Characterisation and detection of mefenoxam sensitivity in *Phytophthora nicotianae* and *Phytophthora citrophthora* from citrus in South Africa

by

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Declaration

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SUMMARY

In South Africa, citrus is of high agricultural and economic importance, representing one of the country's major fruit crops. This sector plays a pivotal role in the nation's economy by substantially contributing to export earnings and employment opportunities. Citrus production is, however, threatened by oomycete pathogens, particularly *Phytophthora*, that can cause citrus diseases resulting in significant economic losses. *Phytophthora nicotianae* and *P. citrophthora* have been reported in every citrus-producing province in South Africa including citrus nurseries. These soil-borne pathogens primarily target the roots and the lower parts of citrus trees, causing root rot, lesions, gummosis, and brown rot of citrus fruit. Infected trees experience a decline in vigour, leading to stunted growth, wilting, and death in severe cases. These diseases also compromise the tree's ability to translocate water and nutrients, resulting in reduced fruit production and poor fruit quality.

Mefenoxam is routinely used in citrus nurseries and orchards to treat *Phytophthora* infections. This chemical inhibits RNA polymerase I, responsible for rRNA synthesis. Its action prevents mycelial growth, sporangia formation, and germ tube growth, but due to its site-specificity, there is a high risk of resistance development. Continuous use of mefenoxam by citrus growers has led to the detection of mefenoxam-resistant *Phytophthora* isolates globally, including in South African nurseries and orchards. The monitoring of resistance to mefenoxam is important to ensure the lasting efficacy of this highly effective chemical and is reliant on the rapid and accurate detection of mefenoxam sensitivity.

In this study, mefenoxam-insensitive and -sensitive *P. nicotianae* and *P. citrophthora* isolates were identified by *in vitro* fungicide sensitivity testing using Ridomil Gold 480 SL. These isolates were subjected to whole genome sequencing (WGS) using an optimised DNA isolation protocol to obtain high-quality, intact DNA from *Phytophthora* mycelia. A complete genome assembly of *P. citrophthora* was generated, for the first time, using PacBio HiFi long-read sequencing and used as the reference genome for WGS obtained by Illumina sequencing. Single nucleotide polymorphisms (SNPs) were detected in ABC transporter and cytochrome P450 genes as well as in RNA polymerase III subunits for *P. nicotianae* isolates and in RNA polymerase II and III subunits for *P. citrophthora* isolates.

A quantitative polymerase chain reaction (qPCR) assay was developed to differentiate between mefenoxam-sensitive and homozygous-resistant *P. citrophthora* isolates. The specificity of this assay for *P. citrophthora* was validated against various other citrus soil-borne pathogens. The low number of insensitive isolates significantly limited the design of qPCR assays for *P. nicotianae*. Additionally, we evaluated a multiplex assay to detect *P. citrophthora* and assess mefenoxam sensitivity, simultaneously, although the amplification products could not be differentiated from each other,

necessitating further optimisation. Overall, this study offers important genetic insights into mefenoxam sensitivity in *Phytophthora*, setting a foundation for the development of diagnostic tools to monitor fungicide resistance and manage citrus diseases caused by oomycetes more effectively.

OPSOMMING

In Suid-Afrika is sitrus een van die belangrikste vrugtegewasse en is daarom van hoë landbou- en ekonomiese belang. Hierdie sektor speel 'n kernrol in die ekonomie van die land deur aansienlik by te dra tot uitvoerinkomste en werksgeleenthede. Sitrusproduksie word egter bedreig deur oomysete patogene, veral *Phytophthora*, wat sitrusiektes kan veroorsaak en kan lei tot aansienlike ekonomiese verliese. *Phytophthora nicotianae* en *P. citrophthora* is reeds in elke sitrusproduserende provinsie in Suid-Afrika gerapporteer, asook in sitruskwekerye. Hierdie grondgedraagde patogene teiken hoofsaaklik die wortels en die onderste dele van sitrusbome, wat wortelverrotting, gomvloei en bruinverrotting van sitrusvrugte veroorsaak. Geïnfekteerde bome ondergaan 'n afname in groei, wat lei tot gebrekkige ontwikkeling, verwelking en selfs dood in ernstige gevalle. Hierdie siektes belemmer ook die boom se vermoë om water en voedingstowwe te translokeer, wat lei tot verminderde vrugproduksie en swak vrugte kwaliteit.

Mefenoxam word gereeld in sitruskwekerye en boorde gebruik om *Phytophthora* infeksies te behandel. Hierdie swamdoder inhibeer RNA polimerase I, wat verantwoordelik is vir rRNA-sintese. Die swamdoder voorkom die groei van miselium, die vorming van sporangia en die groei van kiembuise, maar daar is 'n hoë risiko vir weerstandontwikkeling weens die enkel genetiese teiken. Die voortdurende gebruik van mefenoxam deur sitrusprodusente het gelei tot die opsporing van mefenoxam-weerstandige *Phytophthora* isolate wêreldwyd, asook in Suid-Afrikaanse kwekerye en boorde. Die monitering van weerstand teen mefenoxam is belangrik om die langdurige doeltreffendheid van hierdie hoogs effektiewe swamdoder te verseker en is afhanklik van die vinnige en akkurate opsporing van mefenoxam-sensitiwiteit.

In hierdie studie is mefenoxam-onsensitiewe en -sensitiewe *P. nicotianae*- en *P. citrophthora* isolate geïdentifiseer deur *in vitro* swamdoder-sensitiwiteitstoetse met Ridomil Gold 480 SL uit te voer. Hierdie isolate is onderwerp aan hele genoomvolgordebepaling (HGV) met behulp van 'n geoptimeerde DNS-isolasieprotokol om hoë kwaliteit, intakte DNS van *Phytophthora* miselium te verkry. 'n Volledige genoomsamestelling van *P. citrophthora* is, vir die eerste keer, gegenereer deur gebruik te maak van PacBio HiFi lang-leesvolgordebepaling en is gebruik as die verwysingsgenoom vir HGV wat verkry is deur Illumina-volgordebepaling. Enkelnukleotiedpolimorfismes (ENP's) is opgespoor in “ABC-transporter” en “cytochrome P450” gene, asook in RNA polimerase III subeenhede vir *P. nicotianae* isolate en in RNA polimerase II en III subeenhede vir *P. citrophthora* isolate.

'n Kwantitatiewe polimerase kettingreaksie (kPKR) toets is ontwikkel om te onderskei tussen mefenoxam-sensitiewe en homosigote-weerstandige *P. citrophthora* isolate. Die spesifisiteit van hierdie toets vir *P. citrophthora* is bevestig teen verskeie ander sitrus grondgedraagde patogene. Die

lae aantal onsensitiewe isolate het die ontwerp van kPKR-toetse vir *P. nicotianae* beperk. Daarbenewens het ons 'n multipleks-toets geëvalueer om *P. citrophthora* op te spoor en mefenoxam-sensitiwiteit gelyktydig te assesseer, alhoewel die amplifikasieprodukte nie van mekaar kon onderskei word nie, wat verdere optimisering noodsaaklik maak. Hierdie studie bied belangrike genetiese insigte in mefenoxam-sensitiwiteit in *Phytophthora*, wat as 'n grondslag dien vir die ontwikkeling van diagnostiese hulpmiddele om swamdoderweerstand te monitor en sitrusiektes veroorsaak deur oomycetes meer doeltreffend te bestuur.

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CONTENTS

CHAPTER 1

Reviewing *Phytophthora* citrus pathogens in South Africa

INTRODUCTION

The citrus industry in South Africa plays a pivotal role in the country's economy, contributing significantly to exports and job creation. Citrus pathogens pose a significant threat to this industry, causing diseases which can lead to decreased crop yield, compromised fruit quality, and substantial economic losses for growers and exporters. *Phytophthora nicotianae* and *Phytophthora citrophthora* occur in citrus nurseries and orchards throughout South Africa. Their presence in nurseries is of high concern, as growers purchase citrus nursery material and facilitate the spread of the pathogens to large orchards. Once in orchards, the pathogens cause several diseases that decrease tree productivity or even result in tree death. Citrus nurseries are key role players in the sustainable success of the South African citrus industry. The expert-oriented industry puts high pressure on nurseries to continuously produce nursery material of the highest quality, for growers to meet production targets. Therefore, nurseries are forced to regularly perform pathogen screenings and upon detection, use chemicals to ensure that all materials are free of harmful pathogens. One of the chemicals routinely used in citrus nurseries to treat *Phytophthora* infestations is mefenoxam. The continued use of this chemical serves as a positive selection pressure for resistant isolates and can result in the rapid erosion of fungicide efficiency, which represents a major challenge for the management of *Phytophthora* infections. New methods for the detection of fungicide resistance are needed to prevent the overuse of this chemical and the development of large fungicide-resistant populations. This review focuses solely on the two predominant *Phytophthora* spp. occurring in South African citrus, *P. nicotianae* and *P. citrophthora*. It includes an in-depth review of the pathogens, and current detection and management strategies, to ultimately highlight the need for new molecular detection methods for mefenoxam-resistance in these pathogens. The management of fungicide resistance should be of top priority to prevent any further damage by *Phytophthora* to the South African citrus industry.

CITRUS IN SOUTH AFRICA

Citrus is grown in every province of South Africa, with Limpopo, Eastern Cape, and Western Cape being the largest production areas. These provinces make up 85% of the citrus cultivation areas in the country (Citrus Growers' Association of Southern Africa, 2022). New hectares are actively being

planted in these areas each year, allowing a continued increase in production. South Africa produces oranges, lemons, mandarins, and grapefruit. Oranges are the predominant citrus type produced, yielding 1.65 million metric tons (MT) in the 2020/2021 marketing year, followed by lemons (625 000 MT), mandarins (567 000 MT) and grapefruit (367 000 MT) (Sikuka and Caldwell, 2021). South Africa produced 2 815 000 tons of citrus in the 2020/2021 season, making the country the 12th biggest citrus producer worldwide. However, of the total citrus produced, 2 599 000 tons were exported in the same season, resulting in South Africa being ranked as the second-largest exporter of citrus internationally (Citrus Growers' Association of Southern Africa, 2022). The citrus industry of South Africa is, therefore, largely export-oriented. This can be attributed to the high export market prices, which, in some citrus types are double the price of local markets (Sikuka and Caldwell, 2021). The industry generates an export revenue of approximately R30 billion a year and creates over 120 000 jobs in South Africa (Citrus Growers' Association of Southern Africa, 2022), deeming the industry a key role player in the South African economy.

Citrus nurseries

The chain of citrus production starts in citrus nurseries. Citrus rootstock seeds are sown and allowed to germinate. When the germinated seeds have more than 2 differentiated leaves, the healthy seedlings are transplanted into trays. Seedlings are transplanted into bags when they reach 20 cm in length. The seedlings are then grown in greenhouses until they reach 50 cm in length and stems have a diameter of 8 mm, whereafter grafting or budding takes place. Scions are grafted or budded onto seedlings with the budding union protected with tape. After a few weeks, the bud union is inspected to determine if budding was successful. After seven days, the rootstock is cut just above the bud union and the new tree is secured with a stake and left to grow. The young, healthy trees can then be distributed to citrus growers after 16 to 18 months (Citrus Academy, 2019).

The Citrus Improvement Scheme (CIS) was established in 1973 to ensure that citrus growers were provided with horticulturally superior and disease-free nursery trees (Von Broembsen and Lee, 1988). The CIS, managed by Citrus Research International (CRI), supplies rootstock seeds and budwood to citrus nurseries in Southern Africa. The seeds and budwood, obtained from the Citrus Foundation Block in the Eastern Cape province of South Africa, are disease-free and true-to-type. Nurseries must undergo a series of audits to confirm that they comply with CIS standards, which include pathogen screenings. Upon successful audits, nurseries are registered as certified. When buying from these certified nurseries, growers are assured of pathogen-free nursery trees made of high-quality genetic material. One of the main aims of the CIS is, therefore, to exclude harmful pathogens from citrus nurseries when detected through routine monitoring. One such pathogen, *Phytophthora*, was initially

sporadically reported in South African citrus nurseries (le Roux, 2003). However, continued reports of its detection, despite the implementation of chemical control measures, are cause for concern.

PHYTOPHTHORA IN SOUTH AFRICA

Phytophthora nicotianae Breda de Haan and *P. citrophthora* (R.E.Smith and E.H.Smith) Leonian belong to the kingdom Chromista, phylum Oomycota and family Peronosporaceae, which are a family of water moulds (Cooke *et al*., 2000). These pathogens are classified as oomycetes and although similar in behaviour, they are phylogenetically distinct from other fungal groups belonging to the kingdom Fungi. Based on genes encoding the small ribosomal subunit, actin, and tubulin, it was determined that fungi have a shared ancestry with animals. On the other hand, the closest relatives of oomycetes are the golden-brown algae (Baldauf *et al*., 2000). Oomycetes are diploid during an extensive part of their life cycle, indicating the presence of two sets of chromosomes in each cell of the organism. Their hyphae are aseptate and cell walls are primarily composed of 1,3-β-glucans, along with some 1,6-β-glucans and 1,4-β glucans, which make up cellulose. Although chitin is a significant component of fungal cell walls, it has been identified in only a limited number of oomycetes in small quantities (Erwin and Ribeiro, 1996). Both species produce ovoid, obpyriform (pear-shaped) to limoniform (lemon-shaped) papillate sporangia (Ho, 2018).

Phytophthora nicotianae and *P. citrophthora* are two distinct species within the genus *Phytophthora*. Based on phylogenetic analysis using the 5.8S ribosomal RNA (rRNA) gene and flanking internal transcribed spacers 1 and 2 (ITS1 and ITS2), these species exhibit evolutionarily divergence from each other (Cooke *et al*., 2000). More recent phylogenetic analyses with mitochondrial sequences and other popular nuclear sequences, such as beta-tubulin, elongation factor 1 alpha and heat shock protein 90, confirmed that these species are evolutionary different (Blair *et al*., 2008; Martin *et al*., 2014). *Phytophthora nicotianae* was first described by Breda de Haan in 1896 and placed into clade 1 on the evolutionary phylogenetic tree of *Phytophthora* (Kroon *et al*., 2012). *Phytophthora citrophthora*, first described in 1906 (Smith and Smith, 1906), placed into clade 2a (Blair *et al*., 2008; Kroon *et al*., 2012; Martin *et al*., 2014).

Both species are heterothallic, indicating that the appropriate A1 and A2 mating types must both be present for sexual reproduction to occur (Ho, 2018). In South Africa, mating type analysis of *P. nicotianae* revealed a predominance of the A1 mating type (79%), with A2 comprising only 18%, and 3% being sterile (Meitz-Hopkins *et al*., 2014). These mating types co-existed in multiple regions, primarily in Mpumalanga, Limpopo, and the Western Cape, albeit at a skewed A1:A2 ratio of 4:1. Conversely, *P. citrophthora* exhibited a notable sterility rate (64%), with A1 and A2 mating types

observed in separate orchards, primarily in the Limpopo and Western Cape provinces, respectively. Oospore formation was limited, with observed abnormalities in *P. citrophthora*, further delineating mating patterns within this species.

Distribution of oomycetes

Host range and availability, plant immunity, environmental conditions and interaction with other organisms are all important factors that determine the distribution and spread of oomycetes (Lamour and Kamoun, 2009). In addition, climatic conditions are a key factor influencing the distribution of *P. nicotianae* and *P. citrophthora* in South Africa. These oomycetes are predominantly found in habitats where the soil is moist, as they rely on water for a significant portion of their life cycle (Lamour and Kamoun, 2009). The temperature range of *P. nicotianae* is 12°C to 33°C, with an optimum temperature for mycelial growth between 30°C and 32°C (Matheron and Porchas, 1996; Aglave, 2018). This pathogen is, therefore, prevalent in regions of South Africa where rainfall periods coincide with warmer temperatures (Meitz-Hopkins *et al*., 2014). This frequently occurs in the northern regions of the country, including Gauteng, Mpumalanga, Limpopo and North West (Thompson *et al*., 1995), as well as Kwazulu-Natal (Maseko *et al*., 2007). The temperature range of *P. citrophthora* is 9°C to 27°C, with an optimum temperature between 24°C and 28°C (Matheron and Porchas, 1996; Aglave, 2018). This pathogen has a high incidence in areas where temperatures are cooler during rainfall, including the Western Cape and Eastern Cape provinces (Meitz-Hopkins *et al*., 2014). Meitz-Hopkins *et al*. (2014) also reported that *P. nicotianae* and *P. citrophthora* co-occur in some citrus orchards in South Africa. Both pathogens have a wide temperature range which allows them to survive in less favourable climatic regions and, therefore, it is expected that the two species can be both found in the same citrus orchard.

Disease cycle

The soilborne pathogens*, P. nicotianae* and *P. citrophthora* have a similar disease cycle (Fig. 1). The oomycetes survive in the soil using structures like hyphae, chlamydospores or oospores (Aglave, 2018). *Phytophthora nicotianae* produces chlamydospores and oospores whereas *P. citrophthora* produces chlamydospores but rarely produces oospores (Abad *et al.*, 2022). Oospores are sexual spores and are, therefore, only produced if the A1 and A2 mating types of *P. nicotianae* are present. The disease cycle starts when there is a slight deficit in water potential and hyphae, chlamydospores or oospores germinate to form sporangiophores that bear sporangia (Aglave, 2018). In the presence of free water and after a drop in temperature, sporangia release zoospores. The flagellated zoospores swim through free water in the soil and on the soil surface and accumulate in puddles on the orchard

floor. Rain, irrigation, and mechanical equipment splash zoospores from puddles onto the trunk, lower hanging branches and fruit of the citrus tree. Upon landing on a suitable site, the zoospores encyst and secrete an adhesive that glues them to the infection surface (Hardham, 2001). A germ tube emerges from the cysts and forms penetration hyphae, which allows the pathogen to enter plant cells, infect the citrus plant and obtain the necessary nutrients for further growth. Mycelia can overwinter in infected fruit, roots, trunk, and soil and serve as inoculum in the next disease cycle. As mentioned above, both pathogens can also overwinter in the soil as chlamydospores (Abad *et al.*, 2022). These spores are highly specialised survival spores produced when conditions are unfavourable for fungal growth. This can include temperatures between 15°C and 18°C, depletion of nutrients and low oxygen levels.

Genome information

There is a lack of information on the genomes of *P. nicotianae* and *P. citrophthora,* due to the minimal amount of full genome sequences available for these pathogens. This has resulted in a limited understanding of the species' genome features and their role in its pathogenicity and virulence. To date, there are four assembled genomes of *P. nicotianae* available on the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), but a fully assembled high-quality *P. citrophthora* genome has not been published. The lack of a high-quality genome impedes any genetic and genomic studies on this species.

