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The presence of *Phytophthora infestans* in the rhizosphere of a wild *Solanum* species may contribute to off-season survival and pathogenicity

Ramesh R. Vetukuri[a,1,*], Laura Masini[b,1,2], Rebecca McDougal[c], Preeti Panda[c], Levine de Zinger[b,3], Maja Brus-Szkalej[b], Åsa Lankinen[b,4], Laura J. Grenville-Briggs[b,4]

[a] Dept. of Plant Breeding, Swedish University of Agricultural Sciences, 230 53 Alnarp, Sweden
[b] Dept. of Plant Protection Biology, Swedish University of Agricultural Sciences, 230 53 Alnarp, Sweden
[c] Scion (New Zealand Forest Research Institute), 49 Sala Street, Rotorua, New Zealand

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ABSTRACT

We evaluated oomycete presence and abundance in the rhizosphere of wild perennial *Solanum* species to investigate the presence of plant pathogenic or mycoparasitic species. Furthermore, we investigated whether these plant species could function as hosts, or associated plants, for off-season survival of economically important pathogens. We collected soil samples in Sweden from *Solanum dulcamara* and as a control from *Vitis vinifera* over all four seasons of a year, and in New Zealand from *Solanum nigrum* and *Solanum laciniatum* in the summer. Species identification, confirmed by ITS and Cox2 sequencing, and root infection assays on the crop plant *Solanum tuberosum* and on *S. dulcamara*, suggested the presence of mainly Pythiales species. In Sweden, we also found evidence for the presence of *Phytophthora infestans*, the causal agent of potato late blight, in the rhizosphere of *S. dulcamara*. These *Ph. infestans* isolates had no negative effects on root growth of *S. dulcamara* in Sweden, but were more pathogenic on potato leaves than a common lab strain. Oomycete diversity measures indicated a high similarity between seasons and countries. In conclusion, our study suggests a previously unknown overwintering strategy for the pathogen *Ph. infestans*, indicating a possible influence of the wild species *S. dulcamara* on the epidemiology of potato late blight in Sweden.

1. Introduction

The oomycetes are a unique, and widespread, group of filamentous microbes belonging to the kingdom Stramenopila. Oomycetes are found in a wide variety of ecosystems and many members of this family are destructive pathogens of diverse hosts including crop plants, natural forests, fish, insects and, occasionally, humans (Fawke et al., 2015). Those that are the most economically damaging in agriculture and horticulture include the downy mildews of the genera *Peronospora* and *Plasmopara* and members of the *Pythium, Phytophthora* and *Aphanomyces* genera. The genus *Pythium,* and recently described sister-genera *Globisporangium* and *Elongisporangium* (Uzuhashi et al., 2010), are some of the most important groups of soil-borne plant pathogens, being ubiquitous in soils and having an extremely wide host range, attacking the roots of thousands of different host species. Indeed, many *Pythiaceae* species may be considered “necrotrophic pioneer colonizers” since they aggressively attack young feeder roots and germinating seedlings (Schroeder et al., 2013). They can have a significant impact in terms of plant disease and seedling losses in both agricultural systems and in wild plants (Mills and Bever, 1998; Packer and Clay, 2000, 2004; Nechwatal and Oßwald, 2001). They may also contribute to species diversity within plant communities (Packer and Clay, 2000). Other species of the *Pythium* genus are mycoparasites and may therefore play a role in shaping soil microbial communities through mycoparasitism of soil inhabiting oomycetes and fungi. The identification and characterisation of these mycoparasitic species could also be useful for the development of these species as novel agents for the biological control of plant diseases.

Abbreviations: Ph, *Phytophthora*; *Py*, *Pythium*; *Pp*, *Phytopythium*

* Corresponding author at: Dept. of Plant Breeding, PO Box 101, Swedish University of Agricultural Sciences, 230 53 Alnarp, Sweden.
E-mail address: Ramesh.Vetukuri@slu.se (R.R. Vetukuri).

[1] These authors contributed equally to this work.
[2] Current address: Plant Biotechnology Division, British American Tobacco, 210 Cambridge Science Park Cambridge CB4 0WA, UK.
[3] Current address: Institute of Sugar Beet Research, PO Box 20, 4670 AA Dinteloord, The Netherlands.
[4] These authors contributed equally to this work.

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In the absence of a preferred host plant, pathogenic species must survive in the soil either by feeding saprotrophically, producing environmentally resistant resting spores, or by survival on alternate hosts or in association with other plants. Where the preferred host is a cultivated plant, alternate hosts or other associated plants may be wild plant species found at the borders of, or within, agricultural fields (Van Baarlan et al., 2007). Studies in microbial ecology, including the plant microbiome, are opening up new possibilities for molecular ecological investigations of the survival and interactions of plant pathogenic oomycetes within natural soils and in association with alternate, wild, host species.

One of the most devastating and well-known members of the *Phytophthora* genus is *Phytophthora infestans* the causal agent of potato and tomato late blight. The origin of this pathogen can be traced to either Central Mexico (Grünwald and Flier, 2005), and/or the Andes (Gómez-Alpiza et al., 2007) where it infects several wild *Solanum* species and cultivated potato (*Solanum tuberosum*). Mexican isolates of *Ph. infestans* are characterised by high genetic diversity due to sexual reproduction and hybridization (Grünwald and Flier, 2005). Wild *Solanum* species in the region form a metapopulation, and have been suggested to support the evolution of *Ph. infestans* isolates (Grünwald and Flier, 2005).

Three wild *Solanum* species, *Solanum nigrum* (black nightshade), *Solanum physalifolium* (hairy nightshade), and *Solanum dulcamara* (bittersweed nightshade), can serve as natural hosts, or associated plants, for *Ph. infestans* in Europe (Andersson et al., 2003; Flier et al., 2003; Deahl et al., 2004; Golas et al., 2010). In Sweden, all three species are found within or at the margins of agricultural land predominantly in the southern and central areas of the country (Abreha et al., 2018). *Solanum nigrum* and *S. physalifolium* are self-compatible annual weeds, whereas *S. dulcamara* is a semi-woody, outcrossing perennial climbing shrub. These species differ in susceptibility to *Ph. infestans* infection, where *S. nigrum* is resistant, *S. dulcamara* shows varying resistance and *S. physalifolium* is highly susceptible (Frades et al., 2015; Abreha et al., 2018). *S. physalifolium* thus has the largest potential to directly affect epidemiology of *Ph. infestans,* but it is not as common as the other two species in Sweden. Isolates of *Ph. infestans* collected from *S. physalifolium,* in Sweden, were more aggressive on cultivated potato (*S. tuberosum*) compared to *Ph. infestans* isolates that were isolated directly from potato (Grönberg et al., 2012). Since *S. physalifolium* and *S. nigrum* are annual species in Sweden and *S. dulcamara* is a perennial, the latter species may be of particular importance for off-season survival of *Ph. infestans* or other plant pathogenic oomycetes. For example, in Sweden *S. dulcamara* roots growing close to or in water streams used for agricultural irrigation have been shown to harbour *Ralstonia solanacearum*, causing brown rot disease in potato (Persson, 1998).

While *S. nigrum* is considered an annual weed in Northern Europe, this species can survive as a short-lived perennial in New Zealand. *S. nigrum* was introduced into New Zealand in the 1800s and is typically found in arable or disturbed land being particularly abundant on North Island. Therefore, it is possible that although this species is unlikely to contribute to off-season survival of oomycete or fungal pathogens in the Swedish climate, it may have the potential to do so in other parts of the world, such as New Zealand. In fact, *S. nigrum* was shown to act as a reservoir of begomoviruses causing tomato yellow leaf curl disease (TYLCD) in southern Spain (García-Andrés et al., 2006). *Solanum laciniatum* is a large, native perennial species that is highly abundant throughout both the North and South Island of New Zealand and is also found in close proximity to arable land and at forest borders and thus could potentially contribute to the off-season survival of forest or crop pathogens. In New Zealand, *Phytophthora* species are impacting native and plantation forests (Scott and Williams, 2014), as well as horticultural crops. The inoculum reservoir for the foliar forest pathogens *Phytophthora pluvialis* and *Phytophthora kernoviae* has not yet been identified. Sampling non-host species near plantation forests affected by such pathogens, could yield important information useful for controlling the diseases caused by these species.

