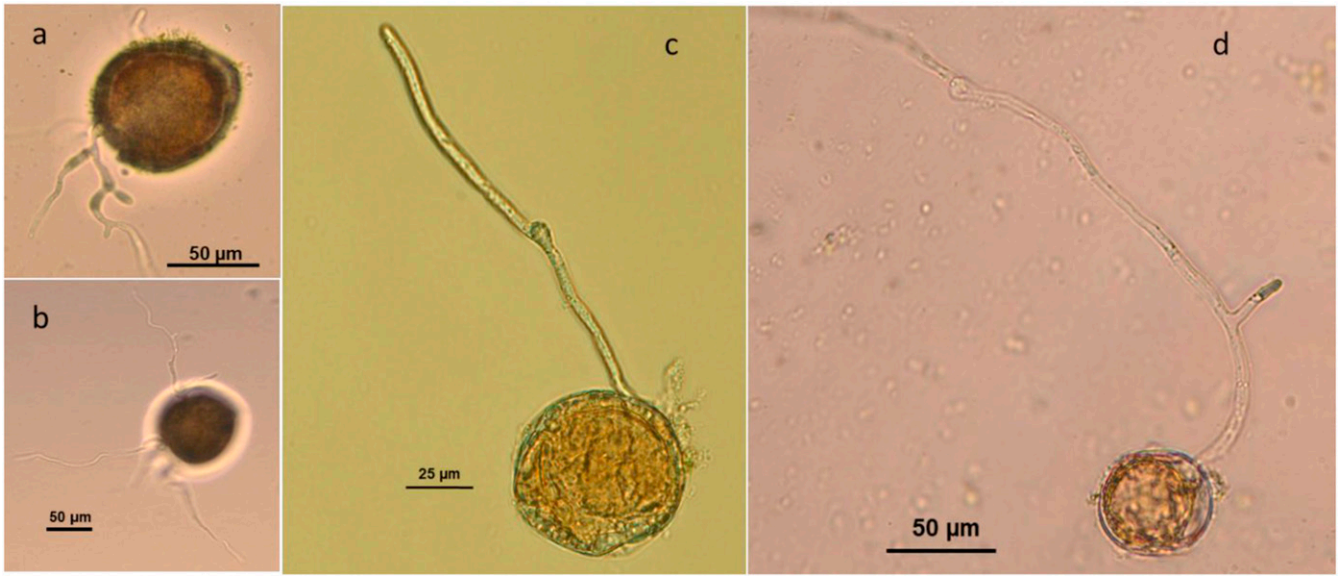

Fig. 5. Germination of the oospores of *Peronospora effusa*. (**a**) and (**b**) Germination of oospores on potato dextrose agar (PDA) after 4 weeks, and 1 week of incubation on PDA at 4°C, respectively. (**c**) and (**d**) Germination of oospores in deionized water following incubation for 35 and 48 days at 4°C, respectively.

