

RESEARCH ARTICLE

Development and application of multiplex targeted-sequencing approaches to identify *Phytophthora* species associated with root rot and wilting complex of red raspberry

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Abstract

Phytophthora species are primary causal agents of raspberry root rot and wilting complex (RRWC), a disease complex that is of major concern to raspberry producers worldwide. Accurate identification of the causal agents is a first step for effective disease management. Advancements in molecular diagnostics can facilitate the detection of multiple pathogen species associated with this disease complex. We developed multiplex targeted-sequencing methods using degenerate primers for heat shock protein 90, elongation factor 1 α and β -tubulin genes to identify *Phytophthora* species causing RRWC. One hundred and twenty-eight isolates recovered during 2018 to 2020 from diverse fields in major raspberry growing areas of British Columbia (BC) were sequenced and identified by comparing with known reference sequences of 142 *Phytophthora* species, 111 *Pythium* species, and nine *Phytophthora* species in the NCBI database. This multiplex targeted-sequencing method was highly specific and identified two species of *Phytophthora* associated with RRWC. These were *P. rubi* (85% of isolates) and *P. gonapodyides* (15% of isolates). *Phytophthora rubi* was predominantly isolated from the cultivars ‘Chemainus’ (51%), ‘Rudi’ (27%) and ‘Meeker’ (15%), whereas *P. gonapodyides* was predominately isolated from the moderately resistant cultivar ‘Cascade Bounty’. Pathogenicity studies on intact plants and detached leaves confirmed that *P. rubi* and *P. gonapodyides* can cause symptoms of RRWC on raspberry, thus fulfilling Koch’s postulates. To our knowledge, this is the first report of *P. gonapodyides* as a causal agent of RRWC on raspberry in BC. This study provides novel insights into the identification and species composition of *Phytophthora* associated with RRWC in raspberry production systems.

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Introduction

Red raspberry (*Rubus idaeus* L.) is an important fruit crop belonging to the family Rosaceae. It is widely cultivated in many geographic areas, including Europe [1], North America [2, 3], and South America [4, 5]. British Columbia (BC) accounts for ~80% of Canadian red raspberry (*Rubus idaeus* L.) production [6].

Diseases or disease complexes caused by oomycetes, fungi, viruses, bacteria and nematodes are known to constrain raspberry production, which adversely impacts plant health, fruit yield, and quality. The root rot and wilting complex (RRWC) caused by *Phytophthora* spp. and other soil-borne pathogens are the most economically important raspberry disease worldwide, including in the Pacific Northwest (PNW) region of North America [3, 7]. RRWC causes millions of dollars in losses due to plant decline and reduced raspberry fruit yields and quality in the PNW. Although several species of *Phytophthora* were reported as causal agents of RRWC, *P. rubi* (Wilcox and Duncan) Man in't Veld (synonym. *P. fragariae* Hickman var. *rubi*) is considered as the most important species associated with RRWC of raspberry in PNW of Canada and USA [3, 8]. Earlier research in Washington indicated that disease complex can cause up to 100% plant mortality under the saturated field conditions [2]. In BC, control measures (such as, fumigation and fungicides application) for RRWC are the single largest non-labor expenses for raspberry growers [3]. However, comprehensive studies to identify causal agents associated with RRWC and their pathogenicity studies were not done previously from BC.

The above-ground symptoms on raspberry plants is the first step for diagnosing RRWC. These symptoms include scorching, chlorosis, reddening, and necrosis of leaves, and partial wilting of stems and the entire plant. Moderate to severe wilting occurs in advanced stages and plants can die. In the root and crown area, discoloration and decay occurs. Typical RRWC root symptoms include production of fewer and weaker rootlets and presence of dark-reddish brown lesion at the crown. To identify the causal agents (*Phytophthora* spp.), colony characteristics on selective media and examination of spore morphology are traditionally used. However, identifying *Phytophthora* using morphological features is challenging, as several species have similar colony or spore morphology. Morphology-based techniques are time and labor-intensive and also require mycological expertise for accurate identification [9, 10].

In recent years, molecular techniques that target specific DNA regions in the *Phytophthora* genome have been developed [10, 11], especially sequencing of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA [12], cytochrome oxidase subunits (I, II), nad1, and nad9 of the mitochondrial genome [13], and nuclear loci (β -tubulin, HSP90) [14]. ITS-based sequencing has some limitations for accurately distinguishing among species of *Phytophthora*. For instance, *P. rubi* and *P. fragariae* have identical ITS sequences [10, 11], making it difficult to distinguish these species without additional genetic data. The majority of available molecular assays were designed to target a single gene to detect a single species in one sample, not allowing for simultaneous identification of multiple species of *Phytophthora* [11]. Moreover, amplifying and sequencing single genetic regions in separate PCR assays is time-consuming and expensive. Therefore, molecular characterization assays providing genetic data on multiple housekeeping genes in one reaction would allow one to amplify and sequence multiple potential variant positions in a single reaction, providing a finer scale understanding of the species involved within a single infected tissue sample.

The main goal of this study was to develop and apply multiplex targeted-sequencing approaches to identify and characterize *Phytophthora* spp. associated with RRWC from diverse fields in major raspberry growing areas of BC. Isolations were made from diseased samples and isolates of *Phytophthora* spp. associated with RRWC were identified and pathogenicity on red raspberry was confirmed.

Materials and methods

Disease monitoring, sampling and pathogen isolation

Eleven, 20, and 16 fields from raspberry growing areas of BC were monitored between April and December of 2018, 2019, and 2020 (Fig 1) and a total of 57, 67, and 76 samples with RRWC infected root and cane as well soil samples were collected in 2018, 2019, and 2020 respectively (Table 1). Each sample was kept in a plastic bag and bags were stored at 4°C until processed for pathogen isolation. The presence of *Phytophthora* spp. on plant tissues was first confirmed using Agdia ImmunoStrip assay (Agdia Inc., Elkhart, IN, USA) following the manufacturer's standard protocol. Positive samples were further processed in the laboratory to isolate the pathogen either directly from root and cane tissues near the crown or from soil and tissue using baiting techniques. For pathogen isolation from plant tissues, tissues were washed in running water and small pieces (~5 mm²) from the margin of the symptomatic and healthy sections of root and cane tissues were obtained. The root tissues were surface sterilized in a 25% Ivory dish soap (Procter and Gamble, Ohio, USA) solution for 2 minutes followed by three rinses in sterile water for 1 minute. The canes pieces were sterilized in 10% commercial bleach (6.25% Javex) for 50 s, blotted dry and sprayed once with 70% ethanol, blotted dry, followed by three rinses in sterile water for 30 sec. Rhododendron leaves and pears were used in baiting techniques to isolate the pathogen from soil and root samples. Briefly, buckets (~2 L) were sterilized using 70% ethanol and filled with ~500 cm³ of a mixture of field soil and fine roots. Sterile water was added to the bucket until the soil was completely flooded and the water level maintained at ~3 to 5 cm above the soil layer. The rhododendron leaves and unripe pears were sterilized using 70% ethanol and dried in sterile paper towels. Two pieces of leaves (cut into half) and a pear fruit were placed on the soil in each bucket. The buckets were placed in a



Fig 1. Overview of the field sites monitored during this study. Reprinted from ArcGIS Online maps under a CC BY license, with permission from ESRI, original Copyright 2018 ESRI. The copyrights belong to ESRI, but according to the terms of use, the copyright holder does not need to apply for permission to use because it is free for academic publications, and can be used freely and commercially under the CC BY 4.0 license.