There is great variation in the genome size of the four available *P. nicotianae* genomes, ranging between 59 Mb and 106,7 Mb. This intraspecies variation in genome size can be attributed to the high levels of repetitive DNA sequences often reported in *Phytophthora* genomes, making it difficult to accurately assemble their genomes (Cox *et al*., 2022). The enhanced read lengths of third-generation sequencing technologies, such as Nanopore and PacBio SMRT, spanning hundreds of kilobases, have been effective in resolving repetitive regions, rendering these technologies valuable in assembling *Phytophthora* genomes (Engelbrecht *et al*., 2021). A large variation in genome sizes can also be seen between different *Phytophthora* species, with *P. infestans* having the largest genome (228.5 Mb) and *P. plurivora* the smallest (40.4 Mb). Repetitive genome content, lengths of intergenic regions and the number of pseudogenes could account for the observed variation in size among *Phytophthora* genomes (Gao *et al*., 2021).

CITRUS DISEASES CAUSED BY *PHYTOPHTHORA* SPECIES

Phytophthora spp. can cause a range of diseases in citrus plants leading to crop production losses in orchards or young tree losses in nurseries.

Damping-off

In nurseries, *P. nicotianae* and *P. citrophthora* can cause the damping-off of citrus seedlings. The pathogen penetrates and infects the stem right above the soil level causing the emerging seedling to collapse (Savita *et al*., 2012). Damping-off and seedling death most often occur in nurseries where seeds are not pre-treated with fungicides, poor phytosanitary measures are used (unsterilised soil; untreated irrigation water) and moisture levels and temperatures are favourable for *Phytophthora* growth (Cacciola and Lio, 2008).

Root rot

Root rot occurs when the pathogens infect the root cortex and cause the decay of fibrous roots. The roots become discoloured, have a water-soaked appearance, and slough off from the stele, leaving the rootlet appearing thread-like (Aglave, 2018). In nurseries, the destruction of feeder roots has a detrimental effect on the young trees (Savita *et al*., 2012) but adult trees can survive with a high percentage of infected roots. Only when the destruction of roots occurs faster than the regeneration of roots do symptoms of decay become visible in the canopy of adult trees due to the limited uptake of water and nutrients (Cacciola and Lio, 2008). These symptoms include yellowing and loss of leaves, twig dieback, and a reduction in fruit size and production. This disease remains extremely difficult to diagnose, as symptoms are similar to those of poor soil aeration due to compaction or flooding and high salt content in the soil or nematode damage (Aglave, 2018).

Foot rot

Foot rot occurs when the pathogens infect the scion near the soil level and cause lesions on the trunk of the tree. Abundant gum exudation occurs from these lesions, which can spread around the circumference of the trunk and eventually girdle the tree. Leaves can turn pale green with yellow veins. The trunks of adult trees are typically partially girdled with some twig dieback and defoliation of the canopy, but nursery and young trees are rapidly killed by this disease (Savita *et al*., 2012).

Brown rot

Brown rot of citrus fruit is caused by *P. nicotianae* and *P. citrophthora*. The disease is primarily observed on mature fruit that grows near the soil level (Savita *et al*., 2012). The first symptoms include firm lesions with a water-soaked appearance on the rind of the fruit. The lesions then turn soft and have a tan to olive-brown colour (Aglave, 2018). The disease can spread to fruit throughout the canopy in warm and wet conditions. Symptoms on the fruit can also emerge post-harvest. The packing of infected fruit could cause brown rot to transmit to nearby fruits in the same container. Infected fruit typically emits a pungent and rancid odour while in storage (Cacciola and Lio, 2008).

Trunk and branch cankers

Phytophthora nicotianae and *P. citrophthora* infiltrate citrus trees, typically exploiting wounds caused by mechanical damage, pruning cuts, insect feeding or natural openings like stomata (Graham and Menge, 1999). Once inside, these pathogens establish themselves within the vascular tissues, including phloem and cambium tissues, causing the development of cankers on the tree's trunk or branches. Cankers manifest as localised regions of necrotic tissue surrounded by healthy tissue, often presenting as sunken, water-soaked lesions on the bark, accompanied by gum exudation (Schutte and Botha, 2010). A comprehensive study by Schutte and Botha (2010) examined trunk and branch cankers affecting Clementine mandarins in South Africa's Western Cape province, pinpointing *P. citrophthora* as the primary causative agent of this disease.

OOMYCETE-CITRUS INTERACTION

When *Phytophthora* spp. infect a citrus plant, the host recognises pathogen-associated molecular patterns (PAMP) by transmembrane pattern recognition receptors (PRRs). The detection of these molecular patterns gives rise to a PAMP-triggered immunity (PTI) in the host plant aimed at preventing further invasion by these pathogens (Oßwald *et al*., 2014). *Phytophthora* spp. can suppress PTI by deploying effectors that promote effector-triggered susceptibility (ETS) in plants and allow them to infect citrus. These effectors include a wide range of molecules that are either apoplastic (in plant extracellular space) or cytoplasmic (inside plant cells) (Kamoun, 2006). Apoplastic effectors in *Phytophthora* include cell wall-degrading enzymes, inhibitors of plant defence enzymes, toxins and elicitins, a group of holoproteins that have only been observed in *Phytophthora* and *Pythium* species (Hardham and Cahill, 2010). Cytoplasmic effectors include RXLR proteins (Boutemy *et al*., 2011) and Crinklers, or CRN effectors (Torto *et al*., 2003).

Phytophthora nicotianae and *P. citrophthora* are hemibiotrophs. The pathogens act as biotrophs during the initial host infection stages but become necrotrophs during colonisation of the host (Oßwald *et al*., 2014). Biotrophic oomycetes, including *Phytophthora*, develop haustoria, which serves as a specialised structure to support biotrophic interactions. When the pathogen penetrates the host cell wall, the plasma membrane is invaginated and haustoria is formed in this intracellular space for the reabsorption of nutrients (Dodds *et al*., 2009). These structures are also responsible for the secretion of apoplastic and cytoplasmic effectors (Wang *et al*., 2017) from the haustorium into the extrahaustorial matrix. From there, the effectors cross the extrahaustorial membrane to enter the plant cytoplasm. Here, the effectors interact with host proteins and manipulate the host resistance response (Dodds *et al*., 2009) (Fig. 2).

PATHOGEN DETECTION

The detection of *Phytophthora* is crucial to prevent its spread in an orchard and to minimise any damage to the citrus plant. Early and accurate detection of these pathogens allows for the rapid implementation of effective management strategies. Several methods have been developed for the detection of *Phytophthora* spp., including biomonitoring, soil-baiting, immunodetection, polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP).

Primary isolation

Phytophthora can be detected by plating infected citrus tissue on selective culture media. The media typically contain antibiotics, that inhibit the growth of bacteria or fungi and only facilitate the outgrowth of *Phytophthora*. The culture can then be purified, and the pathogen can be identified based on morphological characteristics (O'Brien *et al*., 2009). This method can, however, produce false negatives. Plant samples that first test negative can produce a positive result upon washing the tissue in sterile deionized water and replating onto new media (Hüberli *et al*., 2000). The culture plate isolation method can lead to misdiagnosis and should therefore not be used as a stand-alone method for *Phytophthora* detection.

Soil baiting

Phytophthora spp. are soilborne pathogens and can be detected through soil-baiting. Soil samples are flooded with distilled water and sterilised leaf or fruit baits are placed into the water and soil mixture for 24 to 72 hours. The leaf disks are then removed, blotted dry and plated onto selective media, typically containing PARPH, that only facilitates the growth of *Phytophthora* (Jeffers, 1986). The leaf

disks are then incubated and examined for *Phytophthora* growth (Ferguson and Jeffers, 1999). As with culture plate isolation, further detection tests should be performed to prevent misdiagnosis.

Immunodetection

Enzyme-linked immunosorbent assays (ELISA) are regularly used for the detection of plant pathogens. This method relies on an antigen-antibody interaction for the detection of pathogens. As infection is initiated by penetrating zoospores, these structures are used to generate monoclonal antibodies for use in ELISA reactions (Hardham *et al*., 1991). ELISA kits for the detection of *Phytophthora* are commercially available but can only identify the pathogen at the genus level. It has been recommended that ELISA kits should not be used as the only screening method, but rather as a first screen to decrease the number of samples that undergo additional testing (Bulluck *et al*., 2006).

PCR

Molecular techniques are seen as the method of choice for the detection of plant pathogens. These techniques are highly specific, as the DNA structure of pathogens is not affected by environmental conditions as with proteins (O'Brien *et al*., 2009). It is also regarded as more sensitive than culture-based methods or ELISA and can detect pathogens more rapidly (Vincelli and Tisserat, 2008). Traditional PCR reactions involve the use of oligonucleotide primers that flank a DNA region highly specific to a chosen species of *Phytophthora*. After DNA amplification, the PCR products can be viewed by gel electrophoresis and used to detect if the chosen species is present in a sample. Gene targets typically used for the design of *Phytophthora* species-specific primers include *ITS* (Grote *et al*., 2002; Ippolito *et al*., 2002), mitochondrial cytochrome oxidase (*cox*) (Hughes *et al*., 2006) and ras-related GTP-binding protein 1 (*Ypt1*) (Schena *et al*., 2007).

Real-time PCR is currently the gold standard for pathogen detection in plant diagnostic labs. This method allows the quantification of the target product after each cycle of amplification. The amplified region is shorter than in traditional PCR, allowing more efficient amplification and a very high sensitivity (O'Brien *et al*., 2009). Additionally, multiple species can be detected simultaneously in a real-time PCR assay through multiplexing. Several primer pairs, specific for each species, can be added in a reaction to allow the identification of multiple species. Ippolito *et al*. (2004) developed a multiplex real-time PCR assay to allow the simultaneous detection of *P. nicotianae* and *P. citrophthora* from citrus roots and soil. Two specific primer pairs, designed to amplify the ITS2 region of both species, were modified for use in a real-time assay. The respective amplicons were detected by two distinguishable fluorescence signals.

LAMP

LAMP assays are a robust method for pathogen detection. This method also involves DNA amplification, but offers even higher specificity than PCR assays, as two to three primer pairs are used in one reaction that recognises up to eight locations in the target DNA region (Soroka *et al*., 2021). The LAMP method can amplify DNA in less than an hour, under constant temperature conditions (Notomi *et al*., 2000). This simple-to-use assay enables the rapid and specific detection of pathogens, deeming it a promising disease diagnostic tool (Garg *et al.*, 2022). Several LAMP assays have been developed for the detection of *P. nicotianae* (Li *et al.*, 2015; Hieno *et al.*, 2019), but no assays have been reported for *P. citrophthora*. This can be attributed to the lack of genetic information available for this pathogen.

PATHOGEN MANAGEMENT

The most efficient way to prevent *Phytophthora* infection is to avoid the introduction of these pathogens into citrus nurseries and orchards. Citrus nurseries are provided with pathogen-free seeds from the South African Citrus foundation block and these undergo regular screenings to ensure that nursery stock is *Phytophthora*-free (Citrus Research International, 2021). Citrus growers can prevent the planting of infected propagation material by only using citrus plants from certified nurseries. However, *Phytophthora* is often introduced into nurseries or orchards through other sources. Managing these infections in an integrated manner can greatly minimise the damage caused by these pathogens, as no single control strategy can control *Phytophthora*-related diseases. Integrated control strategies include the use of tolerant rootstocks, cultural practises as well as biological and chemical management, most of which are employed to control *Phytophthora* spp. in South Africa.

Tolerant rootstocks

One of the most effective ways to manage the damage caused by *Phytophthora* spp. is with the use of tolerant rootstocks. Citrus rootstock tolerance can be defined as the ability to withstand infection by *Phytophthora spp.* or the ability to remain productive when infected with the pathogen (Graham, 1990). Also, tolerant rootstocks grow new roots and maintain root density in *Phytophthora*-infested soil (Graham, 1995). Therefore, when rootstocks become infected the *Phytophthora* populations remain relatively low and only cause minimal root damage (Graham and Feichtenberger, 2015). In South Africa, widely used tolerant rootstocks include Cleopatra Mandarin, Carrizo & Troyer Citrange, Swingle Citrumelo, C-35 Citrange, Benton Citrange, X639 Hybrid, Minneola × Trifoliate Hybrid (M×T),

and US-812 (Sunki × Benecke) (Citrus Academy, 2017). The use of these rootstocks can be seen as a long-term, effective solution in minimising tree damage caused by *Phytophthora* (Castle, 2010).

Although tolerant to certain pathogens, rootstocks often have other characteristics which lead to poor performance. These characteristics include high susceptibility to other diseases, incompatibility with commercial scions, lack of adaptability to certain soil conditions or decreased tree productivity, which is measured by growth vigour, tree size, yield and fruit quality (Ferguson *et al*., 1990). Therefore, continuous citrus breeding is crucial to develop tolerant rootstocks while retaining good horticultural characteristics. Several breeding programs around the world aim to combine increased rootstock productivity, quality, tolerance to pathogens and tolerance to abiotic stresses (Lima *et al*., 2018). In the past, breeding programs made use of direct crosses to obtain new rootstocks, where breeding and testing of new rootstocks took at least 20 to 30 years (Bowman *et al*., 2021). Modern plant biotechnology, however, greatly accelerates the development of new rootstocks. Currently, breeding programs are focused on genetic mapping to identify and localise quantitative trait loci (QTLs) linked to resistance development in rootstocks (Lima *et al*., 2018). This will lead to more efficient targets which can be used to breed citrus rootstocks with increased tolerance to *Phytophthora* infection whilst preserving good horticultural characteristics.

Cultural practices

A substantial part of the life cycle of *Phytophthora spp*. requires the presence of water, highlighting the importance of proper irrigation management in citrus orchards (Declercq *et al*., 2012). Infested irrigation water is a main source of infection, as run-off water from infected orchards often ends up in streams, rivers or dams nearby which in turn is used for irrigation (Jung and Blaschke, 2004). Bioassays have been developed to detect *Phytophthora* spp. in run-off water (MacDonald, 1994; Hong *et al*., 2002; Kong *et al*., 2003). One interesting example is an *in-situ* baiting assay, where rhododendron leaves are attached to a two-rope bait-deployment system and left in irrigation water containers for 7 days. The baiting leaves are then plated out and *Phytophthora* isolates are confirmed using PCR techniques (Ghimire *et al*., 2009).

The overwatering of citrus trees leads to puddles in which *Phytophthora* oospores can germinate, facilitating the pathogen's spread (Declercq *et al*., 2012). It is recommended to irrigate during the day, extending the intervals between irrigation to allow residual water to evaporate and eliminate the presence of free-water (Cacciola and Lio, 2008). The use of drip irrigation or any localised irrigation methods is not recommended as it leads to the oversaturation of soil and increases the chance of infection by these pathogens (Cacciola and Lio, 2008). Soil preparation is another important aspect of managing *Phytophthora*. Planting sites must include underground drainage for excess water

to drain and runoff. Raised soil beds can also be used to avoid any waterlogging (Cacciola and Lio, 2008). Ultimately, the most important aspect of cultural management is to ensure that the soil in nurseries or orchards is never oversaturated. Without free water, the environment is less favourable for infection by *Phytophthora* spp.

Chemical

The use of chemicals is seen as a highly effective and efficient manner of managing *Phytophthora*. These chemicals can include the use of chlorine in irrigation water or curative chemicals including fosetyl-Al, phosphonates, mefenoxam and metalaxyl.

Chlorine

Chlorination of irrigation water is a control measure used to prevent the introduction of *Phytophthora* spp. in citrus nurseries in South Africa (van Niekerk *et al*., 2020). Chlorine is added to recycled irrigation water, to ensure that the water sources in nurseries are inoculum-free. In South African nurseries, active chlorine concentrations between 3 and 6 ppm are administered to irrigation water. However, it has been reported that mycelium fragments might survive exposure to chlorine at concentrations of up to 8 ppm. To ensure that all propagules of *P. nicotianae* and *P. citrophthora* are eliminated from irrigation water, van Niekerk *et al*. (2020) proved that chlorination is effective when active chlorine is applied at concentrations of 6 ppm, at an exposure time of 60 min, before deactivation of the chemical using sodium thiosulfate.

Curative chemicals

Fosetyl-Al, potassium phosphonate (also referred to as potassium phosphite), metalaxyl, and mefenoxam (metalaxyl-M) are active ingredients in commercial fungicides that have proven to be effective in the treatment of *Phytophthora* (Gisi and Sierotzki, 2008). Fungicides that contain fosetyl-Al and potassium phosphonates are systemic multi-site fungicides that target multiple stages in the lifecycle of *Phytophthora* pathogens. The multi-site nature of these fungicides makes it difficult for pathogens to develop resistance. Common tradenames of these fungicides include Aliette, ProPhyt and K-Phite. These fungicides are often applied as fruit and foliar applications to manage gummosis and brown rot (Gray *et al*., 2018). Metalaxyl and mefenoxam are systemic fungicides belonging to the phenylamine class, that utilise a site-specific action method. The risk of pathogen resistance development to these fungicides is considered as high. Ridomil Gold SL is a common fungicide in this category and it is often applied as a soil drench to manage root rot (Gray *et al*., 2018). This fungicide is most often used in nurseries when *Phytophthora* infestations are detected (van Niekerk *et al*., 2020).

It is recommended that growers use multi-site products either alone or in combination with single-site fungicides to maintain their effectiveness over time.

Mefenoxam sensitivity

Mefenoxam (Ridomil Gold™, Syngenta), as previously mentioned, is an active ingredient in the phenylamine class that is effective for use against oomycete pathogens infecting several hosts, including *Phytophthora* in citrus (Timmer, 1977; Farih, 1981b; Davis, 1982; Sandler, 1989;). Mefenoxam consists only of the active R-enantiomer from the metalaxyl racemic mixture of R- and S-enantiomers and is seen as the more active form of metalaxyl (Monkiedje and Spiteller, 2005). Mefenoxam interferes with RNA polymerase I and inhibits the synthesis of rRNA (Davidse *et al*., 1988). As a result, mycelial growth, the formation of sporangia, chlamydospores and oospores, and germ tube growth of zoospores are prevented (Farih, 1981a).