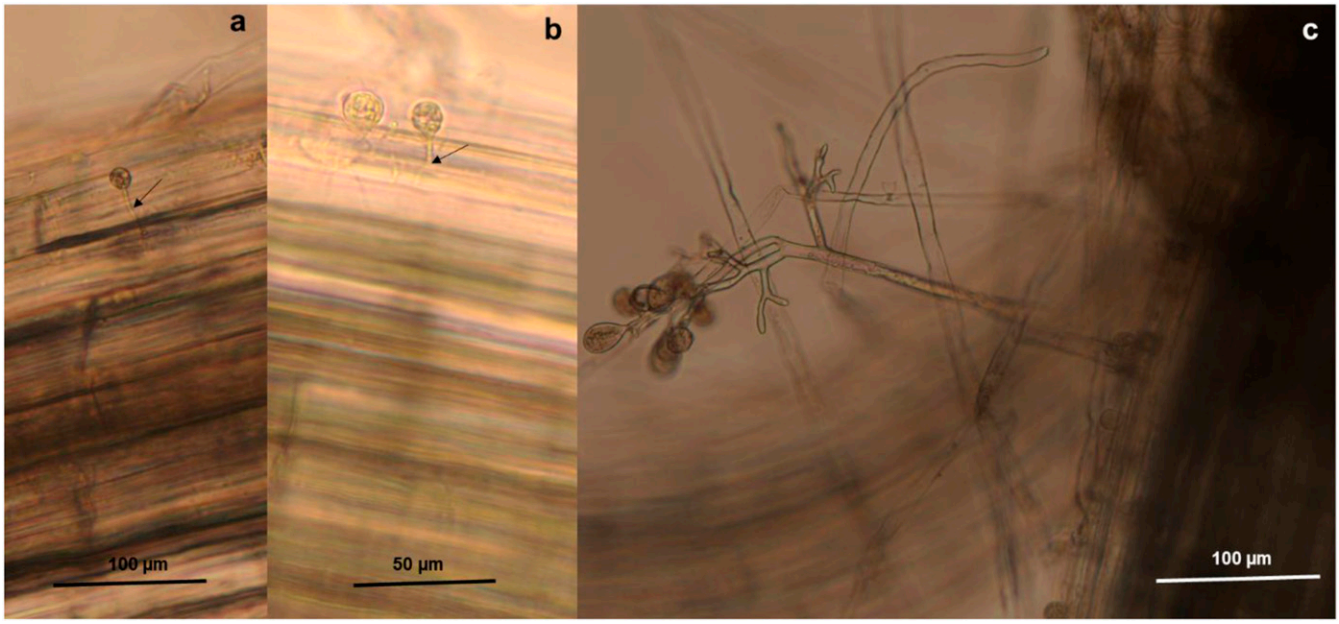

Fig. 6. Germination of the sporangia of *Peronospora effusa*, and infection of spinach. (**a**) and (**b**) germination of a sporangia on the spinach root surface and penetration of germ tubes. The arrows indicate where the sporangial germ tube is infecting root epidermal cells. (**c**) Sporangiophores bearing sporangia emerging from spinach (cultivar Viroflay) roots.

infestation in soil and crop residue and subsequent disease development in poppy plants growing in infested soil was reported under growth chamber and field conditions (Montes-Borrego et al. 2009). Soilborne oospores were also reported as a primary source for downy mildew infection in cucumber (Zhang et al. 2012). The role of oospores as a source of primary inoculum for the disease development has also been also discussed in *P. sparsa*, the causal agent of rose downy mildew (Salgado-Salazar et al. 2018).

Despite the evidence of seed transmission potential of several downy mildew pathogens, further research would be required to understand the epidemiological significance of the seed transmission with respect to transmission rates from infested seeds, longevity and germination of oospores, and their role in the development of epidemics.

Asexual reproduction. Asexual reproduction in *P. effusa* is characterized by the development of sporangiophores bearing sporangia (Fig. 2). Sporangiophores emerge from stomata of infected leaves and are visible on the leaf surfaces as a gray to light brownish mass. Mycelia and sporangiophores are hyaline and the latter have a monopodial branching pattern (Choi et al. 2007). Sporangia, as shown in Figure 2a, are oval and are 21 to 33 μm in length and 18 to 23 μm in width (Choi et al. 2007). The sporangia are likely short lived, based on the evidence from other oomycetes and related species. The half-life of sporangia of *Phytophthora infestans* was estimated to be about 5.5 h at 15 to 20°C and 40 to 80% relative humidity (RH). The sporangial viability was reduced to 1.5 and 3.8 h at 30°C under 40 and 88% RH, respectively (Minogue and Fry 1981). The sporangial survival in *P. destructor* was highest at 10°C and 53 to 95% RH and lowest at 33°C and 33% RH. The half-life of sporangia of *P. destructor* and *P. tabacina* decreased from 83 to 68% on cloudy and 46 to 0% on sunny days following 6 h exposure at these conditions (Bashi and Aylor 1983). Similarly, the sporangial germination of *Pseudoperonospora cubensis* (cucurbit downy mildew pathogen) decreased with increasing exposure on sunny days than on cloudy days (Kanetis et al. 2010). A similar observation of acute decline in sporangial germination under sunny conditions was also reported in *Bremia lactucae* (Wu et al. 2000). Sporangia of *P. effusa* can germinate over a wide temperature range (5 to 25°C), but germination rate decreases with increasing temperatures (Choudhury and McRoberts 2018).