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Table 1. Sampling details and *Phytophthora rubi* and *Phytophthora gonapodyides* isolates from diverse fields, locations and years.

Year	Location	Cultivar	Field surveyed	Samples collected	No. of isolates ^a	
					<i>P. rubi</i>	<i>P. gonapodyides</i>
2018	Abbotsford	Chemainus, Meeker, Rudi, Squamish	9	45	10	0
	Chilliwack	Cascade Bounty, Cascade Harvest	1	5	0	12
	Langley	Chemainus	1	6	0	2
subtotal			11	56	10	14
2019	Abbotsford	Chemainus, Meeker, Rudi, Squamish, Cascade Delight	15	50	3	3
	Chilliwack	Chemainus, Cascade Bounty, Cascade Harvest	2	8	1	2
	Delta	Cascade Delight	1	3	2	0
	Langley	Chemainus	1	4	0	
	Pitt Meadow		1	2	0	0
subtotal			20	67	6	5
2020	Abbotsford	Chemainus, Meeker, Rudi, Squamish	12	59	81	0
	Chilliwack	Chemainus, Cascade Bounty, Cascade Harvest	2	12	8	0
	Delta	Cascade Delight	1	3	0	0
	Agassiz	Chemainus	1	2	4	0
subtotal			16	76	93	0
Total			47	199	109	19

^a *Phytophthora* spp. infecting raspberry were identified using multiplex targeted-sequencing of three nuclear genes: heat shock protein 90 (HSP90), elongation factor 1 α (EF1 α), β -tubulin (β -tub). The isolates were recovered from the cane, root, and baiting isolation methods.

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Convivon growth chamber at 12°C for 10 to 14 days. For pathogen isolation, rhododendron leaves and pear tissues with dark brown lesions were cut into small pieces (~5 mm²) from the edge of infected and healthy sections of the tissue. The tissue pieces were sterilized as described above for processing cane tissues. The tissue pieces were air-dried on sterile paper towels then placed onto corn meal agar (CMA) amended with 200 μ l pimarinic (P), 250 mg ampicillin (A), 400 μ l rifampicin (R), 100 mg pentachloronitrobenzene (P), and 50 mg hymexazol (H) per liter; PARPH) and incubated at 16°C for ~2 weeks and colony growth was monitored. Once sparse colony growth was observed on a plate, a small plug of mycelia was transferred from the edge of the colony onto V8 agar medium amended with 400 μ l pimarinic, 250 mg ampicillin, 400 μ l rifampicin (V8PAR) per liter. These plates were incubated at 16°C for 1 to 2 weeks in darkness. Once the pathogen was fully grown, plates were stored at 10°C for short-term. For long-term storage, each isolate was grown on V8PAR media with popcorn and rye grains placed on the surface. After 2 weeks, the colonized grains containing mycelia were transferred to 30 mL French square vials. Each vial received 8 to 10 rye or popcorn grains, ~15 mL sterile water and four 5-mm V8A plugs. Also, isolates were grown on oatmeal agar slants in 30 mL French square vials for 2 weeks. All isolates of *Phytophthora* spp. were then stored at 10°C in darkness for further use. Colony morphology of all isolates of both species were observed by culturing them on V8A and CMA media. In addition, sporangial morphology of few representative isolates of *P. rubi* ($n = 4$) and *P. gonapodyides* ($n = 2$) were also examined. To induce the pathogen sporulation, 8-day old isolates grown on V8A were transferred onto Petri dishes containing ~15 mL suspension of non-sterile soil extract (1.5 g of soil per liter of sterile water) and were incubated at 18°C under continuous fluorescent light for 2 to 4 days.

Multiplex targeted-sequencing

Suspected oomycete isolates were grown on cellophane placed on top of V8 agar for 10–13 days. The mycelia were then harvested, freeze-dried for 2 to 3 days, and ground to fine powder

with magnetic MixerMill bead beating device (Qiagen) and genomic DNA was extracted using the MagMAX kit (ThermoFisher Scientific Inc.) following the manufacturer's protocols. The DNA from representative samples was visualized on 1% gel and quantified using a high sensitivity QuBit device (ThermoFisher Scientific Inc.). Degenerate PCR primers of *Phytophthora* (S1 Fig) were created using Batch Primer 3 (<https://probes.pw.usda.gov/batchprimer3/>). Primer pairs were developed to target beta tubulin (β -tub), elongation factor 1 alpha (EF1 α), and heat shock protein 90 (HSP90) genes by alignment of a representative set of *Phytophthora* species in CLC Genomics Workbench v.9.5.2 (Qiagen) and use of the redundant consensus sequence for primer designs (S1 Table). Each unknown isolate was barcoded and the targeted loci were amplified in a single multiplexed PCR reaction using an optimized Hi-plex PCR approach [15]. The resulting sample-specific barcoded amplicons were then sequenced on an Illumina HiSEQX next-generation DNA sequencing device following the manufacturer's directions at Admera Health LLC (Plainfield, NJ). The reference sequences ($n = 499$) of all published targeted pathogens (*Phytophthora* spp. $n = 142$; *Pythium* spp. $n = 111$; and *Phytophthora* spp. $n = 9$) for three nuclear genes (β -tub, EF1 α , and HSP90) were obtained from National Center for Biotechnology Information (NCBI) GenBank database and European Molecular Biology Laboratory (EMBL) as of June 2021 [14, 16–18], and used as reference sequences mapping the raw data. The sample-specific raw reads were quality checked using FastQC 0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), then imported into Geneious 2020.2.4 (Biomatter, Auckland, New Zealand; <https://www.geneious.com/>), where they were trimmed using BBDuk and aligned to reference sequences using BMap [19] with a minimum identity of 98%, maximum mismatch per reads of 2%, and exclusion of multiple map reads. The species identification for all unknown samples was determined based on the species of the reference sequences with the highest number of mapped reads across all three target nuclear genes. The multiplex targeted-sequencing steps used in this study to identify *Phytophthora* species associated with root rot and wilting of red raspberry is illustrated in Fig 2.

ITS sequencing

To compare the results from ITS sequencing and multiplex targeted sequencing, selected isolates ($n = 14$) were sequenced using universal primers for ITS1 [20]. Pathogen genomic DNA was extracted from 10 to 12-day-old mycelial cultures (~20 mg) grown on V8PAR using FastDNA Spin kit (MP Biomedical, Burlingame, CA) following the manufacturer's standard protocol. One μ l pathogen DNA was used for PCR in a 20 μ l reaction volume consisting of 10 μ l of 2 \times GoTaq Green Master Mix (Promega, Madison, WI), 0.5 μ l of 10 mM forward and reverse primers, and 8 μ l deionized water. PCR amplification was performed in a Bio-Rad thermal cycler. The thermal cycles were 3 min at 94°C; 35 cycles, of 10 s at 94°C, 45 s at 58°C; and 10 min final extension at 72°C. Five μ l of amplified PCR product of each sample was separated on 1% agarose gels to confirm the amplification and remaining 15 μ l product of each sample were sent to MCLAB (<http://www.mclab.com>) for sequencing. For species identity, the resulting ITS sequence reads were compared to NCBI GenBank database using Basic Local Alignment Search tool (BLAST) in Geneious Prime 2022.0.2.

