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Development and validation of a novel rapid in vitro assay for determining resistance of potato cultivars to root attachment by *Spongospora subterranea* zoospores

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Abstract

Spongospora subterranea f. sp. *subterranea* is a major pathogen of potatoes leading to losses in tuber quality and yield. Disease can be expressed as root infection, root galling and tuber lesions, the latter known as powdery scab. Attachment of zoospores to potato root hairs is the first step before infection of roots and disease development. Root hair infection results in root dysfunction leading to impaired plant productivity and yield. Varieties vary in their susceptibility to root and tuber disease; however, varietal screening is both time and resource intensive. Furthermore, traditional screens assess root galling or tuber disease and not root infection. In this study, we determined optimal conditions for zoospore release and attachment of zoospores to plant roots and used this information to develop an in vitro bioassay to assess resistance to zoospore root attachment among 153 potato lines and cultivars. Optimal zoospore release occurred at 20°C in Hoagland's solution in a rapid and synchronized manner over the first 2 days, followed by a steep decline. The extent of zoospore root attachment varied with cultivar (Iwa > Agria > Russet Burbank > Gladiator), region of the root maturation zone (lower > middle > upper) and temperature (greatest zoospore root attachment occurring at 15°C). Further comparisons suggested efficiency of zoospore root attachment was also generally associated with known variety resistance to powdery scab, zoosporangial infection and root galling, with a few notable exceptions. The bioassay proved to be a rapid and robust method for screening cultivar resistance to zoospore root attachment.

KEYWORDS
host resistance, in vitro assay, root infection, *Spongospora subterranea* f. sp. *subterranea*, zoospore germination, zoospore root attachment

1 | INTRODUCTION

The soilborne pathogen *Spongospora subterranea* f. sp. *subterranea* infects potato roots and tubers, leading to root dysfunction and disease expression as zoosporangial root infection, root galling and powdery scab tuber lesions (Balendres, Tegg, et al., 2016; Falloon et al., 2016). These commonly occurring root and tuber diseases are of great economic importance across most major potato production areas worldwide (Merz

& Falloon, 2009). Root infections can result in diminished root function with subsequent yield losses (Falloon et al., 1996, 2003, 2016; Nielsen & Larsen, 2004; Nitzan et al., 2008); in addition, infected tubers will impact seed tuber and fresh market quality and value, and can reduce the durability of cool storage of tubers prior to processing (Balendres, Tegg, et al., 2016; Falloon et al., 2016; Harrison et al., 1997; Merz & Falloon, 2009). In Australia, for example, a conservative estimate of the economic loss to the Australian potato processing industry alone due to powdery scab is $13.4 million annually (Wilson, 2016).

The disease cycle of *S. subterranea* has been studied extensively. *S. subterranea* persists in the soil as dormant resting spores (Harrison et al., 1997) that can release motile zoospores under conducive environmental conditions, stimulated by the presence of host plant root exudates (Balendres, Nichols, et al., 2016). Zoospores are attracted by chemotaxis to host roots (Amponsah et al., 2021), to which they attach (encyst) and penetrate through the cell wall, inserting their cellular contents to facilitate infection (Merz, 1997). A multinucleate plasmodium then forms, which segments into uninucleate zoosporangia (Kole, 1954). Secondary zoospores form within zoosporangia and are subsequently released into the soil where they can reinfect the roots or developing tubers in a polycyclic manner (Balendres, Tegg, et al., 2016; Clay & Walsh, 1990: Lahert & Kavanagh, 1985; Nitzan et al., 2008). Root infection generally leads to formation of root galls that are filled with sporosori; on root decay, these are released into the soil environment, adding to the soil inoculum load (Balendres, Tegg, et al., 2016; Harrison et al., 1997; Nitzan et al., 2008). Tuber disease results from zoospore infection of young developing tubers (van de Graaf et al., 2007; Hughes, 1980).

Strategies to manage *S. subterranea*-induced diseases are very limited. Depending on market demands, growers may be able to select cultivars with resistance against root and tuber disease caused by *S. subterranea* infection (de Boer, 1991; Falloon et al., 2003, 2016; Hernandez Maldonado et al., 2013; Torres et al., 1995); however, no cultivar has complete immunity to infection, and significant disease may still result in moderately resistant varieties (Merz et al., 2012). Reliable identification of host resistance among commercial cultivars is a critical step forward for management of disease caused by *S. subterranea* infection (Falloon et al., 2003, 2016; Nitzan et al., 2008).

Traditionally, identification of host resistance has been based on the assessment of tuber powdery scab and/or root galling in large, replicated field or glasshouse challenge trials (de Boer, 1991; Falloon et al., 2003, 2016; Nitzan et al., 2008; Torres et al., 1995). These types of assessments are both time (4–6 months) and resource intensive, coupled with the confounding impacts of variable environmental conditions and, in field trials, erratic distribution of soil inoculum (Falloon et al., 2003; Hernandez Maldonado et al., 2013; Nitzan et al., 2008). These glasshouse and field assays also fail to provide direct information on the relative host resistance to root hair infection, which we now know to be critical for impact on plant productivity (Falloon et al., 2016; Shah et al., 2012). Previously, Merz et al. (2004) developed a laboratory bioassay that did examine resistance to root infection by observation of the relative abundance of zoosporangia within root hairs from tissue-cultured plantlets incubated with sporosori inoculum. Whilst also much quicker than glasshouse and field assays, this method still required several weeks for observable infection to occur. Further, when assessing abundance of zoosporangia in roots, care must also be taken to ensure the roots of all test plants first come into contact with inoculum at the same time, as variation in the number of infection cycles over the incubation period can result in a difference in observed root infection levels and could lead to an inaccurate rating (Thangavel et al., 2015).

The need for an efficient in vitro assay for host resistance to root attachment/infection by zoospores is further emphasized by the variation in ratings of host resistance of some potato cultivars depending on the stage of disease being assessed. For example, cultivar Swift produced low levels of tuber powdery scab in the field but high levels of root galling in the glasshouse, suggesting resistance to tuber and root diseases may not necessarily be related (Falloon et al., 2003). Similarly, cv. Russet Burbank shows good resistance to tuber disease but has moderate susceptibility to root infection and galling (Boyd, 1951; Falloon et al., 2016; van de Graaf et al., 2007). It is also known that there are different temperature optima for expression of root infection (11–25°C; van de Graaf et al., 2005, 2007; Kole, 1954) and tuber disease (9–17°C; van de Graaf et al., 2005, 2007; Hughes, 1980; Shah et al., 2012). Thus, where soil temperatures may be warmer, substantial root infection can occur in the general absence of tuber disease, further confounding assessments (Falloon et al., 2003, 2016).

There is increasing concern about the impact of the early phases of root infection on plant productivity and yields, for which demonstrated cultivar resistance data is largely lacking. In the past two decades, root infection has received more attention (Falloon et al., 2003, 2016; Nitzan et al., 2008, 2010), but less is known about the initial mechanisms involved in zoospore root attachment. A potential alternative rapid in vitro assessment method for assessing cultivar resistance to root disease is to observe the relative propensity of initial zoospore root attachment at the very start of the infection process, so avoiding issues of polycyclic infection. We postulate that this would provide a more robust method of screening for host resistance to root infection, with results obtained in significantly less time than previous assessment methods. In this study, we first optimized in vitro conditions for zoospore release and zoospore attachment to potato roots. We then developed a zoospore root attachment assay, which was used to screen 153 potato lines and cultivars for their relative resistance to zoospore root attachment against known standards. We compared the results of zoospore root attachment within selected cultivars against known resistance to root galling and tuber disease, and zoosporangial root infection.

