# BS20148: *Phytophthora* Biodiversity in Kiwifruit

# Report Summary (October 2023)

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*This paper summarises an innovation project, which aimed to understand the diversity of various Phytophthora species associated with Kiwifruit and the risk Phytophthora species pose to the kiwifruit industry. This was achieved by surveying 128 kiwifruit orchards from different kiwifruit growing regions in New Zealand and identifying the Phytophthora species present in leaf, and plant-associated soil and root samples. The survey found no Phytophthora species from leaf samples but often found them in the soil and root samples. The species found are detailed in the summary below.*

*This research supports the kiwifruit industry to enhance our knowledge on biosecurity risks for Phytophthora species, and allows the industry to:*

- *Establish a comprehensive understanding of Phytophthora species present in kiwifruit orchards in New Zealand.*
- *Provide baseline data to rule out potential biosecurity risks from a Phytophthora species that was previously not reported to be present in NZ*

*The project report has been summarised below to remove confidential information, however please contact Zespri Innovation if you would like further information about this project.*

## 1. BACKGROUND

*Phytophthora* species are soil-borne oomycetes (i.e., fungi-like) or "water-mould" that can cause root rot and crown rot in kiwifruit plants. These organisms can be found in most horticultural soils. They survive well in dry soil but need wet soil to infect plants. *Phytophthora* become active when soils are saturated and mobile zoospores which have flagella (tails) are produced. These can swim through water in the soil to seek out plant roots to attack. *Phytophthora* infections generally start in the plant roots and move up through the plant.

In 1999, approximately 55 *Phytophthora* species had been described, which increased to 117 by 2012 (Martin et al. 2012). By 2018 the number of *Phytophthora* species was approximately 313, based on phylogenetic information and traditional morphotyping (Ho 2018). Described species are listed at www.mycobank.org as well as other websites such as www.phytophthoradb.org and www.Phytophthora-ID.org. Changes in *Phytophthora* nomenclature and taxonomy are ongoing.

*Phytophthora* species pose significant production and biosecurity threats to plant production industries. They pose diagnostic and management challenges and there is uncertainty about their diversity in many ecosystems (Scott et al. 2019).

Zespri, KVH and MPI commissioned a literature review (BS1950) into the potential impact of *Phytophthora* species on the New Zealand kiwifruit industry which recommended that New Zealand should be in a "state of alert for *Phytophthora* attacks on kiwifruit"; and "determine the risk of *Phytophthora* species, that are present in New Zealand" (Woodward & Boa 2019). In that review, the authors found that 12 countries had reported at least 15 different *Phytophthora* species on kiwifruit, mostly affecting yellow-fleshed cultivar (*A. chinensis* var. *chinensis)* or green-fleshed cultivar (*A. chinensis* var. *deliciosa)*. The most significant *Phytophthora* species affecting kiwifruit were *P. cactorum, P. cinnamomi, P. citrophthora, P. cryptogea* and *P. megasperma*. The authors concluded that climate change "is likely to result in further development of conditions conducive to disease development".

Recently it has become apparent that Kiwifruit Vine Decline Syndrome (KVDS) in Italy is not just driven by waterlogging stress/biotic stress, it is also associated with several *Phytophthora spp. and Phytopythium* spp. (Donati et al. 2020, Tacconi et al. 2015). A recent paper concluded that Oomycetes seemed to be a key taxa at the onset of KVDS both because they were consistently isolated from diseased plants and because the oomycetes lifecycle is in perfect agreement with the spreading pattern of the disease (Savian et al. 2020). Symptoms associated with KVDS include leaf curl, fruit drop, cane wilting, and plant collapse. Generally affected vines die within 2 years. KVDS affects almost 3000ha of kiwifruit orchards in Italy, which equates to around 10% of all cultivated kiwifruit in Italy (Donati et al. 2020).

Fig 1: *Phytophthora* symptoms on the crown (Right) and roots of kiwifruit vines (left). Credit Ian Horner

## 2. METHODS

Over the duration of this project, 128 kiwifruit orchards in New Zealand were surveyed for presence of *Phytophthora* in Plant-associated-soil, root and leaf samples collected from all major kiwifruit growing regions (Figure 2). Samples were collected in spring 2020 (November 2020; preliminary), autumn 2021 (March–April 2021), spring 2021 (November–December 2021), autumn 2022 (April–May

2022) and spring 2022 (November–December 2022) Approximately 60% of the sites represented green-fleshed and 40% yellow-fleshed varieties.