Pathogenicity studies

To confirm the ability of species of *Phytophthora* to cause root rot of raspberry, pathogenicity trials were conducted by inoculating strains on detached rhododendron leaves in the laboratory and on roots of 'Chemainus' raspberry seedlings in the greenhouse at the Agassiz Research and Development Center, Agassiz, BC. Raspberry seedlings were propagated using

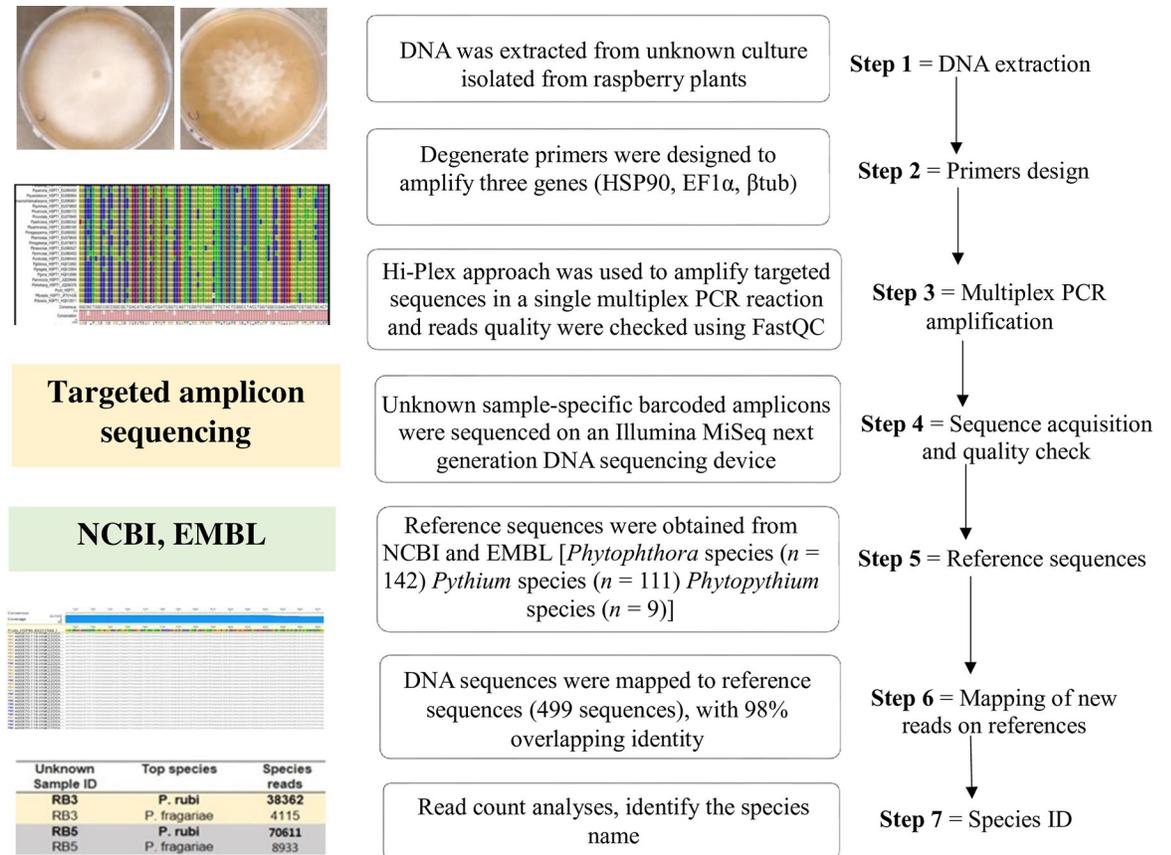


Fig 2. Schematic illustration of the multiplex targeted-sequencing steps used in this study to identify *Phytophthora* spp. associated with root rot and wilting complex of red raspberry.

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tissue cultures from Agriforest Bio-Technologies Ltd. (www.agriforestbiotech.com, Kelowna, BC). In detached rhododendron leaf assays, three isolates each of *P. rubi* and *P. gonapodyides* were assessed. Rhododendron leaves were surface sterilized with 70% ethanol for 1 minute, rinsed three times with sterile water, then dried on sterile paper towels, and placed in a 9-cm Petri dish lined with moist sterile filter papers. Leaves were scratched in the center using a sterile needle. A 5 mm mycelial plug of a 12-day old isolate grown on V8PAR was placed on the wounded section of leaves and the petri dishes were sealed with Parafilm. Dishes were incubated at 16 to 18°C in the dark for 3 to 4 weeks. Leaves treated with V8PAR plugs served as negative controls. For each isolate, three leaves were inoculated using mycelia plug and repeated independently. Once a visible lesion appeared, the lesion length was measured 3 to 4 times at 4 to 6 days interval for lesion progress. The area under the disease progress curve (AUDPC) was calculated for lesion length using the equation by Madden et al. [21].

$$AUDPC = \sum_{i=1}^n [((W_{i+1} + W_i)/2)[T_{i+1} - T_i]]$$

Where W_i represents the percentage wilting index at the i^{th} observation, T_i the time (days) at the i^{th} observation, and n the total number of observations.

Finally, the relative area under disease progress curve (rAUDPC) was calculated as described by Forbes et al. [22].

For root inoculation on seedlings, a mycelial suspension of three isolates of *P. rubi* were inoculated onto 3 to 4-week-old seedlings of raspberry cultivar ‘Chemainus’. Plants were grown in 36-well trays containing a 3:1 ratio of autoclaved potting mix (Sumas Gro-Media Ltd, Chilliwack, BC) and vermiculite (Sun Gro Horticulture Distribution Inc. Washington, USA) and watered and fertilized as needed with a hydroponic solution (0.4 g/L Plant Prod[®] 20-20-20 classic, Terralink, Abbotsford, Canada). Plants were inoculated using mycelial suspension inoculum in 36-well tray method as previously described in Sapkota et al. [23]. Inoculated plants were then placed on a saucer and flooded with distilled water for 24-hour to facilitate zoospore production and their attachment to plant roots. Inoculated plants were then drained and maintained in the greenhouse at $18 \pm 2^\circ\text{C}$, with a 14-h day/night cycle, and $70 \pm 5\%$ relative humidity. Nine raspberry plants received sterile water, flooded with distilled water as described above and used as negative controls. For each isolate, experiments were arranged in a completely randomized design with three replicates. Each treatment had three plants in each replicate. Two independent experiments were carried out. Once first symptoms appeared, plants were assessed 3 to 4 times at 5 to 7 days intervals to record wilting progress using a 0 to 4 rating scale, where the scale 0 = healthy, vigorous growth with no wilting, 1 = wilting (slight) in one or a few leaves with slight foliage symptoms (scorching, yellowing, necrosis), 2 = wilting (slight) started in whole plants, stem lesion present from stem base, 3 = moderate wilting in leaves and stem, stem lesion progressing towards stem tip, and 4 = severe to complete wilting, dead (whole plant dead including leaves, stem). Three to four weeks after inoculation, plants were uprooted, roots were washed, and visually assessed for root rot using a 0 to 4 rating scale, where 0 = 0% infection, healthy roots, 1 = 1 to 12% infection, 2 = 13 to 25% infection, 3 = 26 to 50% infection, 4 = 51 to 75% infection, and 5 = $\geq 76\%$ infection. For whole plant inoculation, the percent wilting index (PWI) was calculated using disease severity of plant wilting progress according to the formula by Cooke [24]:

$$\text{PWI} = ((\sum (\text{scores}, P_0 \times 0 + P_1 \times 1 + P_2 \times 2 + P_3 \times 3 + P_4 \times 4)) / (\text{total numbers of plants rated} \times \text{maximum score on scale})) \times 100$$

where P_0 to P_4 are number of plants with rating scale of 0 to 4.