2 | MATERIALS AND METHODS

2.1 | Preparation of sporosori inoculum

S. subterranea sporosori inocula were obtained from powdery scab diseased potato cultivar Kennebec tubers, harvested from a commercial crop grown in north-west Tasmania, Australia in 2019.

Diseased potato tubers were washed with tap water and left to air-dry in a cool place for 24–48 h. Sporosori were scraped from tuber lesions with a scalpel and then sifted through a 600-μm sieve. The inoculum was stored in a covered container at the ambient temperature in the dark until use.

2.2 | Potato lines and cultivars

In total, 153 potato (*Solanum tuberosum*) lines and cultivars were assessed in this study and compared to four cultivars that differ in their resistance to powdery scab: Gladiator (regarded as very resistant to powdery scab), Russet Burbank (regarded as moderately resistant), and Agria and Iwa (both regarded as highly susceptible) (Falloon et al., 2003; Genet et al., 2007). Tissue-cultured plantlets of each potato variety or line were grown in potato multiplication (PM) medium (composed of Murashige and Skoog [MS] salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; Phytagel, 2.2 g/L; pH 5.8) under a 16 h photoperiod using white fluorescent lamps (65 μmol m^{-2} s^{-1}) at 22°C. For use in experiments, 1-month-old potato plantlets were transferred from PM medium to liquid potato multiplication (LPM) medium (composed of MS salts, 4.43 g/L; sucrose, 30 g/L; casein hydrolysate, 0.5 g/L; ascorbic acid, 0.04 g/L; pH 5.8) and grown for 2 weeks under a 16 h photoperiod using white fluorescent lamps (65 μmol m^{-2} s^{-1}) at 22°C.

2.3 | Optimum temperature for in vitro zoospore release

The optimum incubation temperature for zoospore release from sporosori was determined using a modified method of Balendres, Clark, et al. (2018). Aliquots of 3 mg of dried *S. subterranea* sporosori inoculum (five replicates per temperature treatment) were added to 1.6 ml Eppendorf tubes and suspended in 1 ml Hoagland's solution. The Hoagland's solution (Falloon et al., 2003) composition was KNO_3, 253 mg/L; $Ca(NO_3)_2.4H_2O$, 722 mg/L; KH_2PO_4, 2.3 mg/L; $MgSO_4.7H_2O$, 120 mg/L; NH_4NO_3, 40 mg/L; Fe-EDTA, 20 mg/L; H_3BO_3, 140 μg/L; KCl, 400 μg/L; $MnSO_4.H_2O$, 63 μg/L; $ZnSO_4.7H_2O$, 115 μg/L; $CuSO_4.5H_2O$, 50 μg/L; and $Na_2MoO_4.2H_2O$, 22 μg/L, prepared in sterile deionized distilled water. The tubes were covered with aluminium foil and incubated in darkness at 10, 15, 20, 25 or 30°C (plant growth chamber; Steridium Pty Ltd). This temperature range was selected based on successful zoospore release in field and laboratory studies (Fornier et al., 1996; van de Graaf et al., 2007; Harrison et al., 1997). Zoospore release was assessed by observation of subsamples taken at 2, 4, 7, 13, 20, 30 and 40 days after incubation. At each assessment, the tubes were briefly mixed to ensure a homogenous solution, then 1 μl was pipetted directly onto a glass slide with a coverslip and the numbers of *S. subterranea* zoospores were determined by counting the total zoospore number at 200× magnification (DM 2500 LED; Leica). Three 1 μl samples were taken and counted each time from each tube, and five replicates were included in this experiment. Identification of zoospores used both zoospore morphology (Kole, 1954) and motility behaviour (Merz, 1997).

2.4 | Distribution of zoospore root attachment of four potato cultivars

The density of root hairs on four potato cultivar standards, Iwa, Agria, Russet Burbank and Gladiator, was determined by light microscopy at 400× magnification. Roots from 2-week-old tissue-cultured potato plantlets grown in LPM were washed with deionized water. For each assessment, three primary roots (8–9 cm long) from the same plant were divided into three segments representing the upper, middle and lower parts of the root maturation region (Figure 1). Each segment was trimmed to a length of 10 mm, thus providing a total

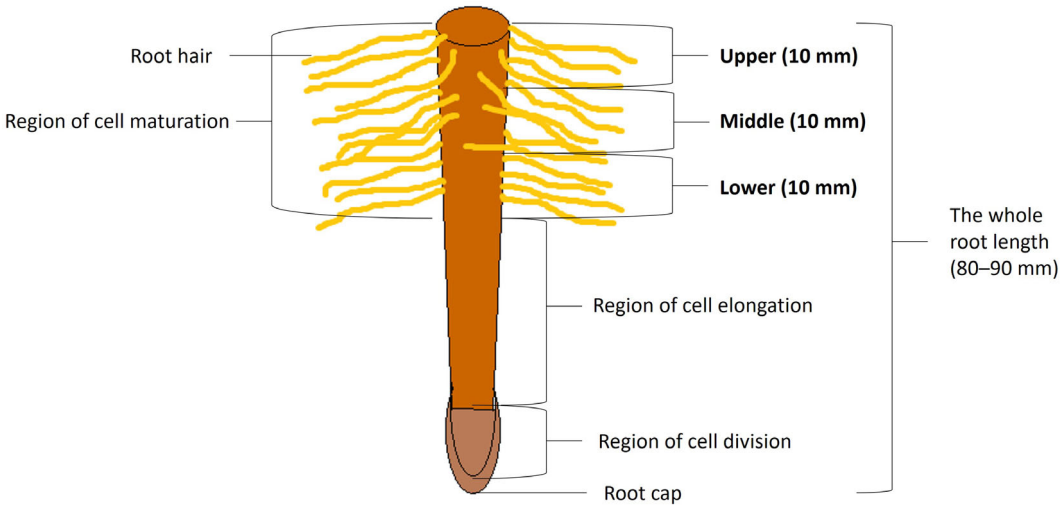

FIGURE 1 Schematic diagram of primary root of potato plantlets, showing the upper, middle and lower regions of the maturation zone from which root samples were taken. [Colour figure can be viewed at wileyonlinelibrary.com]

of nine root segments for each plant. Root segments were placed on a glass slide and covered with a coverslip. The number of root hairs was quantified by randomly scanning five fields of view for each root segment under light microscopy at 400× magnification. For each root zone, the data from the three primary roots were averaged to provide a replicate value. Three plants of each cultivar were assessed, giving three replicates of each root zone per cultivar.

A preliminary study evaluating zoospore attachment to the entire potato primary root revealed that zoospores attached exclusively on the root hairs within the maturation region of the root (Figure 1), and that 1000 zoospores/ml gave more reproducible data for attachment than 200 zoospores/ml. Subsequently, for each of the four cultivars, the preferred location within the root maturation region for attachment of *S. subterranea* zoospores was determined. Root segments were cut from three regions of the maturation region (lower, middle and upper), as described above, and placed in a plastic container. The three root segments of each individual root from each of the four cultivars, with three replicates per cultivar, were placed in a single container separated by a 100-μm mesh. Sixty millilitres of zoospore suspension (1000 zoospores/ml) was added to the treatment container, which was then incubated in the dark at 15°C for 48 h. The number of zoospores attached to each root segment was quantified from five randomly selected fields of view under light microscopy at 400× magnification.