Previous studies have shown that the root pathogenic oomycete *Phytophthora cinnamomi*, which has a very broad host range, can survive hot Mediterranean summers on alternative hosts or in association with other plants that often do not show any symptoms of infection (Crone et al., 2013). It is also able to asymptomatically colonise many annual and herbaceous perennial weed species that are native to the Western Australian forests where this pathogen has been introduced (Crone et al., 2013). These data suggest that some pathogenic oomycetes are able to survive in association with other plants and that native plants may make important contributions to off-season survival of plant pathogens.

While the infection phase on the host is believed to exert the strongest selective force on pathogenicity (McDonald and Linde, 2002), off-season survival could also have an effect, e.g. due to trade-offs between life-history strategies (Laine and Barrès, 2013; Tack and Laine, 2014). This is particularly important in pathogens that alternate between epidemic and survival phases, where the latter can take place in a different organ of their host or outside of the host (e.g. in the soil or in the roots of an overwintering host or associated plant). For example, *Ph. infestans* isolates that overwintered in inoculated tubers in Northern, central or Western France, were both more virulent and had a shorter latent period than isolates that did not overwinter in tubers, suggesting there may even be a positive correlation between survival and virulence (Montarry et al., 2007). Knowledge about the link between virulence and survival phases, such as overwintering, is understudied but can be particularly valuable for understanding the epidemiology of a pathogen and for developing control measures in crops (Andrivon et al., 2013). To this end, the pathogenicity of isolates collected from their potential overwintering hosts, or other associated plants should be evaluated.

The aims of the present study were to identify any oomycete plant pathogens or mycoparasites present in the rhizosphere of common wild *Solanum* species found in proximity to agricultural fields and to investigate the potential of these wild *Solanum* species to contribute to the off-season survival of oomycete plant pathogens in different seasons and latitudes. We compared the presence and distribution of culturable *Pythium* and *Phytophthora* species present in rhizosphere samples taken from both *S. dulcamara* growing in a common garden area at the SLU campus and an adjacent vineyard in southern Sweden, over all four seasons of the year. Seasonal variation revealed a greater number of oomycete species during spring and summer in Sweden. We therefore compared this data to that obtained during early summer from the rhizosphere of *S. nigrum*, *S. laciniatum* and a pond in New Zealand. Oomycetes were obtained by baiting and/or direct plating of soil samples, and species identification was assessed by sequencing the ribosomal internal transcribed spacer (ITS) and mitochondrial cytochrome oxidase subunit 2 (Cox2) regions. Root and detached leaf assays were used to measure the pathogenicity of a selection of the obtained oomycete species.

2. Materials and methods

2.1. Sampling sites

Soil sampling in Sweden was carried out at the Swedish University of Agricultural Sciences (SLU) campus experimental garden in a site with *Solanum dulcamara* (55°66′106.2″N 13°08′198.9″E) and an adjacent vineyard (55°66′148.7″N 13°08′268.6″E) at Alnarp. As the vineyard was located in identical microclimate and with identical soil type, this allowed a comparison of how host plant influence abundance and diversity of oomycetes in the rhizosphere. The *S. dulcamara* site was established in 2012–2013 by planting > 150 genotypes originating from 12 wild populations in south Scania (mainly Lunds Kommun and Lomma Kommun) (Abreha et al., 2018). In 2012, the highly susceptible *Solanum physalifolium* were grown in the same field. *S. physalifolium* but not *S. dulcamara* were heavily infected by naturally occurring late

blight during 2012. In subsequent years, late blight was noted on potato (2013–2014) and *S. physalifolium* (2013) in other parts of the experimental garden. Thus, hypothetically, both the *S. dulcamara* site and the vineyard should have been exposed to natural inoculum.

New Zealand soil samples were collected from rhizosphere soils of *S. nigrum* and *S. laciniatum* in Rotorua at the broader Scion (New Zealand Forest Research Institute) campus (38°09′43.8″S 176°15′47.7″E) and from the sediment of a close by pond, as well as from the Rotorua lakefront area (located in a geothermal area, 25 °C water temperature, 38°07′53.4″S 176°15′43.5″E). The Scion campus site is located near a mixed species forest. Plants were growing together with mixed native and exotic trees and shrubs in a relatively undisturbed area without tillage. Sampling was performed from plants in vegetation and gardens along the lakefront walkways. The pond is located on the Scion campus on the southern side of Lake Rotorua and in the same locality (within 3–10 m) as the sampled plants.

2.2. Soil sampling and baiting for oomycete cultures

Rhizosphere soil samples from the SLU campus common garden where *S. dulcamara* was growing and the adjacent vineyard at the same location were collected in a randomized manner during all the four seasons in a year, starting from March 2015 representing spring samples. Summer, autumn and winter samples were collected in July 2015, September 2015 and January 2016, respectively. Sampling was carried out in a standard walking "W" pattern, meaning five soil cores were taken per sampling occasion. In New Zealand soil sampling was carried out in a similar way but only during the summer season in December 2015. Soil cores containing soil adjacent to and including lateral roots of individual plants (5 cm from the main stem) in each of these sites were sampled to a depth of 15 cm. Each core contained approximately 10 g of soil. The soil samples were each thoroughly mixed by repeated turning and shaking in polyethylene bags.

To isolate soil-borne oomycetes, baiting and direct plating methods were used, since we were interested in identifying living oomycetes that were capable of off-season survival in these soils. To bait the oomycetes from these samples, fresh leaf baits from wild type *S. dulcamara, S. nigrum, Solanum laciniatum, Rhododendron ponticum, Quercus robur* and *Camellia japonica* were used. Soil samples (one litre) were placed in sterile plastic boxes flooded with de-ionized water and several baits were spread over the water surface. After incubation for 1–3 days at room temperature, baits were dried with clean paper towels, cut into small segments and plated onto a selective agar medium consisting of corn meal agar (CMA) supplemented with pimaricin (10 μg ml^{-1}), rifampicin (10 μg ml^{-1}), benomyl (10 μg ml^{-1}), ampicillin (250 μg ml^{-1}) and pentachloronitrobenzene (100 μg ml^{-1}) and grown at 20 °C. For isolation of *Phytophthora* spp. from baits, CMA was supplemented with hymexazol (250 μg ml^{-1}) in addition to the above chemicals. After incubation for 1–3 days, newly emerging colonies were transferred to new CMA supplemented with the above-mentioned antibiotics. In direct plating method, roughly 1 cm^3 of soil was placed on CMA supplemented with antibiotics mentioned above and incubated at 20 °C. Developing colonies were repeatedly sub-cultured, purified from bacterial and fungal contaminants and transferred to V8 agar plates amended with pimaricin (10 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) for further identification and maintenance.

2.3. DNA extraction and species identification

Mycelium of individual isolates was grown for DNA extraction in 10% liquid V8 medium amended with pimaricin (10 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) for 5 days at 20 °C. Mycelium from each liquid V8 culture was harvested by filtration, snap frozen in liquid nitrogen, and stored at −80 °C until required. Total DNA was extracted using a QIAGEN DNeasy® Plant Mini Kit (Qiagen, UK) according to manufacturers' recommendations.

The ITS and Cox2 regions were PCR amplified individually with the universal primer pairs known to amplify a wide range of oomycetes: ITS5 (5′GGAAGTAAAAGTCGTAACAAGG 3′) and ITS26 (5′ATATGCTTAAGTTCAGCGGGT 3′) (White et al., 1990; Howlett et al., 1992), Cox2-F (5′GGCAAATGGGTTTTCAAGATCC 3′) and Cox2-RC4 (5′TGATTWAYNCCACAAATTTCRCT 3′) (Hudspeth et al., 2000; Choi et al., 2015).