As viable sporangia of *P. effusa* land on the moist spinach leaf surfaces, they germinate immediately and germ tubes (Figs. 2b and 2c) penetrate healthy leaves directly. Similar to *P. effusa*, the sporangia of other members of Peronosporaceae do not release zoospores (Thines and Choi 2016) unlike other oomycetes (Fry and Goodwin 1997; Gascuel et al. 2015). Sporangia (Fig. 2a) from infected lesions become airborne and are dispersed locally within the field from which they originate, or potentially across different fields (Choudhury et al. 2017). The spinach downy mildew is a polycyclic disease where airborne sporangia can lead multiple secondary infections in commercial fields. Interestingly, there is a continuous presence of airborne spores of *P. effusa*, as measured indirectly by spore trapping, although there is a notable increase in the detectable DNA of the pathogen during the major spinach growing season from March through October in the Salinas Valley (Choudhury et al. 2016). The cold nights and coastal fog, especially in the morning from spring to early autumn, is common in the major spinach production areas of coastal California (Choudhury et al. 2016; Clemesha et al. 2016), which offer optimal conditions for windborne sporangial survival.

Sporangia and sporangiophores in spinach roots and seeds. When spinach (cultivar Viroflay) seedlings were grown for 12 days in a growth chamber and inoculated with *P. effusa* sporangia as described earlier (Feng et al. 2018c), sporulation was observed in a majority of cotyledons and true leaves at 7 days postinoculation. At 10 days postinoculation, mycelial growth, sporangiophores bearing sporangia, and germinating sporangia were observed in Viroflay roots (Fig. 6). Infection through roots has been reported for other *Peronospora* species (Aegerter et al. 2002; Heist et al. 2002) but not for *P. effusa*. Potentially, *P. effusa* infects leaves, grows within the plant, and may lead to asymptomatic systemic colonization of different parts including roots, but this requires validation. Occurrence of dew on cotyledons, leaves, and stems may transport the pathogen via runoff water to the root systems, where they may colonize roots. Alternatively, root colonization has been observed in field samples as well, and thus some infections may be initiated in the roots and become systemic.

Plant pathogens can colonize and become established in different parts of the crop seeds (Carroll et al. 2018; Gilardi et al. 2018; Landa et al. 2007; Vartanian and Endo 1985). The occurrence of *P. effusa* hyphae in different parts of spinach seeds such as the calyx tube, funiculus, integument, and nucellus has been previously observed (Leach and Borthwick 1934). Sporangiophores and sporangia from commercial spinach seed lot wash-offs have also been detected (Kunjeti et al. 2016b). However, it is still unclear whether these structures have any role in the disease cycle. Based on the current understanding in other *Peronospora* species, it is likely that these propagules lose viability during seed harvest and drying.

Detection and monitoring of airborne sporangia. Many plant pathogens, including downy mildews, are disseminated through airborne spores. The understanding of spatiotemporal influx of spores released in the atmosphere is useful in developing a forecast system to predict disease epidemics (Neufeld et al. 2018; Schmale and Ross 2015). An early warning system informing the levels of airborne inoculum status may be useful to the application of preventative fungicides before the onset of disease or to schedule fungicide application to prevent the spread of diseases. Early detection systems may help determine when and where to apply fungicides, which ultimately saves resources (Gent et al. 2013; Klosterman et al. 2014). The ipmPIPE (integrated pest management information-pest information platform for education and extension) system has been used in cucurbit downy mildew (Neufeld et al. 2018; Ojiambo et al. 2011) and other crop diseases to monitor environmental variables, map the pathway of spore transport and deposit, forecast disease spread, and catalog the disease incidence in different locations (Schwartz et al. 2014; VanKirk et al. 2012).