2.5 | Optimum temperature and root tissue for attachment of zoospores to root hairs of four potato cultivars

Each assessment used three primary roots (8–9 cm long) that had numerous root hairs, excised from a single, 2-week-old in vitro propagated plantlet of cultivars Iwa, Agria, Russet Burbank or Gladiator. Roots were washed, and a 10-mm segment was taken from the lower maturation region of each root, providing a total of three root segments from each individual plant. Washed root segments of each cultivar were equally distributed within the container and a zoospore suspension (1000 zoospores/ml) was added as previously described. The containers were then incubated at 10, 15, 20, 25 or 30°C in the dark for 48 h. After treatment, root segments were mounted on a glass slide with a coverslip and the number of attached zoospores was counted by light microscopy as described above. Data from the three roots of each plant were averaged to provide a replicate value and three plants of each cultivar were assessed, giving three replicates per cultivar.

2.6 | In vitro screening of 153 potato lines and cultivars by zoospore root attachment assay

A total of 153 individual potato cultivars, breeding lines or clonal replicates were obtained from the potato germplasm collections of TIA, Agronico Pty Ltd and Solan Pty Ltd. Three plants of each cultivar, line or clone were incubated for 2 weeks in LPM, as previously described, and roots were harvested. A 10-mm section from the lower maturation region from each root was sampled and used for assessment of zoospore root attachment as previously described, with incubation at 15°C. Cultivars, lines and clones were tested in batches of eight with two reference cultivars (Iwa and Gladiator) included in each batch. This assessment of each individual cultivar or line was performed with three independent biological replicates (three plants of each individual cultivar) and each comprised three technical replicates (three roots from each plant).

Scores of zoospore root attachment for each cultivar/line in the screenings were standardized according to the two reference cultivars, Gladiator and Iwa, present in each batch. The mean scores for zoospore attachment to roots of Gladiator and Iwa in the first batch screened were 1.64 (G1) and 11.6 (I1), respectively, and this served as a reference score (G1 + I1) to adjust for batch differences in each subsequent batch. This was done by calculating a reference point correction coefficient (η_n) for each batch:

$$\eta_n = \left(G_n + I_n\right) / (G1 + I1) \quad (1)$$

where G_n and I_n are the zoospore attachment scores for Gladiator and Iwa in batch *n*. This coefficient was used to linearly scale the attachment score for each cultivar/line.

2.7 | Assessment of susceptibility of potato cultivars to zoosporangial infection

The relative susceptibility to development of zoosporangial root infection was determined in 12 cultivars (Ida Rose, Nicola, Shepody, 10086, Krantz, Iwa, Tolaas, Toolangi Delight, Granola, Gladiator, Russet Burbank, Russet Nugget) that varied in their response to zoospore root attachment assay. In vitro propagated plantlets of the 12 cultivars (with three plantlets per cultivar) were grown for 3 weeks in LPM as described above. Each plantlet was suspended in an individual plastic container (30 × 130 mm) containing 10 ml Hoagland's solution with 25 mg of dried sporosori inoculum prepared as described earlier. A further three plantlets of each cultivar were suspended in a separate container filled with only 10 ml Hoagland's solution as a noninoculated control. The plantlets were incubated in a plant growth chamber (Steridium Pty Ltd) at 15°C for 3 days in darkness. Following inoculation, each plantlet was transferred into a fresh container containing 10 ml of Hoagland's solution only, arranged in a completely randomized pattern and grown in a plant growth chamber (Contherm Scientific Pty Ltd) under a 16-h photoperiod using white fluorescent lamps (65 μmol m^{-2} s^{-1}) at 20 ± 2°C. After 1 month, plantlets were each assessed for root zoosporangial infection as follows.

From each plant, about 0.1 g of fresh, intact washed roots were randomly selected, cut into 10-mm long sections and stained with 0.1% trypan blue for about 15 min. Assessment of *S. subterranea*

zoosporangial infection was conducted for three biological replicates under light microscopy at 200× magnification (Balendres, Tegg, et al., 2018). The intensity of zoosporangial infection of each root segment was assessed according to the rating scale of Merz et al. (2004): 0 = no infection; 1 = <10% of roots infected, sporadic; 2 = 2%–10% of root infected, slight; 3 = 11%–25% of root infected, moderate; 4 = 26%–50% of root infected, heavy and 5 = >50% of root infected, very heavy.

2.8 | Statistical analysis

All data were subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 27 following conformation of normality and homogeneity of variance. Data for zoospore release, root hair density and zoospore root attachment to different root regions were assessed by two-way ANOVA, with a Tukey's honestly significant difference (HSD) test used to determine statistically significant differences between the means at the 5% level ($p = 0.05$). Zoospore root attachment and zoosporangia intensity scores were assessed by one-way ANOVA with a Tukey's HSD test used to determine statistically significant differences at the 5% level ($p = 0.05$). Multiple comparison of means was calculated with Fisher's least significant difference analysis at a 0.05 level of probability.

3 | RESULTS

3.1 | Optimum temperature for in vitro zoospore release

The mean number of zoospores released was significantly influenced by assessment date ($p < 0.001$), incubation temperature ($p < 0.001$) and their interaction ($p < 0.001$; Figure 2). Maximum zoospore release occurred on day 2 at both 20 and 25°C treatments and thereafter decreased across all temperatures. No zoospores were observed after only 2 days of incubation at 10 and 15°C or at any time point at 30°C.

3.2 | Distribution of root hairs in the maturation zone of primary potato roots

Root hair density was significantly influenced by cultivar ($p < 0.05$; Table 1, Figure 3) and root region ($p < 0.05$). However, the interaction between cultivar and root region was not significant ($p = 0.751$). Mean root hair density was higher in the mid-section than in either the lower or upper regions of the maturation zone. Russet Burbank had the highest mean root hair number followed by Iwa, Agria and then Gladiator.