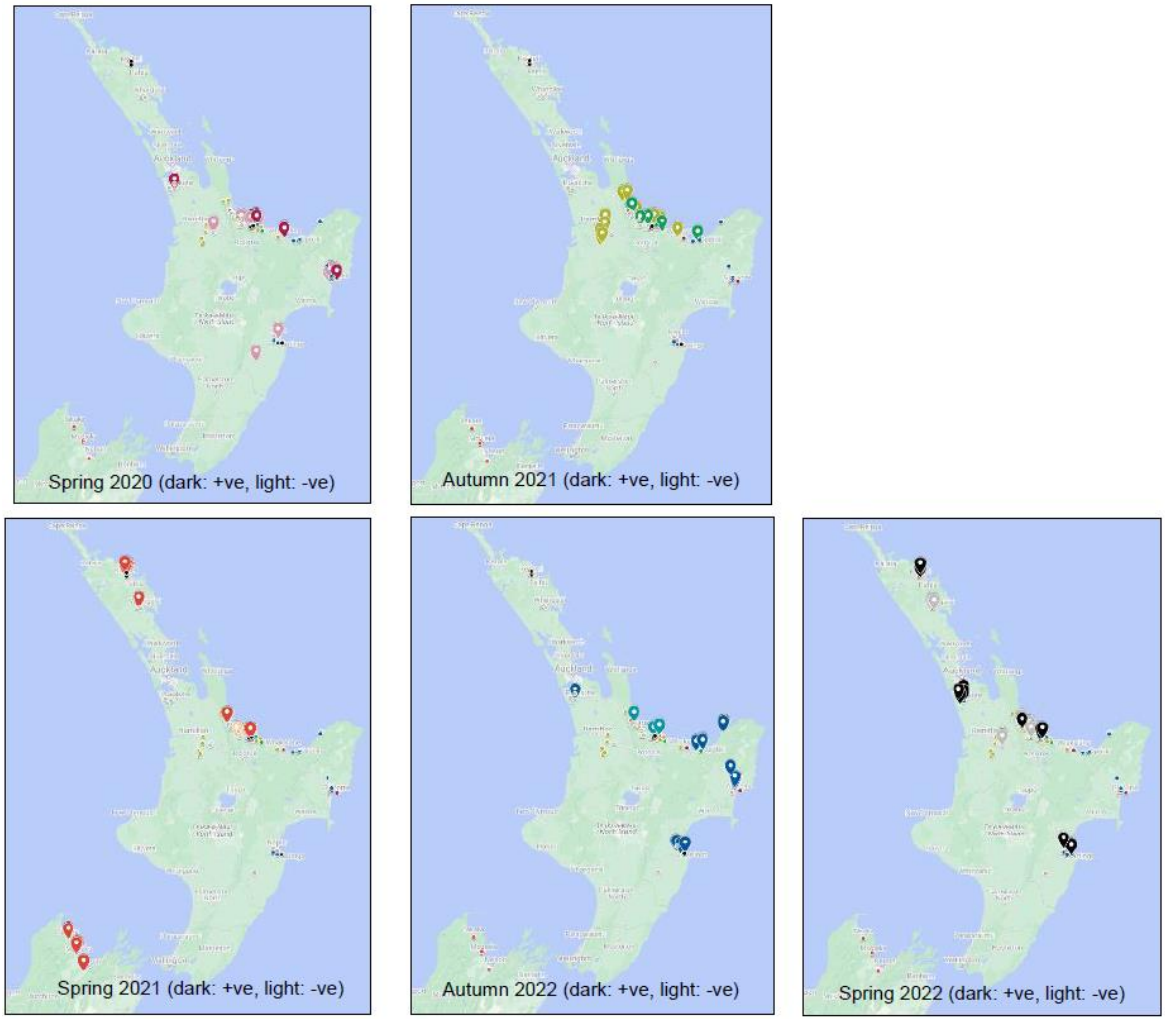


Figure 2. Map of sampling sites of kiwifruit orchards consisting mainly of Gold3 and Hayward orchards. Sampling is shown separately for the spring 2020 (n= 25 sites), autumn 2021 (n=25 sites), spring 2021 (n=26 sites), autumn 2022 (n=26 sites) and spring 2022 (n=26 sites) sampling events. Pins represent sampling sites, with the darker coloured pins representing sampling sites where *Phytophthora* species were detected by soil baiting technique. Sites were selected and identified with the help of KVH staff.