The capture of asexual spores using impaction spore samplers (generically referred to as spore traps) in combination with molecular assays have been used to detect and quantify the airborne spores of downy mildew in hop orchards (Gent et al. 2009), powdery mildew in vineyards (Thiessen et al. 2016), and more recently, downy mildew of lettuce and spinach (Choudhury et al. 2016, 2017; Klosterman et al. 2014; Kunjeti et al. 2016a). The dispersal or migration routes of pathogen propagules or insect vectors at the regional and continental scales are utilized in different ipmPIPE systems (Isard et al. 2006; Ojiambo et al. 2015; VanKirk et al. 2012). The analogous ipmPIPE from the cucurbit downy mildew may not be reproducible in forecasting spinach downy mildew, but monitoring of spinach fields and environmental variables in addition to assessment of airborne spores through spore samplers is likely to be instrumental in forecasting the risk of disease outbreaks.

The past 20 years have seen remarkable strides in the improvement of molecular detection of plant pathogens, such that the role of conventional morphometric characteristics such as shape and size of sporangia, sporangiophores, and oospores to delineate species has diminished (Choi et al. 2007; Richards 1939). Molecular techniques are more accurate and offer higher throughput than conventional methods. In vitro amplification and quantification of distinct regions of nuclear or mitochondrial genomes can offer the ability to characterize and distinguish the downy mildew pathogens (Crandall et al. 2018; Kunjeti et al. 2016a). Klosterman et al. (2014) developed molecular detection techniques for airborne spores of *P. effusa* using a combination of spore traps and quantitative amplification of pathogen DNA. In this assay, a strong correlation was reported between physical spore counts and the corresponding DNA copy number amplified by qPCR. *P. effusa* shares high sequence homology with *P. schachtii* (beet downy mildew pathogen) and both crops are usually grown in the same region. Therefore, single nucleotide polymorphisms (SNPs) were detected in *P. effusa* and *P. schachtii* 18S rDNA, which were utilized in a qPCR assay to determine the frequency of the SNPs from both species (Klosterman et al. 2014). As genomic sequences become available for these and related species, additional

species-specific sites may be selected in the future. Recent advances on genomics of cucurbit downy mildew pathosystem have enabled development of molecular diagnostic tools and provided insights on population genetics of the pathogen (Rahman et al. 2017; Thomas et al. 2017a).

Genomic resources and genetic diversity of isolates. The genomes of several downy mildew pathogens, including *Pseudoperonospora cubensis* (cucurbit downy mildew), *P. tabacina* (tobacco downy mildew), and *Plasmopara viticola* (grape downy mildew), have been published (Derevnina et al. 2015; Dussert et al. 2016; Thomas et al. 2017a). The availability of sequence data has contributed to the development of diagnostic markers for pathogen detection and to the understanding of pathogen biology, and effectors related to pathogenicity and virulence. Recently, the genomes of three *P. effusa* pathotypes (races 12, 13, and 14) were made available in the National Center for Biotechnology Information database (GenBank accession nos. NPIT00000000, NPIU00000000, and NPIV00000000, respectively) (Feng et al. 2018b). A publication describing the sequencing and genome analysis of separate race 13 and race 14 pathotypes of *P. effusa* is also recently available (Fletcher et al. 2018). The access to these genome sequences will provide the necessary resources to elucidate the genetic basis for virulence, colonization, mating type, evolutionary pathways for emergence of new pathotypes, etc., which can be utilized to improve the host resistance and disease control measures (Aylward et al. 2017). Additionally, because the costs of whole genome-sequencing have dropped precipitously over the last decade, genome sequencing of multiple isolates of a species can provide resources to develop an accurate and reproducible diagnostic assay for closely related species (Studholme et al. 2011; Withers et al. 2016).

