AHDB Horticulture

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Project leader:	Charlotte Nellist, NIAB-EMR
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

Phytophthora may not be the only culprit in raspberry root rot.

Background

Root rot of the European red raspberry (*Rubus idaeus*), caused by a yet-unknown consortium of *Phytophthora* species, is a recurring and destructive disease of this commodity fruit. The disease is most frequently observed during persistent periods of high rainfall and humidity and when the crop is in high productivity. This timing corresponds with the most economically important stage of raspberry growing, thus severely impacting a grower's ability to profit from this work-intensive crop. As such, root rot is a significantly limiting factor in UK raspberry production. Current control strategies rely on cultural practices due to the lack of fungicide efficacy. Infection prevention is employed through securing clean planting material, maintenance of freely draining soil and sterilising irrigation lines. Infection risks have led to ~70% of UK raspberry growers moving from field to pot-based cultivation which involves more consumables and labour, increasing the costs involved in raspberry production.

Raspberry root rot is an understudied field of research. Much is to be gained from further understanding the species involved in the disease.

This project seeks to investigate whether factors such as location, agronomy, and variety affect the diversity of root rot-causing pathogens and whether *Phytophthora* is not the only pathogen causing root rot symptoms. Additionally, in the upcoming years of this project, the potential of meristem culture to eradicate *Phytophthora* from raspberry plants will be investigated. This method could potentially reduce the spread of root rot from propagator to grower.

Summary

<u>Surveys and Sampling</u>

In the first year of this project, 13 UK raspberry grower sites were sampled across England and Scotland. Root and cane tissue were taken from healthy plants and plants exhibiting root rot symptoms i.e., wilting, chlorosis, cane lesions. Additionally, a questionnaire was distributed which collected information agronomy details and the Raspberry root rot experiences of individual growers.

<u>Root and cane isolations</u>

Diseased cane and roots taken from grower sites was plated onto *Phytophthora*-specific media using a protocol adapted from Stewart et al. (2014) which consisted of cornmeal agar

amended with antibiotics (rifampicin and ampicillin) and fungicides (pimaricin PCNB and hymexazol) which reduced the growth of fast-growing fungi and bacteria. Isolates were sub-cultured onto fresh agar plates and grown in the dark at 18°C for 14 days. DNA was extracted from the isolates and they were sequenced to determine their identity. Over 300 samples of roots and canes were plated out, from which 24 isolates were obtained which had similar characteristics to *Phytophthora*. Sequencing results showed the isolates were a consortium of fungal species, mainly beneficial soil fungi such as *Morteriella* and *Trichoderma*. Notably; two of the isolates were known pathogens of other soft fruit plants; *Diaporthe eres* (a.k.a *Phomopsis*) - associated with dieback and fruit rot in other Rosaceae species, and *Cadophora luteo-olivacea* - a vascular pathogen of grapevine (Gramaje et al., 2014). Pathogenicity testing of these isolates on a range of commercially relevant cultivars is ongoing. Further tests will be conducted using these pathogens to investigate how they affect plants and if they cause visible symptoms and reduce productivity.

Financial Benefits

Over 16 thousand tonnes and 146.8 million pounds worth of raspberries were produced in the UK in 2019, a figure which is steadily growing with the popularity of the fruit (DEFRA, 2020). However, root rot can have a devastating financial impact on raspberry growers due to the cost of replacing diseased canes and lost fruit crop. This project seeks to increase our understanding of root rot in raspberry and the effects of emerging pathogens on the crop. Through extensive pathogenicity screening and sampling, we hope to reduce the financial loss associated with raspberry root rot and improve upon rapid screening processes for new varieties and develop a new method of irradicating *Phytophthora* from meristem cultured plants.

Action Points

At this early stage of the project, recommendations to change-of-practice cannot be given.

SCIENCE SECTION

Introduction

The reported health benefits of raspberry consumption are numerous, and several studies note their beneficial effects in disease prevention and organ health associated with their high antioxidant and anthocyanin content. These health benefits coupled with their attractive colouring and shine make raspberries a valuable commodity, however these heavily-fruiting plants have fine root systems which make them vulnerable to disease.

Phytophthora, the reported causal genus for root rot in European raspberry (*Rubus idaeus*) are oomycete pathogens which have been attributed with significant losses in productivity throughout the U.K. Recent observations by U.K. plant pathologists, coupled with reports from the U.S., South America and Europe have suggested there may be other species of *Phytophthora* responsible for causing the disease other than *Phytophthora rubi*. The incidence of the disease across the UK and pathogenicity of these additional *Phytophthora* species is also unclear.