Due to its site-specificity, there is a high risk for fungicide resistance development in these pathogens when using mefenoxam. As a result of the effectiveness of mefenoxam, citrus nursery growers have continuously employed this product, leading to the progressive detection of mefenoxam resistance in *Phytophthora* spp. recovered from nurseries. The occurrence of *Phytophthora* isolates with decreased sensitivity to mefenoxam has been globally documented (Hu *et al*., 2008; Aiello *et al*., 2018) and has recently been reported in South African nurseries (van Niekerk *et al*., 2020). However, the molecular mechanism involved in mefenoxam resistance development in *Phytophthora* spp. remains unclear.

Several gene areas have been reported to be associated with mefenoxam insensitivity. The *RPA190* gene, encoding the largest subunit of RNA Polymerase I, has been linked to mefenoxam resistance (Randall *et al*., 2014; Chen *et al*., 2018; Wang *et al*., 2021). However, the authors consistently suggest that other genes may also be involved in this mechanism. Childers *et al*. (2015) found several genes that can contribute to a resistant genotype, including a phospholipase D, ATP-binding cassette superfamily (ABC) transporters and mannitol dehydrogenase. They also reported that changes in RNA polymerases may not be the only mechanism behind resistance development but suggested that *Phytophthora* spp. possibly undergo changes to increase the biodegradation of mefenoxam, which agrees with the findings by Chen *et al*. (2018). Vogel *et al*. (2021) found that the homolog of yeast protein Rrp5 might play a role but failed to locate any candidate genes in or near RNA polymerase I subunits. It has long been assumed that the target site of mefenoxam is RNA Polymerase I but attempts to associate mutations in the subunit of RNA polymerase I with mefenoxam insensitivity have been inconclusive (Randall *et al.*, 2014; Matson *et al.*, 2015; Vogel *et al.*, 2021; González-Tobón *et al.*, 2022; Marin *et al.*, 2023;). A few other plausible candidate genes have been

identified, but further research is needed to elucidate the full mechanism of mefenoxam resistance development in *Phytophthora*.

Sensitivity testing

Upon isolation of *Phytophthora* isolates from infected citrus material, their sensitivity to mefenoxam can be tested *in vitro*. Standard culture media, typically corn meal agar (CMA), is amended with the fungicide at different concentrations. Isolates are then plated onto media and after incubation, their growth is measured in two perpendicular directions with a calliper. Their sensitivity can then be classified according to EC_{50} values, as done by van Niekerk *et al*. (2020) or according to their relative growth at a specific concentration, as by Hu and Li (2014). This traditional method of sensitivity typing, however, is time-consuming and laborious, especially when testing many samples.

With advancements in molecular biology, it is possible to rapidly detect fungicide-resistant genotypes once the mechanisms of resistance have been elucidated at a molecular level. Various molecular techniques, including PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), allele-specific PCR, and allele-specific real-time PCR, have been successfully used to detect fungicide-resistant genotypes of several important pathogens affecting fruit-bearing plants, including *Penicillium* (Hamamoto *et al*., 2001), *Monilinia* (Ma *et al*., 2005) and *Botrytis* (Luck and Gillings, 1995). Real-time PCR is not only a powerful method for the detection of a pathogen, but also for the rapid detection of fungicide-resistant isolates. When a mutation indicative of mefenoxam-resistance is identified, primers can be designed to detect resistant isolates and the number of resistant alleles in a tested sample can then be quantified using the standard curve. This method can be utilised in the citrus industry to rapidly detect the prevalence of mefenoxam-resistant isolates and subsequently advise growers to discontinue the use of phenylamine fungicides for the management of *Phytophthora*-related diseases.

CONCLUSION

Phytophthora nicotianae and *P. citrophthora* are two pathogens that present a serious threat to the current success of the citrus industry in South Africa. The proper management of these pathogens is a key strategy in preventing large-scale effects on the citrus industry. Integrated control strategies, including the use of tolerant rootstocks, suitable irrigation practices and chemical control, are of utmost importance to reduce the impact of these pathogens. Citrus growers heavily rely on the use of chemicals, especially mefenoxam, to control *P. nicotianae* and *P. citrophthora*, but resistance towards mefenoxam has been detected in South African isolates. Considering how highly effective mefenoxam

is for the control of *Phytophthora* spp. its use cannot simply be discontinued, as there are only a few registered active ingredients for the treatment of *Phytophthora* infestations. The focus should then rather be shifted to the responsible use of mefenoxam. Monitoring resistance to active ingredients is crucial in determining the longevity of specific chemicals and thus, the accurate detection of resistant isolates is the first step in ensuring the responsible use of this chemical. *In vitro* sensitivity testing, the current method for fungicide-resistance detection in *Phytophthora* isolates is a time-consuming and labour-intensive process. There is a need for a rapid and accurate method to detect fungicide resistance, which can be done through the development of an optimised real-time PCR assay. To develop this assay the first objective of **Chapter 2** was to perform whole genome sequencing of mefenoxam-sensitive and -insensitive isolates to identify SNPs associated with mefenoxam sensitivity. Following the identification of SNPs in genes associated with mefenoxam resistance in other *Phytophthora* species, the second objective of research **Chapter 2** was to develop an optimised real-time PCR assay capable of distinguishing between mefenoxam-sensitive and -insensitive isolates. Quantitative detection of resistance to mefenoxam will not only enable the detection of resistance but also help determine the rate at which resistance is occurring so that growers can be advised to halt the use of mefenoxam and diversity their management with other chemicals and control strategies to prevent further disease. A qPCR diagnostic assay for resistance detection has been successful in several other pathogens and can greatly contribute to responsible chemical use and the improvement of management strategies in the South African citrus industry.

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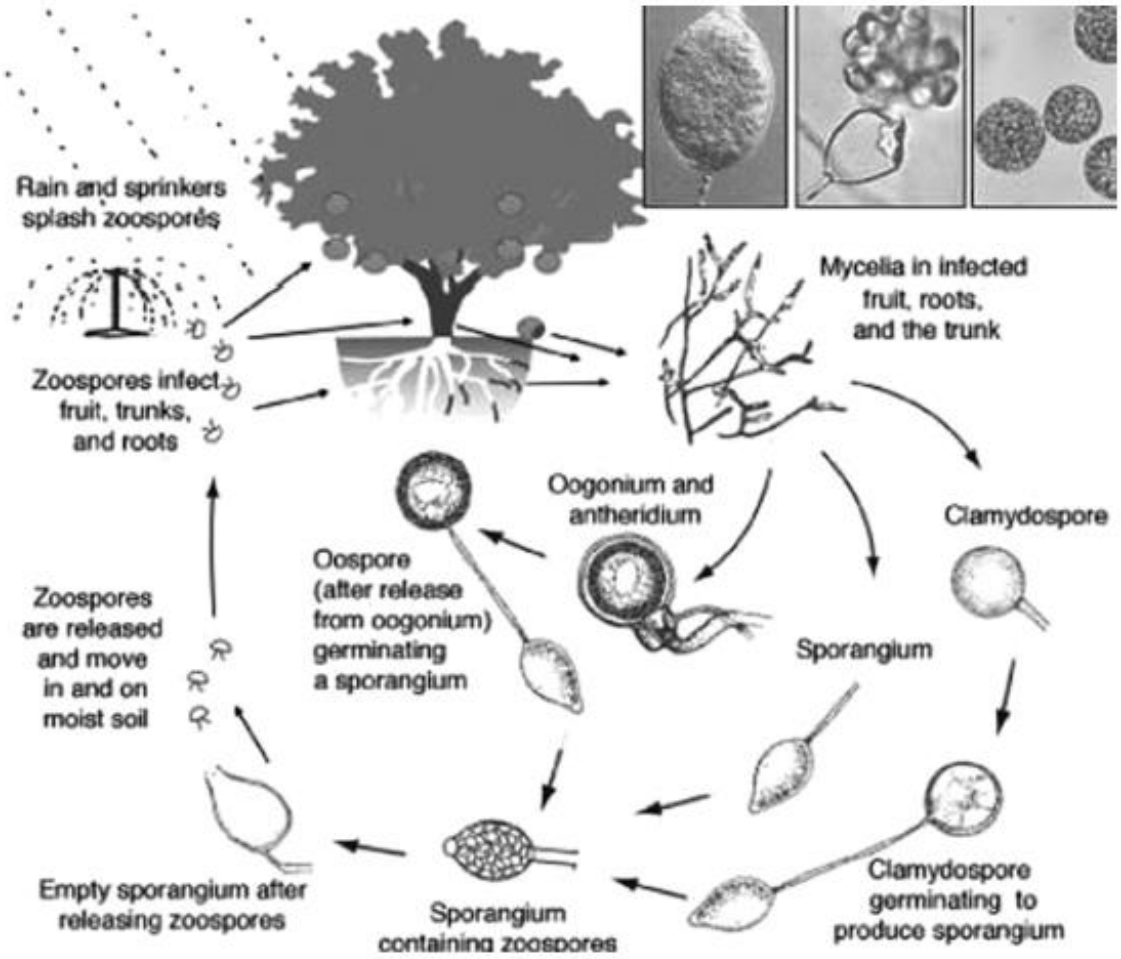

Figure 1. Disease cycle of *Phytophthora nicotianae* and *Phytophthora citrophthora* on citrus. (Aglave, 2018).

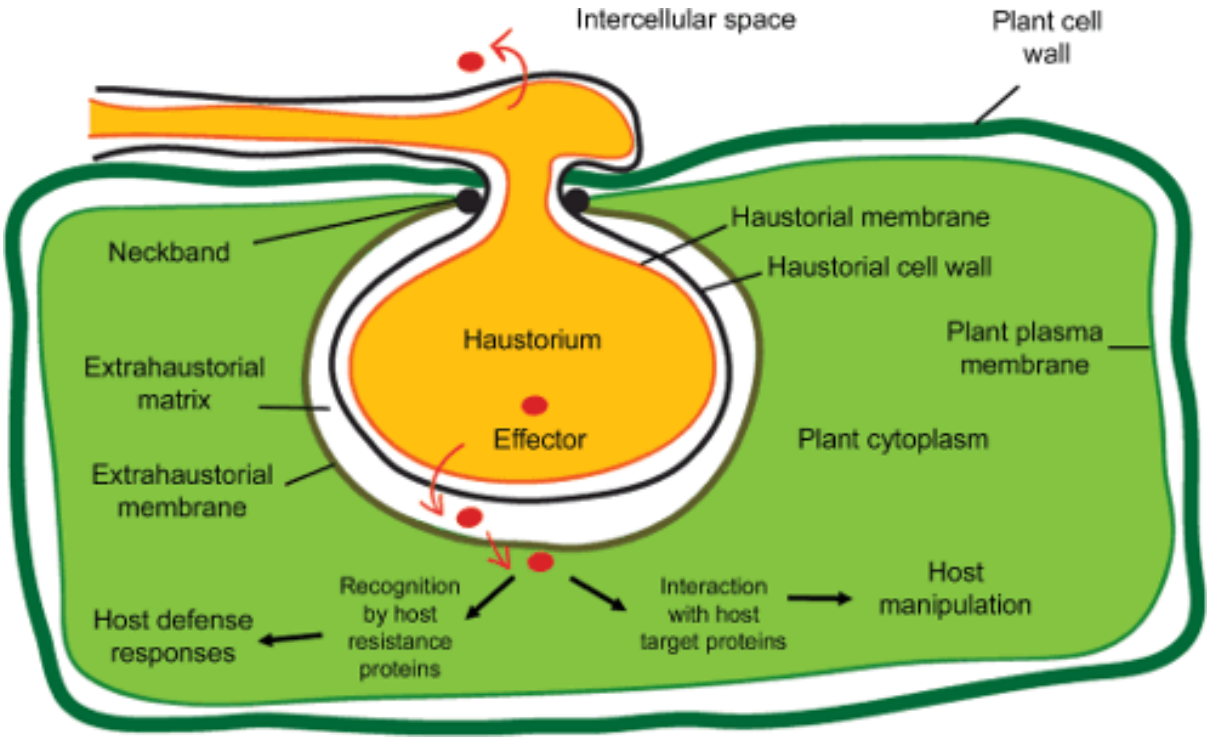

Figure 2. The interaction between pathogen haustoria and citrus host cell. (Dodds, 2009).

CHAPTER 2

Characterisation of mefenoxam sensitivity in *Phytophthora* species from South African citrus

ABSTRACT

Phytophthora nicotianae and *Phytophthora citrophthora* are of the most important oomycete pathogens of South African citrus. These pathogens are routinely detected in citrus nurseries and orchards across South Africa, despite preventative and curative control measures. Mefenoxam is used to treat *Phytophthora* infections, however, instances of resistance to mefenoxam have been documented in South Africa. Isolates of *P. nicotianae* and *P. citrophthora* were evaluated for mefenoxam sensitivity *in vitro* with sensitive and insensitive isolates being subjected to whole genome sequencing (WGS) to identify genetic markers associated with sensitivity. A complete genome sequence of *P. citrophthora* was generated using PacBio HiFi long-read sequencing to serve as a reference genome for WGS obtained by Illumina sequencing. The 48.5 Mb genome was annotated and contained 16,409 protein-coding genes, 423 CAZymes and 713 effectors. Single nucleotide polymorphisms (SNPs) were identified in genes including the ABC transporter and cytochrome P450 genes, suggesting the involvement of minor genes with subtle effects on fungicide sensitivity. Several primers were designed and evaluated for the quantitative detection of mefenoxam sensitivity. The limited availability of insensitive *P. nicotianae* isolates hindered the design of qPCR assays. For *P. citrophthora*, primer pair *QPC2* and *QPC4* showed good accuracy in discriminating between mefenoxam-sensitive and -insensitive isolates. Furthermore, the *QPC2* primer pair was shown to be specific for *P. citrophthora* but was only able to differentiate sensitive and homozygous-resistant *P. citrophthora* isolates. Efforts were made to create a multiplex assay that simultaneously detected *P. citrophthora* and assessed mefenoxam sensitivity. While this assay held promise for rapid pathogen detection and mefenoxam sensitivity screening, the amplification products could not be distinguished when evaluating the melt curve. These limitations underscored the need for further optimisation and validation of the multiplex assay. In conclusion, this study provides valuable insights into the genetic basis of mefenoxam sensitivity in *Phytophthora* species and lays the groundwork for developing diagnostic tools to monitor mefenoxam resistance in oomycete citrus pathogens.

INTRODUCTION

The production of citrus in South Africa holds substantial significance as a major agricultural commodity and a pivotal component of the country's economy. Renowned for its vibrant citrus industry, South Africa ranks among the top 10 citrus-producing nations globally (Citrus Growers' Association of Southern Africa, 2022) with oranges, lemons, grapefruits, and mandarins among the primary citrus varieties cultivated nationwide. This fruit is a crucial export crop, contributing significantly to the country's revenue and employment opportunities. However, the citrus industry in South Africa faces significant challenges due to pests and pathogens including oomycete pathogens, like *Phytophthora* species, which can cause devastating citrus diseases that diminish the overall productivity of citrus orchards and often lead to substantial yield losses. Oomycetes thrive in moist, waterlogged, or poorly drained soils, common conditions in citrus-growing regions. Managing oomycete pathogens in South African citrus involves implementing integrated pest management strategies. This approach includes cultural practices such as well-drained soil systems, appropriate irrigation methods, and the use of resistant rootstocks. Additionally, farmers employ chemical control to effectively combat these pathogens and protect citrus crops from diseases associated with oomycetes.

Fungicides are an essential agricultural tool used to ensure crop health, enhance crop quality, and safeguard yields. However, its sustained use has placed immense pressure on pathogen populations and has resulted in the emergence of fungicide resistance. Fungicides may block a specific metabolic pathway of a pathogen, thereby impeding its growth and ability to reproduce. Single-site fungicides exclusively target one point in a metabolic pathway (McGrath, 2004) so when mutations occur in the encoding gene the pathogen can overcome its effects and showcase its ability to adapt (Deising *et al.*, 2008). The phenylamides are a class of single-site fungicides used to control oomycetes. Phenylamides hinder the biosynthesis of ribosomal RNA (rRNA) in pathogens, thereby inhibiting various life stages in the oomycetes, such as hyphal growth, haustoria development, and sporangia formation (Stevenson *et al.*, 2019). Active ingredients that are classified as phenylamides include metalaxyl, mefenoxam (metalaxyl-M), furalaxyl, oxadixyl, benalaxyl, kiralaxyl (benalaxyl-M) and ofurace (Gisi and Ziegler, 2002).

In the citrus industry, Ridomil Gold 480 SL (Syngenta, Switzerland) is primarily used for the management of *Phytophthora*, including in South African nurseries (Farih, 1981b; van Niekerk *et al*., 2020). It is a soluble, systemic fungicide formulated with mefenoxam that is xylem-mobile, enabling it to translocate upward within the citrus plant following application as a soil drench (Gray *et al*., 2020). It can be applied just before or during the planting of seedlings, to young citrus trees during

transplanting or to bearing trees showing symptoms of decline due to infection. Preventative application creates a protective barrier around seedling roots and safeguards the young plants against infection. When *Phytophthora* is detected in the citrus nursery, mefenoxam is also used as a curative treatment to suppress the disease and prevent further spread.

While highly effective, the exclusive use of mefenoxam carries a significant risk of resistance development. Mefenoxam resistance in *Phytophthora* spp. has been reported in various countries around the world, including China (Chen *et al.*, 2018), Italy (Aiello *et al.*, 2018), and the United States (Hu *et al.*, 2008). In South Africa, citrus is predominantly affected by *Phytophthora nicotianae* and *Phytophthora citrophthora* and a recent South African study has indicated a reduced sensitivity to mefenoxam in both species (van Niekerk *et al.*, 2020). The molecular mechanism underlying the development of mefenoxam resistance in *Phytophthora* has, however, not been determined. Several studies have failed to identify mutations associated with decreased sensitivity in genes encoding RNA Polymerase I (Matson *et al.*, 2015; Vogel *et al.*, 2021; González-Tobón *et al.*, 2022; Marin *et al.*, 2023). Research involving sexual crosses of *Phytophthora* isolates has revealed that mefenoxam resistance is primarily determined by a dominant gene, while differences in sensitivity are influenced by minor genes (Lee *et al.*, 1999). Childers *et al.* (2015) proposed that among these minor genes are ABC transporters, phospholipase D, mannitol dehydrogenase and RXLR effectors. Furthermore, Wang *et al.* (2020) suggested that the cytochrome P450 protein may also play a role. Given the known impact of minor genes, there is a growing need to conduct comprehensive genome screening of *Phytophthora* isolates to identify mutations linked to mefenoxam resistance. This is, however, hampered by the notable lack of genetic data and molecular knowledge of *P. citrophthora* that is needed to determine the underlying genetic drivers of fungicide resistance within this oomycete species.