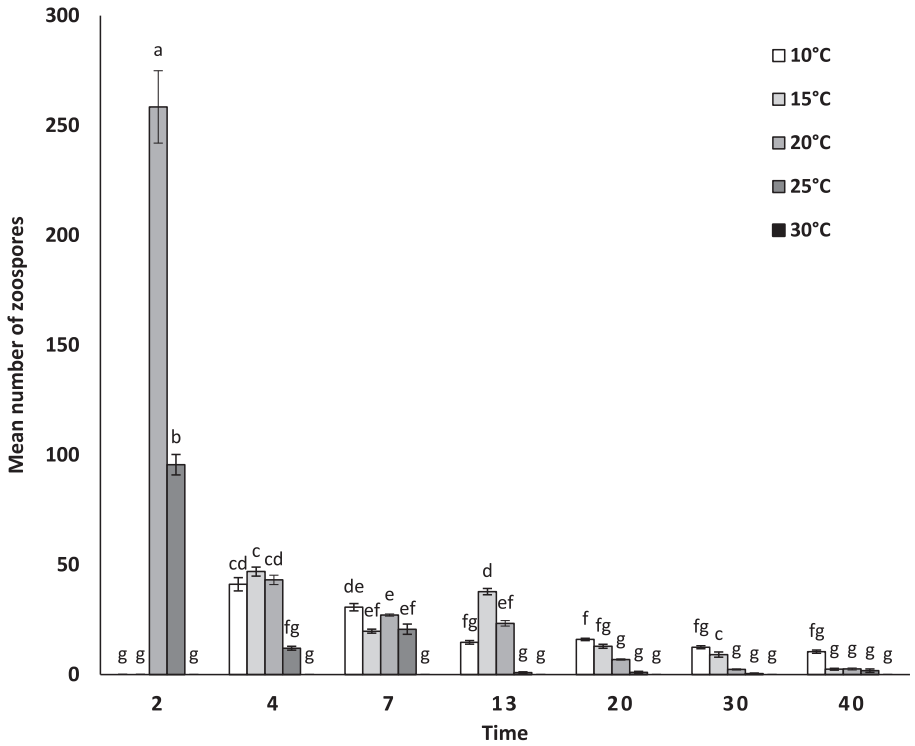

FIGURE 2 The impact of temperature treatment (10, 15, 20, 25 and 30°C) on release of *Spongospora subterranea* zoospores in Hoagland's solution, assessed at 2, 4, 7, 13, 20, 30 and 40 days after incubation. Vertical bars represent standard error ($n = 5$). p (temperature) < 0.001; p (time) < 0.001; p (time × temperature) < 0.001. Different letters above bars indicate significant temperature × time interaction effect as determined by least significant difference (0.05) = 8.6.

TABLE 1 Root hair density in upper, middle and lower regions of the maturation zone of primary roots in potato cultivars Iwa, Agria, Russet Burbank and Gladiator ($n = 3$).

Cultivar	Root hair number per field of view	Region	Root hair number per field of view
Iwa	50.9 b	Upper	48.2 b
Agria	49.5 c	Middle	50.5 a
Russet Burbank	52.5 a	Lower	49.5 a
Gladiator	44.6 d		
LSD ($p < 0.05$)	1.26		1.09

Note: Field of view was at 400× magnification. Different letters indicate significant treatment effects ($p < 0.05$) of the cultivar and root region as determined by LSD (cultivar) = 1.26 and LSD (region) = 1.09.

Abbreviation: LSD, least significant difference.

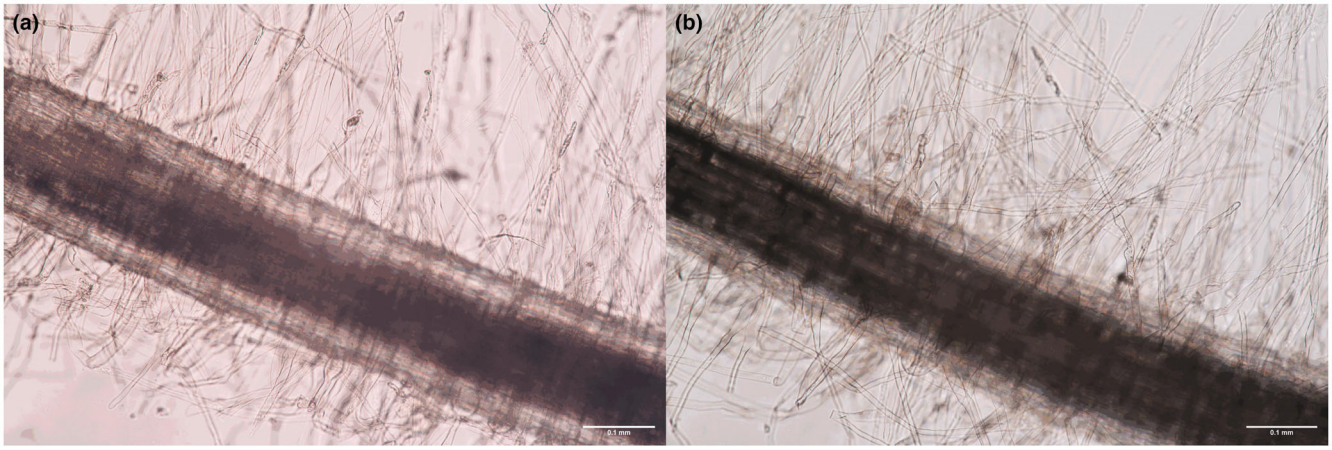

FIGURE 3 Representative images of root hairs from the maturation region of a resistant Iwa (a) and susceptible Gladiator cultivar (b). The root length of each field of view is 1.0 mm; scale bar = 0.1 mm. [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Optimum temperature for and distribution of zoospore attachment to roots of four cultivars (Iwa, Agria, Russet Burbank and Gladiator)

Zoospore root attachment was significantly influenced by potato cultivar ($p < 0.001$), root region tested ($p < 0.001$) and their interaction ($p < 0.001$; Figure 4). Zoospore root attachment for Iwa ($p < 0.001$) was significantly greater (8.9 attached zoospores ± 0.3) than the other three cultivars (0.3 ± 0.1 to 4.9 ± 0.2) in the lower region of the primary root maturation zone. Zoospore root attachment was significantly higher in the lower region than in the upper and middle regions of root maturation zones of the cultivars Iwa, Agria and Russet Burbank.

Zoospore root attachment was significantly influenced by temperature ($p < 0.001$), cultivar ($p < 0.001$) and their interaction ($p < 0.001$; Figures 5 and 6). After 48 h incubation, zoospore root attachment was significantly higher in Iwa and Agria than Gladiator and Russet Burbank at all incubation temperatures except 30°C. In addition, for all cultivars, attachment was significantly higher at 15°C than at other incubation temperatures and very little zoospore root attachment was observed at 30°C.

3.4 | Zoospore root attachment in response to potato cultivars with different resistance

The mean zoospore root attachment scores for the two standard cultivars Iwa and Gladiator differed across all the batches in which they were tested. The mean score for Gladiator varied between 0.8 and 2.2 (standard deviation [*SD*] = 0.5), whereas the mean score for Iwa varied between 9.9 and 15.4 (*SD* = 1.9). All cultivar screening for zoospore root attachment included these two standards as a reference point; hence, linear scaling (see methods above) was required to account for batch-to-batch differences in zoospore root attachment, as observed for the two standards.

Figure 7 displays the scaled mean zoospore root attachment scores for the 153 cultivars/lines of potato assessed using an in vitro bioassay. The zoospore root attachment score (scaled mean severity score) of all cultivars/lines exhibited a continuum of susceptibility from very susceptible to very resistant. The cultivars were arbitrarily classified as very resistant to zoospore root attachment (scaled mean severity score ≤3.3), moderately resistant (score 3.4–6.6), moderately susceptible (score 6.7–9.9) and very susceptible (score ≥10) (Figure 7). This categorization classified 11.8% of the