This project aims to determine the incidence of *Phytophthora* root rot (PRR) in U.K. raspberry production, identify the species causing the disease, and determine their pathogenicity on commercially relevant raspberry cultivars. In addition, the potential of meristem culture as a method of excluding *Phytophthora* from infected raspberry stocks will be evaluated.

Phytophthora in raspberry is an understudied field of research. This project will employ traditional methods of *Phytophthora* identification such as isolation with selective media, and morphology, in addition to molecular methods such as PCR, qPCR, Sanger sequencing and High Throughput Sequencing. These results will improve our understanding of PRR in U.K. raspberry production, help provide a method to produce *Phytophthora*-free planting material and inform growers on how best to reduce the disease.

Materials and methods

Sampling

Raspberry plants exhibiting symptoms of *Phytophthora* and those which appeared healthy were sampled in late October 2020. Samples were collected from 13 grower sites in England and Scotland. A total of 50 samples were taken per site. Two to three canes and roots with substrate soil attached were taken and placed in 1 L plastic bags. Trowels, secateurs, and handsaws were thoroughly disinfected using 70% ethanol between samples. Canes and soil were maintained separately to minimize soil pathogen contamination. Samples were placed in coolers during transport and held in a 4°C cold store until processing. Due to COVID-

19 imposed restrictions, some samples were held in cold store for up to six weeks before processing.

Isolation of symptomatic tissue onto selective media:

Isolation was performed according to the method outlined in Stewart et al. (2014). Diseased roots, i.e., those which had signification browning or apparent lesions, were placed in a sieve and rinsed in running tap water to remove soil/substrate. The roots were cut into 10 mm sections and transferred into a 70% ethanol for 10 seconds and allowed to dry on sterile filter paper for 1 minute. After rinsing with sterile distilled water, roots were placed on sterile filter papers to dry for 30 seconds. Five root sections per sample were aseptically transferred to 9 cm Petri dishes containing CMA (Difco cornmeal agar, 17 g/1000 ml of deionized water) amended with pimaricin (0.2 ml of a 2.5% (w/v) stock), ampicillin-Na (0.250 g), rifampicin (0.4 µL of 2.5% aqueous solution), pentachloronitrobenzene (PCNB 5 ml) and hymexazol (0.05g) (CMA-PARPH).

Diseased cane tissue was cut into 10 mm sections, soaked in sodium hypochlorite (1.2% available chlorine) for 2 minutes and rinsed in sterile distilled water three times and 4 pieces of cane per sample were carefully submerged in Petri dishes containing CMA-PARPH. The plates were sealed with Parafilm and incubated in the dark at 18°C until mycelial growth was observed (5-7 days after isolation). The hyphal tips of growing colonies were transferred onto fresh CMA-PARPH. Cultures were routinely transferred to PDA media to ensure no contaminating fungi were present which could affect pathogenicity and sequencing results.

Sequencing of cultures

To determine the identity of cultures, a rapid fungal DNA extraction was performed using the Sigma-Aldrich extraction and dilution buffers (Sigma-Aldrich, U.K), following manufacturer's protocol. The extracted DNA was stored at -20°C in preparation for downstream analysis. For Sanger sequencing, the internal transcribed spacer 1 (ITS1) region (>900 bp) was amplified from the DNA using ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3'') and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers. ITS4 and ITS6 primers were used as they amplify the ITS region in *Phytophthora* (Grünwald et al., 2011; Schena et al., 2008). Sterile MilliQ water was used as a negative control, genomic DNA extracted from *Phytophthora idaei* and *Cladosporium cladosporioides* were used as oomycete and fungal positive controls, respectively.

PCR amplifications were carried out in 25 µL reaction volumes. Each reaction tube contained 0.6 µL of both ITS6 (forward primer) and ITS4 (reverse primer) at 10µM, 8.8 µl of sterile MilliQ water, 12 µl of 2x PCR MyTaq Red Mix (containing 5 mM dNTPs and 15 mM

$MgCl_2$) (Bioline), and 5 µL of DNA template or MilliQ H_2O. Thermo-cycling reaction was carried out in a BioRad C1000 Touch Thermal Cycler. Parameters were as follows; 1 cycle at 94°C for 3 min; 35 cycles for 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 1 min of extension at 72°C, followed by 1 extension cycle at 72°C for 10 min (Grünwald et al., 2011). The PCR amplification products were separated by electrophoresis in 1% agarose gels stained with GelRed (Biotium) in 1 X TAE (40 mM Trisacetate pH 8.0, 1 mM EDTA) buffer at 100V for 1 hr and visualised under a UV trans-illuminator (Bio-Rad ChemiDoc™ MP Imaging System). Images were taken with Image Lab™ (version 5.2) image acquisition and analysis software. Amplification product size was determined by comparison with 500 bp hyper ladders (Bioline). PCR products were submitted for Sanger sequencing, along with 5 µL of either ITS4 or ITS6 at 10µM concentration (5′-GAAGGTGAAGTCGTAACAAGG-3′) as the sequencing primer. Sanger sequencing was performed by Eurofins Genomics, London, U.K. A consensus sequence was produced using a de novo assembly on Geneious^{tm} software and run through BLAST (Basic Local Alignment Search Tool) on NCBI and matches to fungal and oomycetes were obtained.