Traditionally, bioassays are used to evaluate a pathogen's sensitivity to fungicides. While these methods do not necessitate a full understanding of the genetic resistance mechanism, they have several drawbacks that include being time-consuming, labour-intensive and a result may take several days to obtain. Molecular assays offer a faster, more reliable, and cost-effective alternative to traditional methods. Polymerase chain reaction (PCR) techniques can rapidly detect SNPs linked to resistance (Beckermann, 2013) while molecular assays can facilitate the screening of large populations and determine the proportion of resistant isolates (Massi *et al*., 2021). DNA-based detection of fungicide resistance in various oomycetes has been demonstrated for quinone outside inhibitors (QoI) and carboxylic acid amide (CAA) resistance in *Plasmopara viticola* (Huang *et al*., 2020) and oxathiapiprolin resistance in *Phytophthora capsici* (Miao *et al*., 2016). No molecular assays have been developed to detect mefenoxam resistance in *Phytophthora*. This can be attributed to a limited

understanding of the full mefenoxam resistance mechanism or the absence of identified SNPs that can serve as resistance markers.

Thus, this study aimed to design a molecular diagnostic tool to detect and validate mefenoxam insensitivity in populations of *P. nicotianae* and *P. citrophthora* sampled from citrus nurseries and orchards in South Africa. The first objective of this study was to carry out whole-genome sequencing and analyse the complete genome sequences of both *P. nicotianae* and *P. citrophthora* to identify single nucleotide polymorphisms (SNPs) potentially associated with mefenoxam sensitivity. The second objective of this study was to develop a real-time PCR assay for the quantitative detection of mefenoxam resistance in *P. nicotianae* and *P. citrophthora*, respectively.

MATERIALS AND METHODS

Origin and purification of *Phytophthora* isolates

Phytophthora nicotianae isolates were obtained from the disease clinic of Citrus Research International (CRI) (Table 1). Thirty-three isolates, received as actively growing fungal cultures on half-strength potato dextrose agar (PDA; 19,5 g L^{-1}) were plated onto PARPH media (18 g L^{-1} CMA, 10 mL PARPH antibiotics) (Jeffers and Martin, 1986) and incubated at 29°C in the dark for three days. Colonies were purified through hyphal tipping onto corn meal agar (CMA; 18 g L^{-1}) and were incubated at 29°C.

Phytophthora citrophthora isolates were obtained from the *Phytophthora* collection of Dr J. van Niekerk at the Department of Plant Pathology (Stellenbosch University, South Africa) (Table 1). The isolates were previously collected from South African citrus nurseries in the Eastern Cape, Limpopo, Mpumalanga, North West and Western Cape (van Niekerk *et al*., 2020). The isolates were plated onto PARPH media and incubated at 25°C for three days. Colonies were purified from the PARPH media through hyphal tipping onto CMA and then incubated at 25°C.

Isolates from both species were maintained by regular sub-culturing on CMA media. The isolates were repurified after every two rounds of subculturing on CMA. This involved plating onto PARPH media, a three-day incubation period and subsequently hyphal tipping onto CMA media. For long-term storage, 10 mycelial plugs per isolate were added to 10 mL of sterilised, distilled water (dH_2O) in a 30 mL McCartney bottle.

Fungicide sensitivity typing

The *P. nicotianae* isolates (n=33) were subjected to mefenoxam sensitivity testing. Agar plugs from the edges of actively growing cultures were plated onto CMA amended with mefenoxam (Ridomil Gold

480 SL; Syngenta) at 0, 1, 2, 5, 15, 30, 60 and 100 ppm. The plates were incubated at 29°C for three days. Each isolate and concentration combination was repeated three times. The fungal growth of each isolate was measured twice at perpendicular angles to calculate the average growth diameter for each isolate at each concentration. An isolate's relative growth (RG_{100}) was calculated by dividing the average growth diameter of amended plates at 100 ppm by the average growth diameter of control plates. Isolates were classified as sensitive or insensitive to mefenoxam according to a classification protocol described by Hu and Li (2014). Isolates were classified as sensitive if their RG_{100} was less than 40% and insensitive if their RG_{100} was above 40%.

Eleven *P. citrophthora* isolates were subjected to mefenoxam sensitivity testing. The sensitivity levels of the isolates were previously determined (van Niekerk *et al*., 2020) and, therefore, testing was repeated to confirm the stability of the mefenoxam sensitivity levels. Agar plugs from the edges of actively growing cultures were plated onto CMA amended with mefenoxam at 0, 15, 30, and 60 ppm and the plates were incubated at 25°C for three days. Each isolate and concentration combination was replicated three times. The average growth diameter of amended plates at 60 ppm was used to calculate the relative growth of *P. citrophthora* isolates.

Statistical analysis was performed to determine significant differences between the mefenoxam-sensitive and -insensitive isolates of both species. A non-parametrical Wilcoxon test was conducted to statistically verify the differences in RG between the two sensitivity groups.

DNA extraction for whole genome sequencing

Isolates from each sensitivity category (sensitive and insensitive) of both species were subjected to DNA extraction. For each isolate, 5 mycelial plugs were added to 100 mL of potato dextrose broth (PDB; 24 g L^{-1}) in a 250 mL Erlenmeyer flask. Mycelia were grown in a shaking incubator for three to five days at 29°C (*P. nicotianae*) and 25°C (*P. citrophthora*). After incubation, mycelia were removed from the broth, washed with sterilised dH_2O, dried off by placing mycelia onto sterile tissue paper and frozen at -80°C until extraction.

High-quality DNA was extracted from mycelia through a two-step extraction process using the CTAB/PVP pre-extraction method, followed by the QIAGEN DNeasy Plant Mini Kit protocol (QIAGEN, Germany). Frozen mycelia were ground to a fine powder in liquid nitrogen. In the pre-extraction step, 75 mg of ground tissue was added to a 2 mL Eppendorf tube containing sterilised glass beads. The samples were disrupted twice for 30 s at 30 hertz (Hz) using a TissueLyser (QIAGEN, Germany). Subsequently, 1 mL of pre-warmed CTAB/PVP extraction buffer (1.4 M NaCl, 2% CTAB (w/v), 0.1 M Tris (pH 8), 0.02 M EDTA (pH 8), and 1% PVP (pH 8)) was added to each sample. The PVP was added to the extraction buffer just before use. The samples were disrupted twice again for 30 s at high speed.

Following incubation, 4 µL of proteinase K (10 mg mL^{-1}) was added to the solution and incubated at 60°C for 30 min, with the tubes being inverted every 10 min. Then, 3 µL of RNase (100 mg mL^{-1}) was added to the solution and incubated at 60°C for 30 min, with the tubes being inverted every 10 min. After centrifugation for 10 min at 13,000 rpm, the lysate was transferred to new 2 mL tubes, and the samples were further processed using the QIAGEN DNeasy Plant Mini Kit protocol starting from step 2.

Quality assessment of the DNA was performed using a NanoDrop spectrophotometer (ThermoFisher Scientific, USA), Qubit (ThermoFisher Scientific, USA), and BioAnalyzer (Agilent Technologies, USA). Qubit and BioAnalyzer assessments were performed by the Central Analytical Facility (CAF) of Stellenbosch University. The DNA was stored at -20°C for further analyses.

Species confirmation

The identification of all isolates was confirmed molecularly using species-specific primers as described by Carelse (2021). Primers, designed to amplify a portion of the ras-related GTP-binding protein 1 gene (*Ypt1*) specific to *P. nicotianae* and *P. citrophthora*, respectively, were used to confirm oomycete identities. The primers that were used to confirm *P. nicotianae* isolates were forward and reverse *PN_YPT1_FW17* (5'-GTGTGTGTCTGTAGTGGGACACG-3') and *PN_YPT1_RV17* (5'-GGATCTTCTCTCGATAAGTCGGAC-3'), while for *P. citrophthora,* the forward and reverse primers were *PC_YPT1_FW2* (5'-GAAGAAGAGATCCAGTGAGGTTC-3') and *PC_YPT1_RV2* (5'-GGTGGATGACGAGTCTTAAACA-3') (Carelse, 2021). Each 20 µL reaction contained 10 µL 2× Kapa Taq ReadyMix (Kapa Biosystems, USA), 0.5 µM of each primer and 4 µL of DNA (25 ng μL^{-1}). PCR reactions were conducted in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA) with the following amplification conditions: an initial denaturing step of 3 min at 95°C, followed by 35 cycles consisting of 30 s at 95°C, 30 s at 55°C and 50 s at 72°C and a final extension step of 1 min at 72°C. A non-template control and negative control were also included. The PCR reactions involving *P. nicotianae* isolates had *P. citrophthora* DNA as the negative control, and conversely, *P. citrophthora* DNA was used as the negative control in *P. nicotianae* assays.

The PCR products were visualised on a 1% agarose gel (1 g agarose powder (Cleaver Scientific, England)) to 100 mL of a 1× TAE buffer solution (0.4 M Tris, 0.05 M acetic acid, and 0.01 M EDTA, pH 7.5) and SYBR™ Safe DNA Gel Stain (Invitrogen, USA) was added. A GeneRuler 100 bp DNA ladder (Thermo Scientific, USA) was included and visualised alongside 10 µL of each DNA sample. Gel electrophoresis was conducted at 100 V for approximately 45 min and the resulting bands were visualised using UV light in the Bio-Rad Gel Doc XR+ Imaging System (Bio-Rad, USA).

Whole genome sequencing

PacBio sequencing

For the generation of a reference genome for *P. citrophthora*, isolate *Pc249* was selected and renamed as *STE-U-9442* (hereafter referred to as such). The isolate has been stored in the culture collection of the Department of Plant Pathology (Stellenbosch University, South Africa). Isolate *STE-U-9442* was submitted to Macrogen for PacBio long-read sequencing (Pacific BioSciences, USA). The sequencing reads were assembled to create a high-quality reference genome (Möller *et al*., 2024). The assessment of genome completeness was performed with BUSCO (v5.3.0) (Manni *et al*., 2021) using the eukaryota_odb10.2019-11-20 lineage for evaluation, consisting of 70 genomes and 255 BUSCOs.

MAKER (v3.01.03) was employed for gene location prediction, while Protein BLAST+ (v2.7.1+) was performed against UniProt Swiss-Prot (201806) to identify proteins using several databases, including GO (Gene Ontology Consortium, 2004), Interpro (v69.0) (Paysan-Lafosse *et al*., 2023), Pfam (v31.0) (Mistry *et al*., 2021), and EggNOG (v4.5.1) (Huerta-Cepas *et al*., 2019). The predicted proteins underwent searches utilising HMMER and DIAMOND tools on the dbCAN server (https://bcb.unl.edu/dbCAN2/index.php) (Yin *et al*., 2012; Zhang *et al*., 2018), scanning through the dbCAN, dbCAN-sub, and CAZy databases. Proteins identified through at least two databases were categorised as carbohydrate-active enzymes (CAZymes). Proteins possessing a signal peptide (predicted via signal version 6 (Teufel *et al*., 2022)) but lacking transmembrane helices (determined through TMHMM version 1.0.20 (Hallgren *et al*., 2022)) were recognised as candidate effectors. These candidate effectors underwent screening with EffectorP version 3 (Sperschneider and Dodds, 2022). Subsequently, protein sequences underwent a BLAST search (with a percent query coverage and identity cut-off set at 35, E-value cut-off at 1.0 x 10-5) against the Pathogen-Host Interactions database (Urban *et al*., 2019) to identify proteins associated with pathogenicity. The identification of the mitochondrial (mtDNA) genome contig relied on its similarity to a previously sequenced *P. citrophthora* mitochondrial genome (Genbank accession number NC_067066.1). Genome assembly and the prediction of protein-coding genes were conducted using MFannot (https://megasun.bch.umontreal.ca/apps/mfannot/).

Illumina sequencing

Eight *P. nicotianae* and nine *P. citrophthora* isolates were subjected to whole genome sequencing (WGS) on the Illumina platform at Macrogen (South Korea). Additionally, for library construction, DNA was isolated and quality control (QC) was performed. QC criteria included a minimum DNA quantity of 1,000 ng and a DNA integrity number (DIN) of seven or higher out of 10. The TruSeq® DNA PCR-Free Kit was used for the preparation of high-quality, PCR-free DNA libraries before sequencing.

Illumina sequencing-by-synthesis technology was employed to generate sequencing data. The data was then transformed into raw data and underwent a quality control phase. Trimmomatic (Bolger *et al*., 2014) was used to remove adapter sequences and low-quality reads. The filtered reads were aligned to a reference genome using the BWA-MEM (Burrows-Wheeler Aligner) tool (v0.7.17). For *P. nicotianae* a publicly available reference sequence was used (NCBI accession number ASM332846v1). For *P. citrophthora* the genome assembly generated from PacBio sequencing was used as the reference sequence (NCBI accession number JASMQC000000000). After alignment, duplicated reads were eliminated using Sambamba (Tarasov *et al*., 2015). Variants, including single nucleotide polymorphisms (SNPs) and short insertions/deletions (indels), were detected using SAMTools (Danecek *et al*., 2021). The program SnpEff (v4.3t) (Cingolani et al., 2012) was used to annotate and determine the potential effects of the detected variants on genes and proteins. The aforementioned bioinformatics were conducted by Macrogen.

Analysis of variant calling data

To identify potential SNPs associated with mefenoxam insensitivity, a bibliographic review of mefenoxam insensitivity in other oomycetes, including *P. infestans*, was conducted to identify target gene areas that can potentially be involved in the development of mefenoxam resistance in *P. nicotianae* and *P. citrophthora*. The Variant Call Format (VCF) file of each isolate and the accompanying General Feature Format (GFF) file, containing genome annotation information, were incorporated into Integrative Genomics Viewer (IGV) (v2.16.0) (Thorvaldsdottir *et al*., 2013). On IGV, the data sets from different isolates were integrated to allow the visual exploration of genomic data. The SNP profiles were visually analysed, with a focus on the identified target gene areas. SNPs indicative of mefenoxam sensitivity were subsequently identified in *P. nicotianae* and *P. citrophthora* isolates by comparing the genome data sets of the sensitive and insensitive isolates.

Validation of SNPs by Sanger sequencing

To validate the presence of the SNPs obtained from WGS, primers were designed to amplify the gene regions that contain the identified SNPs. Primer pairs were designed with Primer3web (v4.1.0) (Untergasser *et al*., 2012) using all default parameters. Five primer pairs per species that amplified SNPs in different gene regions, were evaluated. This included *PN2*, *PN3*, *PN5*, *PN9* and *PN10* for *P. nicotianae,* and *PC3*, *PC6*, *PC7*, *PC11* and *PC16* for *P. citrophthora*. DNA from the originally sequenced isolates were used to validate the presence of the SNPs and was amplified in a 20 µL reaction containing 10 µL 2X Kapa Taq ReadyMix, 0.5 µM of each primer and 4 µL of DNA (25 ng μL^{-1}). PCR reactions were conducted in an Applied Biosystems Veriti™ 96-Well Fast Thermal Cycler with

amplification conditions of an initial denaturing step of 3 min at 95°C, followed by 35 cycles consisting of 30 s at 95°C, 30 s at 53°C and 1 min at 72°C and a final extension step of 5 min at 72°C. A non-template control (PCR-grade water) was also included.

The PCR products (5 µL) were visualised on a 1% agarose gel conducted at 100 V for approximately 45 min. The resulting amplification products were then visualised using UV light in the Bio-Rad Gel Doc XR+ Imaging System. The amplified PCR products were purified using the MSB® Spin PCRapace Kit (Invitek, USA) and submitted for Sanger sequencing (CAF, Stellenbosch University) of both the forward and reverse primers. The resulting nucleotide sequences and original sequences generated from WGS were incorporated into Geneious Prime 2023.0.4 (https://www.geneious.com). For each isolate, the Sanger-generated sequence was aligned with the original sequence obtained from WGS and then visually inspected to confirm the presence of a SNP at a specific position. The alignment process utilised Geneious alignment, employing a global alignment approach with free end gaps and a specified identity threshold of 1.0/0.0.

Quantitative PCR assay development for mefenoxam sensitivity

Primer design

Primers for quantitative PCR (qPCR) were designed using Primer3web (v4.1.0). The primer design incorporated an overlap of the SNP with the 3' end in either the forward primer, the reverse primer, or both. Additional primer design factors that were considered included primer length, primer melting temperature (T_M), guanine-cytosine content (GC), annealing temperature (T_A), product size, and the potential for hairpin/dimer formation. Primer specificity was evaluated *in silico* using the ipcress command line tool (v2.2.0) (part of Exonorate package) (Slater and Birney, 2005). Primers that only allowed amplification of one product were ordered and synthesised by Inqaba Biotechnical Industries (South Africa).

The designed primers were evaluated and optimised using the previously sequenced isolates. Five sensitive and one insensitive isolate was used for *P. nicotianae* assay development and three sensitive and insensitive isolates, respectively, were used for *P. citrophthora*. The qPCR assays were conducted on a Rotor-Gene Q real-time PCR cycler (QIAGEN, Germany) and the conditions were optimised for each primer set using a gradient of annealing temperatures of 65–68°C for *P. nicotianae* and 55–65°C for *P. citrophthora*. Primers that amplified only the sensitive isolates were selected for subsequent qPCRs.

Validation of qPCR assays

Following the optimisation of qPCR assays, uncharacterised *Phytophthora* isolates were subjected to the assay. New *P. nicotianae* and *P. citrophthora* isolates were obtained from the disease clinic of CRI, the Department of Plant and Soil Sciences (University of Pretoria, South Africa) and the Department of Plant Pathology (Stellenbosch University, South Africa) (Table 2).