The knowledge of genetic variability in *P. effusa* is helpful to develop spinach varieties that are resistant to downy mildew. However, information regarding the genotypic diversity of *P. effusa* populations is largely unavailable. Lyon et al. (2016) studied the population structure of *P. effusa* isolates from two major spinach producing areas in the U.S.: Salinas, CA, and Yuma, AZ. Single nucleotide polymorphic sites were identified in *P. effusa* genomes, and different isolates were assigned as individual genotypes depending on the presence of the distinct loci. Many identical genotypes were observed in both locations and the authors suggested that there was possibility of cross movement of pathogens during spinach seasons due to anthropogenic activities or dispersal of identical primary inoculum. In the majority of isolates of *P. effusa*, higher levels of gene flow (0.24 to 83.78) and a greater degree of genetic diversity was detected, indicating the considerable intraspecific polymorphisms. Furthermore, the majority of genotypes detected in field samples did not match with historical race types (Lyon et al. 2016). These data indicate that *P. effusa* is quickly evolving, and that sexual and asexual reproduction are driving genetic diversity in populations, providing the pathogen the resources to overcome resistance in existing spinach cultivars.

Disease Management

Host resistance. Deployment of genetic resistance is an effective approach to manage many plant diseases. Resistant cultivars in combination with chemical fungicides have been used to effectively manage downy mildew for conventional production (Correll et al. 2011; Klosterman 2016). Indicative of their importance, fungicide usage and downy mildew symptoms were recently evaluated in 68 commercial spinach varieties in Yuma, AZ, and none of the varieties tested had resistance to all of the pathogen races present (Correll et al. 2015b). Heritable resistance in landraces, wild relatives, and breeding lines have been characterized and utilized to develop resistance against many plant pathogens. Open pollinated and hybrid downy mildew resistant cultivars of spinach are grown in different parts of the world. In California, essentially all commercial spinach cultivars are hybrid varieties with resistance against various downy mildew races. As described earlier, sexual recombination in oomycete pathogens can quickly introduce unique pathotypes in the population (Hausbeck and Lamour 2004; Wang et al. 2017). The changes in the populations through sexual recombination or genetic mutation in *P. effusa* genome is likely to compromise resistance in newly bred varieties that are otherwise immune to infection (Correll et al. 2011).

A review of evolving races of *P. effusa* (Correll et al. 2011) suggested that there are now 17 recognized races (Feng et al. 2018c; Correll, *unpublished data*). Race 1 of *P. effusa* was reported in the early 1800s, and since then, 16 additional races have been documented. In the U.S., P. G. Smith, a researcher at the University of California, Davis, initiated the systematic approach to understand the genetic basis of resistance in spinach. In 1950 and 1961, his team published results on a single dominant gene in spinach that conferred resistance to races 1 and 2 (Smith 1950; Smith et al. 1961) that formed the basis of subsequent resistant cultivars. Eenink (1974) in the Netherlands later discovered that resistance to race 1 was governed by two closely associated major genes. Irish et al. (2008) characterized the resistance locus (now designated as *RPF1* locus) and identified a dominant gene that conferred resistance to downy mildew by analyzing the inheritance pattern in a set of near-isogenic spinach lines. The *RPF1* locus is effective against multiple races including 1 to 7, 9, 11, and 13 (Irish et al. 2008). Additional resistant loci, including *RPF2* to *RPF10*, likely confer resistance to multiple races of *P. effusa* that have been documented during the past two decades (Correll et al. 2011; Irish et al. 2008). It is likely that the RPFs include the resistance genes against races 1 and 2 found by Smith (Smith 1950; Smith et al. 1961) and Eenink (1974).

Molecular markers linked to important traits including disease resistance have been utilized to map various genes and to study inheritance patterns in breeding materials (Sthapit et al. 2014; Wiesner-Hanks and Nelson 2016). However, only a few such markers have been developed for disease resistance selection in spinach (Correll et al. 2011; Irish et al. 2008). The spinach genome has been recently sequenced and genomic information of spinach will provide resources to improve the spinach cultivars against different diseases including downy mildew (Xu et al. 2017). Shi et al. (2017) characterized more than 300 spinach genotypes, including commercial hybrids, aiming to gain the information about the genetic diversity and the genetic markers useful in spinach breeding programs.