The AUDPC was calculated for PWI using the equation by Madden et al. [21]. Finally, the rAUDPC was calculated as described by Forbes et al. [22]. In addition, root rot rating was converted to percent root rot index (PRAI) using similar formula used to calculate PWI. The pathogen was re-isolated from infected tissues of both pathogenicity assays by culturing on CMA-PARPH medium. Species of *Phytophthora* were confirmed by morphological observation and the HiPlex targeted sequencing approach.

Statistical analyses. Pathogenicity data were analyzed using SAS v. 9.4 (SAS Institute, Cary NC). The data were tested for normality using Shapiro-wilk test and non-normal data were transformed using square root and log transformation prior to further statistical analyses. Brown-Forsythe test was used to determine significance of variance homogeneity among data sets to decide whether or not data from two or more experiments could be combined for analyses. Analysis of variance was performed for disease assessment and other traits recorded in both detached and intact pathogenicity assays. Treatment means were separated using Fisher’s protected least significant difference (LSD) at $\alpha = 0.05$.

Results

Disease distribution, symptoms, and pathogen isolation

A total of 11, 20, and 16 raspberry fields were monitored in 2018, 2019, and 2020, respectively, from diverse growing areas of BC (Table 1). RRWC disease was observed in all three years;

however, disease and symptom expression varied among years and fields. Incidence of RRWC in raspberry fields was higher in 2020 compared to the other two years. Typical symptoms of RRWC were observed on plants infected with *P. rubi* (Fig 3A to 3F). Diseased plants exhibited symptoms of wilting of cane and leaves, leaf chlorosis, scorching, reddening, and necrosis (Fig 3A to 3C). Plants in severely infected fields were stunted and had very poor vigor with yellowing, necrosis and moderate to severe wilting (Fig 3D). When plants were uprooted, dark reddish-brown lesions at roots and crown were observed and most had few rootlets (Fig 3E and 3F).

ImmunoStrip assays revealed that 56, 70, and 100% of monitored fields had *Phytophthora* positive samples in 2018, 2019, and 2020, respectively (Fig 4A). Similarly, 43, 69, 89% of collected samples tested positive for *Phytophthora* in 2018, 2019, and 2020, respectively (Fig 4B). Greater percentage of *Phytophthora* recovery from *Phytophthora* positive fields (69%) and samples (50%) was observed in 2020 than in 2018 and 2019 (Fig 4A and 4B).

Colony morphology of isolates of *P. rubi* was similar, as sparse colonies were on CMA (Fig 5A), and white colonies with relatively abundant aerial fluffy mycelia at the center on V8A (Fig 5B). *Phytophthora rubi* sporangia were terminal, non-papillate, and ovoid to ellipsoid in shape (Fig 5C) with an average size of $40.1 \pm 1.5 \mu\text{m}$ long and $27.7 \pm 1.1 \mu\text{m}$ wide. Colony pattern of isolates of *P. gonapodyides* on CMA was radiate sparse (Fig 5D) and on V8A was stellate to rosette with relatively less mycelia at the edge (Fig 5E). *Phytophthora gonapodyides* produced non-papillate, ellipsoid to ovoid sporangia (Fig 5F) with an average size of $33.3 \pm 1.0 \mu\text{m}$ long and $19.7 \pm 0.8 \mu\text{m}$ wide.

Species identification and composition

The HiPlex targeted-sequencing approach, as illustrated in Fig 2, amplified and produced sequences across three nuclear genes (HSP90, EF1 α , and βtub) and was able to identify the species of *Phytophthora* infecting raspberry (S2 Table). The species ID for all unknown samples was determined based on the species of the reference sequences with the highest number of mapped reads across all three target nuclear genes (S2 Table). The multiplex targeted-sequencing method was highly specific and generally showed greater differences in total number of mapped reads between top species. An example of an exact alignment of targeted region of *P. rubi* and *P. gonapodyides* with multiplex targeted Illumina sequences for unknown raspberry samples can be seen in S2 Fig. Using representative isolates recovered from infected raspberry, we compared multiplex-targeted sequencing results with ITS sequencing. Based on BLAST search, ITS sequencing results showed the identical percentage identity (99 to 100%) for both *P. rubi* and *P. fragariae*. Further, ITS-based sequencing could not distinguish the majority of *P. gonapodyides* isolates from *P. megasperma* (percentage identity was similar (99.60 to 99.85%) for four isolates. ITS showed less than a 0.5% percent identity difference between *P. gonapodyides* and *P. megasperma* for remaining three isolates.

Out of 260 isolates of suspected Oomycete isolate, 109 and 19 isolates were identified as *P. rubi* and *P. gonapodyides*, respectively, from multiplex targeted-sequencing approach (Table 1). All *P. gonapodyides* isolates, except one, were recovered from the baiting isolation method. Seventy-four percent of *P. gonapodyides* isolates were recovered from 2018 and the remainder were from 2019. About 57% of isolates of *P. rubi* were isolated from roots and 42% isolates were from canes and 1% of isolates were recovered from baiting techniques (Fig 6A). The majority of *P. rubi* isolates (85%) were recovered in 2020, while 9 and 6% of isolates were from 2018 and 2019, respectively (Fig 6B; Table 1). In 2018, *P. rubi* was most frequently recovered from the cultivar 'Chemainus' (51%), followed by 'Rudi' (27%) and 'Meeker' (15%) (Fig 6C; Table 1), whereas *P. gonapodyides* was predominately isolated from moderately resistant cultivar 'Cascade Bounty'.