PCRs were carried out using 10 ng of DNA as described (White et al., 1990; Choi et al., 2015). Yield and integrity of the DNA was assessed using a NanoDrop Micro Photometer (NanoDrop Technologies, UK), and agarose gel electrophoresis, respectively. The size of the PCR products of ITS and *cox*2 are approximately 1000 bp and 581 bp, respectively. PCR products were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, UK). Sanger sequencing for species identification was carried at the GATC biotech AG sequencing facility (Germany) using ITS and Cox2 primers. DNA star software (*DNASTAR*, USA) was used to manually analyse and trim (based on sequencing quality) nucleotide sequences obtained from Sanger sequencing. Resulting sequences with complete ITS and Cox2 regions were searched for matching hits against the National Center for Biotechnology Information (NCBI) GenBank non-redundant nucleotide database (BLASTn; Altschul et al., 1990). Search hits to sequences from records in the database were evaluated for coverage and identity and the best-matched NCBI accession recorded. All the sequence data are submitted to NCBI and GenBank Accessions are provided (Supplementary Table S1).

2.4. Mating type determination

In order to assess whether it was likely that collected isolates of *Phytophthora infestans* represented a sexually outcrossing population, the mating type of the *Ph. infestans* isolates collected from Alnarp during three seasons (winter, spring, and summer) was determined. Individual test isolates were cultured adjacent to known A1 and A2 isolates and examined microscopically for oospore formation following the established methods of Cvitanich and Judelson (2003). Dutch isolates A1 (88069) and A2 (Ipio) were used as *Ph. infestans* reference isolates.

2.5. Sequence alignment and phylogenetic analysis

DNA sequences for individual isolates were aligned and molecular operational taxonomic unit (MOTU) numbers were assigned to sequences with nucleotide variations. DNA sequences with at least 99% sequence identity were classified as identical MOTUs and the longest sequence available for that MOTU number was used as the reference. MOTUs were aligned, together with appropriate reference sequences using Geneious (v 10.2.4) (Kearse et al., 2012) and the MAFTT multiple alignment (v1.3.5) plugin (Katoh et al., 2002). Phylogenetic trees were constructed using the Geneious FastTree plugin (Price et al., 2009), where support values are calculated using the Shimodaira-Hasegawa (SH) test (Anisimova and Gascuel, 2006) Trees were visualized and edited in iTol (Letunic and Bork, 2016). Reference sequences were chosen based on tentative identifications of isolate MOTUs from BLAST-N analysis, and type strain sequences were used where available (Supplementary Tables S2, S3).

2.6. Detached leaf assay

To investigate pathogenicity of the three collected *Ph. infestans* isolates (one each from winter, spring, summer) on susceptible *S. tuberosum* cv. Désirée, the isolates and the commonly used lab isolate 88069 were maintained on rye-pea medium amended with 2% sucrose at 20 °C. For inoculation, sporangia were harvested both directly from the plates and after one round of inoculation on *S. tuberosum* cv. Désirée leaves. Seven days after inoculation, sporangia were washed from the surface of these leaves and counted. These were used directly for detached leaf assays. All sporangial suspensions were counted on a

haemocytometer, and the final concentration adjusted to 1.5×10^4 sporangia per ml. The inoculum was then placed at 4 °C for 1–2 h to promote zoospore release. After two hours zoospore samples were collected, and counted using a haemocytometer, to allow adjustment of inoculum to 1×10^5 zoospores per ml.

Eight- to ten-week-old *S. tuberosum* cv. Désirée plants grown in controlled conditions were used to assess late blight disease development (Vetukuri et al., 2011). Three experiments were conducted using inoculum grown in vitro on rye sucrose plates. Two further experiments were conducted, using inoculum derived from leaves, to ensure that all test isolates were at maximum pathogenicity before testing. Since it is possible that *Ph. infestans* may become attenuated in virulence after repeated in vitro cultivation, inoculum from our laboratory reference strain 88069, recently revived from liquid nitrogen stocks, was passaged both through in vitro cultivation and through Désirée leaves prior to detached leaf assays. In each of these experiments 4–8 leaves were inoculated per test isolate (total N_{plates} = 22–23 per isolate, total N_{leaves} = 15–16 per isolate). Counting from the top, the second to fourth fully expanded leaflets of a compound leaf were detached and placed abaxial side up on moist paper in clear plastic containers, and inoculated with approximately 2000 zoospores in a 20 µl droplet on either side of the leaf midrib. Six to eight leaflets were inoculated with either *Ph. infestans* isolate. Plastic containers were tightly sealed and placed at 20 °C with 16 h photoperiod. Pathogenicity phenotypes were assessed at 7 days post inoculation (DPI) by measuring the maximum length and width of the lesion size of each leaf. The lesion size (LS) was calculated as LS = 1/4 π x length x width (Vleeshouwers et al., 1999). Lesion size was then normalized against the total leaf area, and expressed as a percentage.

2.7. Root infection assay

To assess root pathogenicity of a subset of the oomycete species on *S. tuberosum* and *S. dulcamara*, five isolates i) *Aphanomyces cladogamus/Leptolegnia caudata*, ii) *Pythium heterothallicum*, iii) *Pythium intermedium*, vi) *Pythium rostratifingens*, and v) *Pythium sylvaticum* were selected based on their occurrence in the Swedish soil samples (Fig. 1, Supplementary Table S1). Stem cuttings of *S. tuberosum* cv. Bintje and *S. dulcamara* accession Sd 3:6 (Frades et al., 2015) were used in root infection assays. We used cv. Bintje rather than cv. Désirée because this cultivar was previously shown to be more susceptible to the root pathogenic fungus *Colletotrichum coccodes* compared to cv. Désirée (Andrivon et al., 1998). Additionally, root infection assays on *S. dulcamara* with the *Phytophthora infestans* isolates collected during winter and spring were conducted to explore a potential pathogenic effect on this host species.

Plants used in the root infection assays were generated by in vitro clonal micropropagation (Burra et al., 2015). Stem cuttings were transferred to shoot-inducing medium, containing half-strength MS supplemented with vitamins (Duchefa Biochemie, NL), 2% sucrose, and 0.5 $mg \cdot l^{-1}$ IBA (indole-3-butyric acid), pH 5.8, under long days conditions (day/night: 16 h - 23 °C/8 h - 18 °C). After seven days, stem cuttings showing initial root development were transferred to a sterile infection box, made of a small size tip box, filled with half-strength MS with vitamins, and 2% sucrose to cover the roots. Stem cuttings/seedlings were suspended in a sealed inoculation box and kept at 20 °C with a 16 h photoperiod. After three days, the stem cuttings/seedlings were inoculated by the addition of agar plugs of an actively growing plate culture of the different isolates (1 plug per stem cutting/seedling). Isolates were grown on CMA supplemented with ampicillin