### 2.1 Sampling

Vines located in *Phytophthora*-favourable conditions were targeted for sampling when possible. These conditions included wet and waterlogged areas, low-lying dips and hollows, and areas with heavier soils. Vines were also selected for sampling based on their physical conditions, favouring those that showed symptoms of poor health, such as trunk swelling, yellowing leaves and overall vine decline. In some cases a vine was re-sampled in a following year if further vine decline occurred to understand whether this was associated with the presence of *Phytophthora*.

Leaf, root and soil samples adjacent to plants were collected from four kiwifruit vines at each orchard. At each vine four healthy leaves were collected randomly from each corner of the vine. Fully expanded leaves were collected, from actively growing shoots. Four points around the vine trunk within 30 cm of the trunk were identified for soil sampling (Figure 2.).

Figure 2: An example of soil sampling around a kiwifruit trunk

## 2.2 Isolating and identifying *Phytophthora* species from surveyed sites

Samples were analysed in the laboratory by either baiting (all soil plus some leaf samples), immunostrip (all leaf samples) or by using eDNA techniques (all root segment samples) (Table 1).

Table 1: Description of methods employed to identify *Phytophthora* species from leaf and soil/root samples

| Sample Type | Method of analysis | Description of method |
|---|---|---|
| Leaf | Immunostrip | An on-site assay for *Phytophthora* spp. was performed using Agdia *Phytophthora* immunostrips (ImmunoStrip® for *Phytophthora* (Phyt); Agdia Inc., IN, USA). A positive test result was recorded if two lines appeared on the immunostrip and a negative test result was recorded if there was only one line. |
| Leaf | Baiting | For detecting aerial *Phytophthora* spp., kiwifruit leaves were submerged in sterile water using water weights. The below mentioned plant bait species and Hayward tissue cultured leaves were floated as baits. Half the baits from the leaf baiting were tested using the Agdia *Phytophthora* immunostrips test kit. |
| Soil/root | Baiting | Soil baiting was always done using four plant substrates (lupin seedlings, pine needles, rhododendron and ivy leaves) with more bait substrates added in autumn and spring 2022 (azalea, cedar, Michelia spp. and/or *Metrosideros* spp. leaves). The additional bait substrates recovered more *Phytophthora* isolates, although the same species were generally |

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|---|---|---|
| | | attracted to the different bait tissues. Isolates were cultured using selective media and the ribosomal internal transcribed spacer (ITS) and Cytochrome c oxidase subunit I (cox) gene regions of each isolate were amplified in PCR reactions. Sequences were compared to nr/GenBank database from the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). Similarity hits against *Phytophthora* were reported only where the %ID (identity) was >99% with a coverage >90% of the targeted gene/intergenic region. |
| Soil/Root | eDNA | DNA was extracted from the root samples with attached soil and a PCR was completed using oomycete specific primers tagged with adapters to amplify the internal transcribed spacer 1 (ITS1) region of the ribosomal RNA (rRNA) gene, spanning from the 18S to the 5.8S ribosomal genes. PCR products were sequenced on Illumina MiSeq using paired-end sequencing, analysed and a phylogenetic tree was generated to infer a species identification. |

## 2.3 Testing Pathogenicity of Phytophthora species to kiwifruit

Four *Phytophthora* pathogenicity tests were conducted. The methods for each are detailed below:

**Assay 1:** The initial pathogenicity assay was carried out on **apple fruit** using six isolates of non-kiwifruit host, including apple. Isolates were H78, H85, H586, H595 (all *P. cactorum*), PC3910 and PC20270 (both *P. cinnamomi*). A small mycelial plug (ø 5 mm) from a 1-week-old culture grown on PDA was used for inoculation of surface-sterilised (70% ethanol) red apple 'Braeburn'. A small part of the apple skin was aseptically removed, and the plug placed with mycelium facing the flesh. Agar-only inoculation served as the control. There were three fruit replicates per oomycete isolate. All inoculated apples were kept inside a plastic container in the dark at high humidity and room temperature. Lesion diameter was measured after 1 week of inoculation and ranged from 29.1 to 42.2 mm.

**Assay 2:** the same six isolates were also assessed in a **detached kiwifruit shoot** assay. Shoots (n=8 shoots per isolate and cultivar) were about 3 months old (green lignified) collected from 'Zesy002' or 'Bounty71' rootstock plants (Khdiar et al. 2020). A small mycelial plug (ø 5 mm) from a 1-week-old culture grown on PDA was used for inoculation of surface sterilised (70% ethanol) shoot. A small part of the shoot epidermis was aseptically removed, and the plug placed with the mycelium facing the tissue. Agar-only inoculation served as the control. Shoots were kept in plastic trays in the dark, at high humidity and room temperature. Lesion measurement was carried out weekly for 2 weeks.