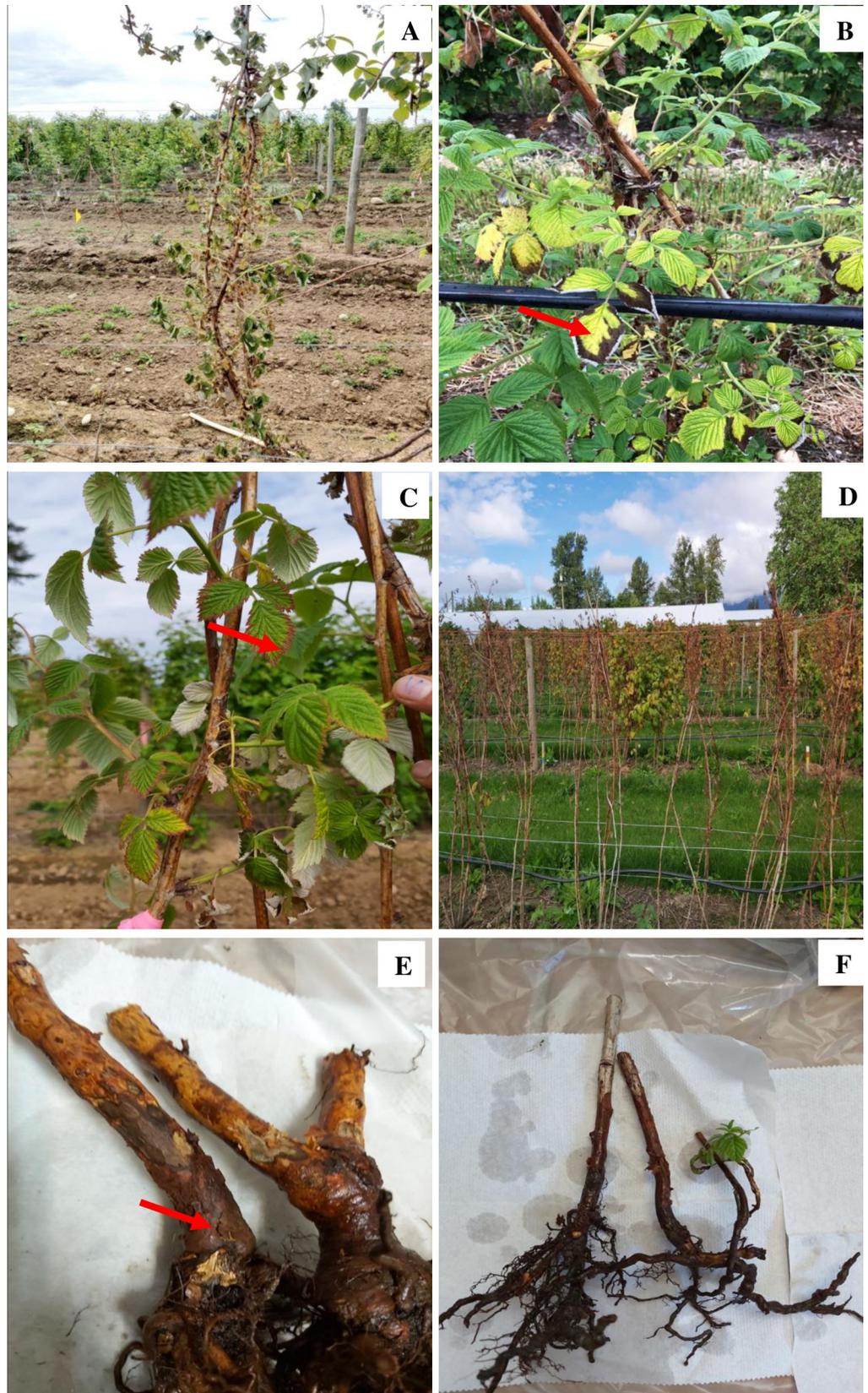


Fig 3. Symptoms associated with *Phytophthora rubi* infection on red raspberry. (A) Cane and leaves wilting. (B) Leaves scorching and chlorosis. (C) Leaves reddening and wilting. (D) Plants showing chlorosis, necrosis, and moderate to severe wilting. (E and F) Distinctive dark reddish-brown lesions at roots and crown with very few new rootlets.

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Pathogenicity studies

Isolates of *P. rubi* and *P. gonapodyides* produced distinct dark expanding lesions on rhododendron leaves (Fig 7A and 7B) which did not develop on control leaves (Fig 7C). For *P. rubi*, the

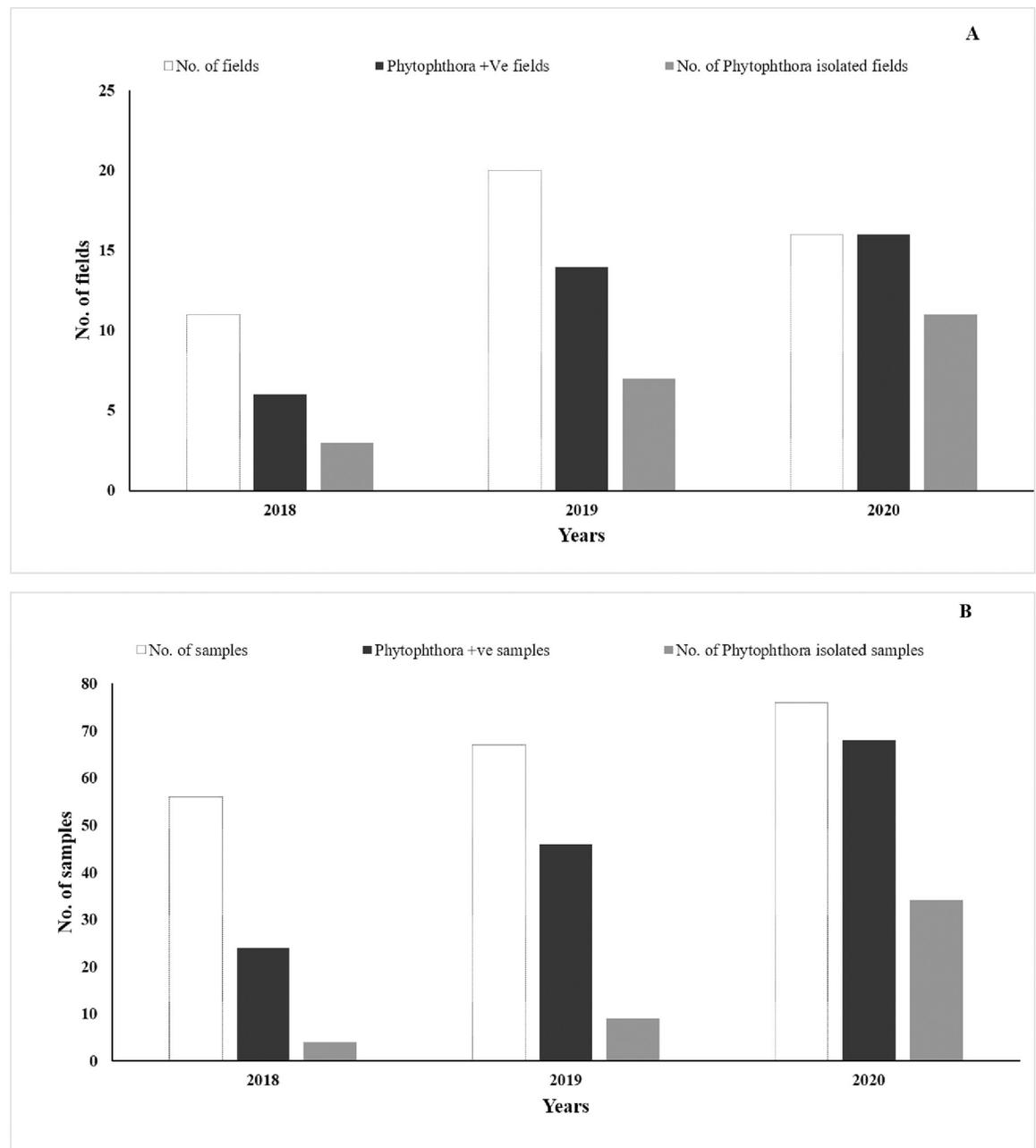


Fig 4. Numbers of *Phytophthora* positive and *Phytophthora* isolated fields (A) and samples (B). *Phytophthora* positive fields were confirmed with Agdia ImmunoStrip assay and species of *Phytophthora* were identified using HiPlex targeted-sequencing approach.