The discovery of resistant genetic loci and linked molecular makers will be useful to incorporate resistance genes into advanced breeding lines. Conventional breeding techniques rely on scoring of superior plant phenotypes from either inoculated experiments or natural pathogen pressure in the field conditions for the selection and crossing to develop the resistant cultivars. These methods are lengthy, laborious, and costly. Instead, molecular breeding with modern tools and techniques would expedite breeding efforts to select for important phenotypic traits (Shi et al. 2017) by genotyping. Furthermore, new phenomic tools can supplement the findings of molecular and genetic analysis and allow plant pathologists and breeders to screen hundreds of breeding lines for multiple traits, including that conferring disease resistance (Nelson et al. 2018). The discovering of polymorphic regions in spinach genome and use of marker assisted breeding and selection will possibly advance cultivar improvement efforts in the future.

New races of the spinach downy mildew pathogen have been emerging at an alarming rate (Feng et al. 2018c; Irish et al. 2007). As resistant cultivars rapidly lose their effectiveness against the newly emerged races, it is hard for breeders to keep up with the evolution of the pathogen. Gene pyramiding is a strategy that can be used to breed spinach cultivars and hybrids with durable resistance (Mundt 2018). Multiple resistance loci RPFs may be introduced into cultivars or parental lines for final combination into hybrids. Molecular markers linked to the RPFs could be employed to facilitate the pyramiding process, as markers linked to RPFs 1, 2, and 3 have been reported (Feng et al. 2018a; Irish et al. 2008). Besides the major RPF genes, quantitative trait loci (QTLs) for downy mildew resistance have not received enough attention in spinach. The combined use of both major genes and QTLs in commercial cultivars and hybrids should provide more durable downy mildew resistance.

Molecular markers can also be used to help identify spinach downy mildew races rapidly, which will aid breeders and researchers in the fight against the disease. Currently, spinach downy mildew races are identified by inoculating a set of differential varieties with the pathogen spores and subsequently observing their disease development, a laborious and time-consuming process (Feng et al. 2018c; Irish et al. 2007). Although there are PCR markers that can distinguish different downy mildew species (Klosterman et al. 2014), molecular markers linked to specific races of spinach downy mildew are not available yet.

Cultural practices, biological, and chemical controls. The removal of volunteer spinach plants and infected roots and leaves may decrease inoculum levels, both in terms of sporangia and oospores in the system. We recently observed profuse sporulation in senescing leaf tissues, which displayed symptoms and sporulation at an earlier (five or six true leaves) stage of development (Fig. 7). Therefore, proper disposal of older infected leaves and crop residue after harvesting can reduce the production and movement of sporangial inoculum. The knowledge gap on survival of oospores in soil and seed transmission occurring in field plots complicates repeated cultivation of spinach in these fields because of inoculum augmentation. Based on the knowledge gained from other oomycete pathogens, crop rotation can minimize the risk of new epidemics (Hannukkala et al. 2007; Ristaino and Johnston 1999). Airborne sporangia are always a concern, especially in areas similar to the Salinas Valley, where airborne detection of the pathogen has been noted year-round (Choudhury et al. 2016; 2017; Klosterman et al. 2014). The existence of disease on garden plants and/or volunteer plants is likely contributing to the pervasive presence of airborne sporangia. In the future, the knowledge of spatial or regional distribution of different races will be valuable to deploy resistant cultivars in individual spinach production fields.

In conventional spinach production systems, effective fungicides are available to control the disease provided they are applied before the appearance of symptoms (Subbarao et al. 2018). Coupling of early warning with effective detection systems can guide fungicide sprays, which can halt the early infection process and may eliminate the impending epidemics. Hence, recent efforts have focused attention also on spore trapping and quantification of pathogen DNA to advise growers on conditions optimal for increased airborne inoculum loads (Choudhury et al. 2016, 2017; Klosterman et al. 2014). Minimal use of overhead irrigation can help to reduce wetness in leaf canopies and therefore limit infections and symptom development as prolonged leaf wetness and high humidity favor spinach downy mildew (Choudhury et al. 2016; Klosterman 2016). Furthermore, early morning irrigation may allow sufficient time during the day to dehydrate the leaf boundary layer. The positive correlation between nitrogen fertilizer and the incidence of downy mildew was observed in grape, camelina, and pearl millet (Bavaresco and Eibach 1987; Jiang and Caldwell 2016; Zarafi et al. 2005). Nitrogen fertilizers often boost lush vegetative growth, which may create an environment conducive to higher downy mildew sporulation and infection. Therefore, the timing and dose of nitrogen fertilizers may also be an important consideration to manage downy mildew.