Molecular analysis of *Phytophthora* species in symptomatic and healthy looking Raspberry material

Due to a high volume of samples and the time-intensive nature of isolation, a subset of roots and canes were taken from each farm for molecular testing. The roots of each subset sample were thoroughly washed with tap water to remove residual soil or substrate. Canes were cut into 10 x10 mm sections using secateurs and scalpels which were disinfected with 70% ethanol between samples. Between 1-2 g of root sample were placed into 2 mL Eppendorf tubes and stored at -80°C.

To determine the sample state from which optimum DNA quantity can be achieved; a comparison was performed between samples frozen (FR) and those which had been freeze-dried (FD) prior to DNA extraction using the Qiagen PowerSoil Pro Kit (Qiagen). Two aliquots each from a random selection of five samples were prepared as described above. One aliquot was freeze-dried, following which the DNA from both sets of samples was extraction using the PowerSoil Pro kit according to the manufacturer's instructions. DNA was quantified by spectrophotometry (Nanodrop,Thermo Scientific) diluted to a 1:10 and 1:100 concentration and stored at -80°C.

To ensure that all DNA samples were of sufficient quality to be amplified by PCR, 2 µL of each DNA sample (undiluted and ten times diluted) was amplified using the universal ITS primers ITS4 and ITS5 (White et al., 1990). The PCR protocol was performed as above using sterile MilliQ water as a negative control and *P. idaei* gDNA as a positive control. The PCR

amplification products were separated by electrophoresis as above. Samples which exhibited an amplicon band were chosen for a nested PCR amplification using *Phytophthora* genus and species-specific primer pairs 18Ph2F/5.8S-1R and ITS6/5.8S-1R were used in the first and second rounds, respectively as outlined in (Scibetta et al., 2012). In nested PCR, the first and second rounds of PCR amplifications were performed in a volume of 15 µL and 25 µL respectively. One microlitre of the first round-product was added in the second-round mix. Amplification conditions for *Phytophthora* spp. specific primers consisted of 1 cycle of 95°C for 2 min, 40 cycles (1st round) or 35 cycles (2nd round) of 95°C for 20 s, 61°C for 25s, 72°C for 30 s and a final cycle of 72°C for 5 min. For ITS4 and ITS5 primers, similar thermocycling conditions were used, however, annealing temperature was reduced to 55°C.

Results

Grower Survey

Information obtained about U.K. grower's raspberry production are outlined in Table 1-3. The survey showed that 100% of growers chose a *Phytophthora*-resistant cultivar as the primary method of disease control on their farms, and 82% of the 18 growers surveyed used a fungicide regime.

Table 1: Results from the 2020 Phytophthora Disease Management Survey completed by 18 U.K. raspberry growers detailing the substrate type.

Substrate Type	Number of Growers (%)
Compost	17
Coir	47
Soil	35

Table 2: Results from the 2020 Phytophthora Disease Management Survey completed by 18 U.K. raspberry growers detailing the length of plant production in years.

Length of Plant Production (years)	Number of Growers (%)
4-6	31
3-4	31
2-3	37
1-2	1

Table 3: Results from the 2020 Phytophthora Disease Management Survey completed by 18 U.K. raspberry growers detailing control methods they use to prevent Phytophthora root rot.

Control Methods	Number of Growers (%)
Fungicide (Dimethomorph/Metalaxyl)	82
Chemical water sterilization	11
UV water sterilization	5
Growing resistant cultivar	100

Isolates obtained from grower sampling.

Through plating symptomatic root tissues from sampling onto *Phytophthora*-selective agar, and subsequent isolation of growing hyphae, twenty-three cultures were obtained which had colony morphology comparable to *Phytophthora*. The ITS region of each isolate was sequenced using Sanger technology. The identities of the isolates are listed in Table 4 below:

Table 4: Identities of isolates obtained from grower sampling and subsequent plating on *Phytophthora*-specific media. The ITS region of each isolate was sequenced using Sanger sequencing.