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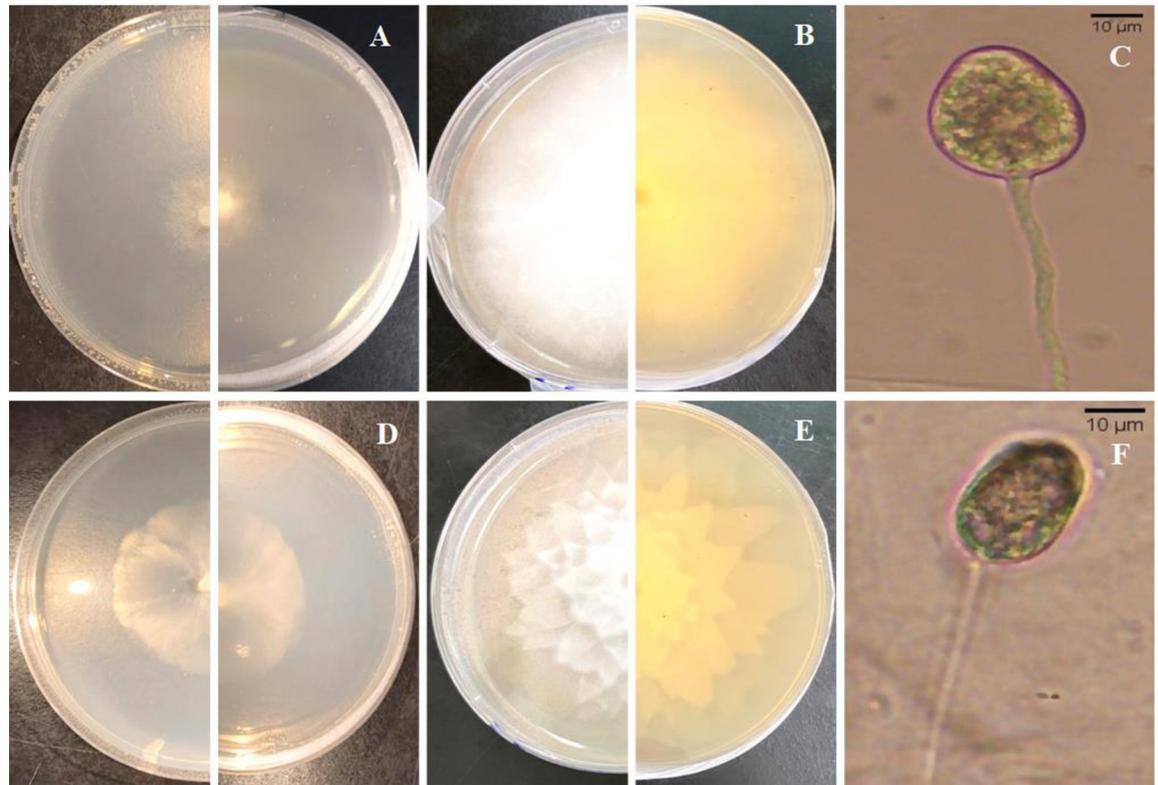


Fig 5. *Phytophthora rubi* (A to C) and *Phytophthora gonapodyides* (D to F) morphological characteristics. (A and D) Seven-day-old colony on corn meal agar medium. (B and E) 12-day-old-colony on V8A medium. (C and F) Sporangia.

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first visible lesion was observed at 11 to 12 days after inoculation (DAI) and 8 to 10 DAI for *P. gonapodyides*. The mean rAUDPC of lesion length in *P. rubi* isolates ranged from 0.11 to 0.13 and 0.14 to 0.17 for *P. gonapodyides*.

In seedling assays inoculated with isolates of *P. rubi*, wilting of leaves and cane symptoms started at 9 to 12 DAI (Fig 8A to 8D). After 3 to 4 weeks following inoculation, severe wilting and root rot occurred (Fig 8E and 8F) in both experiments. The mean rAUDPC of wilting index ranged from 0.35 to 0.55. In addition, the PRAI values (92 to 100%) were very high among isolates of *P. rubi*. Infected leaves of rhododendron and raspberry roots tested positive for *Phytophthora* in the Agdia ImmunoStrip assay and *Phytophthora* was re-isolated from the infected tissues, confirming Koch's postulates.

Discussion

This study showed that RRWC was present in diverse fields of major raspberry growing areas in BC during 2018 to 2020. Foliage symptoms and wilting occurred in several fields across three years. Foliage symptoms and initial wilting began in late spring or early summer and severe wilting and dying of plants occurred in mid-summer and through the fall season. Higher disease severity and plant death occurred in the cultivars 'Chemainus', 'Meeker', and 'Squamish' than 'Cascade Bounty' and 'Cascade Harvest'. Isolation of *Phytophthora* spp. associated with RRWC is difficult because it can co-occur with other soil-borne pathogens and grows slowly in culture media. In 2018 and 2019, we used baiting techniques and direct isolation from infected roots, crowns and stem bases, since direct isolation from infected tissues was not very successful. However, isolation of *P. rubi* directly from infected tissue was

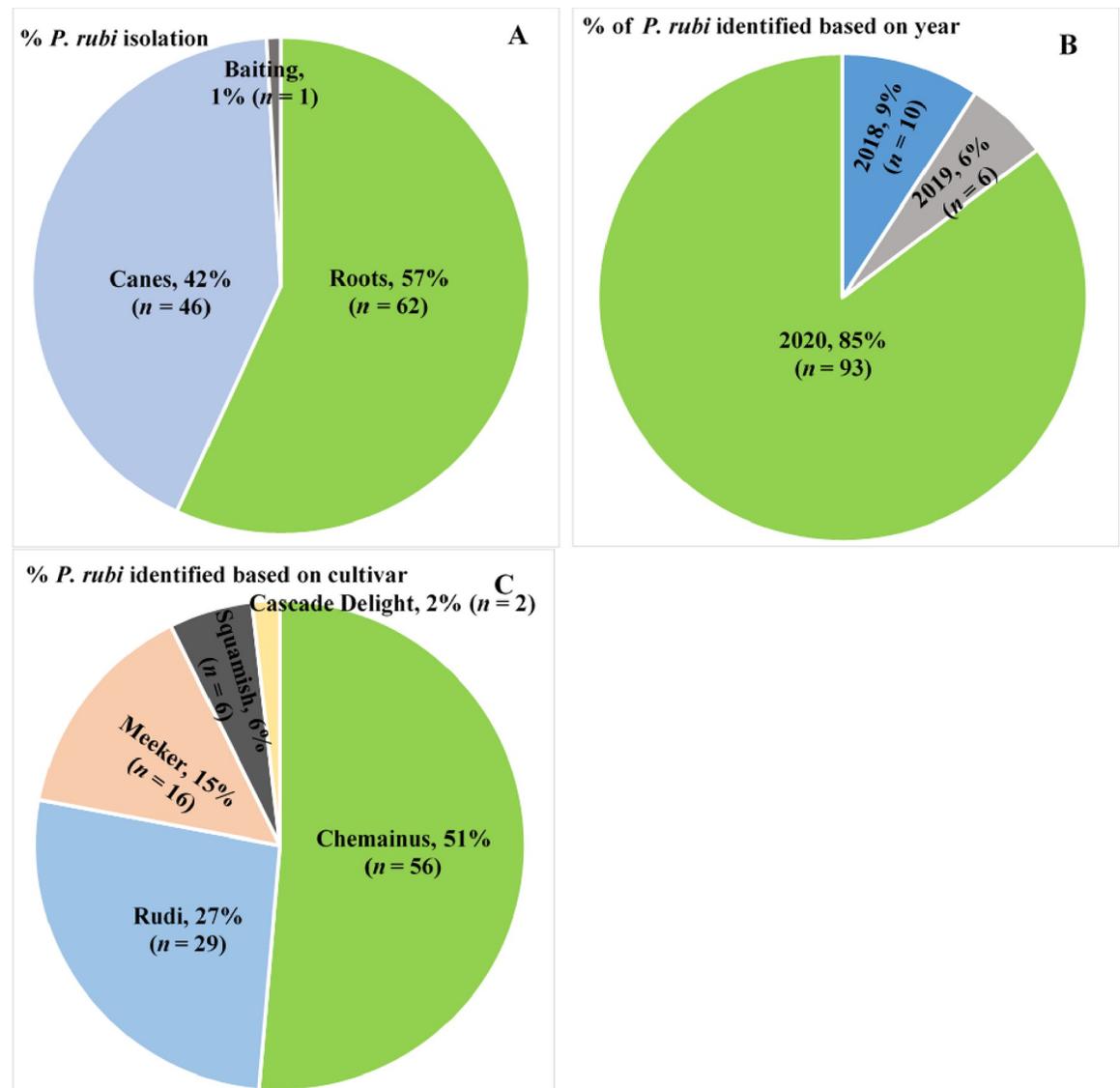


Fig 6. Frequency distribution of *Phytophthora rubi* infecting raspberry recovered from BC. (A) based on method of isolation. (B) by year. (C) by raspberry cultivars. Data were combined from all three years to compare isolation methods and raspberry cultivars.