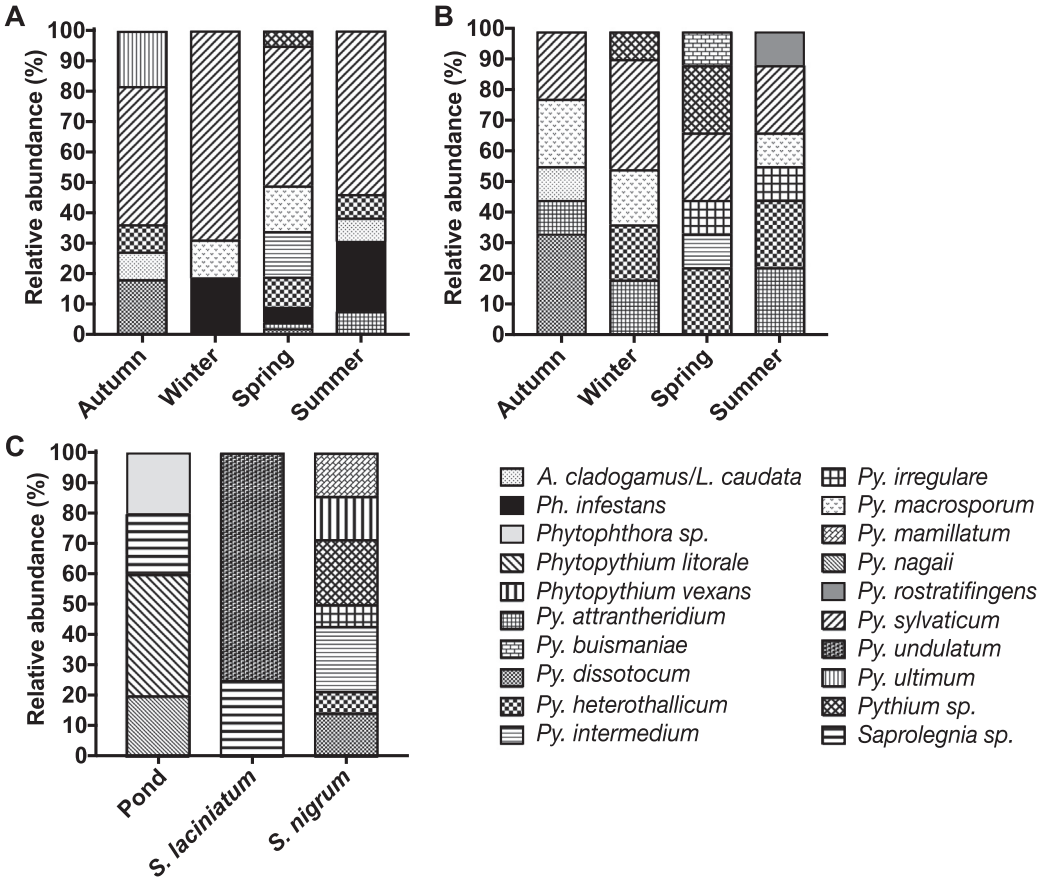

Fig. 1. Identities and frequencies of oomycete species isolated from the rhizosphere of (a) *Solanum dulcamara*, and (b) *Vitis vinifera*, in Sweden over four seasons, and of (c) *S. laciniatum, S. nigrum* and from a pond in New Zealand over the summer.

(250 μg ml^{-1}), rifampicin (10 μg ml^{-1}), and pimaricin (10 μg ml^{-1}), except for *Phytophthora* isolates that were grown on rye-pea agar supplemented with pimaricin (10 μg ml^{-1}), and ampicillin (100 μg ml^{-1}).

Three root assay experiments were conducted per oomycete isolate and plant species, usually involving 3–5 inoculated replicates and untreated control plants, respectively (total $N_{s.\ tuberosum}$ = 12–15 per isolate, total $N_{s.\ dulcamara}$ = 9–13 per isolate). Control plants were kept in separate boxes per plant species. Disease was evaluated 7 DPI by measuring the root length of each stem cutting/seedling.

2.8. Data analysis

Data from the detached leaf assays and root infection assays were analysed using ANOVAs (type III sums of squares). For lesion size data from the detached leaf assays on *S. tuberosum* involving *Ph. infestans* from both plates and leaves, a two-way ANOVA involving the fixed factor isolate and the random factor experiment were used. Differences between isolates were analysed using Tukey's post-hoc test. Data on root length from the root infection assays were analysed separately for each of the tested isolates per plant species, because root assays were performed at different occasions. We used a two-way ANOVA involving the fixed factor treatment (inoculated vs. control plants) and the random factor root assay experiment.

To get an indication of the oomycete community structure across species, seasons and countries species richness and measures of diversity were calculated for each sampling event and plant species/control site. It should be noted that the small sample size makes the diversity measures less reliable. Species richness was calculated by summing the number of detected species (or genus for species that could only be determined to genus level). Simpson's dominance ($D = \Sigma p_i^2$) and Shannon's diversity ($H = -\Sigma p_i \ln(p_i)$) were used for diversity measures taking both number of species and their occurrence into account, where p_i is the frequency of occurrence of each species. Evenness (E), i.e. how close in numbers each species is in a sample, was calculated as $^{e^H}/_S$ ($0 < E \leq 1$), where e is the base of the natural logarithm. To test for differences between Swedish host species and between countries a non-parametric U test was used. Differences among seasons in Sweden for both host species were tested with a non-parametric Kruskal-Walls test. Because diversity did not differ significantly among seasons (see Results and discussion), the comparisons between host species in Sweden and between countries were based on all Swedish samples. All statistical tests were performed in SPSS (2017).

3. Results and discussion

3.1. Identification and phylogeny of oomycetes in rhizosphere samples in Sweden and New Zealand

To determine the role of wild *Solanum* spp. as potential associated plants of oomycete species at different latitudes, we carried out a comparative study of oomycete species diversity in the rhizosphere of *Solanum dulcamara*, and an adjacent vineyard in southern Sweden, as well as of *Solanum nigrum* and *Solanum laciniatum* and from sediment in an adjacent pond in New Zealand. To assess the seasonal variation of active soil dwelling oomycetes, samples were taken across all four seasons of the year in southern Sweden. In Alnarp, Sweden, we collected 81 oomycete isolates from the *S. dulcamara* rhizosphere, and 38 isolates from the grapevine (*Vitis vinifera*) rhizosphere (Table 1, Supplementary Table S1) from spring 2015 till winter 2016. These isolates were collected by either baiting or direct plating from rhizosphere soils. Sequencing of amplified products from both ITS and Cox2 regions was performed. For 11 isolates, the species identity was not resolved by ITS sequences, and they were instead classified according to their Cox2 sequence. Sequence matches for three isolates belonging to the order of Saprolegniales were not resolved with reference to the sequences available on the NCBI database, indicating only that they were closely related to *Aphanomyces cladogamus* and *Leptolegnia caudata*. In total, the isolates were assigned to 12 oomycete species, of which 11 belong to the order of Pythiales, and one to the order of Saprolegniales (Fig. 1a, b). While sister genera of *Pythium* spp. have been described, the uptake of use of the new names for these genera has not been consistent in the literature, hence we have continued to use the *Pythium* genus in our identifications, since these are listed as the current names in the Mycobank database (http://www.mycobank.org/). In addition, five isolates had high similarity to previously unclassified *Pythium* species (Supplementary Table S1). According to the classification by LéVesque and De Cock (2004), a large number of the *Pythium* species isolated belong to clade F (68%, 81/199 isolates), with the remaining isolates assigned to clades B2 (six isolates), E (one isolate), I (14 isolates), and J (one isolate).

Baiting of oomycetes from Rotorua, New Zealand yielded four isolates from the *Solanum laciniatum* rhizosphere, 14 isolates from the *S. nigrum* rhizosphere and five from the pond sediment (Table 1, Supplementary Table S1). The total of 23 isolates belonged to ten different species, the majority being *Pythium* spp. (six isolates could not be identified to species level) (Fig. 1c, Fig. 2, Supplementary Table S1). Additionally, three *Pythium* isolates, two *Saprolegnia* isolates, and one *Phytophthora* isolate were not able to be identified to species-level using ITS or Cox2 DNA sequences. 43% of isolates identified from the *S. nigrum* rhizosphere (6/14) were of *Pythium* species from clade F with the remainder from clade B2, clade I or unclassified since they were not identified to the species level. From *S. laciniatum,* 4/5 isolates were of *Pythium* species belonging to clade H, with the remaining isolate being of a *Saprolegnia* species.

The species of *Pythium* that group within the phylogenetic clade F are characteristically important plant pathogens with a worldwide distribution. Clades H and I also harbour some globally distributed and destructive plant pathogens (LéVesque and De Cock, 2004) and therefore the prevalence of species from these clades within our samples is expected. Subclade B2 consists of species that are less commonly isolated, but members of this clade have been isolated from soil, algae and the roots of *Pinus* species (LéVesque and De Cock, 2004). Although we did not specifically sample *Pinus* in this study, there were several stands of *Pinus* very close to the area where the wild *Solanum* species were grown and thus the isolation of these rarer species may be explained by the proximity of suitable host species. Interestingly we did not recover any species from Clade D which contains several *Pythium* species that are mycoparasitic towards other oomycetes and fungi and/or which can adopt endophytic lifestyles within several host plants (Horner et al., 2012).