Because new pathogen "races" are outpacing the current host resistance almost every year (Feng et al. 2018c), the use of nonsynthetic biopesticides for disease control in organic production is especially appealing. However, biopesticides evaluated for efficacy are yet to show promise (Correll et al. 2015a). The integrated approach including both preventive and curative intervention in organic and conventional spinach production systems would be optimal to manage downy mildew. Preventive measures are especially valuable to manage downy mildew in organic spinach production systems. Sowing of clean seeds with the use of oospore-free fields will help reduce some primary inoculum; thereby preventing some outbreaks of disease in the field.

Effective seed treatment can be helpful in preventing dispersal of oospores through spinach seeds. Seed treatments and other phytosanitary procedures can be applied depending upon the seed morphology and the nature of seedborne pathogen (Mancini and Romanazzi 2014). Rather than discarding the contaminated seed lots, a recent study (Choudhury et al. 2017) suggested that seed meant for organic production system could be treated and switched over to conventional production systems. Despite the labor-intensive procedures and associated additional costs with the currently available options, seed treatment would be the optimal solution. Seed treatments not only benefit the organic production but are also equally viable in conventional spinach production systems to decrease overall inoculum loads and to curb fungicide resistance in *P. effusa* populations by switching to other products with different modes of action. Seed treatment with metalaxyl was highly effective to minimize the downy mildew incidence in 1-week-old spinach plants (Correll and Koike 2018). Hot water, botanicals, and antagonistic microbial strains are acceptable for the treatment of seeds for organic production (Gatch 2016), which can be useful in minimizing the downy mildew inoculum in spinach seeds.

Closing Remarks and Future Prospects

Global consumption of spinach is increasing, particularly in the organic sector, but downy mildew presents a major problem in maintaining increased production. To meet these growing demands for spinach, solutions to better manage downy mildew are required. Research on taxonomy, race identification, host range specificity, and molecular identification of *P. effusa* have resolved some of the vexing issues over the past decade. Further research on the molecular basis of recognition between virulent isolates and spinach cultivars and population biology of these isolates can provide the genetic basis of new pathotype or race emergence and host resistance. Undoubtedly, comparative and functional genomics projects that take advantage of the newly available *P. effusa* genome sequences will shed light into these areas. We currently have a limited understanding of the epidemiological roles of seedborne oospores, mycelium, etc. The relatively recent surge of downy mildew epidemics in cucurbit crops and basil suggest that movement of oospores on seeds facilitates movement of the pathogen to new geographical regions via infested seed lots (Cohen et al. 2015; Pintore et al. 2016; Thomas et al. 2017a; Wyenandt et al. 2015). Nevertheless, longevity of oospore viability may vary depending upon the pathogen, environmental

Fig. 7. *Peronospora effusa* sporulation on senescing and necrotic spinach (cultivar Viroflay) leaves. Arrows indicate areas of sporulation.

variables, and species. Potentially, careful monitoring of oospore infestation in commercial spinach seed lots and the development of seed sanitation methods can minimize the widespread dispersal of the pathogen through contaminated seeds. Furthermore, research on genes or mechanisms conferring resistance to infection, inoculum sources, inoculum longevity, and improved fungicides will continue to be the priority to manage downy mildew. The coordinated efforts of seed companies, growers, and researchers will facilitate the development of durable solutions for management of downy mildew on spinach.

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Shyam Kandel is a research plant pathologist (research associate) with Drs. Steve Klosterman and Beiquan Mou at the USDA-ARS Crop Improvement and Protection Research Unit, Salinas, CA. His research projects include genetics, epidemiology, and management of downy mildew of spinach. He received his M.S. in plant pathology from Washington State University in 2012 and his Ph.D. in environmental and forest sciences from the University of Washington, Seattle, in 2016. For his Ph.D. dissertation, he worked on plant-endophyte interactions and biological control of soilborne plant pathogens. He is interested in both basic and applied research to find solutions to minimize the damage caused by plant pathogens.