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successful in 2020, which may be favoured by frequent rainfall in spring and summer months. In previous studies, recovery of *Phytophthora* species directly from infected tissues was reported for *P. rubi* from cane and root tissues of red raspberry in western USA [8], and for *P. cinnamomi* and *P. citrophthora* from roots of blueberry in Chile [25]. Recovery of *P. gonopodydes* was more difficult directly from diseased tissues but not when a baiting method was used.

In this study, we developed and applied an Illumina-based multiplex targeted-sequencing approach to accurately identify *Phytophthora* species associated with RRWC. Our approach used degenerate primers designed to amplify and sequence multiple potential variant positions within three nuclear genes (HSP90, EF1 α and β tub), which are unique to different *Phytophthora* species. Most previous studies used single genes or genetic regions for pathogen identification which may not be robust for accurate species identification or detect the presence of multiple species. Moreover, amplifying and sequencing single genes (one set of primers

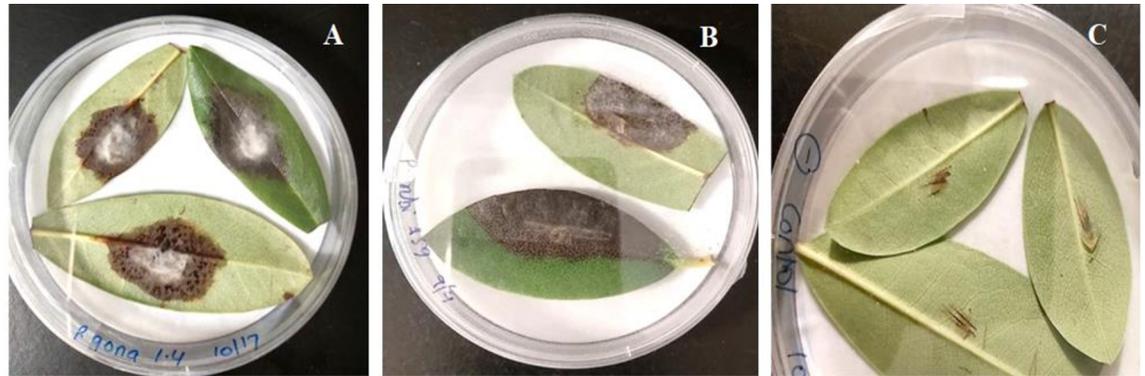


Fig 7. Dark lesions produced on rhododendron leaves by isolates of *P. gonapodyides* (A), *P. rubi* (B). Lesions did not develop in non-inoculated control leaves (C).

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per reaction per sample) is time consuming and expensive. We also compared multiplex-targeted sequencing results with ITS sequencing using representative isolates recovered from infected raspberry. However, the ITS sequencing results were not able to distinguish among isolates of *P. rubi* and *P. fragariae*, and *P. megasperma* and *P. gonapodyides*. Other researchers also reported that *P. rubi* shares identical ITS sequences with *P. fragariae* [10, 11]. In addition with molecular characterization, colony and sporangial morphology of *P. rubi* and *P. gonapodyides* could be supportive for pathogen characterization, though similarity in morphological features can be observed among the species of *Phytophthora*. However, traditional methods including the use of morphological features for identifying species of *Phytophthora* can be slow and labor-intensive, and in the case of isolates with poor spore formation, can be inconclusive [3, 10]. Given the limitations of existing identification methods, the method described here was highly specific, and clearly distinguished among species of *Phytophthora* and could be used to monitor the introduction of previously undescribed species. Also, this technique successfully identified *Phytophthora* species that are known to attack blueberry, for example *P. cinnamomi* (unpublished data), suggesting the potential of this method to identify different *Phytophthora* pathogens infecting different berry hosts. A recent study also reported that multiplex targeted-sequencing effectively identified common fungal, bacterial and other pests associated with *Megachile rotundata* brood cells [26]. Targeted sequencing is now often less expensive and will likely a powerful tool in species detection.

The majority of isolates (85%) collected from major raspberry growing areas of BC were *P. rubi*. Previous studies also reported that *P. rubi* is a major causal agent associated with the RRWC and is widespread in most raspberry producing fields in the Pacific Northwest of USA [8, 27]. *Phytophthora rubi* has been reported as a major pathogen from raspberry-growing regions worldwide [2, 3, 5, 7, 28]. However, we also identified isolates of *P. gonapodyides* in our study, the majority of which were predominately recovered from the cultivar ‘Cascade Bounty’ in one raspberry field. This cultivar was reported as moderately resistant to *P. rubi* at the time of release [29]. Isolation of *P. gonapodyides* from this cultivar suggests that the resistance to one species of *Phytophthora* may not be effective against another species. Wilcox and Latorre [5] also reported that some red raspberry cultivars with resistance to *P. rubi* showed susceptibility to *P. megasperma*. Therefore, knowledge of species composition of *Phytophthora* populations associated with RRWC is critical while identifying the sources of resistance and release of resistant cultivars. Several other species of *Phytophthora* have been sporadically reported to attack raspberry plants, such as *P. gonapodyides* (Petersen) Buisman and *P. megasperma* Drechs in Chile [5] and New York state [7], *P. cryptogea* Pethyb. and Leth., in New