When comparing isolates between the two countries, five out of the ten species retrieved in New Zealand showed similarity to those collected in Sweden, including (i) *Pythium coloratum/dissotocum/monospermum* (Clade B; Moralejo et al., 2017) (ii) *Pythium heterothallicum* (iii) *Pythium intermedium*, (iv) *Pythium irregulare* and (v) *Pythium buismaniae* (Fig. 1, Fig. 2). All of these species were sampled from the *S. nigrum* rhizosphere in New Zealand (Fig. 1c). *Py. heterothallicum* and *Py. intermedium* were isolated from the *S. dulcamara* rhizosphere in Sweden however, *Py. irregulare* and *Py. buismaniae* were only found in the grapevine rhizosphere in Sweden (Fig. 1a,b). *Pythium nagaii*, which was detected in the pond samples (Fig. 1c), was also present in Sweden, although it was isolated from *S. dulcamara* outside our study area in a wild population close to a wetland (data not shown).

3.2. Isolation of Phytophthora infestans from the rhizosphere of Solanum spp.

Wild *Solanum* spp. are commonly found within or at the boundaries of agricultural fields and managed forests in Sweden and New Zealand, and they can harbour potential pathogens for crop species (Persson, 1998; García-Andrés et al., 2006; Crone et al., 2013). The detection of

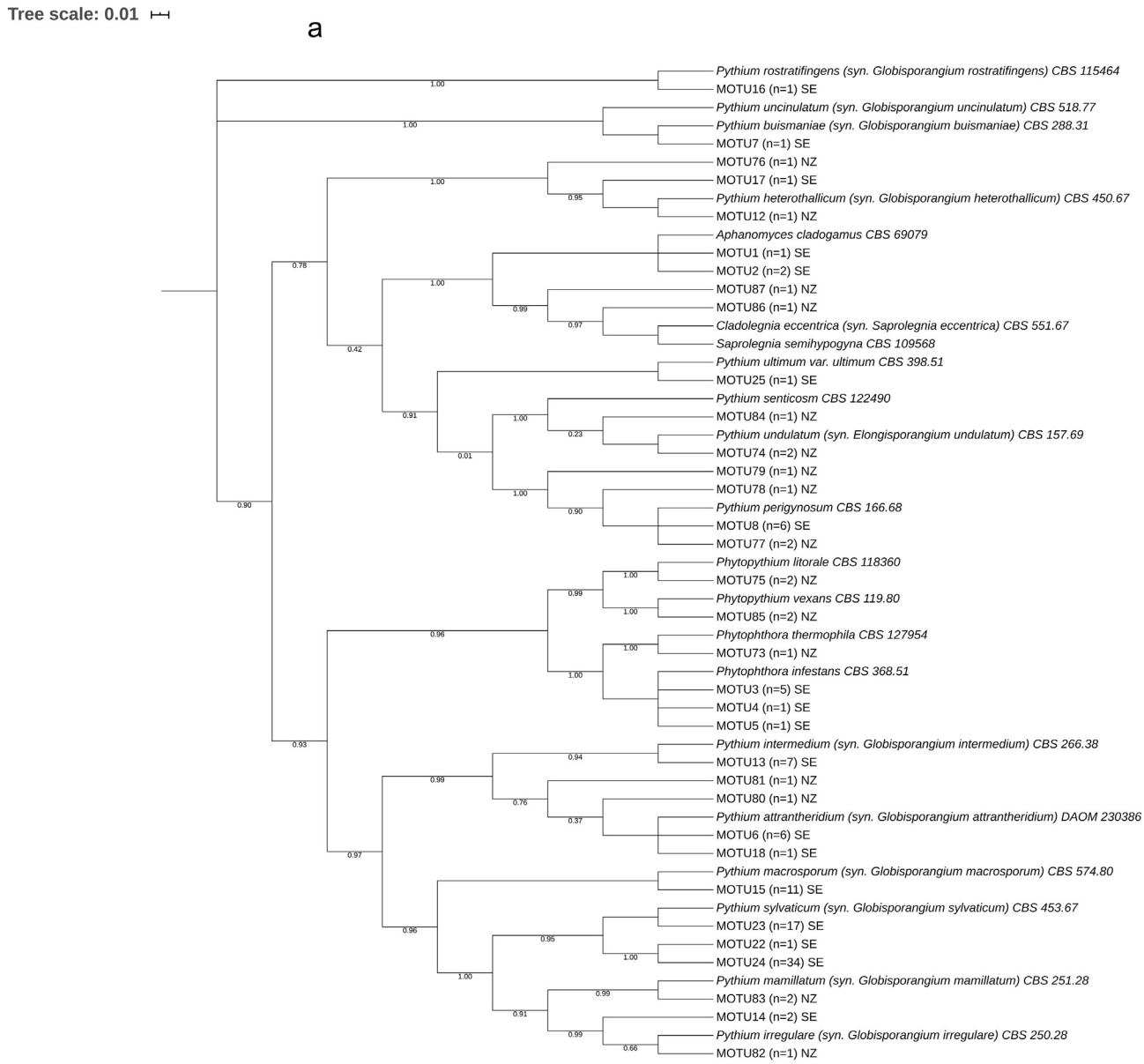

Fig. 2. Phylogenetic analysis of the oomycete species identified from Swedish and New Zealand rhizosphere and soil samples. A: Internal transcribed spacer region, B: mitochondrial cox2 region. Reference sequences were chosen from public available databases and type strain sequences were used where available.

Ph. infestans in our winter (three isolates), spring (two isolates) and summer (three isolates) *S. dulcamara* samples (Fig. 1a), but not in any *V. vinifera* samples (Fig. 1b) in an experimental garden exposed to natural inoculum of *Ph. infestans*, suggests that *Ph. infestans* can overwinter in the rhizosphere of *S. dulcamara* in Sweden. It is also possible that *Ph. infestans* can overwinter inside the roots of *S. dulcamara*, but that was beyond the scope of the present study. While it is well known that *Ph. infestans* can survive the winter as living mycelium in potato tubers in climate zones where winter temperatures do not often dip below freezing (Mariette et al., 2016; Andrivon et al., 2013), few studies have investigated overwintering survival in wild non-tuberous *Solanum* species. In an experimental garden in the Netherlands there was no evidence for overwintering in three *S. dulcamara* plants naturally infected in the previous year, but this study did not sample the rhizosphere but rather screened emerging shoots for signs of infection under artificial humid conditions in a plastic tent covering the plants (Golas et al., 2010).

It is possible that surviving the winter in the rhizosphere of *S. dulcamara* could influence the pathogenicity of *Ph. infestans* on potato. Pathogenicity could be reduced due to a trade-off between pathogenicity and adaptation to off-season survival with alternative associated plants (Laine and Barrès, 2013; Tack and Laine, 2014) or increased as indicated when surviving in potato tubers (Montarry, et al., 2010). Alternatively, specific isolates could develop host genotype-specific virulence or isolate-specific virulence. For example, it has recently been shown that the specificity some *Ph. infestans* isolates display towards potato ro tomato hosts is a stable biotrophy related trait. (Kröner et al., 2019). We therefore tested the pathogenicity of three wild isolates (collected from winter, spring and summer, respectively) on the susceptible potato cultivar Désirée using detached leaf assays. Infection with all three wild isolates resulted in larger lesions than when infected with the standard lab strain 88069 both when inoculated from plates ($F_{3,82} = 12.9$, $P < 0.001$) and from leaves ($F_{3,45} = 53.5$, $P < 0.001$) (Fig. 3). Thus, our result does not support the hypothesis of a trade-off between pathogenicity in potato and off-season survival on another host. Instead, our results could potentially suggest an increase in pathogenicity following winter survival, as seen in potato tubers (Montarry et al., 2010). Our results are also in accordance with an observed