**Assay 3:** In Assay 3, potted 'Bruno' and 'Bounty' rootstock plants (1 year old) were obtained from a commercial nursery. A *Phytophthora cryptogea* isolate was obtained from the New Zealand Culture Collection (Landcare Research, ICMP 7300) and grown on PDA for three days prior to the inoculation of sterilised, water-soaked, un-hulled oat grain for four weeks (Ian Horner, pers. comm.) Plants were maintained at the PFR Te Puke glasshouse at 22°C. Roots were inoculated with the colonised grain by making two vertical 10 cm pencil holes into the growth substrate, each then was refilled with approximately 1 teaspoon of *Phytophthora*-grain inoculum. Non-inoculated plants (n= 10; five each of 'Bruno' and 'Bounty') served as a control. *Phytophthora*-treated shoots (n=6) were inoculated with a mycelial plug as described above in the detached shoot assay. Control shoots (n= 6) were inoculated with agar only. All inoculation points were wrapped with Parafilm® to provide moisture. All experimental plants (n=24) were placed individually in 5L buckets and exposed to fortnightly flood

treatments (Conn et al. 1991) which involved filling each bucket with water to 2 cm below the rim for 48 hours. Nil control plants were not inoculated and not flooded. Plants were scored for symptoms after 12 weeks using a score of 0–4, with 0 = healthy plant, 1 = some symptoms of decline (yellowing, lack of growth, leaf drop, wilt), 2 = 50% decline, 3 = 75% decline, and 4 = >90% decline. Shoots and roots from treated and corresponding controls were plated onto oomycete selective agar PARPH (Jeffers & Martin 1986). There were six replicate plants per soil or shoot inoculation treatment for each cultivar. Results are presented descriptively.

**Assay 4:** was done on detached 'Bounty71' and 'Bruno' autumn shoot cuttings, which were collected from spare potted plants (not flooded, not inoculated) as described in Assay 3. A total of 144 shoots, approximately 40 cm long, were cut on 16 May 2023 from the potted plants. Shoots were de-leafed, and the bottom ends of the shoots were placed in 500 mL sterile reverse osmosis water in 1 L Schott's bottles. The upper part of shoots were inoculated with mycelial plugs as described in Assay 2. For each cultivar there were 11 shoots in one bottle with six replicate bottles per cultivar. The 11 shoots represented a treatment each, which were

1. Negative control (no wounding and no inoculation)

2. Agar only inoculation (V8 juice agar)

3. Agar only inoculation (PARPH agar)

4. *P. cryptogea* (isolate 2692-3-7)

5. *P. citrophthora* (isolate 6609-3-1)

6. *P. cinnamomi* (isolate 7939-3-1)

7. *P. megasperma* (isolate 2433-2-2)

8. *P. cryptogea* (isolate 8444-2-3)

9. *P. pseudocryptogea* (isolate 2062-5-4)

10. *P. chlamydospora* (isolate 4824-2-1)

11. *P. multivora* (isolate Sp_21_Phy29)

Lesion length was measured after 3 weeks of incubation at 20°C and natural light in the containment glasshouse. Analysis of variance (ANOVA) was determined using Minitab®, general linear model, and Fisher pairwise comparisons.

# 3. RESULTS

## 3.1 *Phytophthora* Survey Results

No *Phytophthora* species were detected in the leaf samples from the 128 sites using baiting and/or the immunostrip assays. Oomycete detection in soil (bait) and root samples (eDNA) was common. Only a few sites were positive for *Phytophthora* spp. by isolation in the first two sampling periods, but *Phytophthora* were more common thereafter. The *Phytophthora* recovered over time are detailed below:

Table 2. Putative identification of *Phytophthora* species isolates from soil/root samples from New Zealand kiwifruit orchards in 2020-2023

| Year | Season | No. vines sampled | No. vines positive for *Phytophthora* species by baiting | *Phytophthora* species identified |
|---|---|---|---|---|
| 2020 | Spring | 100 | 5 | *Phytophthora citrophthora, P. chlamydospora*, *P. cryptogea*, *P. megasperma* |
| 2021 | Autumn | 100 | 7 | *P. citricola, P. cryptogea*, *P. plurivora* |
| | Spring | 104 | 33 | *P. citrophthora*, *P. cryptogea*, *P. chlamydospora*, *P. plurivora*/*citricola* complex, *P. multivora* |
| 2022 | Autumn | 104 | 33 | *P. cactorum*, *P. cinnamomi*, *P. citrophthora*, *P. chlamydospora*, *P. cryptogea*, *P. plurivora*/*citricola* complex, *P. megasperma* |
| | Spring | 103 | 24 | *P. cryptogea*, *P. plurivora*, *P. pseudocryptogea* |

DNA metabarcoding targeting oomycetes and *Phytophthora* has been completed for the samples. Bioinformatics analyses identified a more diverse and larger group of *Phytophthora* taxa than detected by the traditional isolation methods, although there was also a high degree of agreement between the two approaches. In most cases, species detected by DNA metabarcoding were also represented in the outcomes from the isolation approach. Nevertheless, the DNA metabarcoding detected *Phytophthora* species in larger numbers of samples than indicated by the isolation approach. In some species, there were considerably larger numbers of detections by metabarcoding (e.g. *P. megasperma*), suggesting that these species were more difficult to isolate.

### 3.2 *Phytophthora* Pathogenicity Results

**Assay 1:** In the apple assay, lesions advanced fast (about 32 to 57 mm), by 1 week for all the *Phytophthora* isolates, compared with the agar-only controls, where no lesions developed.

**Assay 2:** In the detached shoot assay, none to very minimal lesions could be observed on 'Bounty71' shoots during the 2 weeks of incubation. This was similar to the agar-only controls. Results on Gold3 detached shoots were inconclusive, as all canes deteriorated within a few days of incubation.

**For Assay 3**, the **potted plant assay**, there was a clear effect of the fortnightly flooding treatment on both 'Bruno' and 'Bounty' rootstocks compared with the nil-control plants, with plant health scores of 2.3, 1.6 and 0, respectively (0 = healthy plant, 2 = 50% decline). This was not affected by root or shoot inoculation with *P. cryptogea*. The lack of effect from soil inoculation with *P. cryptogea* on plant health (as well as root biomass, data not shown) was surprising, therefore re-isolations were conducted for selected plants (n=17).

From a total of 17 plants chosen for re-isolations from both roots and shoots, eight were positive for *Phytophthora* species. All eight recovered *Phytophthora* spp. from root and six from shoot re-isolations. From root re-isolations, in the control plants (with flooding, no or agar-only inoculation), we isolated *P. cryptogea, P. pseudocryptogea* and *P. cinnamomi.* In non-flooded control plants (no inoculations), we isolated *P. cinnamomi,* In the treated plants (flooded and inoculated with *P. cryptogea*) we isolated *P. cryptogea* and *P. cinnamomi,* Results strongly indicate that roots of potted plants in Assay 3 were already infected/carried *Phytophthora* isolates, primarily *P. cryptogea*, but *P. cinnamomi* and *P. pseudocryptogea* were also found. This is not an extensive survey, but was conducted to understand the unexpected results, i.e. the lack of treatment effect from root inoculations with *P. cryptogea*. 'Bounty71' rootstocks yielded fewer *Phytophthora* isolates and were less affected by flooding than 'Bruno' plants.

**Assay 4,** in the **detached shoot assay** the average lesion length of 'Bruno' was higher (19.8 mm) than in 'Bounty71' (6 mm, $p<0.001$) – discounting the 5 mm wound made to inoculate with the mycelial plug. The largest lesions were formed by *P. pseudocryptogea* (35.8 mm) followed by *P. cryptogea* (28.7 mm), *P. citrophthora* (24.2 mm), *P. chlamydospora* (16.8 mm), *P. megasperma* (14.0 mm), and *P. cinnamomi* (4 mm). The V8 agar, PARPH agar and nil controls did not feature any lesions or wound expansions. Treatment differences were significant ($p<0.001$), so was the cultivar interaction ($p<0.05$; Figure 4). *Phytophthora pseudocryptogea* caused the largest lesions in both cultivars ('Bruno' 46.0 mm; 'Bounty71' 25.6 mm)., whereas *P. cryptogea* caused a large lesion only in 'Bruno' but not 'Bounty71' ('Bruno' 49.0 mm; 'Bounty71' 8.4 mm).
This shoot assay clearly indicated that *Phytophthora* spp. can infect kiwifruit vines, and that there are species and cultivar effects. The assay might lend itself to resistance phenotyping.