Dr. Beiquan Mou is a research plant geneticist with the Agricultural Research Service (ARS), U.S. Dept. of Agriculture in Salinas, CA. He obtained his Ph.D. degree in plant breeding and genetics from Oregon State University and completed postdoctoral training at Iowa State University. Dr. Mou conducts research on the genetics and breeding of lettuce and spinach. He has authored more than 80 peer-reviewed journal articles, two book chapters, and a utility patent, and edited one technical book. He has released 35 lettuce and spinach germplasm lines with improved quality and/or disease/insect resistance. He has obtained extramural grants totaling more than $8 million to ARS. He has chaired the Leafy Vegetable Crop Germplasm Committee of the National Plant Germplasm System for the past 13 years. He is an adjunct professor at Shanghai Normal University, China. He currently serves as the consulting editor of HortScience journal to edit vegetable cultivar descriptions for North America. He has received numerous international invitations to present his research at conferences including keynote speeches and to serve as journal or book editors. He is widely sought out for advice, consultation, and/or collaboration nationally and internationally by other researchers, government agencies, industry, media, and the public.

Nina Shishkoff has a B.S. in botany from the University of Michigan and a Ph.D. in mycology from Cornell University. She has worked with obligate parasites (powdery mildews) since 1994, researching the taxonomy and host range of *Podosphaera xanthii*, the mildew causing powdery mildew of squash and melons. She studied their pesticide resistance, mating behavior, and biological control as a research associate at Cornell's Long Island Horticultural Research and Extension Center in Riverhead, NY, also doing work on *Phytophthora capsici* on pumpkin and *Pythium* in greenhouse settings. When she joined the USDA Agricultural Research Service, her main research goal was to investigate the implications for eastern nurseries of the spread of sudden oak death disease from the western U.S.

Dr. Ainong Shi is an associate professor and vegetable breeder at the Department of Horticulture, University of Arkansas. Dr. Shi has over 20 years of experience in plant breeding and genetics, led several USDA funded grants, and has published over 100 articles. He has devoted time in field, greenhouse, and lab for crop germplasm evaluation, variety development, and cultivar release. He has also more than 15 years of experience in molecular breeding including QTL and association mapping, SNP discovery and genotyping, DNA and RNA sequencing data analysis, marker-assisted selection, and genomic selection and breeding.

Dr. Krishna Subbarao is a professor of plant pathology at UC Davis but is located at the USDA station in Salinas, CA. His research has covered both airborne and soilborne pathogens affecting cool-season vegetable crops. His recent work has elucidated the likely origins, global diversity, genomics of pathogens, and the nature of microbiomes associated with inputs that suppress Verticillium wilt. His previous work on lettuce and spinach downy mildews has encompassed developing and validating forecasting systems, analyzing disease spread using geographical information systems, and using statistical and molecular techniques to elucidate sources and dynamics of inoculum in coastal California.

Dr. Steven J. Klosterman is a research molecular biologist at the United States Department of Agriculture, Agricultural Research Service (USDA ARS), Crop Improvement and Protection Unit, in Salinas, CA. He has nearly 20 years of research experience focusing primarily on the molecular genetics and functional genomic analyses of plant pathogens and plant defense gene expression. Currently, his main interests include genetic and functional genomics analyses of *Verticillium dahliae*, molecular diagnostics of downy mildew diseases affecting lettuce and spinach, and application of these new diagnostics in epidemiological studies. Of these studies with *Peronospora effusa*, he is especially interested in the worldwide transport of viable oospores of the downy mildew pathogen on spinach seed, the different genotypes of the pathogen carried on seed, and how this affects disease development in the U.S. Dr. Klosterman earned a B.Sc. in biology at Wright State University, Dayton, OH, and a Ph.D. in plant pathology in 2002 at Washington State University, Pullman, WA, followed by postdoctoral work at the University of Georgia, before beginning with USDA ARS in 2006.

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