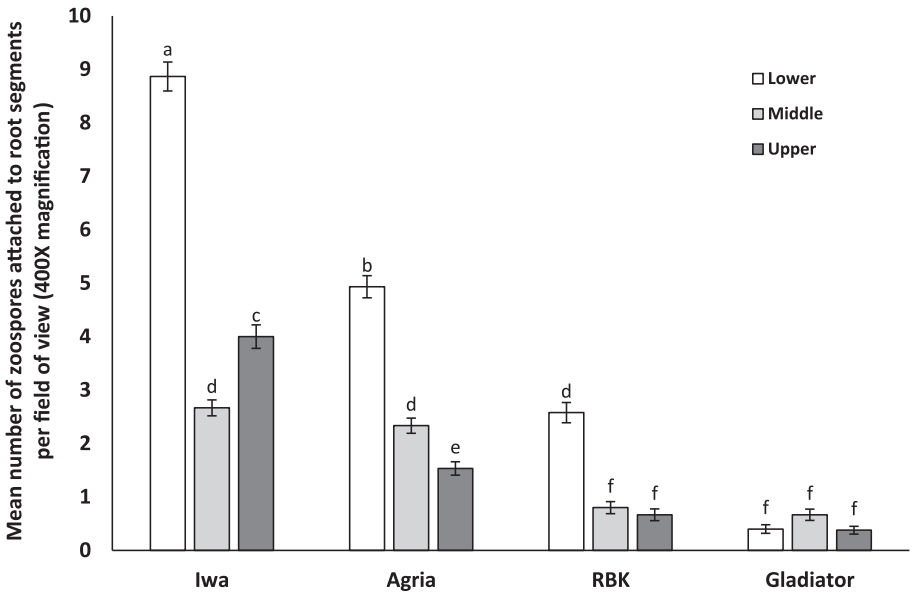

FIGURE 4 The mean number of *Spongospora subterranea* zoospores observed per field of view at 400× magnification attached to each of three root regions (lower, middle and upper) of the maturation zone of roots from potato cultivars Iwa, Agria, Russet Burbank (RBK) and Gladiator. Vertical bars represent standard error ($n = 3$). p (cultivars) < 0.001; p (region of root) < 0.001; p (cultivar × region of root) < 0.001. Different letters above bars indicate significant region × cultivar interaction effect as determined by least significant difference (0.05) = 0.44.

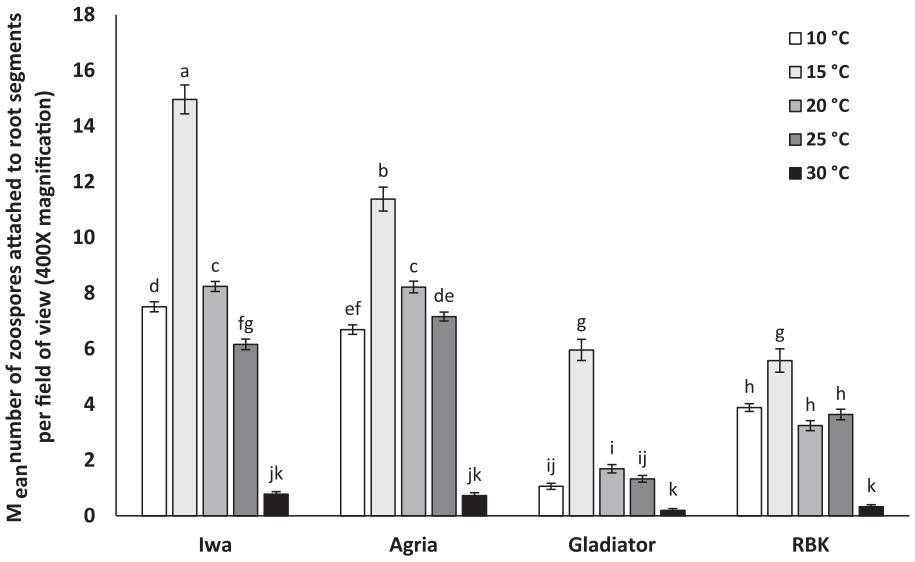

FIGURE 5 The mean number of *Spongospora subterranea* zoospores observed per field of view at 400× magnification attached to the lower root maturation region of potato cultivars Iwa, Agria, Russet Burbank (RBK) and Gladiator at 10, 15, 20, 25 and 30°C. Vertical bars represent standard error ($n = 3$). p (temperature) < 0.001; p (cultivar) < 0.001; p (temperature × cultivar) < 0.001. Different letters above bars indicate significant temperature × cultivar interaction effect (least significant difference [0.05] = 0.67).

cultivars as very resistant, 49.7% as moderately resistant, 28.8% as moderately susceptible and 9.8% as very susceptible. Comparison of zoospore root attachment results of 13 cultivars measured in this study with root galling intensity scores published in previous studies (Bittara et al., 2016; Falloon et al., 2003, 2016) showed that cultivars Gladiator, Gold Kennebec, Yukon, Russet Ranger and Iwa were categorized into the same levels of resistance with both assessment methods (Table 2). In contrast, cultivars Nicola, Agria and Shepody were categorized as moderately susceptible, very susceptible and very susceptible, respectively, based on root galling intensity scores, but were assessed as very susceptible, moderately susceptible and moderately susceptible, respectively, in the zoospore root attachment bioassay. Similarly, cultivars Umatilla Russet, Alturas and Russet Burbank were considered moderately susceptible by the root galling scores but were rated as very resistant in the zoospore root attachment assay, whilst Summit Russet was categorized as very

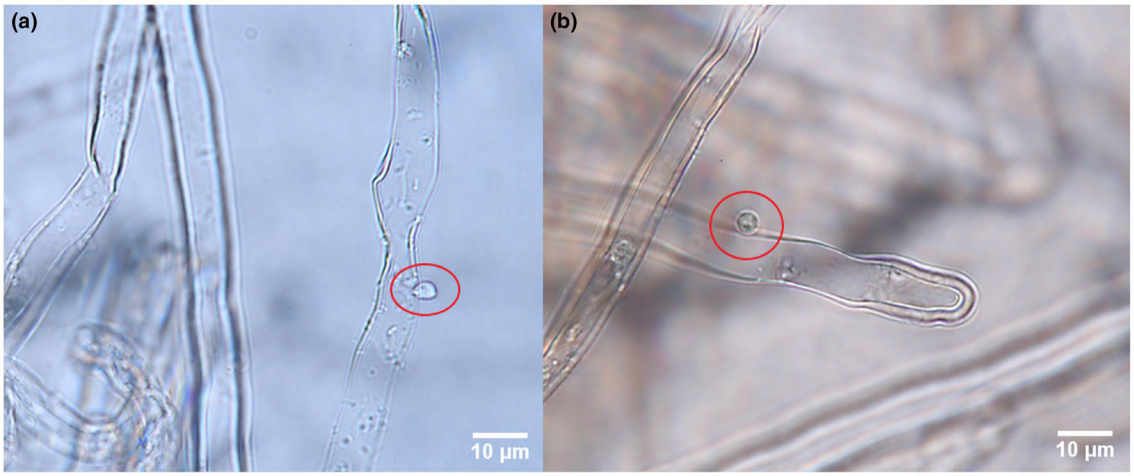

FIGURE 6 Attachment of *Spongospora subterranea* zoospores to potato root-hairs (lower zone of root maturation region) of potato cultivars Iwa (a) and Gladiator (b), indicated by red circles. [Colour figure can be viewed at wileyonlinelibrary.com]

resistant to root galling but moderately resistant to zoospore root attachment. Linear regression analysis of zoospore root attachment assay results with published field potato powdery scab (tuber disease) resistance scores revealed a negative linear relationship with a relatively weak R^2 value (0.42) (Figure 8). Notable outliers from the relationship were Nicola (highly susceptible to zoospore root attachment but moderately resistant to tuber disease), MacRusset (moderately resistant to zoospore root attachment but highly susceptible to tuber disease) and Nooksac (moderately susceptible to zoospore root attachment but moderately resistant to tuber disease).