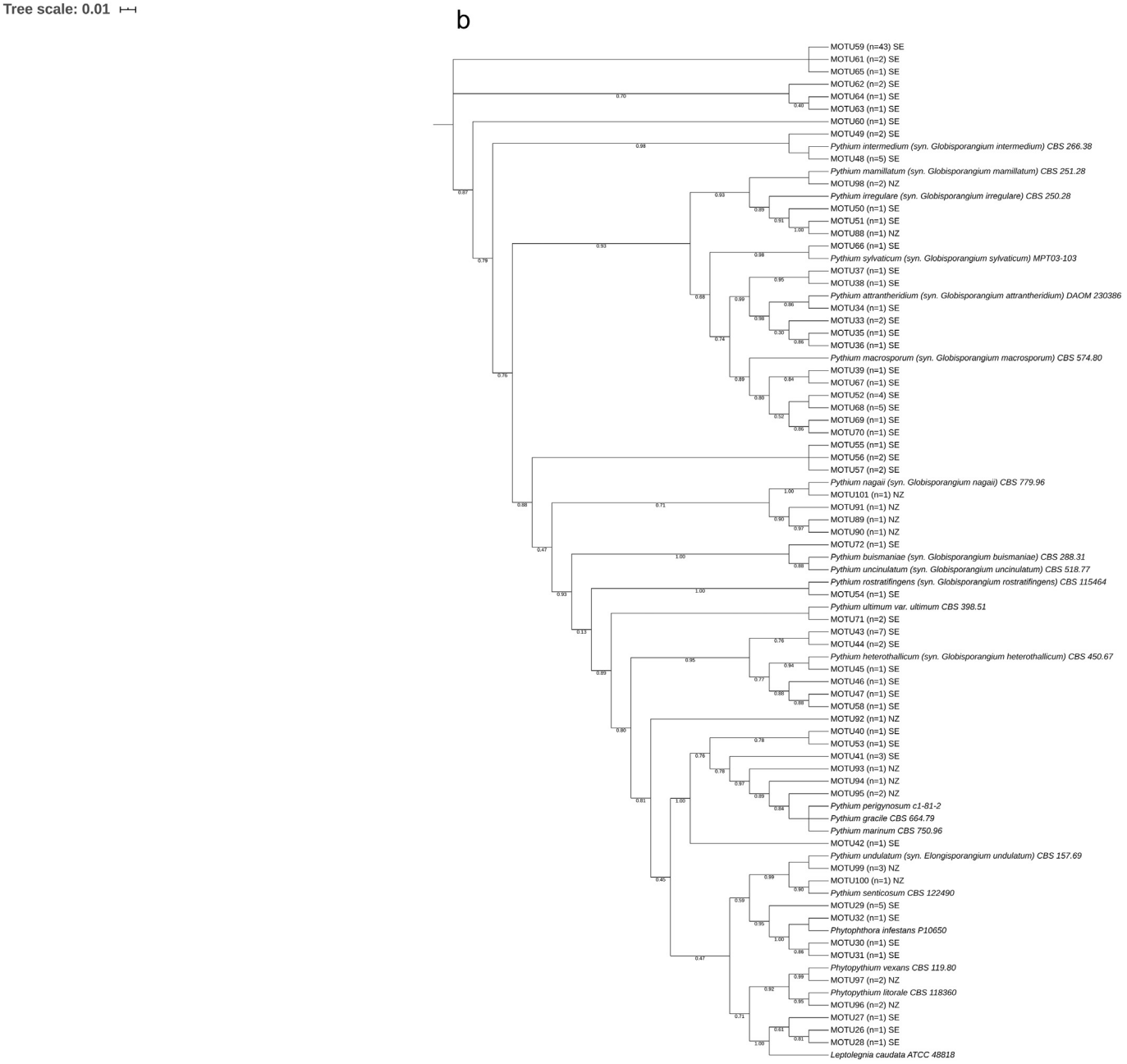

Fig. 2. (*continued*)

increase in foliar pathogenicity on potato of *Ph. infestans* isolates collected from the wild relative *S. physalifolium* (Grönberg et al., 2012). However, further testing of these isolates under more natural conditions, and in comparison to field isolates of *Ph. infestans* collected from potato, may reveal deeper insights into these processes.

Because studies suggest sexual reproduction of *Ph. infestans* in the Nordic countries (e.g. Brurberg et al., 2011), we also checked whether the *Ph. infestans* isolates that we obtained from the *S. dulcamara* rhizosphere were A1 or A2 mating type. All isolates collected were A2 and were able to form oospores in culture when grown in mixed cultures with A1 isolates but not A2 isolates. This data suggests that these wild *Ph. infestans* isolates are capable of sexual reproduction, but given that all three isolates were of the A2 mating type, there was no clear evidence that they are part of a sexually outcrossing population. However, identification and more detailed genotyping of these and further *Ph. infestans* isolates from similar wild associated plants and proximal potato fields is needed before conclusions as to the sexual nature of these populations can be drawn. Interestingly, low abundance *Ph. infestans* sequences were recently identified in the rhizosphere of *Arabidopsis thaliana* suggesting that this oomycete may occasionally be able to inhabit the rhizosphere as an endophyte, (Sapp et al., 2018). Our results from *S. dulcamara* suggest that overwintering in the rhizosphere of a wild relative available in the (agro)ecosystem may be a previously overlooked part of the *Ph. infestans* lifecycle and an off-season survival strategy.

Our root infection assays with two isolates of *Ph. infestans* did not suggest a negative impact on root growth of *S. dulcamara* (mean ± SE; winter isolate: 6.55 cm ± 0.35 cm, spring isolate: 5.83 cm ± 0.70 cm, control: 6.02 cm ± 0.56 cm, $F_{2,23} = 0.030$, $P = 0.97$), suggesting that *Ph. infestans* is not an effective root rot pathogen. Even though up to 38% of the *S. dulcamara* plants growing in the investigated field were previously found to be susceptible to *Ph. infestans* in detached leaf assays, no natural infections were reported during a related three-year study despite the presence of natural inoculum on potato and *S.*

Table 1
Diversity measures of oomycete species isolated from the rhizosphere of (a) *Solanum dulcamara*, and (b) *Vitis vinifera*, in Sweden over four seasons, and of (c) *S. laciniatum*, *S. nigrum* and from a pond in New Zealand over the summer.

Sweden	*S. dulcamara*				*V. vinifera*			
Diversity measure	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Number of isolates	16	41	13	11	11	9	9	9
Species richness	3	8	5	5	5	6	6	5
Simpson's dominance	0.523	0.273	0.361	0.289	0.240	0.185	0.185	0.235
Shannon's diversity	0.831	1.62	1.26	1.41	1.52	1.74	1.74	1.52
Evenness	0.757	0.780	0.785	0.879	0.942	0.968	0.968	0.946

New Zealand	*S. laciniatum*	*S. nigrum*	Pond
Diversity measure	Summer	Summer	Summer
Number of isolates	4	14	5
Species richness	2	7	4
Simpson's dominance	0.625	0.163	0.280
Shannon's diversity	0.562	1.87	1.33
Evenness	0.811	0.961	0.961

physalifolium in or nearby this field (Abreha et al., 2018). Thus, *Ph. infestans* does not appear to have strong pathogenic effects on *S. dulcamara* leaves (Abreha et al., 2018*)*. We conclude that despite the low occurrence of natural *Ph. infestans* infection on *S. dulcamara* leaves, this species could impact the epidemiology of late blight as an overwintering associated plant in Sweden.

In contrast to our results in Sweden, we were unable to detect *Ph. infestans* in the rhizosphere of the two candidate wild *Solanum* species *S. nigrum* and *S. laciniatum* investigated in New Zealand in the summer (Fig. 1c). *Ph. infestans* has previously been associated with the native *Solanum* species *S. aviculare* and *S. laciniatum*, in native forest, botanical gardens and urban areas in New Zealand and as such these species have been suggested as overwintering hosts (Driver, 1957). It is conceivable that the investigated species do not provide suitable off-season survival possibilities, or because the sites were not approariate for detection (non-agricultural). Moreover, no other *Phytophthora* species were cultured from the soil-rhizosphere of any of the wild *Solanum* plants, even though previous studies isolated *Phytophthora citricola*, *Phytophthora erythroseptica*, and *Phytophthora cryptogea* from *Solanum* hosts in New Zealand (Johnston et al., 2017). However, since sampling was carried out in a limited fashion, at sites in a single season, it is not possible to discount the possible presence of *Ph. infestans* or other *Phytophthora* species in association with these plants. It is possible that soil samples were not taken at the optimum proximity to lateral roots to allow detection of *Phytophthora* samples, however, the soil samples did contain roots and these were included in the baiting, therefore we believe that if active *Phytophthora* spp. were present it is likely that we would have detected them.