Mycelial DNA was extracted using the crude extraction protocol described by González-Mendoza *et al.* (2010). Approximately 100 mg of lyophilized mycelia was added into a 2 mL Eppendorf tube with sterile glass beads. Following this, 250 µL of prewarmed extraction buffer (3% SDS (w/v), 0.5 mM EDTA, 1.0 M NaCl, and 0.1 M Tris (pH 8)) was added and the samples were subjected to high-speed disruption for 2 min using a Tissuelyser (QIAGEN, Germany). An additional 250 µL of extraction buffer was added, and the samples were vortexed for 15 s. Following lysis, 500 µL of chloroform:phenol:isoamyl alcohol (25:24:1) mixture was added and the samples were inverted and then incubated at 65°C for 5 min. After cooling to room temperature, the samples were centrifuged at 11,600 rpm for 5 min. Three hundred µL of the supernatant was transferred to a new 1.5 mL Eppendorf tube, and 300 µL of ice-cold 100% ethanol was added and thoroughly mixed by pipetting. The samples were placed in a -20°C freezer for 30 min to allow DNA precipitation. Following centrifugation for 10 min at 11 600 rpm, the supernatant was carefully removed, and 500 µL of ice-cold 75% ethanol was added. This step was repeated with samples finally being centrifuged at 11 600 rpm for a final 5 min, and the supernatant was discarded. The resulting DNA pellet was air-dried at room temperature. Finally, 100 µL of dH_2O and 3 µL of RNase A (10 mg mL^{-1}) (QIAGEN, Germany) were added, and the sample was incubated at 65°C for 10 min. The quality and quantity of the DNA were assessed using a NanoDrop spectrophotometer. The DNA was then diluted to a concentration of 25 ng μL^{-1} with sterilised dH_2O.

The uncharacterised *Phytophthora* isolates were subjected to the qPCR assays to detect their mefenoxam sensitivity level. The DNA was amplified in a 20 µL reaction, containing 10 µL 2X SensiFAST™ SYBR® No-ROX (Bioline, USA), 2 µL of DNA and 0.5 µM of each primer from the primer pairs that were selected during assay development. The qPCR conditions were as follows: initial denaturation at 95°C for 3 min; followed by 40 cycles of 95°C for 5 s, optimised annealing temperature for 10 s and 72°C for 20 s, concluding with a melt curve. For this stage, the reaction temperature is increased from 72°C to 95°C, rising 1°C at each step.

In vitro mefenoxam sensitivity screening

Consequently, a total of 29 *P. nicotianae* and 16 *P. citrophthora* isolates were subjected to fungicide sensitivity testing, as previously described, to validate the results obtained from the qPCR assays. For

this assessment, a concentration of 30 ppm was used for RG measurements (RG_{30}). Isolates were classified as sensitive if their RG_{30} was less than 40% and insensitive if their RG_{30} was above 40%. The outcomes obtained from the qPCR assays and the *in vitro* sensitivity screening were compared, and subsequently, the accuracy of the assays was determined.

Validation of SNPs with Sanger sequencing

Following the mefenoxam sensitivity qPCR assay, the presence of the specific SNPs was validated by Sanger sequencing as previously described. For *P. citrophthora*, primer pairs *PC3* and *PC6* were used. The DNA was amplified in a 20 µL reaction and each reaction volume contained 10 µL 2X Kapa Taq ReadyMix, 0.5 µM of each primer and 4 µL of DNA (25 ng μL^{-1}). PCR reactions were conducted in an Applied Biosystems Veriti™ 96-Well Fast Thermal Cycler. The amplification conditions were an initial denaturing step of 3 min at 95°C, followed by 36 cycles consisting of 30 s at 95°C, 30 s at 53°C and 1 min at 72°C, and a final extension step of 5 min at 72°C. A non-template control (PCR-grade water) was also included.

The PCR products were visualised on a 1% agarose gel as previously described and the amplification products were submitted to CAF for the purification of amplification products and Sanger sequencing using both the forward and reverse primers. The resulting nucleotide sequences and original sequences generated from WGS were incorporated into Geneious Prime 2023.0.4 (https://www.geneious.com). For each isolate, the amplified gene region was visually inspected to confirm the presence of a SNP at a specific position.

Primer specificity testing

The specificity of mefenoxam sensitivity assays was tested using a range of closely related oomycete species as well as other citrus pathogens and included *Phytophthora nicotianae*, *P. cactorum*, *P. infestans*, *P. capsici*, *P. citricola*, *P. mengei*, *Pythium irregulare s.s.*, *Neocosmospora solani*, *Neocosmospora citricola* and *Neocosmospora ferruginea*. The DNA was amplified in a 20 µL reaction, containing 10 µL 2X SensiFAST™ SYBR® No-ROX (Bioline, USA), 2 µL of DNA and 0.5 µM of both primers. The qPCR conditions were as follows: initial denaturation at 95°C for 3 min; followed by 40 cycles of 95°C for 5 s, optimised annealing temperature for 10 s and 72°C for 20 s, concluding with a melt curve wherein the reaction temperature is increased from 72°C to 95°C, rising 1°C at each step.

Multiplex real-time assay

A multiplex, real-time assay was evaluated for the simultaneous identification of oomycete species and sensitivity to mefenoxam. This was performed by combining species-specific primers (Carelse, 2021) with a primer set that can detect mefenoxam-sensitive isolates (*QPC2)*. DNA amplification was carried out in a 20 µL reaction volume, consisting of 10 µL of 2X SensiFAST™ SYBR® No-ROX (Bioline, USA), 2 µL of DNA, and 0.5 µM of each primer from both primer sets. The qPCR protocol began with an initial denaturation step at 95°C for 3 min, followed by 40 amplification cycles at 95°C for 5 s, an optimised annealing temperature for 10 s, and 72°C for 20 s. The analysis was concluded with a melt curve analysis, in which the reaction temperature was incrementally increased from 72°C to 95°C, rising by 1°C at each step.

RESULTS

Species confirmation

The chosen isolates were subjected to species-specific PCRs according to Carelse, (2021). In the case of *P. nicotianae*, the selected primer pair (*PN_YPT1_FW17* and *PN_YPT1_RV17*) exclusively amplified a 147 bp band (Fig. 1) with no amplification observed for the non-template control or the negative control (*P. citrophthora)***.** Similarly, for *P. citrophthora*, the designated primer pair (*PC_YPT1_FW2* and *PC_YPT1_RV2*) selectively generated a 113 bp amplification product for *P. citrophthora* isolates (Fig. 2) with no amplification in the control samples.

Fungicide sensitivity typing

For *P. nicotianae*, 32 of 33 isolates displayed RG_{100} values below the 40% threshold and were classified as sensitive to mefenoxam (Table 3). The RG_{100} values within the sensitive group ranged from 1.65% to 25.43%. A single isolate (*Pn211*) showed insensitivity (resistance) to mefenoxam, registering a RG_{100} value of 195.35% and consequently was categorised as insensitive. The statistical analysis of *P. nicotianae* was hindered due to the small sample size of the insensitive group (1 insensitive isolate), however, the analysis revealed a discernible difference between the RG_{100} of the two sensitivity groups (Fig. 3).

In the case of *P. citrophthora*, three of the 11 isolates exhibited RG_{60} values below 40%, signifying their sensitivity to mefenoxam. These sensitive isolates displayed RG_{60} values ranging from 0% to 32.47% (Table 3). Conversely, eight isolates demonstrated RG_{60} values above 40%, indicating their insensitivity to mefenoxam. RG_{60} values within this insensitive group ranged from 80.74% to 151.85%. These isolates were categorised as insensitive. The statistical analysis showed a significant

($P \leq 0.05$) difference between the RG_{60} of the mefenoxam-sensitive and insensitive groups ($P = 0.012$) (Fig. 4).

DNA extraction and quality optimisation

In-house quality control

DNA isolated from selected *P. citrophthora* isolates ranged from 310.80 ng to 2,400.00 ng per isolate while genome quality scores (GQS) ranged from 3.5 to 4.3 of 5 (Table 4). The DNA yield of two isolates (*Pc239* and *Pc253*) was below 800.00 ng and did not meet the minimum DNA requirements for WGS. Successful DNA extraction was carried out on eight *P. nicotianae* isolates, yielding DNA quantities ranging from 1,284.00 ng to 2,052 ng per sample. The genome quality scores spanned from 3.8 to 4.5, signifying good DNA integrity. A second round of DNA extraction was conducted on a single *P. citrophthora* isolate, *STE-U-9442*, in preparation for long-read PacBio sequencing. This process resulted in a DNA concentration of 29.4 ng/µL, yielding a total of 11,466 ng of DNA in the sample, accompanied by a high GQS of 4.3.

Macrogen quality control results

Following quality control (QC) assessment by the genomics service provider several noteworthy disparities between in-house and Macrogen QC were shown. Specifically, for *Pc249*, *Pc232*, *Pc251*, *Pc253*, and *STE-U-9442*, the sample concentrations exhibited variations exceeding 5 ng μL^{-1}. Among these, the most substantial difference in sample concentration was observed in the case of *Pc232*, which displayed a decrease of 28.01 ng μL^{-1} in concentration. To assess the integrity values between these assessments, the genome quality score (GQS) of the isolates, generated by the in-house assessment, were doubled to derive a DNA integrity number (DIN) value. Interestingly, all DIN values were lower than anticipated by in-house assessment, except for isolate *Pc285*, which had a DIN of 7.5. Furthermore, the DIN values of *Pc249*, *Pc242*, and *Pc253* displayed variations greater than one when compared to the in-house assessment. A DIN value was not provided for *STE-U-9442*. Furthermore, isolates *Pc249*, *Pc232*, *Pc253*, and *Pn7* failed to meet the minimum DNA requirements and could not be subjected to Illumina sequencing. Despite not meeting the minimum DNA quantity for PacBio sequencing, isolate *STE-U-9442* met the QC criteria due to its satisfactory DNA integrity and was consequently subjected to long-read PacBio sequencing.

Whole genome sequencing

PacBio sequencing

The long-read sequencing yielded 2,432,934 HiFi reads, with an average read length of 10,393 bp. The final assembled genome of *STE-U-9442* measured approximately 48.5 Mb in size. The genome comprised 155 contigs, with an N50 length of 908.6 Kb. The assessment of completeness revealed that within the 255 BUSCO groups investigated, the assembly of *STE-U-9442* contained 233 complete and singular BUSCOs (91.37%), 6 complete and duplicated BUSCOs (2.35%), 7 fragmented BUSCOs (2.75%), and 9 missing BUSCOs (3.53%). Genome attributes, including the total number of protein-coding genes (16,409) with 1,157 of them predicted to be associated with pathogenicity, were determined (Table 5). The protein groups containing the largest number of genes were post-translational modification, protein turnover and chaperones (630 genes), signal transduction mechanisms (559 genes) and replication, recombination and repair (525 genes) (Fig. 5). From the predicted proteins, 423 were identified as CAZymes and 713 as effectors. The raw sequencing reads were submitted to the NCBI and are available in the NCBI Sequence Read Archive under the accession number SRR24235859. The genome of *P. citrophthora STE-U-9442* was successfully submitted to the NCBI database under BioProject PRJNA945341 with accession number JASMQC000000000.

Illumina sequencing

Sequencing yielded between 19.6 million and 33.3 million reads per isolate for the seven *P. nicotianae* and seven *P. citrophthora* isolates (Table 6). Substantial coverage was achieved during the read assembly process ranging from 44× to 77×. The G+C content of the isolates was between 47.58% to 49.27%, indicating no substantial differences in G+C content (Fig. 6). Variant calling produced an exhaustive number of single nucleotide polymorphisms (SNPs), inserts and deletions (Table 7). The average total number of variants found in *P. nicotianae* isolates (456,963) was higher than those found in *P. citrophthora* (122,644) while SNPs were the most common variant type for both species. Genes associated with mefenoxam insensitivity in *P. infestans* included RNA polymerase I (Randall *et al*., 2014; Chen *et al*., 2018; Wang *et al*., 2021), phospholipase D, ABC transporters, mannitol dehydrogenase, RXLR effectors (Childers *et al*., 2015) and cytochrome P450 (Wang *et al*., 2020). These genes were initially investigated for variants that could be used to develop a qPCR assay for *P. nicotianae* and *P. citrophthora*. No variants were identified in RNA polymerase I, phospholipase D, mannitol dehydrogenase and RXLR effector genes in *P. nicotianae* or *P. citrophthora* isolates. For both species, SNPs were identified from the sensitive isolates in several ABC transporter and cytochrome P450 genes (Table 8). Additionally, SNPs were identified in RNA polymerase II and III genes when comparing sensitive and resistant isolates of *P. citrophthora*.

Validation of SNPs

Sanger sequencing confirmed the presence of SNPs. A total of 17 primer pairs were designed for *P. nicotianae* and 18 for *P. citrophthora* (Table 9). Five sets of primers were used to amplify the SNPs in different gene regions in *P. nicotianae* isolates, including *PN2* (*polr2a*) (Fig. 7)*, PN3* (*ABC transporter A family member 1*)*, PN5* (*ABC transporter A family member 1*)*, PN9* (*cytochrome P450 86A2*) and *PN10* (*RPC3*). Amplification products of the target gene regions, ranged from 218 bp to 795 bp in size. Five primer sets were used to amplify different gene regions in the *P. citrophthora* isolates, which included *PC3* (*polr3a*) (Fig. 8), *PC6* (*RPC3*), *PC7* (*ABC transporter G family member 13*), *PC11* (*ABC transporter B family member 11*) and *PC16* (*cytochrome P450 86A2*). Amplification products of 469 bp, 558 bp, 624 bp, 514 bp and 455 bp were amplified by the respective primer sets. The obtained sequences were aligned to the original sequences generated from WGS and the presence of SNPs were visually confirmed.

Real-time assay development for mefenoxam sensitivity screening

Thirteen sets of primers were designed for quantitative detection of *P. nicotianae* (Table 10). The optimal annealing temperature for all *P. nicotianae* primers was 65°C with primer set *QPN4* exhibiting specific amplification for sensitive *P. nicotianae* isolates (Fig. 9). Insensitive isolates amplified after 32 cycles with amplification considered as unspecific. No isolates were amplified with primer set *QPN1*. All isolates were amplified with primer sets *QPN2*, *QPN3*, *QPN5*, *QPN6*, *QPN7*, *QPN8*, *QPN9*, *QPN10*, *QPN11*, *QPN12* and *QPN13*. Primer sets that failed to exclusively amplify sensitive isolates were excluded from further analysis.

Ten sets of qPCR primers were designed for *P. citrophthora* (Table 10), and like *P. nicotianae*, the optimal annealing temperature for all primer sets was 65°C. Primers could be designed with SNPs close to or at the 3' end of primers as indicated for *QPC2* (Fig. 10). Primer pairs *QPC1* (Fig. 11), *QPC2* (Fig. 12) *and QPC4* (Fig. 13) exclusively amplified sensitive isolates, while insensitive *P. citrophthora* isolates only amplified after 33 cycles or more. No isolates were amplified when using primer sets *QPC5*, *QPC6*, *QPC7*, *QPC9*, *QPC10* and *QPC11* while all isolates were amplified with primer set *QPC8*.

Validation of qPCR assays

When employing primer set *QPN4*, seven of 29 uncharacterised *P. nicotianae* isolates were amplified. In the case of primer set *QPC2*, 15 of 16 uncharacterised *P. citrophthora* isolates were amplified while primer set *QPC4* resulted in the amplification in 13 of 16 isolates uncharacterised *P. citrophthora*

isolates. Notably, no amplification was detected in any of the non-template control samples across all assays.

In vitro mefenoxam sensitivity screening of uncharacterised isolates

Isolate *Pn112* registered an RG_{30} value of 116.99% and was categorised as insensitive. All remaining *P. nicotianae* isolates were classified as sensitive, displaying RG_{30} values below the 40% threshold (Fig. 14). Among the 28 sensitive isolates, only two exhibited minimal growth on mefenoxam-amended plates, displaying RG_{30} values of 4.60% (*Pn182*) and 10.76% (*Pn302*). The remaining 26 isolates displayed no growth on the amended plates.

In the case of *P. citrophthora*, five isolates displayed RG_{30} values exceeding 40%, ranging from 43.10% to 108.49% (Table 11). The remaining 13 isolates were classified as sensitive due to RG_{30} values below 40%. Among the sensitive isolates, a greater degree of variability was observed in their RG_{30} values, when comparing the results to the sensitive *P. nicotianae* isolates. Three isolates registered an RG_{30} of 0%, two isolates exhibited RG_{30} values between 10% and 20%, and five isolates exhibited RG_{30} values between 20% to 40%. The statistical analysis showed a significant ($P \leq 0.05$) difference between the RG_{30} of the two sensitivity groups ($P = 0.0038$) (Fig. 15).

Comparing qPCR and in vitro sensitivity results

The *in vitro* mefenoxam response of *P. nicotianae* isolates correlated with the qPCR results in eight of the 29 isolates when using primer set *QPN4* (Table 12). However, 72.41% of isolates that were sensitive to mefenoxam failed to amplify during qPCR. Primer set *QPN4* was not used in further analyses. With primer set *QPC1,* the *in vitro* mefenoxam response of *P. citrophthora* isolates correlated with the qPCR results in only five isolates (Table 13). Primer set *QPC1* was excluded from subsequent analyses due to the lack of amplification observed in 81.82% of sensitive isolates during qPCR. The *in vitro* mefenoxam response of isolates correlated with the qPCR results in 12 out of 16 *P. citrophthora* isolates (Table 14) when using primer pair *QPC2*, where 100% of the sensitive isolates were amplified. However, four insensitive isolates were also amplified, despite having RG_{30} values ranging from 43.10% to 91.75%. The *in vitro* mefenoxam response of isolates and qPCR with *QPC4* correlated for 12 of 16 *P. citrophthora* isolates (Table 15). All sensitive isolates were amplified except for one (*Pc279*; 9.09%) while three insensitive isolates were also amplified.

Validation of SNPs with Sanger sequencing

The unintended amplification of insensitive *P. citrophthora* isolates was further investigated by sequencing the gene regions containing the SNPs used to develop primers *QPC2* and *QPC4*. The

sequence results indicated heterozygosity for the SNPs included in *QPC2* (base pair 252 and 335) (Table 16). The SNPs at base pair 162 and 329 indicated that insensitive isolates amplified with *QPC4*, displayed heterozygosity for the SNP at either one or both loci, resulting in amplification in the qPCR assay (Table 17). In contrast, the sensitive isolate *Pc279* did not contain either of the SNPs associated with potential sensitivity and as a result, it did not amplify in the assay. However, these SNPs were present in four insensitive isolates namely *Pc249*, *Pc275*, *Pc244* and *Pc2241*.

Primer specificity and multiplex real-time PCR

Primer set *QPC2*, designed to detect mefenoxam-sensitive *P. citrophthora* isolates, was shown to be specific to only *P. citrophthora*. Three *P. citrophthora* isolates were amplified from cycle 23 and one isolate from cycle 28. All other species were only amplified from cycle 34 while no amplification was observed in the non-template control (Fig. 16). The simultaneous identification and discrimination between mefenoxam-sensitive and homozygous-resistant *P. citrophthora* isolates could not be achieved. The qPCR melt curve analysis for the species-specific primer set and *QPC2* indicated a peak at 83°C for both primer sets and did not allow distinction between the two sets (Fig. 17).