3.5 | Assessment of susceptibility of 12 potato cultivars to *S. subterranea* zoosporangial root infection

Zoosporangial infections were not observed in uninoculated control plants of any cultivar 30 days after inoculation. In contrast, all plants inoculated with *S. subterranea* showed zoosporangial root infections, but the infection rate varied among cultivars. Mean zoosporangial intensity scores were significantly greater in cultivars assessed as susceptible to zoospore root attachment (i.e., cultivars Iwa, 10086, Krantz, Nicola, Shepody, Ida Rose) than in cultivars that had greater resistance to zoospore root attachment (i.e., cultivars Russet Burbank, Russet Nugget, Gladiator, Granola, Tolaas, Toolangi Delight) (Figure 9).

4 | DISCUSSION

With an absence of effective control measures, host resistance is regarded as a critical tool in the management of root and tuber diseases caused by *S. subterranea* infections (Bittara et al., 2016). However, the traditional methods used for cultivar screening for disease resistance involve replicated glasshouse or field trials that are both time and resource intensive (Merz et al., 2004), and subject to variation of environmental conditions that can affect disease expression and assessment efficiency. These field and glasshouse trials assess resistance to root galling or tuber disease only, and the lack of information on resistance to root infection can be a limitation, as this phase of the disease is critical for impact on potato yield (Falloon et al., 2016; Shah et al., 2012). Prior in vitro assays that assess plasmodia or zoosporangia within infected roots do provide this data (Merz et al., 2004) but can still require several weeks for completion. Results from in vitro assays that do not control timing of inoculation of roots may also be confounded by differing numbers of infection cycles between cultivars (Thangavel et al., 2015). This study has developed a novel in vitro assessment method that is very rapid (results within 48 h), allows control over experimental environmental conditions and assesses cultivar susceptibility to zoospore root attachment at the first point of pathogen interaction, thus avoiding issues associated with polycyclic infection.

This study provided data on the effect of temperature conditions for *S. subterranea* zoospore release and identified the optimal location on root tissue and temperature incubation conditions for zoospore root attachment. Maximum zoospore release occurred at 20°C in Hoagland's solution, while the highest zoospore root attachment occurred at 15°C. Many studies have reported optimal temperatures for *S. subterranea* root infection or tuber disease development (van de Graaf et al., 2005, 2007; Hughes, 1980; Kole, 1954; Shah et al., 2012), but this is the first to specifically determine the optimal temperature for zoospore root attachment. These results are consistent with the findings from previous studies. For example, germination of zoospores was shown to occur at temperatures between 9 and 17°C in aqueous solution (Fornier et al., 1996) or soils (van de Graaf et al., 2005; Shah et al., 2012). Similarly, root galling and tuber infection were promoted by soil temperatures of 11–25°C and 9–17°C, respectively, with more severe tuber infection at 12°C (van de Graaf et al., 2005; Hughes, 1980; Shah et al., 2014).

This study showed that the zoospore root attachment was generally higher in root hairs from the lower (younger) rather than the

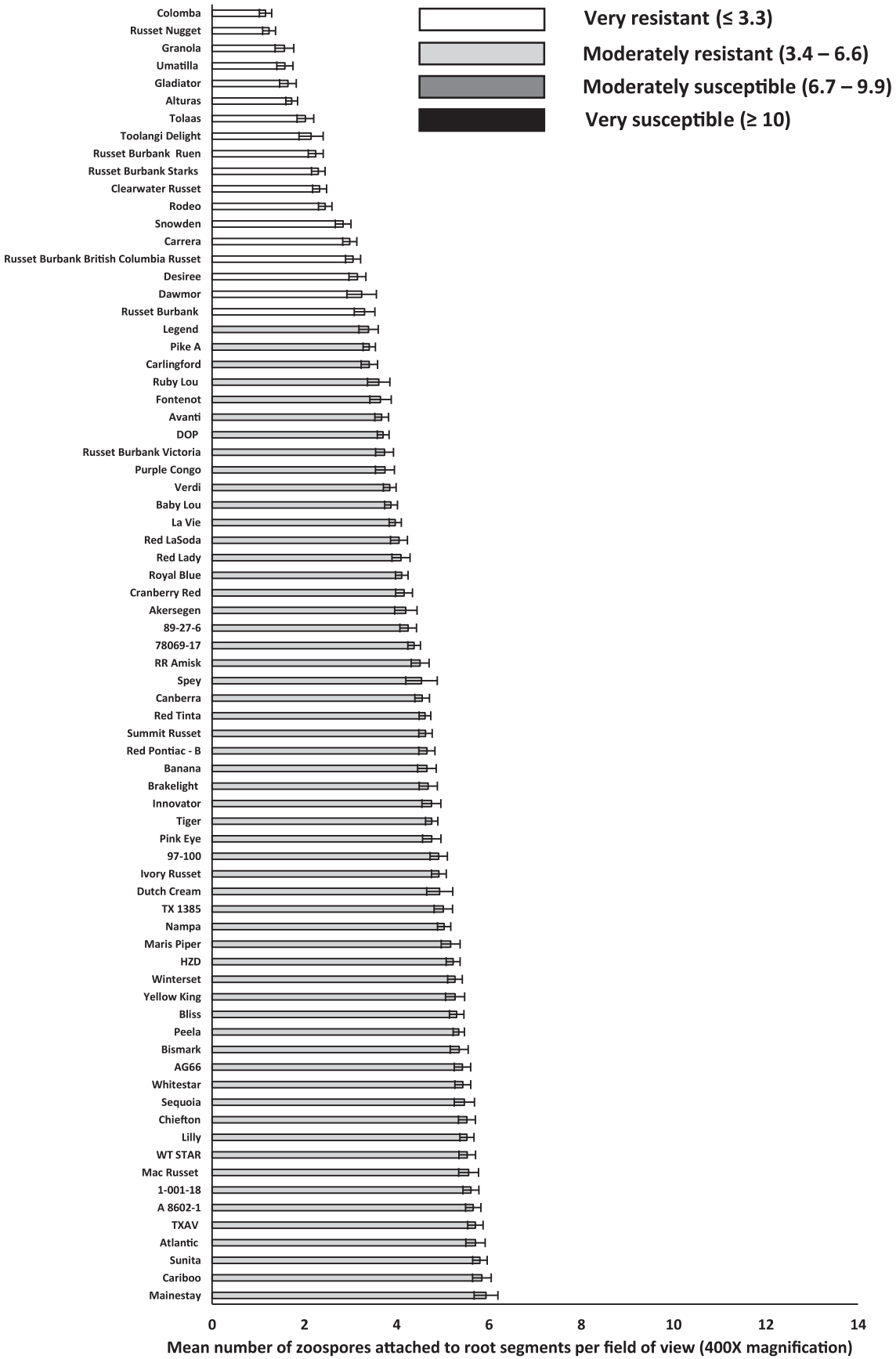

FIGURE 7 Scaled mean scores for *Spongospora subterranea* zoospore root attachment of 153 potato cultivars and lines assessed by the in vitro root attachment assay ($n = 3$) with $p < 0.001$. The cultivars were arbitrarily classified as very resistant (score ≤3.3), moderately resistant (score 3.4–6.6), moderately susceptible (score 6.7–9.9) and very susceptible (score >10). Horizontal bars represent standard error ($n = 3$).