## DISCUSSION

Relatively few sites yielded *Phytophthora* isolates (five and seven, respectively) during the first two sampling rounds from New Zealand kiwifruit orchards, despite targeting *Phytophthora*-prone sites (i.e. weak plants, hollows, heavy and wet soil). Isolations during spring 2021 period yielded more than a four-fold increase, with 45 *Phytophthora* specimens out of a total of 110 isolates identified by DNA sequencing. The majority of samples were obtained from Northland and Tasman regions, which yielded more oomycetes than from the Bay of Plenty region. The autumn 2022 isolations yielded another step up in the number of *Phytophthora* specimens. The regional effect, including soil types and climatic differences, should be further explored.

Different bait plants attracted different *Phytophthora* spp. For example, lupin roots baited the most *Phytophthora* spp. (36%), followed by pine needles (25%), rhododendron leaves (16%), ivy leaves (9%), azalea leaves (7%), *Michelia* spp. leaves (4%) and the other plant tissues (3%). However, between lupin roots, pine needles and rhododendron leaves, all *Phytophthora* species detected in this survey were baited by at least one of these baits.

*Phytophthora cryptogea* by far was the most frequently isolated *Phytophthora* spp. (n=100 isolates) followed by species from the *P. plurivora/citricola* complex (n=27 isolates) and *P. citrophthora* (n=17 isolates). Other species were baited at low frequencies. It is noteworthy that some species were baited multiple times on different plant substrates from a single soil sample. From a total of 441 oomycetes recovered by baiting, 173 isolates belonged to the *Phytophthora* genus.

The Hawke's Bay isolates used in the apple and detached shoot pathogenicity assay were mainly from the apple host. They were able to (nearly uniformly) colonise apple fruit but were less successful in colonising detached kiwifruit canes. For the potted plant bioassay, *P. cryptogea* was then selected, as this was the most frequently isolated species from orchards in this 2-year survey and was the most pathogenic species to kiwifruit in previous New Zealand (Stewart & McCarrison 1991) and American (Conn et al. 1991) studies. The American methodology adopted here, with the frequent flooding-drain cycles, affected both rootstocks, but 'Bruno' more severely than 'Bounty', as observed in by the plant heath score but also rootstock size (Figure 4, Appendix 4). 'Bounty' is well known for its increased wetness tolerance compared with that of 'Bruno' (Mian et al. 2022). 'Bounty' also showed greater resilience to shoot and soil inoculations than 'Bruno'. It is unclear if our soil inoculations indeed made any impact, as oomycetes could be re-isolated at the same frequency from inoculated and non-inoculated plants, with or without water-logging treatments. The potted plant shoot assay could be a useful and simple tool to study pathogenicity as well as evaluating plant resistance.

The detached shoot Assay 4, from shoots sampled in autumn, has shown the infection potential of different isolates to two common rootstocks. The assay may lend itself to phenotyping of resistance. *Phytophthora pseudocryptogea*, *P. cryptogea* and *P. citrophthora* were the most pathogenic, with the latter two isolates the most frequently found.

The inability to re-isolate *Phytophthora* spp. from resampled vines suggests that the vine decline and deaths observed are due to causes other than *Phytophthora,* as has been seen elsewhere in vines with reported symptoms of Kiwifruit Vine Decline Syndrome (KVDS) (Donati et al. 2020). Lack of detection could also be due to sampling errors/variations and seasonality, particularly if low inoculum is present. While oomycetes are associated with KVDS (Savian et al. 2022), additional investigations will be required to ascertain the state and causes of vine decline in this country.

**In summary,** the current occurrence of diseases caused by or associated with *Phytophthora* remains low in New Zealand. More *Phytophthora* and more diverse species were found in the smaller kiwifruit production regions, which is generally associated with heavier soils. Both baiting and eDNA methods were seen to be complementary when understanding *Phytophthora* biodiversity and pathology. Taken together, this baseline survey provides a benchmark for changes in *Phytophthora*-induced vine decline and species diversity in New Zealand kiwifruit orchards. Climate changes will probably increase kiwifruit vine stresses and declines owing to extra wetness and pathogen pressures (Tacconi et al. 2015).

As *Phytophthora* has been associated with diseased kiwifruit vines and vine decline, particularly in poorly drained soils it is important to implement good management practices to minimise the risk and potential impact of *Phytophthora*. For more information visit the KVH website at kvh.org.nz or the *Phytophthora* page within the Zespri Canopy.

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