DISCUSSION

Phytophthora nicotianae and *P. citrophthora* are amongst the most important oomycete pathogens of citrus worldwide and occur in citrus nurseries and orchards throughout South Africa. Despite numerous preventative and curative control measures that include the use of mefenoxam in South African citrus nurseries, these oomycetes are consistently detected. Their continuous presence is cause for concern as contaminated nursery material can facilitate the spread of the pathogens to large orchards. Infection by these oomycetes can cause several diseases that decrease tree productivity or even result in tree death. The regular detection of *Phytophthora* spp. in nurseries, despite chemical control, indicates potential problems with the curative measures, more especially fungicide resistance.

In this study, the initial assessment of mefenoxam sensitivity revealed that the majority of evaluated *P. nicotianae* isolates exhibited sensitivity to mefenoxam with only a single isolate classified as mefenoxam insensitive. This finding was corroborated in a subsequent sensitivity test, where only one of an additional 29 *P. nicotianae* isolates demonstrated insensitivity to mefenoxam. These results align with a prior study on mefenoxam sensitivity among South African *Phytophthora* isolates conducted by van Niekerk *et al.* (2020) in which one of 61 *P. nicotianae* isolates displayed insensitivity

to mefenoxam. These collective findings imply that while some insensitive isolates exist, the vast majority of *P. nicotianae* isolates from South Africa exhibit high sensitivity to mefenoxam.

Notably, the ratio of sensitive to insensitive isolates observed for *P. nicotianae* stands in stark contrast to that of *P. citrophthora* isolates. During the initial sensitivity evaluation, eight out of 11 *P. citrophthora* isolates were categorised as insensitive and their sensitivity levels were confirmed as previously reported (van Niekerk *et al*., 2020). Following additional sensitivity assessments, five out of 16 isolates exhibited insensitivity with six of the 11 isolates classified as sensitive, displaying some degree of insensitivity to the fungicide. *Phytophthora citrophthora* isolates were additionally described as 'intermediately insensitive' to mefenoxam by van Niekerk and co-workers (2020). The distinct difference in sensitivity between these two species to mefenoxam has not been studied and requires further investigation as the sensitivity to mefenoxam can be influenced by a combination of genetic, environmental, and historical factors. The history of fungicide usage plays a crucial role in the development of mefenoxam resistance (González-Tobón *et al*., 2020). *Phytophthora citrophthora* was the first *Phytophthora* species reported on citrus in South Africa in 1920 (Doidge, 1920) with the presence of *P. nicotianae* reported nearly 60 years later in 1977 (Gorter, 1977). However, it is important to consider that mefenoxam was only commercially available in 1977 (Leadbeater, 2014). It is, therefore, unlikely that the sensitivity differences may be due to *P. citrophthora* populations being exposed to mefenoxam for a longer period.

Another factor that contradicts the observed difference in sensitivity between the two species is the prevalence of *P. nicotianae* on citrus in South Africa. Based on the most recent assessment of species distribution within the country, *P. nicotianae* emerged as the most prevalent species, occurring in 80% of citrus orchards (Meitz-Hopkins *et al*., 2014). Considering the prevalence of *P. nicotianae* it would be reasonable to assume that a greater selection pressure would be exerted by mefenoxam on this species. However, the differences in sensitivity may be attributed to a multitude of factors, and a clearer understanding may emerge when the molecular mechanisms underlying mefenoxam resistance development are better understood. Furthermore, oomycetes are a highly plastic group of organisms and are notorious for their quick response to human-mediated selection pressures, whether it is fungicide use or deployment of plant resistance genes (Erwin and Ribeiro, 1996). Considering this genetic plasticity, it is also plausible that *P. citrophthora* might have a greater inclination towards resistance development on a molecular level.

The relative growth (RG) of isolates was initially measured at 100 ppm but later reduced to 30 ppm to more realistically simulate the concentration of mefenoxam that oomycetes may be challenged with *in planta*. When a systemic fungicide is applied a decrease in fungicide concentration occurs due to redistribution and dilution within growing plant tissues, as well as breakdown through

plant metabolism (Caffi and Rossi, 2018). Additionally, chemical dilution can occur when the fungicide doesn't have adequate time to dry in the soil, which may happen due to watering in nurseries or rainfall in orchards. Consequently, *Phytophthora* isolates typically do not encounter the exact concentration initially applied. This raises questions about the validity of using high concentrations during *in vitro* fungicide sensitivity testing. Furthermore, the measurement of RG at 30 ppm was warranted as South African citrus nurseries typically apply the recommended dosage of 45 ppm (0.1 mg L^{-1}) of mefenoxam.

The identification of sensitive and insensitive *P. nicotianae* and *P. citrophthora* isolates was utilised in a whole genome sequencing approach for the identification of SNPs potentially indicative of mefenoxam sensitivity. To facilitate this objective, PacBio HiFi long-read high-throughput sequencing technology was used to generate the first complete genome sequence of *P. citrophthora* (Möller *et al*., 2024). The assembled genome was resolved to contig level and forms the basis for further research on the genomics and molecular mechanisms governing pathogenicity within this species including the molecular mechanism underpinning the development of resistance to mefenoxam. Gaining a comprehensive understanding of this resistance mechanism is significant as it holds the key to the prevention of further resistance buildup and effective disease management. Additionally, the full genome sequence serves as a resource for the discovery of novel targets for disease control, which can lead to practical applications that stand to benefit the citrus industry significantly. Furthermore, this genome sequence can be used in comparative genomic studies to reveal evolutionary relationships and shared genetic attributes between *P. citrophthora* and related organisms.

In this study, the whole genome sequences of seven *P. nicotianae* and *P. citrophthora* isolates, respectively, were generated using Illumina sequencing. Genetic variation among different isolates of each species was identified, when compared to the respective reference genomes, with SNPs representing the most common genomic variant. A substantial difference in the number of variants was detected between the *P. nicotianae* and *P. citrophthora* isolates and can be attributed to the origins of the reference genomes. Specifically, the reference genome for *P. nicotianae* originated from an isolate in China, whereas all the *P. nicotianae* isolates analysed in this study were sourced from South Africa. Conversely, the reference genome for *P. citrophthora* was generated using a South African isolate. This divergence in geographic origins between the isolates and the respective reference genome may account for the elevated number of variants observed in *P. nicotianae*.

The variant calling data was used to identify SNPs between sensitive and insensitive isolates. A limitation of this study, however, is that the sequencing and variant calling process was executed using a limited number of isolates per phenotypic category. Notably, when comparing SNP data

between sensitive and insensitive *P. nicotianae* isolates, only a single insensitive isolate could be included in the analysis. This limitation made it difficult to verify variants associated with the insensitive phenotype. To enhance the robustness of SNP identification linked to insensitivity, it becomes essential to increase the sample size representing each phenotypic group as this will allow for a more representative range of variants that can be identified. This can also easily be facilitated by the primers designed within this study to confirm the presence of SNPs in potentially mefenoxam-sensitive isolates.

As previously mentioned, the underlying mechanism for mefenoxam resistance development has not been determined. Mefenoxam's mode of action is centered on its selective inhibition of ribosomal RNA (rRNA) synthesis, which is achieved by modulating the activity of RNA polymerase I (Davidse *et al*., 1983). Therefore, it would be expected that genetic variations in genes encoding RNA Polymerase I, which transcribes rRNA, are associated with reduced sensitivity to mefenoxam. However, no SNPs were detected in any of the RNA Polymerase I subunits in either *P. nicotianae* or *P. citrophthora* in this study. This result is in agreement with previous research conducted on *P*. *infestans* and *P*. *capsici*, where investigators were unable to identify any potential genes associated with mefenoxam resistance within the subunits of RNA Polymerase I (Vogel *et al*., 2021; González-Tobón *et al*., 2022). Prior research on *P. infestans* has emphasised the significance of the major gene *RPA190* as the primary determinant of mefenoxam insensitivity (Randall *et al*., 2014; Chen *et al*., 2018; Wang *et al*., 2021). However, several researchers suggest that multiple genes or additional loci with minor effects play a role in mefenoxam resistance development (Fabritius *et al*., 1997; Judelson and Roberts, 1999; Lee *et al*., 1999). Furthermore, the multiple genes involved likely have a non-additive or combinatorial effect on resistance. This adds significant complexity to understanding the resistance development mechanism.

Furthermore, SNPs were found in other regions that have been reported to play a role in mefenoxam resistance in *P. infestans*, including ABC transporter and cytochrome P450 genes. Both are examples of minor genes that have more subtle effects on pathogen fungicide sensitivity (Judelson and Senthil, 2005). The ABC (ATP Binding Cassette) transporters are described as efflux pumps and typically facilitate the ATP-dependent movement of a wide range of organic compounds, spanning from small molecules to polypeptides (Judelson and Senthil, 2005). As a result, they help to decrease the concentration of fungicides within pathogen cells. ABC transporters are well-known for causing resistance to multiple drugs in animal cells. SNPs were also found in cytochrome P450 genes, which are described as biotransformation enzymes (Yoo and Lee, 2011). These enzymes play a crucial role in maintaining cell homeostasis by metabolising endogenous and exogenous compounds (Ramamoorthy

et al., 2001). Mutations in cytochrome P450 genes may enhance the ability of the enzyme to metabolise or neutralize mefenoxam, making the pathogen less sensitive to the fungicide.

Mefenoxam can affect messenger and transfer RNAs (mRNA and tRNA), although its impact on rRNA is more significant (Randall *et al*., 2014). It has been proposed that mefenoxam exerts its effect when the RNA polymerase complex is bound to the pathogen DNA (Davidse *et al*., 1983). Eukaryotic RNA polymerases are multi-subunit complexes, with some subunits shared among all three enzymes (Barba-Aliaga *et al*., 2021). This complicates the task of identifying the exact RNA Polymerase subunit targeted by mefenoxam. For this reason, subunits of RNA Polymerase II and III were also analysed in this study. For *P. nicotianae*, variants were identified in the *polr2a* subunit of RNA Polymerase II, while for *P. citrophthora* variants were observed in the *polr3a* of RNA Polymerase III. Additionally, variants were detected in their respective *polr3c* subunits (also known as *RPC3*) of RNA Polymerase III for both species.

Monitoring mefenoxam resistance is vital to ensuring the efficacy and longevity of chemical control measures. The quantitative detection of fungicide resistance offers several advantages over the time-consuming, labour-intensive traditional assays that are generally performed. In this study, primer pair *QPN4* for *P*. *nicotianae* and primer pairs *QPC1*, *QPC2* and *QPC4* all amplified mefenoxam-sensitive isolates and could quantitatively discriminate sensitive and insensitive isolates. This finding was validated on uncharacterised oomycete isolates and correlated with fungicide sensitivity *in vitro*. Primer pairs *QPN4* and *QPC1* proved ineffective in amplifying sensitive isolates indicating that the SNPs utilised in both primer pairs did not represent mefenoxam sensitivity. Primer pairs *QPC2* and *QPC4* demonstrated high accuracy in discriminating between sensitive and insensitive *P*. *citrophthora* isolates. However, some of the insensitive isolates were also amplified while sequence analysis of the erroneously amplified isolates revealed that they exhibited heterozygosity for the SNPs linked to mefenoxam sensitivity. Oomycetes are diploid during their vegetative state. Therefore, when extracting DNA from oomycete mycelia, two DNA bases are present at a single locus (Heffer *et al*., 2002). In the case of the insensitive isolates that did not amplify, they were identified as homozygous for the original allele and did not contain the specific SNP. On the other hand, the sensitive isolates that were successfully amplified were either homozygous or heterozygous for the SNP. This observation confirmed the specificity of primer pairs *QPC2* and *QPC4*, as both primer pairs only amplified isolates where the SNP was present, albeit in a homozygous or heterozygous state.

Based on sequencing information the SNPs employed in *QPC4* were not considered to be associated with mefenoxam sensitivity, as one of the sensitive isolates did not contain either of the SNPs. For *QPC2*, all sensitive isolates contained at least one of the SNPs. Additionally, the insensitive isolates that were amplified were found to be heterozygous for at least one of the SNPs, while the

insensitive isolate that remained unamplified did not have either of the SNPs. The two SNPs targeted by *QPC2* are located within the *polr3a* subunit of RNA Polymerase III. Although no specific mechanisms directly linking *polr3a* to fungicide resistance have been documented, it is possible that mutations within the *polr3a* gene could cause structural changes in the RNA Polymerase III enzyme that might impact the transcription of other genes, potentially influencing the binding site of mefenoxam. Nonetheless, additional research is required to increase confidence in the connection between the SNPs targeted by *QPC2* and mefenoxam sensitivity. Presently, this assay serves as a limited tool for discerning between sensitive and homozygous resistant isolates of *P. citrophthora*.

The inability to develop a quantitative detection assay for mefenoxam-sensitive *P. nicotianae* isolates can be attributed to the limitation of only having a single insensitive isolate available in this study. In the absence of a varied collection of *P. nicotianae* isolates, it becomes challenging to differentiate between the genetic variations responsible for mefenoxam sensitivity and those that are only specific to the single available isolate. This limitation most likely led to the selection of irrelevant SNPs in the design of qPCR assays, which in turn, resulted in a lack of assay success. To increase the chances of successfully identifying SNPs indicative of mefenoxam sensitivity, a larger and more diverse cohort of *P. nicotianae* isolates should be analysed.

Lastly, *P. citrophthora* species-specific primers could not be combined with primers to detect mefenoxam sensitivity as the melt analyses of the multiplex assay could not discriminate between amplification products generated by the two primer sets. This drawback can be addressed by introducing fluorescent probes alongside the sequence-specific PCR primers, which would enable the identification of the species and discriminate between mefenoxam-sensitive and -insensitive isolates. Furthermore, it is feasible to redesign one of the primer sets to yield distinct melt curve profiles, thus enabling differentiation. Furthermore, primer set *QPC2* can only distinguish between sensitive and homozygous-resistant isolates and may fail to detect resistant isolates that are heterozygous. This limitation underscores the necessity for further validation of the SNPs used in primer set *QPC2*, to ensure its effectiveness in accurately identifying the full spectrum of mefenoxam resistance in *P. citrophthora* isolates. However, it is important to recognise that this assay represents an essential foundational step and lays the groundwork for future optimisation. Future research efforts should also focus on determining the performance of the assay to directly detect mefenoxam sensitivity in DNA isolated from citrus roots and nursery soils and water. This approach will reduce both time and resource requirements by eliminating the need for soil baiting.

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Table 1. List of *Phytophthora nicotianae* and *Phytophthora citrophthora* isolates that were cultured for use in fungicide sensitivity typing.

Isolate	*Phytophthora*	Received from	Sampled from	Province	Previously determined mefenoxam sensitivity level
Pn211	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn137	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn147	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn377	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn106	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn146	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn145	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn337	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn262	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Limpopo	n/a
Pn222	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn193	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn138	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn115	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Limpopo	n/a
Pn167	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn226	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn59	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn217	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn367	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn96	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn98	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Limpopo	n/a
Pn184	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn302	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Eastern Cape	n/a
Pn121	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn139	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn239	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn366	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn60	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn228	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn97	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Limpopo	n/a

Table 1. List of *Phytophthora nicotianae* and *Phytophthora citrophthora* isolates that were cultured for use in fungicide sensitivity typing (continued).

Isolate	*Phytophthora*	Received from	Sampled from	Province	Previously determined mefenoxam sensitivity level
Pn89	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn194	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pn183	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Mpumalanga	n/a
Pn7	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown	n/a
Pc274	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc245	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc239	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc249	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc244	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc232	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc250	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc242	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Insensitive
Pc251	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Sensitive
Pc253	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Sensitive
Pc285	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape	Sensitive

Table 2. The uncharacterised *Phytophthora* isolates that were used to validate the developed quantitative polymerase chain reaction assay that aims to detect mefenoxam sensitivity.

Isolate	*Phytophthora*	Received from	Sampled from	Province
Pn98	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Limpopo
Pn182	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Mpumalanga
Pn302	*nicotianae*	Department of Plant and Soil Sciences (UP)	Nursery	Eastern Cape
Pn357	*nicotianae*	Department of Plant and Soil Sciences (UP)	Nursery	Eastern Cape
Pn97	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Limpopo
Pn 112	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Limpopo
Pn2788	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Western Cape
Pn184	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Mpumalanga
Pn1928	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Eastern Cape
Pn183	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Mpumalanga
Pn2744	*nicotianae*	Disease clinic of Citrus Research International	Nursery	North West
Pn56	*nicotianae*	Department of Plant and Soil Sciences (UP)	Nursery	Northern Cape
Pn2387	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Eastern Cape
Pn262	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Limpopo
Pn2475	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pn115	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Limpopo
Pn2939	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Eastern Cape
Pn55	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Western Cape
Pn89	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown
Pn366	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown
Pn3061	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pn2739	*nicotianae*	Disease clinic of Citrus Research International	Nursery	North West
Pn228	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown
Pn217	*nicotianae*	Disease clinic of Citrus Research International	Unknown	Unknown
Pn2541	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pn2982	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Mpumalanga
Pn3062	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pn2185	*nicotianae*	Disease clinic of Citrus Research International	Commercial	Mpumalanga
Pn50	*nicotianae*	Department of Plant and Soil Sciences (UP)	Nursery	Eastern Cape
Pn2084	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Eastern Cape
Pn226	*nicotianae*	Department of Plant and Soil Sciences (UP)	Commercial	Limpopo

Table 2. The uncharacterised *Phytophthora* isolates that were used to validate the developed quantitative polymerase chain reaction assay that aims to detect mefenoxam sensitivity (continued).

Isolate	*Phytophthora*	Received from	Sampled from	Province
Pn2377	*nicotianae*	Disease clinic of Citrus Research International	Nursery	Eastern Cape
Pc2476	*citrophthora*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pc266	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc275	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc226	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc 234	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc244	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc268	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc251	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc229	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc2534	*citrophthora*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pc2241	*citrophthora*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pc2231	*citrophthora*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pc267	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc276	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc272	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc2539	*citrophthora*	Disease clinic of Citrus Research International	Commercial	Eastern Cape
Pc279	*citrophthora*	Department of Plant Pathology (SU)	Nursery	Eastern Cape
Pc3060	*citrophthora*	Disease clinic of Citrus Research International	Commercial	Eastern Cape

Table 3. Relative growth (RG) values of *Phytophthora* isolates at 100 ppm of mefenoxam.