3.3. Oomycete community variability across plant species, seasons and countries

In Sweden the highest number of isolates were collected in spring from *S. dulcamara* and in winter in the vineyard, and the species richness was the highest in spring in *S. dulcamara* ($N = 8$) and in spring and summer in the vineyard ($N = 6$) (Table 1). Out of the 12 species retrieved in total, six *Pythium* species (*Py. attrantheridum*, *Py. dissotocum*, *Py. heterothallicum*, *Py. intermedium*, *Py. macrosporum*, *Py. sylvaticum*), and one Saprolegniales (*A. cladogamus/L. caudata*) were common for both sites (Fig. 1a,b). For the remaining five species, two were unique for the *S. dulcamara* rhizosphere (*Py. ultimum* and *Ph. infestans*) and three were unique for the grapevine rhizosphere (*Pythium buismaniae*, *Py. irregulate*, and *Py. rostratifingens*) (Fig. 1a,b). Simpson's dominance index, D (U test $= 29$, $P < 0.05$), and evenness, E (29, $P < 0.05$), suggested higher diversity and more evenly distributed species in the vineyard than in the *S. dulcamara* rhizosphere (Table 1). No significant difference was found for Shannon diversity, H (114, $P > 0.05$), indicating that the difference in diversity between host species is mostly related to a relatively higher dominance of a few species in *S. dulcamara*. Among all the isolates, *Py. sylvaticum* was the species recovered with the highest frequency, and this species was particularly common in *S. dulcamara* (42/81 isolates) compared to in the vineyard (10/38 isolates) (Fig. 1a,b).

Oomycete communities did not change significantly in diversity with season during the year of the experiment (Kruskal-Wallis test $>$ 367, $P > 0.05$ for all diversity measures) (Table 1). *Pythium sylvaticum* was the only species recovered throughout the year at both sites, with a tendency to a higher relative abundance in winter (69% and 36% in *S. dulcamara* and grapevine rhizosphere, respectively) (Fig. 1a,b). Three other species, *Py. attrantheridium*, *Py. heterothallicum* and *Py. macrosporum* were also found throughout the year when pooling the two sites. Some species were detected in all seasons except winter (*A. cladogamus/L. caudata*) or 1–2 of the three seasons spring, summer and autumn (*Py. dissotocum*, *Py. intermedium*, *Py. ultimum*, *Py. irregulare*, *Py. buismaniae*, *Py. rostratifigiens*). Given the relatively small sample size of the present study, we do not currently know if the observed variability in species occurrence over the year is caused by chance or reflects seasonal variation in diversity.

The ten species collected from the New Zealand samples in early summer were all unique to either of the associated plants *S. laciniatum* (*Py. undulatum*), *S. nigrum* (*Phytopythium vexans*, *Pythium dissotocum, Py. heterothallicum, Py. intermedium, Py. irregulare, Py. mamillatum*,) or to the pond (*Phytopythium litorale*, *Pythium nagaii*) (Fig. 1c). Only the *Saprolegnia* spp., which could not be determined to species level, was retrieved in both the rhizosphere of *S. laciniatum* and the pond sediment. While an overall comparison of diversity in Swedish and New Zealand samples did not show any significant difference for any of the diversity measures (U test $>$ 630, $P > 0.05$), the marked differentiation in oomycete communities and also species richness among the associated plants *S. laciniatum* ($N = 2$), *S. nigrum* ($N = 7$) and the pond ($N = 4$) in New Zealand was not seen in Sweden, where more than half of the oomycete species were shared between the two plant species. The low abundance and unique oomycetes associated with *S. laciniatum* compared to *S. nigrum* may reflect the fact that this species is endemic to New Zealand, while *S. nigrum* is a widely occurring weed in a large part of the world, including agricultural locations in Sweden.

Culture-based methods for the detection of oomycetes within soil communities have advantages in that active microorganisms are

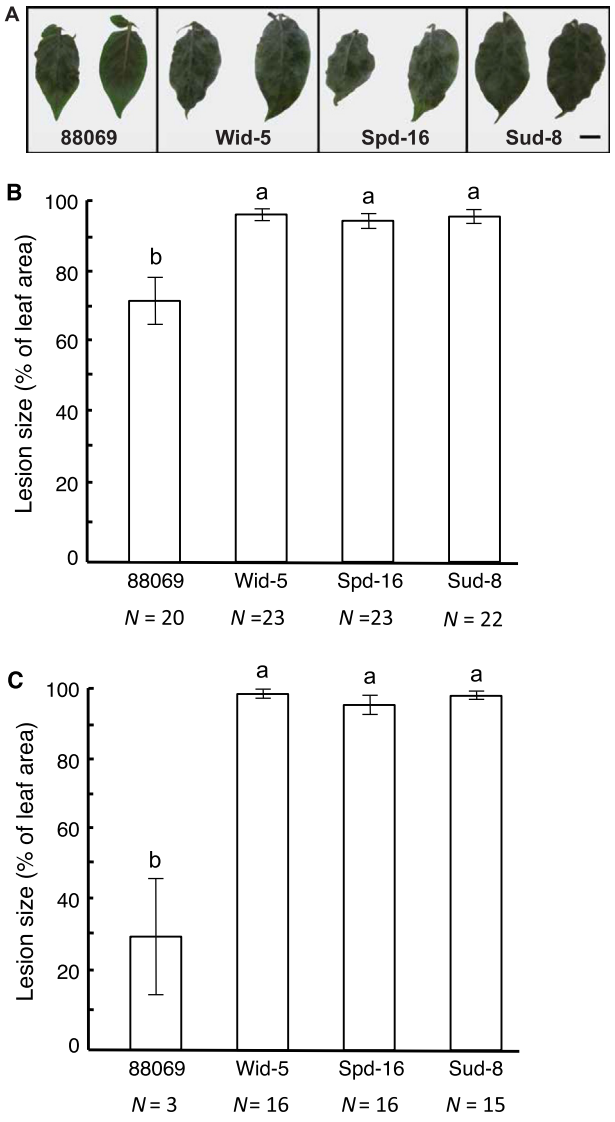

Fig. 3. Pathogenicity of *Phytophthora infestans* isolates evaluated at 7 DPI by detached-leaf assay (DLA) on *Solanum tuberosum* cv. Desirée leaves. (a) Representative leaves. (b) Bars are means of the lesion size relative to the total leaf area ± SE following inoculation from plates. (c) Bars are means of the lesion size relative to the total leaf area ± SE following inoculation from leaves. Different letters indicate a significant ($P < 0.05$) difference. 88069 = lab isolate, Wid-5 = winter isolate, Spd-16 = spring isolate, Sud-8 = summer isolate. N = number of replicate leaves.

detected and detailed characterisation of the isolates is possible, however these methods may not capture the full species diversity or richness present in the rhizosphere (Bik et al., 2012). Although we concentrated on a culture-based approach and had a smaller sample size, alpha diversity in our study was comparable to that seen in recent culture-based (Rojas et al., 2017) and next generation metabarcoding based studies (Sapp et al., 2018). Rojas et al. (2017) identified predominantly *Pythium* species from the rhizosphere of soybean in the USA using culture-based methods. In contrast metabarcoding of oomycetes from the rhizosphere of *A. thaliana* identified many species of the *Globosporangium* genus, as well as several *Pythium* species (Sapp et al., 2018). Most of the *Pythium* species obtained by culturing from soybean rhizosphere were present in all of the 125 tested soybean fields representing the soybean growing regions of 11 states within the USA. The community composition was significantly different by state, but states in close proximity to each other geographically exhibited more similar community composition (Rojas et al., 2017). In our study more than half of the *Pythium* species detected in *S. dulcamara* were also present in the adjacent *V. vinifera* field. Previously *Py. sylvaticum* and *Py. intermedium* have been identified as the dominant active oomycete species present in a range of agricultural and orchard soils with or without tillage in Sweden (Löbmann et al., 2016). The dominance of Pythiaceae and of several individual *Pythium* species across the sampling sites in Sweden and New Zealand is not surprising given their presence in association with a wide range of plants and agricultural soils (Arcate et al., 2006; Schroeder et al., 2012; Rojas et al., 2017; Sapp et al., 2018) and can largely be explained by their wide host range and ability to also survive as saprotrophs (Mao et al., 2014).