Sample Number	Identity	Query Cover%
F01/26	*Mortierella fatshedera*	99.18
F01/24	*Mortierella fatshedera*	99.67
F01/39	*Linnemannia exigua*	92.16
F01/16	*Mortierella alpina*	99.69
F02/43	*Mortierella fatshedera*	99.5
F02/46	*Mortierella fatshedera*	99.65
F02/10	*Mortierella fatshedera*	99.83
F02/15	*Mortierella hyalina*	100
F03/10	*Cadophora luteo-olivacea*	99.35
F05/19	*Mortierella elongata*	99.84
F05/35	*Mortierella fatshedera*	99.5
F05/17	*Clonostachys rosea f. catenulata*	99.45
F05/19	*Mortierella elongata*	99.84
F05/48	*Mortierella fatshedera*	99.5
F01/39	*Linnemannia exigua*	92.16
F02/20	*Mortierella fatshedera*	99.83
F01/46	*Mortierella alpina*	99.69
F01/12R	*Diaporthe eres*	99.64
F07/36	*Trichoderma hamatum*	98.83

Optimisation of raspberry root DNA extraction

Following extraction of DNA from symptomatic raspberry roots with the Qiagen PowerSoil Pro kit, samples which has been freeze-dried prior to extraction had higher DNA concentrations than those which had been frozen only. In addition, samples which had been freeze-dried had consistent 260/280 ratios of ca. 1.8, whilst the frozen samples had 260/280 values ranging from 1.7-2.0 (Table 5).

Table 5: Nanodrop results including DNA concentration (ng/µL), and ratios of contamination (260/280 and 260/230) of DNA extracted from raspberry roots which had either been freeze dried (FD) or frozen (FR) prior to extraction with a commercial kit.

Sample ID	ng/µl	260/280
1/11 FR	63.87	1.75
1/11 FD	79.82	1.89
1/16 FR	52.59	2.03
1/16 FD	150.44	1.88
1/35 FR	25.09	1.78
1/35 FD	89.73	1.83
4/26 FR	32.03	1.9
4/26 FD	56.97	1.82
8/40 FR	93.32	1.86
8/40 FD	61.05	1.94

Discussion

The preliminary findings of this work, while still in development, are informative for both the project as a whole and for the industry in which it is directly involved with. The isolation of fungal species which exhibit *Phytophthora*-like symptoms, such as *Diaporthe, and Cadophora,* in UK plants is of note, as root rot treatment regimes focused on *Phytophthora* such as mefenoxam or dimethomorph may not be as effective in their control leading to unchecked disease which has potentially devastating consequences for growers, particularly those in soil-based production. This finding is further highlighted by the survey responses collected which noted that 82% of growers surveyed relied on chemical biocides to control *Phytophthora,* which would not be effective in the control of fungal root pathogens. Whilst the cultures obtained are morphologically similar to *Phytophthora*, they were identified as fungal.

The work thus far has determined that freeze-drying samples prior to DNA extraction is more efficient and produces DNA of higher quantity and quality than frozen samples. The 260/280 value describes the ratio between the absorbance of the sample at 260 and 280 nm which denotes the purity of the nucleic acid. In double stranded DNA, a 260/280 ratio of 1.8 indicates pure DNA (Pachchigar & Khunt, 2017). The more consistent 260/280 ratio of the freeze-dried samples imply they have lower contaminants present in comparison to the frozen samples in which the ratio is higher. Frozen samples did not form a compact pellet after the first centrifugation step, this meant that soil and other contaminants are difficult to exclude from the next steps which may explain their less-consistent 260/280 ratios. In contrast, freeze-dried samples readily formed pellets which enabled supernatant transfer free of visible contaminants.

Furthermore, freeze-dried samples are more easily stored and processed than those frozen, as additional steps must be taken to extract DNA from frozen samples, i.e., freezing Geno-grinder modules, maintaining samples on ice prior to preparation to prevent thawing.

Conclusions

The preliminary results outlined in this report constitute an important step in developing a more efficient and reliable protocol for DNA extraction from raspberry roots. Through achieving a purer DNA template, more reliable detection via PCR can be achieved which in turn allows for less error rates during sequencing. A culture bank of isolates from UK raspberry grower sites has also been developed. This work also described the isolation of twenty-three cultures from symptomatic raspberry roots and canes which are currently being sequenced.

Future Work

- Perform pathogenicity screening of the fungal and oomycete isolate panel.
- Sequencing of samples from grower surveying to determine fungal and oomycete species diversity in UK raspberry production.
- Continual isolation from symptomatic tissue to obtain more isolates for subsequent years' work.
- Develop a meristem culture method to exclude *Phytophthora* from raspberry.

Knowledge and Technology Transfer

Fruit Focus 2020 – oral presentation

AHDB Soft Fruit Day 2020 - poster presentation

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