Phytophthora nicotianae				***Phytophthora citrophthora***			
Sensitive		**Insensitive**		**Sensitive**		**Insensitive**	
Isolate	**RG (%)**	**Isolate**	**RG (%)**	**Isolate**	**RG (%)**	**Isolate**	**RG (%)**
Pn377	1.67	*Pn211*	195.25	*Pc251*	2.43	*Pc274*	95.01
Pn147	1.77			*Pc285*	32.47	*Pc245*	101.93
Pn137	1.78			*Pc253*	0.00	*Pc239*	151.85
Pn217	1.83					*Pc249*	91.11
Pn337	1.87					*Pc244*	98.46
Pn146	1.88					*Pc232*	80.74
Pn138	1.92					*Pc250*	87.48
Pn121	1.92					*Pc242*	81.59
Pn98	2.22						
Pn97	2.35						
Pn193	2.75						
Pn366	3.45						
Pn367	3.47						
Pn145	3.67						
Pn59	3.94						
Pn96	4.71						
Pn226	4.93						
Pn106	5.98						
Pn60	6.97						
Pn167	7.17						
Pn262	7.51						
Pn302	7.72						
Pn184	7.85						

Isolates highlighted in grey were selected for whole genome sequencing.

Table 3. Relative growth (RG) values of *Phytophthora* isolates at 100 ppm of mefenoxam (continued).

Phytophthora nicotianae				***Phytophthora citrophthora***			
Sensitive		**Insensitive**		**Sensitive**		**Insensitive**	
Isolate	**RG (%)**	**Isolate**	**RG (%)**	**Isolate**	**RG (%)**	**Isolate**	**RG (%)**
Pn228	8.00						
Pn222	8.02						
Pn239	8.69						
Pn139	9.63						
Pn115	9.72						
Pn183	13.16						
Pn89	14.56						
Pn194	16.32						
Pn7	25.43						

Isolates highlighted in grey were selected for whole genome sequencing.

Table 4. Quantitative and qualitative DNA parameters as determined by Central Analytical Facility (Stellenbosch University) and genomic service provider, Macrogen.

		CAF				Macrogen		
Species	**Isolate**	**DNA concentration (ng µL^{-1})**	**DNA amount (ng)**	**Genome quality score (0 - 5)**	**DNA integrity number (0 - 10)**	**DNA concentration (ng µL^{-1})**	**DNA amount (ng)**	**DNA integrity number (0- 10)**
P. nicotianae	*Pn211*	15.00	1,800	3.9	7.8	17.14	1,885	7.6
	Pn7	15.10	1,812	3.8	7.6	11.33	**906**	7.3
	Pn89	10.70	1,284	4.3	8.6	13.66	1,502	7.7
	Pn194	17.10	2,052	4.5	9.0	17.25	1,897	8.3
	Pn146	11.40	1,368	4.0	8.0	13.70	1,507	7.6
	Pn137	13.20	1,584	4.0	8.0	13.82	1,534	7.6
	Pn147	11.90	1,428	4.2	8.4	14.23	1,565	7.6
	Pn377	6.42	1,541	4.0	8.0	9.87	2,200	7.3
P. citrophthora	*Pc274*	11.90	1,428	4.1	8.2	14.25	1,468	7.0
	Pc249	20.00	2,400	3.9	7.8	7.50	**832**	7.0
	Pc245	6.30	756	4.1	8.2	10.30	1,154	7.7
	Pc239	5.20	624	4.0	8.0	9.49	1,557	7.6
	Pc232	34.60	4,152	3.6	7.2	6.59	**731**	**6.4**
	Pc250	10.70	1,284	4.0	8.0	11.82	1,288	7.2
	Pc242	6.56	1,805	4.3	8.6	10.97	2,511	7.4
	Pc251	3.28	946.00	3.8	7.6	8.90	1,985	7.4
	Pc285	6.74	809.00	3.5	7.0	9.56	1,013	7.5
	Pc253	1.23	311.00	3.9	7.8	6.90	1,635	**6.7**
P. citrophthora (Long-read sequencing)	*Pc249 (PacBio)*	29.40	11,466	4.3	8.6	12.74	4,841	

Isolates indicated in red did not meet the minimum DNA requirements for sequencing by Macrogen and were discarded.

Table 5. Genome attributes of the newly assembled genome of the *Phytophthora citrophthora* isolate *STE-U-9442*.

Attributes	***STE-U-9442***
Genome information	
Quality reads	2,432,934
Times coverage	521x
Mean read length (bp)	10,393
Total assembled contigs	155
N50	908,581
Assembled genome size (bp)	48,478,215
G+C content (%)	50.7
Assemble completeness (BUSCO) (%)	91.37
NCBI accession number	JASMQC000000000
Annotation information	
Predicted protein-coding genes	16,409
Predicted pathogenicity-related genes	1,157
CAZymes	423
Cytoplasmic effectors	420
Apoplastic effectors	293

Table 6. The total number of reads, mapped reads and mean depth of Illumina sequencing of *Phytophthora nicotianae and Phytophthora citrophthora* isolates.

Species	Sample name	Total reads	Mapped reads	Mean depth
Phytophthora nicotianae	*Pn211*	21,424,378	20,003,021	44.29
	Pn89	24,657,196	23,006,276	50.15
	Pn194	29,857,868	27,889,704	61.27
	Pn146	33,304,592	31,139,717	67.06
	Pn137	26,258,816	24,563,622	54.11
	Pn147	28,470,076	26,513,079	57.90
	Pn377	24,394,716	22,614,251	49.84
Phytophthora citrophthora	*Pc274*	19,562,496	19,415,887	54.39
	Pc245	27,826,202	26,608,275	72.95
	Pc239	24,926,174	24,740,464	69.16
	Pc250	24,054,808	23,831,354	66.45
	Pc242	25,871,442	25,615,960	71.59
	Pc251	28,080,128	27,949,333	77.34
	Pc285	21,607,720	21,499,420	60.13

Table 7. The total number of single nucleotide polymorphisms (SNPs), insertions, and deletions generated by variant calling from the Illumina sequencing data.

Species	Sample name	Number of SNPs	Number of insertions	Number of deletions	Total number of variants
Phytophthora nicotianae	*Pn211*	399,870	18,169	20,392	438,431
	Pn89	406,399	19,034	21,209	446,642
	Pn194	421,599	20,227	22,646	464,472
	Pn146	431,015	20,935	23,235	475,185
	Pn137	419,026	19,768	22,086	460,880
	Pn147	424,523	20,204	22,516	467,243
	Pn377	405,704	19,030	21,153	445,887
	Average	415,448	19,624	21,891	456,963
Phytophthora citrophthora	*Pc274*	111,685	4288	4195	120,168
	Pc245	115,299	4393	4382	124,074
	Pc239	112,667	4377	4330	121,374
	Pc250	112,594	4324	4295	121,213
	Pc242	113,322	4380	4301	122,003
	Pc251	116,429	4468	4441	125,338
	Pc285	115,514	4434	4392	124,340
	Average	113,930	4381	4334	122,644

Table 8. The variants found in isolates from this study in gene regions reported to be associated with mefenoxam insensitivity in *Phytophthora infestans*.

Species	Gene family	Gene	Location	Base pair position
P. nicotianae	ABC transporters	*ABC transporter A family member 1*	scaffold 71	46958, 57140, 64329, 102315
		ABC transporter B family member 11	scaffold 189	23587, 29201
	Cytochrome P450	*Cytochrome P450 86A2*	scaffold 189	23587, 25486, 29201
	RNA Polymerase II	*polr2a*	scaffold 251.1	58264
	RNA Polymerase III	*RPC3*	scaffold 515.1	8925
P. citrophthora	ABC transporters	*ABC transporter G family member 13*	contig 37	334175
		ABC transporter A family member 1	contig 41	194597
		ABC transporter B family protein tagD	contig 8	1230106
		ABC transporter B family member 11	contig 12	212110
		ABC transporter I family member 20	contig 12	489438
		ABC transporter C family member 3	contig 14	68226
	Cytochrome P450	*Cytochrome P450 86B1*	contig 8	347892
		Cytochrome P450 86A2	contig 12	206790, 209352
		Cytochrome P450 86A2	contig 6	1030423
	RNA Polymerase III	*polr3a*	contig 12	691403
		RPC3	contig 12	940777

Scaffolds for P. nicotianae refer to a publicly available reference genome (ASM332846v1) and contigs for P. citrophthora refer to reference genome STE-U-9442 (JASMQC000000000).

Table 9. Primers designed for the validation of single nucleotide polymorphisms (SNPs) identified through data mining of whole genome sequencing data.

Phytophthora nicotianae				
Primer pair	**Gene**	**Forward primer (5' to 3')**	**Reverse primer (3' to 5')**	**Product size (bp)**
PN1	*polr2a*	*PN1F* AAGACACTCAAAGCCATGGG	*PN1R* ATCGTTGATGACCAGCTCGT	343
PN2	*polr2a*	*PN2F* GGTGAAGCTGTTTGTGGCA	*PN2R* CGACGTTGAATTCCTGTCCG	632
PN3	*ABC transporter A family member 1*	*PN3F* CGAGCAGCTATCACAGTCCT	*PN3R* GCCGGCATCCATTGTTGTAT	218
PN4	*ABC transporter A family member 1*	*PN4F* CCATAGGACAACAGACCCAGT	*PN4R* CCTTTGTGCAGTCGTACGTG	155
PN5	*ABC transporter A family member 1*	*PN5F* GCGAGAAGATAGCCGTCAAG	*PN5R* ATCACCCAAGATGTCGGCTT	564
PN6	*ABC transporter B family member 11*	*PN6F* TCGTTGGATCACTGGTGTCA	*PN6R* GCAGTTCGACCACTTTAGCA	173
PN7	*ABC transporter B family member 11*	*PN7F* CACTTGGCTGCTCTTGTGG	*PN7R* TCCCGTAACTTGCACTCCTT	156
PN8	*Cytochrome P450 86A2*	*PN8F* CGTCAACCGGGCAAGAAC	*PN8R* AGTTCTCGCACTTGGGGTC	111
PN9	*Cytochrome P450 86A2*	*PN9F* CTGGCGGTGGATGAGAGTTA	*PN9R* CTCGGGGTTGAATTCAGCAG	250
PN10	*RPC3*	*PN10F* GCAATCAAGCGCGTTTCTTC	*PN10R* GTACGCGTCGAGTTTGTGG	795
PN11	*RPC3*	*PN11F* CGCACCTCCAGTCCTATCAG	*PN11R* GATACTGAAACGGCGGCATC	153
PN12	*ABC transporter A family member 1*	*PN12F* TCTCCGCTGAATGGACTTGT	*PN12R* TTCATCCTGGACACCGTTGA	168
PN13	*ABC transporter A family member 1*	*PN13F* CAATGAGTAGAACCGGCAGC	*PN13R* CGGGTTCGGATGGGTTTTAT	160
PN14	*ABC transporter A family member 1*	*PN14F* CTATCTGCCTCGGACCTGAC	*PN14R* GCGAAGTAGTTGCGAGTGAA	168
PN15	*ABC transporter A family member 1*	*PN15F* ACATTCCTCGAGCTACACCT	*PN15R* GCATATCCGTGAGTACAGCG	102
PN16	*ABC transporter B family member 11*	*PN16F* TCCATCAGCGACTCGGATTT	*PN16R* GTCCGTTGTTGCCCATATCC	828
PN17	*Cytochrome P450 86A2*	*PN17F* CTGGCGGTGGATGAGAGTTA	*PN17R* CCCTTAGCGACAAAAGTGCC	168

The primers highlighted in grey were tested and used to validate SNPs.

Table 9. Primers designed for the validation of single nucleotide polymorphisms (SNPs) identified through data mining of whole genome sequencing data (continued).

Phytophthora citrophthora						
Primer pair	**Gene**	**Forward primer (5' to 3')**		**Reverse primer (3' to 5')**		**Product size (bp)**
PC1	*polr3a*	*PC1F*	GTGCAAGACTGACGAACGTT	*PC1R*	CGCCGTGATGTTCAAGTGTA	170
PC2	*polr3a*	*PC2F*	TGAACTACATCATGAGCGCG	*PC2R*	TCTGAGTGGATGACAGCACA	472
PC3	*polr3a*	*PC3F*	GGAGAGTATGGCTTCACTGC	*PC3R*	ACGGATGCTTAGTGTCAGGAT	558
PC4	*polr3a*	*PC4F*	CACTCAGCGGCAAGAAGATG	*PC4R*	CGTCGCTTAGGAAATCTGCC	513
PC5	*RPC3*	*PC5F*	AGATCCACCATACAAACGCC	*PC5R*	TGGAGGCGTTAGAAGTCAGA	216
PC6	*RPC3*	*PC6F*	GCCGACCGTGTACTGATCTA	*PC6R*	CCACCTCAAAATGGCAAGACA	624
PC7	*ABC transporter G family member 13*	*PC7F*	ATTGCATTGTCGTCGGAGC	*PC7R*	ACAGCTCACTTAGTCGAGAGG	469
PC8	*ABC transporter G family member 13*	*PC8F*	AAGAGCTCGCCACTTCCC	*PC8R*	AGATGCACGTGTCGGTCAT	334
PC9	*ABC transporter B family protein tagD*	*PC9F*	CATCGCCATGCAGTAGACTAC	*PC9R*	GCACTTTCTATTTTCTGTTCGCA	413
PC10	*ABC transporter B family member 11*	*PC10F*	CCGTGCACATGTTCAATTCC	*PC10R*	CCAAAGTACATACCGCCAGC	164
PC11	*ABC transporter B family member 11*	*PC11F*	ACGTGAAGATCGGCATTGTC	*PC11R*	TGAGATTGTTTCCGTCCAGC	514
PC12	*ABC transporter I family member 20*	*PC12F*	TCTGCATTTTACCAACACCACT	*PC12R*	GAAGTTAAGGCGACGGAGC	540
PC13	*ABC transporter I family member 20*	*PC13F*	CTATGGCCTACTCAGCGGAC	*PC13R*	GCTGCGATGGTCTCAAAAGA	189
PC14	*ABC transporter C family member 3*	*PC14F*	CCAACATGCGCTCATTAGTCA	*PC14R*	GATGTTGACCAGCGCCAC	594
PC15	*Cytochrome P450 86A2*	*PC15F*	CCAGCGCGTTTGATCGTAG	*PC15R*	GTGAGCCGATTCCACTTGG	261
PC16	*Cytochrome P450 86A2*	*PC16F*	CGCCAGCTTGTAAGAAATCAATA	*PC16R*	ACAGTGGTGGAAGTGCTGAA	455
PC17	*Cytochrome P450 86A2*	*PC17F*	AAAGCAAGCCCAAGGTAGTG	*PC17R*	TCTGACCGGGAAGATACAGC	157
PC18	*Cytochrome P450 86A2*	*PC18F*	ACCTTGCGTAATGAGTACTGTG	*PC18R*	GATACCGACAGCATTGCACC	177

The primers highlighted in grey were tested and used to validate SNPs.

Table 10. Primers designed for quantitative polymerase chain reaction (qPCR) assays to detect mefenoxam sensitivity.

Phytophthora nicotianae

Primer pair	Gene	Based on validation primer	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)
QPN1	*polr2a*	*PN2*	*QPN1F* TCTACCAACTGTCTGTA**A**AC	*QPN1R* TCCAGCACCTGGACGCT**C**TG	205
QPN2	*polr2a*	*PN2*	*QPN2F* GGCAGCAAAGCCGTAGG**A**AC	*QPN2R* CCGAACTCTTACCGACGTTC	123
QPN3	*polr2a*	*PN2*	*QPN3F* GCTCTACCAACTGTCTGTA**A**	*QPN3R* TGGAGTGAAGTACGGCAGTT	179
QPN4	*polr2a*	*PN2*	*QPN4F* GTGGCAGCAAAGCCGTAGG**A**	*QPN4R* CTCCTGCATGACCTTGGTGT	201
QPN5	*ABC transporter A family member 1*	*PN5*	*QPN5F* CAGCACGTGGGAGAT**T**CTG**T**	*QPN5R* GTCAAGTTGTAACCCGCACC	181
QPN6	*polr2a*	*PN2*	*QPN6F* TTCTGGTGCTTGTAGCGCAGT	*QPN6R* TGTCCAGCACCTGGACGCT**C**	153
QPN7	*polr2a*	*PN2*	*QPN7F* TCACTCCACCTCCGGACCA**G**	*QPN7R* GTATCCGAGTCCGGTAGAAA	127
QPN8	*ABC transporter A family member 1*	*PN5*	*QPN8F* GTCAGCACTGAGCTTCAACA	*QPN8R* GGTGATCTGATCCTTGTAC**A**	141
QPN9	*ABC transporter A family member 1*	*PN3*	*QPN9F* ACAAACTGATCAAGATCAG**C**	*QPN9R* GCACCAGCAACTGGTCTCTG	116
QPN10	*ABC transporter A family member 1*	*PN3*	*QPN10F* GCACAAACACACGGGAACA**C**	*QPN10R* CTTGGTCAAGGAGAAACAGG	132
QPN11	*ABC transporter A family member 1*	*PN3*	*QPN11F* GGGAACAGGTACATCACGA**G**	*QPN11R* TGCCGGCATCCATTGTTGTA	142
QPN12	*Cytochrome P450 86A2*	*PN9*	*QPN12F* GGCTCCGTGCGTCACGCA**G**	*QPN12R* CGCTCGGGGTTGAATTCAGC	153
QPN13	*Cytochrome P450 86A2*	*PN9*	*QPN13F* CTAAGGGCACCGACACGAC**T**	*QPN13R* CGGTCCAGCACTGAAGGCAA	161

Single nucleotide polymorphisms on the 3' end of the forward or reverse primers are indicated in red bold text.

Table 10. Primers designed for quantitative polymerase chain reaction (qPCR) assays to detect mefenoxam sensitivity (continued).