The lack of mycoparasitic species such as *Pythium oligandrum,* which is reported as both a ubiquitous soil dwelling mycoparasite oomycete (Gerbore et al., 2014a) and as an endophyte associated with *V. vinifera* and other plants (Gerbore et al., 2014b; Yacoub et al., 2018), is somewhat surprising. *P. oligandrum* has been previously isolated from New Zealand soils (Berry et al., 1993; Godfrey et al., 2003) and although not reportedly identified in Sweden, has also been found in the neighbouring country Denmark which has a similar temperate maritime climate (Madsen and de Neergaard, 1999). However, the culture-based methods together with small sample sizes may have biased the sample collection for more dominant species and culture independent deep sequencing methodologies, or a wider geographical sampling covering other locations, may reveal the presence of this species in these soils.

3.4. Root pathogenicity

Because of the importance of the genera *Pythium* and *Aphanomyces* as soil pathogens (LéVesque and De Cock, 2004; Postma et al., 2000; Gaulin et al., 2007), we evaluated root pathogenicity of one *A. cladogamus/L. caudata* isolate and four *Pythium* isolates collected in Sweden in in vitro root assays involving the susceptible potato cultivar Bintje and *S. dulcamara* seedlings. All five isolates caused a severe reduction in root length on potato seedlings (*A. cladogamus/L. caudate*; $F_{1,25} = 29.4$, $P < 0.0001$, *Py. rostratifingens*; $F_{1,42} = 94.3$, $P < 0.001$, *Py. intermedium*; $F_{1,21} = 89.5$, $P < 0.0001$, *Py. heterothallicum*; $F_{1,24} = 128$, $P < 0.001$, *Py. sylvaticum*; $F_{1,27} = 70.7$, $P < 0.0001$ (Fig. 4a,b). The largest reduction resulting in a root length of < 40% of control plants was caused by *Py. intermedium* and *Py. sylvaticum*. These two isolates also caused a similar root reduction in *S. dulcamara* seedlings (*Py. intermedium*; $F_{1,1} = 12.3$, $P = 0.003$, *Py. sylvaticum*; $F_{1,25} = 58.4$, $P < 0.001$ (Fig. 4c,d), suggesting that these species may be equally pathogenic to both the crop and wild *Solanum* species. Interestingly, *Py. sylvaticum* was the most common species in all Swedish soil samples, but not found in New Zealand, while *Py. intermedium* was found in *Solanum* samples in both Sweden and New Zealand (Fig. 1). *Py. sylvaticum* is indeed a common species in soil (Blok, 1970) and has been reported as the most common *Pythium* species in Dutch soils (Van der Plaats-Niterink, 1981) and in cereal crops in Sweden (Larsson, 1994) and causes stem and root rot in wide variety of crops including carrot, spinach and Chinese silver grass (White, 1986; Larsson, 1994; Ahonsi et al., 2011). In southern Sweden, *Py. sylvaticum* has previously been shown to be the causal agent of root rot on spinach seedlings and may cause symptoms as part of a species complex in association with other root rot *Pythiums* (Larsson, 1994). *P. intermedium* causes stunting of apple seedlings and decreased plant biomass (Mazzola et al., 2002).

The three isolates with less pronounced effects on potato roots did not significantly reduce the root length in *S. dulcamara* (*A. cladogamus/L. caudate*; $F_{1,18} = 2.88$, $P = 0.11$, *P. rostratifingens*, $F_{1,18} = 1.50$, $P = 0.24$, *Py. heterothallicum*; $F_{1,17} = 0.066$, $P = 0.80$) (Fig. 4c,d). Since there was not a statistically significant effect with these isolates it may indicated that infection did not occur or was not fully established in these cases. It is possible that these isolates are better adapted to infect potato leaves compared to roots. *A. cladogamus/L. caudate* and *Py. heterothallicum* were repeatedly detected in the rhizosphere of *S.*

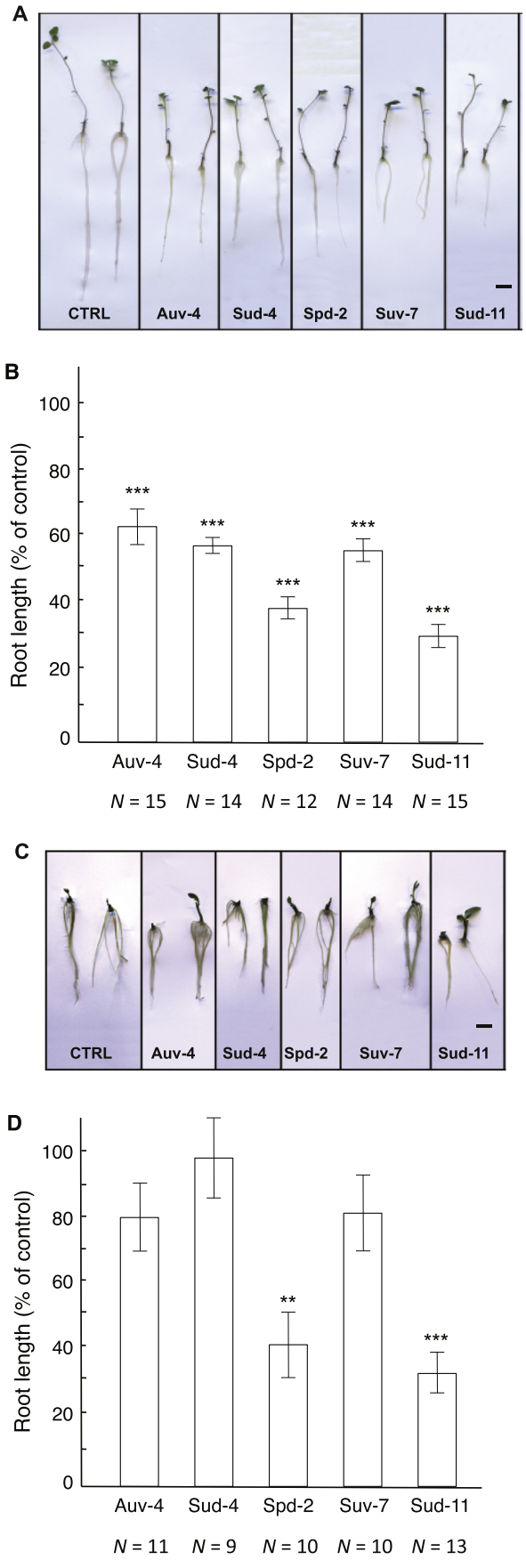

Fig. 4. Root pathogenicity of oomycete isolates evaluated at 7 DPI on (a, b) *Solanum tuberosum* cv. Bintje, and (c, d) *S. dulcamara* Sd 3:6 seedlings. (a, c) Representative seedlings. (b, d) Bars are means of the root length relative to uninoculated control seedlings ± SE. ** $P < 0.01$, *** $P < 0.001$. Isolates: Auv-4 = *Aphanomyces cladogamus/Leptolegnia caudata*, Suv-7 = *Phytium heterothallicum*, Spd-2 = *P. intermedium*, Sud-4 = *P. rostratifigiens*, Sud-11 = *P. sylvaticum*. *N* = number of replicate roots.

dulcamara, (Fig. 1)., *S. dulcamara* could function as an alternative host, or associated plant for these species as well, even though their pathogenicity on potato is not well described.

4. Conclusions

Culture-based approaches allowed the identification of microbial species within the rhizosphere and we demonstrated that the rhizosphere of diverse *Solanum* species and *Vitius vinifera* harbour *Pythium* species with potential to act as root rot pathogens. The abundance of such species within the rhizosphere of the different associated plants and across different latitudes points to their ubiquitous nature and may contribute to epidemiology of root rot diseases by providing a constant reservoir of potential pathogens.

We have also shown, for the first time, that the oomycete potato pathogen *Ph. infestans* is able to overwinter in the wild *Solanum*, *S. dulcamara*. Our data suggest such off-season survival may enhance pathogenicity in potato and provide an additional mechanism to facilitate survival in soils from cold climates, which experience repeated winter freezing. Since the sampling was limited to a few selected sites, how important this is to the general epidemiology of *Ph. infestans* in Sweden still remains to be seen.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.103475.

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Declaration of competing interest

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

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