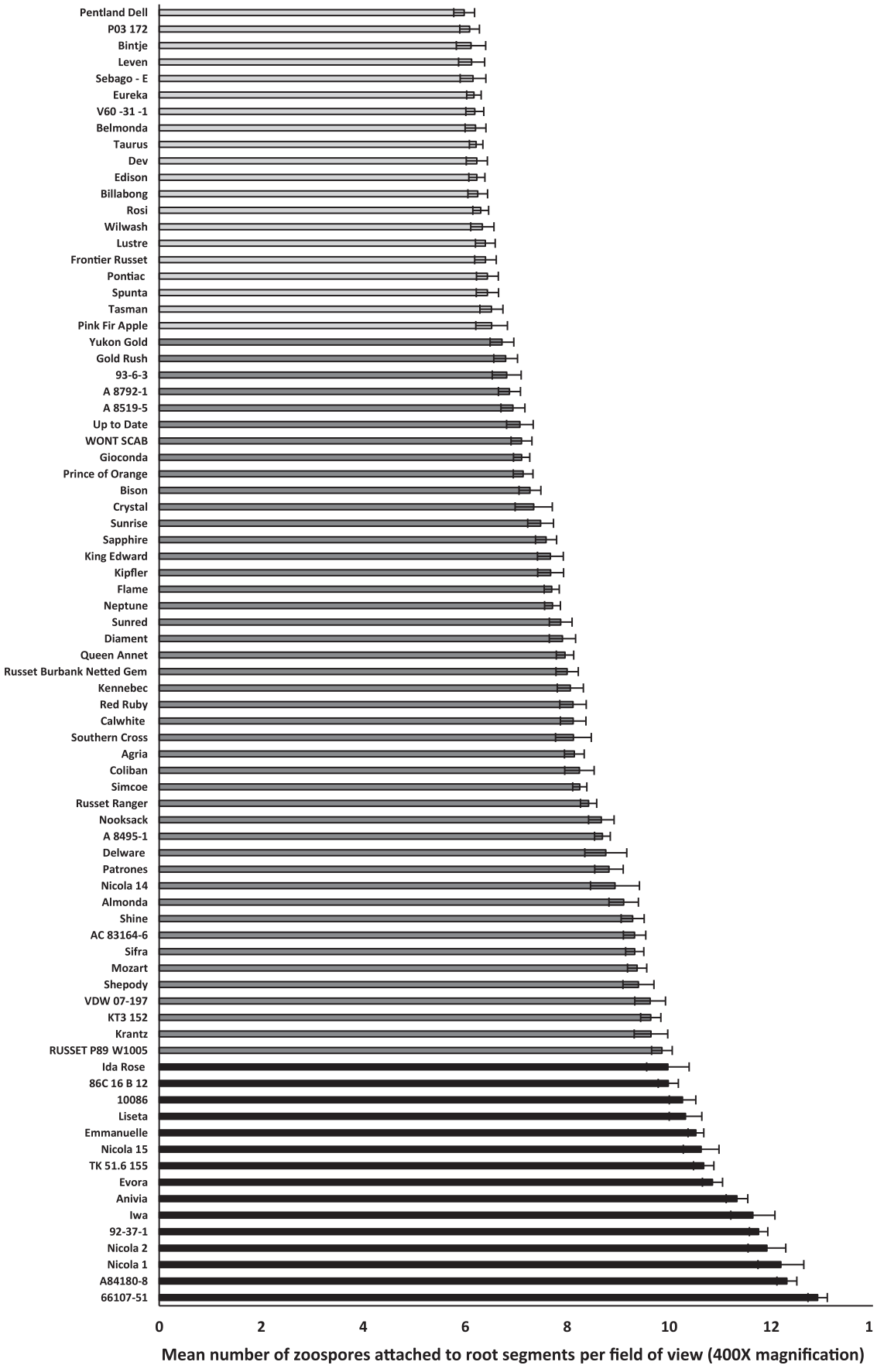

FIGURE 7 (Continued)

TABLE 2 Relationship between categorization of 13 potato cultivars and lines as very resistant to very susceptible by *Spongospora subterranea* zoospore root attachment assay and similar published root gall resistance categories.

	Root galling (Bittara et al., 2016; Falloon et al., 2003, 2016)			
Root attachment (this study)	Very resistant	Moderately resistant	Moderately susceptible	Very susceptible
Very resistant	Gladiator	Desirée	Umatilla Russet, Alturas, Russet Burbank	
Moderately resistant	Summit Russet			
Moderately susceptible			Kennebec, Yukon Gold, Russet Ranger	Agria, Shepody
Very susceptible			Nicola	Iwa

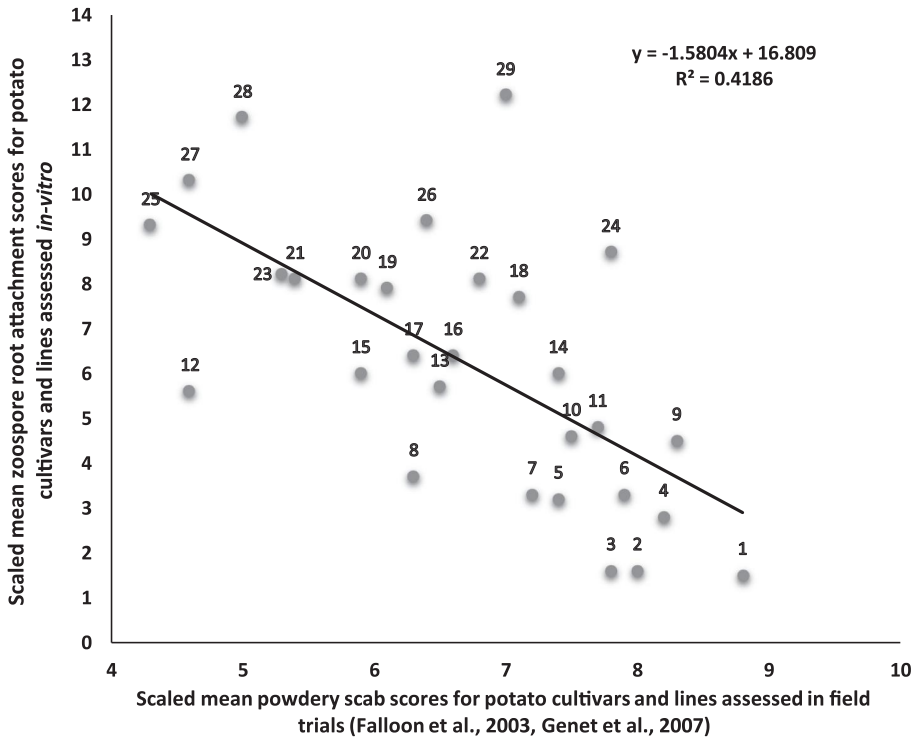

FIGURE 8 Relationship between scaled mean scores for *Spongospora subterranea* zoospore root attachment of 29 potato cultivars and the published powdery scab disease severity scores of these cultivars assessed in field trials (Falloon et al., 2003; Genet et al., 2007). For zoospore root attachment, the cultivars were arbitrarily classified as very resistant (score ≤3.3), moderately resistant (score 3.4–6.6), moderately susceptible (score 6.7–9.9) and very susceptible (score >10). For powdery scab diseases, the cultivars were classified as very resistant (score ≥8), moderately resistant (score 7.0–7.9), moderately susceptible (score 6.0–6.9) and very susceptible (score ≤5.9). 1 Gladiator, 2 Granola, 3 Umatilla Russet, 4 Snowden, 5 Desirée, 6 Russet Burbank, 7 Sebago, 8 Victoria, 9 Spey, 10 Summit Russet, 11 Innovator, 12 MacRusset, 13 Atlantic, 14 Pentland Dell, 15 Concorde, 16 Spunta, 17 Frontier Russet, 18 Flame, 19 Diamant, 20 Kennebec, 21 Agria, 22 Red Ruby, 23 Coliban, 24 Nooksac, 25 Shine, 26 Shepody, 27 Liseta, 28 Iwa, 29 Nicola.