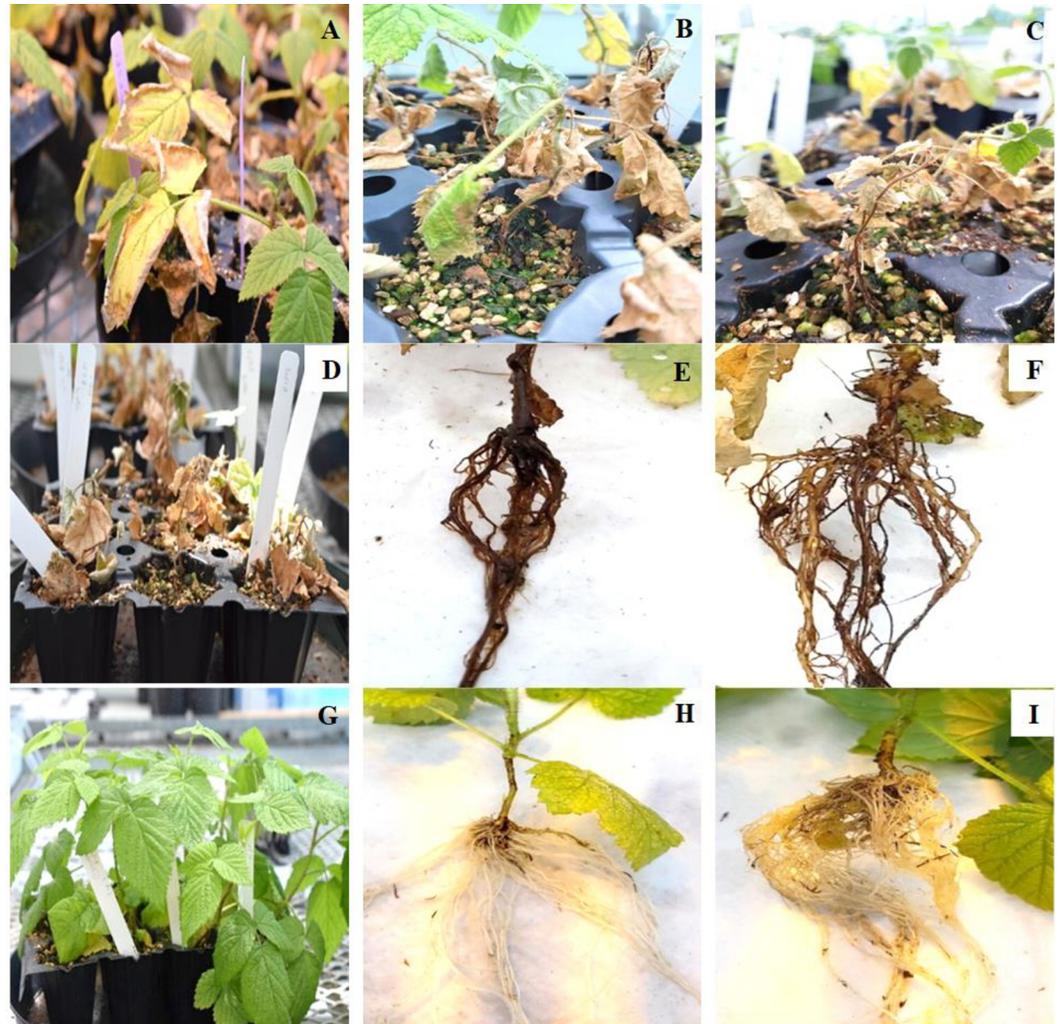


Fig 8. Wilting and root rot symptoms produced in the raspberry cultivar ‘Chemainus’ after root inoculation with *P. rubi*. (A to C) Yellowing, scorching, and necrosis of foliage and wilting of leaves and canes. (D) Severe wilting and dying of seedlings. (E and F) Severe root rot infection with no new roots. (G) Asymptomatic non-inoculated (negative) control plants. (H and I) Healthy root systems of non-inoculated (negative) control plants.

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York state [7], Chile [5], Australia [30] and New Zealand [31]. In addition, *P. citricola* Sawada was reported from New York state [7], Chile [4, 5] and Bulgaria [32] and *P. citrophthora* (R.E. Sm. and E. H. Sm.) Leonian from Chile [4]. This is the first report of recovery of *P. gonapodyides* from red raspberry in BC.

Compared to 2018 and 2019, the frequency of recovery of *P. rubi* isolates was higher in 2020 and isolation was successful from several fields throughout the growing season. In contrast, the recovery of *Phytophthora* isolates was only successful either in late spring or late fall in 2018 and 2019. The availability of high soil moisture levels and optimal soil and ambient temperature in Pacific Northwest including Fraser Valley areas of BC in 2020 (Fig 9) may have greatly favored pathogen spread, rapid multiplication in host tissues, and disease symptoms development of *P. rubi*, which is favored by high rainfall or soil moisture and average soil temperatures of 15 to 20°C [9, 33].

The comparison of species composition among raspberry cultivars indicated that the frequency of *P. rubi* was highest in ‘Chemainus’ followed by ‘Rudi’ and ‘Meeker’. These cultivars

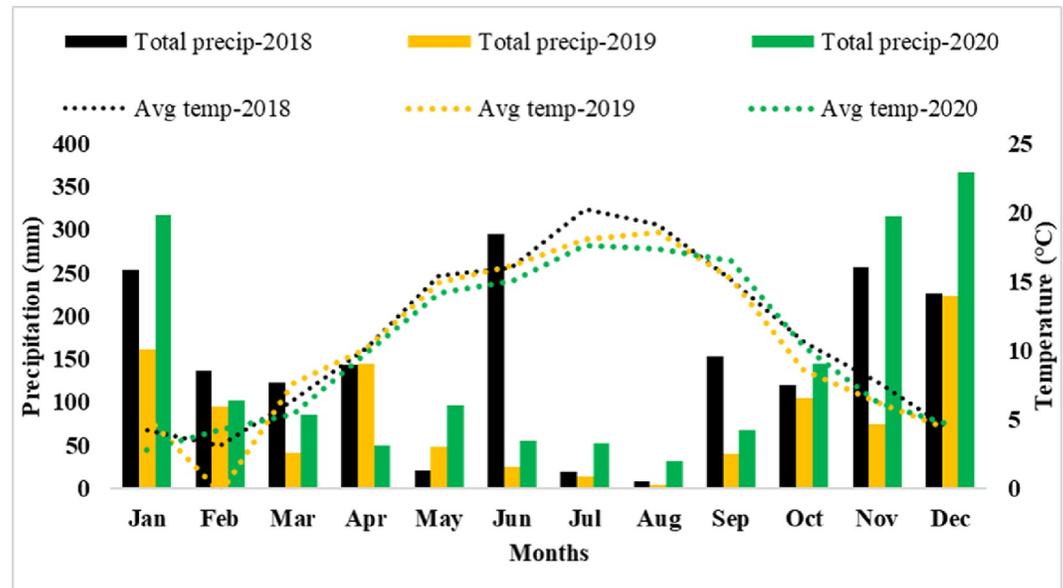


Fig 9. 2018, 2019, and 2020 monthly total precipitation (mm), and average temperature (°C) at Abbotsford, BC.

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are commonly grown in BC. We also found severe wilting and foliage symptoms on the cultivar ‘Cascade Delight’ from which *P. rubi* was isolated. This cultivar was reported as moderately tolerant to RRWC [34]. Our findings suggest that resistance levels of raspberry cultivars may be declining over time. Cultivars ‘Chilliwack’ and ‘Meeker’ were considered to have some level of resistance to root rot when they were released but they are now considered susceptible [35, 36]. There is the possibility that *P. rubi* populations in BC could be different among different sites where the cultivar was evaluated for resistance. It is therefore critical to study the temporal dynamics of pathogen populations collected from diverse fields and cultivars.

We also demonstrated that representative isolates of *P. rubi* and *P. gonapodyides* were pathogenic on rhododendron leaves, producing dark brown lesions. Moreover, *P. rubi* isolates caused severe wilting and root rot on ‘Chemainus’ raspberry roots, suggesting that *P. rubi* isolates were highly virulent to raspberry. Additional studies are in progress to evaluate additional isolates of *P. rubi* and *P. gonapodyides* obtained from diverse fields/regions to understand the virulence diversity of the pathogen population.

In conclusion, this study is the first to investigate a multiplex targeted-sequencing approach using different nuclear genes for accurate identification and characterization of *Phytophthora* spp. associated with RRWC from diverse fields of major raspberry growing areas of BC. The detection capability of different *Phytophthora* species in a single reaction can reduce the overall costs and significantly facilitate species detection over current ITS-based identification approaches. *P. rubi* was identified as the main *Phytophthora* species associated with RRWC of raspberry in BC. We also identified *P. gonapodyides* associated with the disease complex in BC. This species was isolated from moderately resistant raspberry cultivars, indicating that the current sources of resistance to RRWC may not be adequate against all species of *Phytophthora*.

Supporting information

S1 Fig. Example alignment of HSP90 gene region for representative *Phytophthora* species.

Note: each sequence is slightly different (unique to that species).

(TIF)

S2 Fig. Exact sequence alignment of targeted region of *Phytophthora rubi* (A and B) and *Phytophthora gonapodyides* (C and D) with Illumina sequences (multiplex targeted-sequencing) obtained from unknown samples.

(TIF)

S1 Table. List of primers used in multiplex PCR approach of *Phytophthora* spp. identification.

(DOCX)

S2 Table. Summary data for multiplex targeted-sequencing of unknown isolates obtained from red raspberry infected with RRWC.

(DOCX)

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Writing – review & editing: Sanjib Sapkota, Rishi R. Burlakoti, Kurt Lamour, Mark Lubberts, Zamir K. Punja.

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