medium and the upper maturation regions of the root. However, it would appear that the zoospore root attachment response is not associated with root hair density, given that root hair density was similar across the three root regions tested. Potentially, zoospores may have had greater attraction to younger root hairs. A similar observation noted that zoospore adhesion of *Pythium aphanidermatum* occurred largely in the younger root hairs (Jones et al., 1991).

The results of screening 153 potato lines and cultivars demonstrate that susceptibility to zoospore root attachment follows a continuum from very resistant to very susceptible, suggestive of control by polygenetic resistance factors similar to those observed for tuber disease (Falloon et al., 2003; Genet et al., 2007). There was a clear significant correlation between zoosporangial infection severity and zoospore root attachment for the 12 cultivars evaluated in this study; for example, Iwa, 10086 and Krantz were very susceptible to both zoosporangial infection and root attachments, while Tolaas and Toolangi Delight were very resistant in both assessments.

Although relationships between root infection (zoosporangial infection and root galling) and tuber infection (powdery scab) have been established with varying degrees of association, no previous studies have demonstrated a relationship between zoospore root

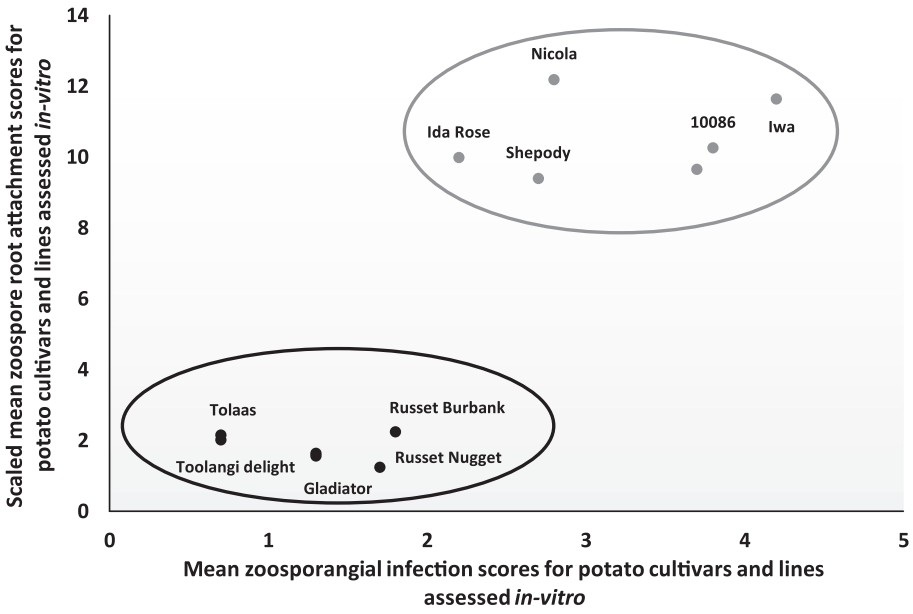

FIGURE 9 Relationship between scaled mean of *Spongospora subterranea* zoospore root attachment scores and zoosporangial infection severity score 0 = no infection; 1 < 10% sporadic; 2 = 2%–10% slight; 3 = 11%–25% moderate; 4 = 26%–50% heavy and 5 > 50% very heavy (Merz et al., 2004) of 12 selected potato cultivars.

attachment and cultivar resistance to both root and tuber infections. Here we showed that the resistance response of most potato cultivars was generally consistent regardless of the method of assessment. This was best demonstrated by the response of cultivars Iwa and Gladiator whereby Iwa had higher zoospore root attachment scores than cultivar Gladiator reflecting the known higher susceptibility of Iwa and the higher resistance of Gladiator to zoosporangial infection, root galling and tuber powdery scab (Falloon et al., 2003, 2016). This is unsurprising as we would expect that zoospore root attachment would impact the rate of root infection and thus the development of later disease expression such as root galls and tuber disease (Thangavel et al., 2015).

There are notable exceptions, however, that emphasize the difference between resistance expression to the root and tuber diseases. Among the 29 cultivars with known resistance to powdery scab, there are three outliers. MacRusset was rated as moderately resistant to zoospore root attachment but moderately susceptible to tuber powdery scab. Nooksac was rated as moderately susceptible to zoospore root attachment but moderately resistant to powdery scab. Additionally, Nicola has been reported as possessing moderate resistance to powdery scab but is highly susceptible to zoospore root attachment in our study with four independent clones tested (Nicola 1, Nicola 2, Nicola 14 and Nicola 15).

Five of the 13 cultivars with documented resistance to root galling were in agreement with their resistance to zoospore root attachment. However, Umatilla Russet, Alturas and Russet Burbank were graded as resistant to zoospore root attachment but susceptible to root galling. Notably, Nicola is rated as very susceptible to zoospore root attachment and moderately susceptible to root galling. Thus, while growers often consider cultivar Nicola as a cultivar with minimal impact from *S. subterranea* due to a lack of tuber disease, root disease may be prevalent, resulting in poor plant performance (Merz et al., 2012). These exceptions mentioned indicate the complexity of the diseases caused by *S. subterranea* and differences in optimal environmental conditions and genetic resistance for tuber and root infections (Harrison et al., 1997; Nitzan et al., 2008).

In conclusion, the in vitro screening technique developed in this study provides a novel and comprehensive assessment method of the relative susceptibility of potato cultivars to zoospore root attachment by *S. subterranea*. Results from the screening of 153 cultivars and lines by the zoospore root attachment bioassay generally agreed with the cultivar resistance scores obtained from published potato powdery scab severity scores field trial data, with a few notable varietal exceptions (Falloon et al., 2003, 2016). This in vitro bioassay only requires potato root tissue samples. In the present study, we used tissue-cultured plantlets, but in unpublished studies we have also successfully used root tissues harvested from glasshouse-grown plants for the in vitro screen. This increases the bioassay's flexibility. For example, breeders could germinate true seed from select crosses and directly test roots from these seedlings without the need for first introducing these into tissue culture. This method avoids any confounding impact of polycyclic infections, which can result in subsequent variations in root infection scores, and the bioassay can be conducted under strictly controlled conditions in the laboratory, which minimizes variable environmental influences. We propose that breeders may find the use of this rapid screen highly valuable to rapidly screen for resistance to root attachment/infection, a trait of considerable interest to the commercial potato industries, decreasing generation time and improving screening efficiency; growers may find the varietal

assessment cultivar rankings for root infection valuable in the selection of suitable planting material according to their disease risk.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data sets generated in this study are available on request to the corresponding author.

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