Phytophthora citrophthora

Primer pair	Gene	Based on validation primer	Forward primer (5' to 3')	Reverse primer (3' to 5')	Product size (bp)
QPC1	*polr3a*	*PC3*	*QPC1F* GCAGGGACTTCCACG**TA**AAG	*QPC1R* A**A**CAGAGCAAGTATCTA**T**CCC	149
QPC2	*polr3a*	*PC3*	*QPC2F* AAGCAAATGGGAAGGG**A**TAG	*QPC2R* GCGCCTTCTTGTATCCA**C**TG	117
QPC4	*polr3c*	*PC3*	*QPC4F* GAGAATTGCAGGTTTAG**C**AG	*QPC4R* GTAAACTCCTCACATCT**T**CC	201
QPC5	*ABC transporter G family member 13*	*PC7*	*QPC5F* TTACTACTTGACTACTATT**A**	*QPC5R* CAAATTCACTCTTATCACAG	93
QPC6	*ABC transporter G family member 13*	*PC7*	*QPC6F* TAAGAGTGAATTTGAAAAA**T**	*QPC6R* GTACAGCTCACTTAGTCGAG	72
QPC7	*ABC transporter G family member 13*	*PC7*	*QPC7F* TGAATTTGAAAAAATCATA**G**	*QPC7R* GTACAGCTCACTTAGTCGAG	66
QPC8	*ABC transporter B family member 11*	*PC11*	*QPC8F* TGTTGGAGCGCTTCTACGA**C**	*QPC8R* CGTGTTCACGTAGCCACTGC	93
QPC9	*ABC transporter B family member 11*	*PC11*	*QPC9F* CTACGATCCTGCTGCCGGA**G**	*QPC9R* CTCACAAGGGAGACGTGTTC	93
QPC10	*Cytochrome P450 86A2*	*PC16*	*QPC10F* TTGAAATCACTCTATTTTG**A**	*QPC10R* GAGGATATTCAATATTTATC	105
QPC11	*Cytochrome P450 86A2*	*PC16*	*QPC11F* TTGGAGTTGATAGTCGGTC**A**	*QPC11R* CTGTCCGATGGCAACTGTTC	87

Single nucleotide polymorphisms on the 3' end of the forward or reverse primers are indicated in red bold text.

Table 11. Number of the *uncharacterised Phytophthora nicotianae* and *Phytophthora citrophthora* classified as sensitive and insensitive to mefenoxam following *in vitro* fungicide sensitivity testing.

Species	Mefenoxam sensitivity classification	No. of isolates in category	Percentage of population (%)
Phytophthora nicotianae (n=29)	Sensitive	28	96.55
	Insensitive	1	3.45
Phytophthora citrophthora (n=16)	Sensitive	11	68.75
	Insensitive	5	31.25

Table 12. Comparison of the *in vitro* mefenoxam sensitivity screening and the quantitative polymerase chain reaction (qPCR) assay results of new *Phytophthora nicotianae* isolates when using primer set *QPN4*.

Isolate	RG at 30 ppm (%)	Sensitivity level	Did qPCR amplify	Pass or fail
Pn50	0.00	sensitive	No	Fail
Pn55	0.00	sensitive	Yes	Pass
Pn56	0.00	sensitive	No	Fail
Pn97	0.00	sensitive	No	Fail
Pn98	0.00	sensitive	No	Fail
Pn112	116.99	insensitive	No	Pass
Pn115	0.00	sensitive	No	Fail
Pn182	4.60	sensitive	No	Fail
Pn183	0.00	sensitive	No	Fail
Pn184	0.00	sensitive	No	Fail
Pn217	0.00	sensitive	Yes	Pass
Pn226	0.00	sensitive	No	Fail
Pn228	0.00	sensitive	Yes	Pass
Pn262	0.00	sensitive	Yes	Pass
Pn302	10.76	sensitive	Yes	Pass
Pn1928	0.00	sensitive	No	Fail
Pn2084	0.00	sensitive	No	Fail
Pn2185	0.00	sensitive	No	Fail
Pn2377	0.00	sensitive	No	Fail
Pn2387	0.00	sensitive	No	Fail
Pn2475	0.00	sensitive	No	Fail
Pn2541	0.00	sensitive	No	Fail
Pn2739	0.00	sensitive	No	Fail
Pn2744	0.00	sensitive	No	Fail
Pn2788	0.00	sensitive	Yes	Pass
Pn2939	0.00	sensitive	Yes	Pass
Pn2982	0.00	sensitive	No	Fail
Pn3061	0.00	sensitive	No	Fail
Pn3062	0.00	sensitive	No	Fail

Table 13. Comparison of the *in vitro* mefenoxam sensitivity screening and the quantitative polymerase chain reaction (qPCR) assay results of new *Phytophthora citrophthora* isolates when using primer set *QPC1*.

Isolate	RG at 30 ppm (%)	Sensitivity level	Did qPCR amplify	Pass or fail
Pc226	47.08	insensitive	No	Pass
Pc272	0.00	sensitive	No	Fail
Pc268	0.00	sensitive	No	Fail
Pc275	43.10	insensitive	No	Pass
Pc244	91.75	insensitive	Yes	Fail
Pc267	20.01	sensitive	No	Fail
Pc276	21.32	sensitive	No	Fail
Pc279	14.52	sensitive	No	Fail
Pc234	17.93	sensitive	No	Fail
Pc229	0.00	sensitive	No	Fail
Pc2231	35.49	sensitive	Yes	Pass
Pc2241	51.86	insensitive	Yes	Fail
Pc2476	0.00	sensitive	No	Fail
Pc2539	108.49	insensitive	No	Pass
Pc2534	31.68	sensitive	Yes	Pass
Pc3060	0.00	sensitive	No	Fail

Table 14. Comparison of the *in vitro* mefenoxam sensitivity screening and the quantitative polymerase chain reaction (qPCR) assay results of new *Phytophthora citrophthora* isolates when using primer set *QPC2*.

Isolate	RG at 30 ppm (%)	Sensitivity level	Did qPCR amplify	Pass or fail
Pc226	47.08	insensitive	Yes	Fail
Pc272	0.00	sensitive	Yes	Pass
Pc268	0.00	sensitive	Yes	Pass
Pc275	43.10	insensitive	Yes	Fail
Pc244	91.75	insensitive	Yes	Fail
Pc267	20.01	sensitive	Yes	Pass
Pc276	21.32	sensitive	Yes	Pass
Pc279	14.52	sensitive	Yes	Pass
Pc234	17.93	sensitive	Yes	Pass
Pc229	0.00	sensitive	Yes	Pass
Pc2231	35.49	sensitive	Yes	Pass
Pc2241	51.86	insensitive	Yes	Fail
Pc2476	0.00	sensitive	Yes	Pass
Pc2539	108.49	insensitive	No	Pass
Pc2534	31.68	sensitive	Yes	Pass
Pc3060	0.00	sensitive	Yes	Pass

Table 15. Comparison of the *in vitro* mefenoxam sensitivity screening and the quantitative polymerase chain reaction (qPCR) assay results of new *Phytophthora citrophthora* isolates when using primer set *QPC4*.

Isolate	RG at 30 ppm (%)	Sensitivity level	Did qPCR amplify	Pass or fail
Pc226	47.08	insensitive	No	Pass
Pc272	0.00	sensitive	Yes	Pass
Pc268	0.00	sensitive	Yes	Pass
Pc275	43.10	insensitive	Yes	Fail
Pc244	91.75	insensitive	Yes	Fail
Pc267	20.01	sensitive	Yes	Pass
Pc276	21.32	sensitive	Yes	Pass
Pc279	14.52	sensitive	No	Fail
Pc234	17.93	sensitive	Yes	Pass
Pc229	0.00	sensitive	Yes	Pass
Pc2231	35.49	sensitive	Yes	Pass
Pc2241	51.86	insensitive	Yes	Fail
Pc2476	0.00	sensitive	Yes	Pass
Pc2539	108.49	insensitive	No	Pass
Pc2534	31.68	sensitive	Yes	Pass
Pc3060	0.00	sensitive	Yes	Pass

Table 16. Sanger sequencing results of new *Phytophthora citrophthora* isolates to validate the presence of SNPs in primer set *QPC2*.

				Sanger sequencing			qPCR	
Isolate	Resistance status	Relative growth at 30 ppm	Position	Nucleotide at position	SNP present	Homozygous or heterozygous	qPCR amplification	Pass or fail
Pc274	Insensitive	96.48	252	G	No	Homozygous	No	Pass
			335	C	No	Homozygous		
Pc249	Insensitive	97.09	252	G	No	Homozygous	No	Pass
			335	C	No	Homozygous		
Pc245	Insensitive	110.54	252	G	No	Homozygous	No	Pass
			335	C	Yes	Homozygous		
Pc251	Sensitive	11.34	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc285	Sensitive	34.25	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc253	Sensitive	0.00	252	A	Yes	Homozygous	Yes	Pass
			335	A	Yes	Homozygous		
Pc226	Insensitive	47.08	252	G/A	Yes	Heterozygous	Yes	Fail
			335	C	No	Homozygous		
Pc272	Sensitive	0.00	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		

SNPs were located in contig 12 in the range of 696300-696900 bp.

Table 16. Sanger sequencing results of new *Phytophthora citrophthora* isolates to validate the presence of SNPs in primer set *QPC2*.

				Sanger sequencing			qPCR	
Isolate	Resistance status	Relative growth at 30 ppm	Position	Nucleotide at position	SNP present	Homozygous or heterozygous	qPCR amplification	Pass or fail
Pc268	Sensitive	0.00	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc275	Insensitive	43.10	252	G/A	Yes	Heterozygous	Yes	Fail
			335	C/G	Yes	Heterozygous		
Pc244	Insensitive	91.75	252	G/A	Yes	Heterozygous	Yes	Fail
			335	C/G	Yes	Heterozygous		
Pc267	Sensitive	20.01	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc276	Sensitive	21.32	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc279	Sensitive	14.52	252	G	No	Homozygous	Yes	Pass
			335	C	No	Homozygous		
Pc234	Sensitive	17.93	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc229	Sensitive	0.00	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		

Table 16. Sanger sequencing results of new *Phytophthora citrophthora* isolates to validate the presence of SNPs in primer set *QPC2*.

				Sanger sequencing			qPCR	
Isolate	Resistance status	Relative growth at 30 ppm	Position	Nucleotide at position	SNP present	Homozygous or heterozygous	qPCR amplification	Pass or fail
Pc2231	Sensitive	35.49	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc2241	Insensitive	51.85	252	G/A	Yes	Heterozygous	Yes	Fail
			335	C/G	Yes	Heterozygous		
Pc2476	Sensitive	0.00	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc2539	Insensitive	108.49	252	G	No	Homozygous	No	Pass
			335	C	No	Homozygous		
Pc2534	Sensitive	31.68	252	G/A	Yes	Heterozygous	Yes	Pass
			335	C/G	Yes	Heterozygous		
Pc3060	Sensitive	0.00	252	A	Yes	Homozygous	Yes	Pass
			335	G	Yes	Homozygous		

Table 17. Sanger sequencing results of new *Phytophthora citrophthora* isolates to validate the presence of SNPs in primer set *QPC4.*

				Sanger sequencing			qPCR	
Isolate	Resistance status	Relative growth at 30 ppm	Position	Nucleotide at position	SNP present	Homozygous or heterozygous	qPCR amplification	Pass or fail
Pc274	Insensitive	96.48	162	T	No	Homozygous	No	Pass
			329	G/A	Yes	Heterozygous		
Pc249	Insensitive	97.09	162	T/C	Yes	Heterozygous	Yes	Fail
			329	G/A	Yes	Heterozygous		
Pc245	Insensitive	110.54	162	T	No	Homozygous	No	Pass
			329	G/A	Yes	Heterozygous		
Pc251	Sensitive	11.34	162	T/C	Yes	Heterozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc285	Sensitive	34.25	162	T/C	Yes	Heterozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc253	Sensitive	0.00	162	T/C	Yes	Heterozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc226	Insensitive	47.08	162	T	No	Homozygous	No	Pass
			329	G	No	Homozygous		
Pc272	Sensitive	0.00	162	C	Yes	Homozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		

SNPs were located in contig 12 in the range of 941000-941700 bp.

Table 17. Sanger sequencing results of new *Phytophthora citrophthora* isolates to validate the presence of SNPs in primer set *QPC4* (continued).

				Sanger sequencing			qPCR	
Isolate	Resistance status	Relative growth at 30 ppm	Position	Nucleotide at position	SNP present	Homozygous or heterozygous	qPCR amplification	Pass or fail
Pc268	Sensitive	0.00	162	T	No	Homozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc275	Insensitive	43.10	162	T	No	Homozygous	Yes	Fail
			329	G/A	Yes	Heterozygous		
Pc244	Insensitive	91.75	162	T/C	Yes	Heterozygous	Yes	Fail
			329	G/A	Yes	Heterozygous		
Pc267	Sensitive	20.01	162	T/C	Yes	Heterozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc276	Sensitive	21.32	162	T	No	Homozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc279	Sensitive	14.52	162	A	No	Homozygous	No	Fail
			329	G	No	Homozygous		
Pc234	Sensitive	17.93	162	G/C/A	Yes		Yes	Pass
			329	G/C/A	Yes			
Pc229	Sensitive	0.00	162	T	No	Homozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		

SNPs were located in contig 12 in the range of 941000-941700 bp.

Table 17. Sanger sequencing results of new *Phytophthora citrophthora* isolates to validate the presence of SNPs in primer set *QPC4* (continued).

				Sanger sequencing			qPCR	
Isolate	Resistance status	Relative growth at 30 ppm	Position	Nucleotide at position	SNP present	Homozygous or heterozygous	qPCR amplification	Pass or fail
Pc2231	Sensitive	35.49	162	T/C	Yes	Heterozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc2241	Insensitive	51.85	162	T/C	Yes	Heterozygous	Yes	Fail
			329	G/A	Yes	Heterozygous		
Pc2476	Sensitive	0.00	162	T	No	Homozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc2539	Insensitive	108.49	162	T	No	Homozygous	No	Pass
			329	G	No	Homozygous		
Pc2534	Sensitive	31.68	162	A	No	Homozygous	Yes	Pass
			329	G/A	Yes	Heterozygous		
Pc3060	Sensitive	0.00	162	T/C	Yes	Heterozygous	Yes	Pass
			329	A	Yes	Homozygous		

SNPs were located in contig 12 in the range of 941000-941700 bp.

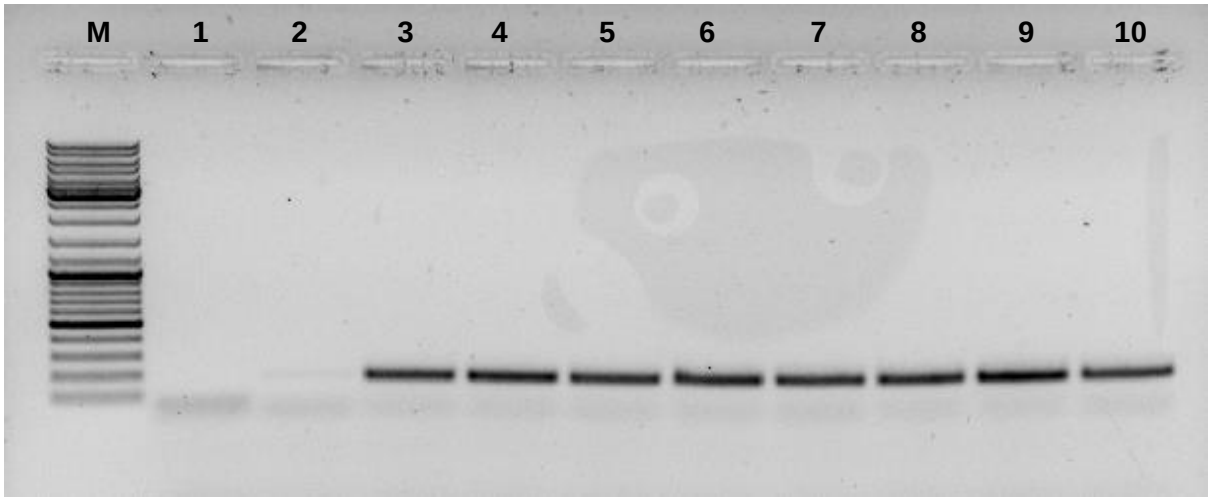

Figure 1. Detection of *Phytophthora nicotianae* using primer set *PN_YPT1_FW17* and *PN_YPT1_RV17*. The PCR products were electrophoresed on 1% (w/v) agarose gel for 45 min at 100 V. Lane M represents a 100 bp DNA ladder. Lane 1 contained the non-template control (PCR-grade water) and Lane 2 contained the negative control (*Phytophthora citrophthor*a DNA). Lanes 3 to 10 represent *Phytophthora nicotianae* isolates.

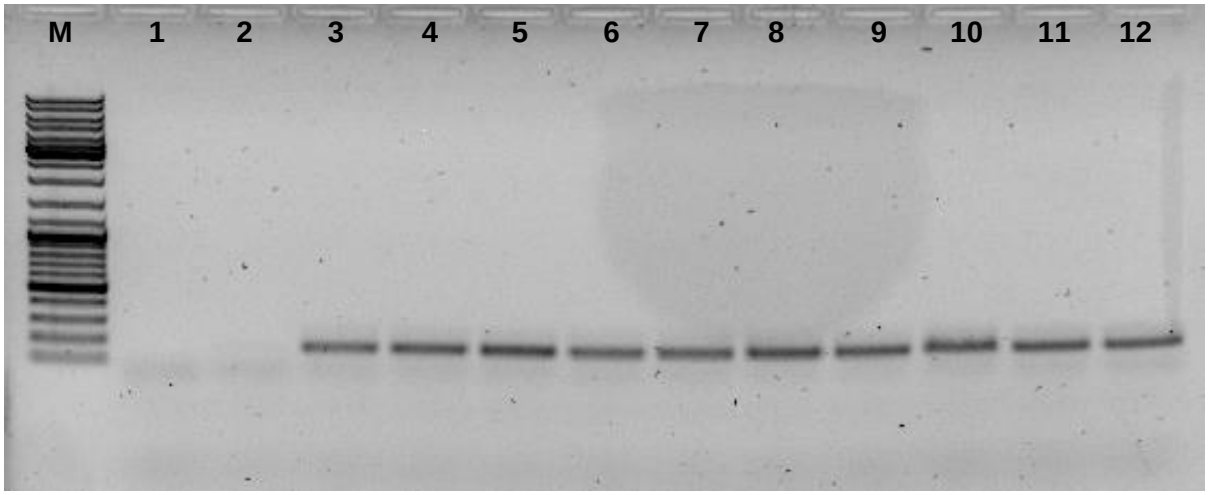

Figure 2. Detection of *Phytophthora citrophthora* using primer set *PC_YPT1_FW2* and *PC_YPT1_RV2*. The PCR products were electrophoresed on 1% (w/v) agarose gel for 45 min at 100 V. Lane M represents a 100 bp DNA ladder. Lane 1 contained the non-template control (PCR-grade water) and Lane 2 contained the negative control (*Phytophthora nicotianae DNA*). Lanes 3 to 12 represent *Phytophthora citrophthora* isolates.

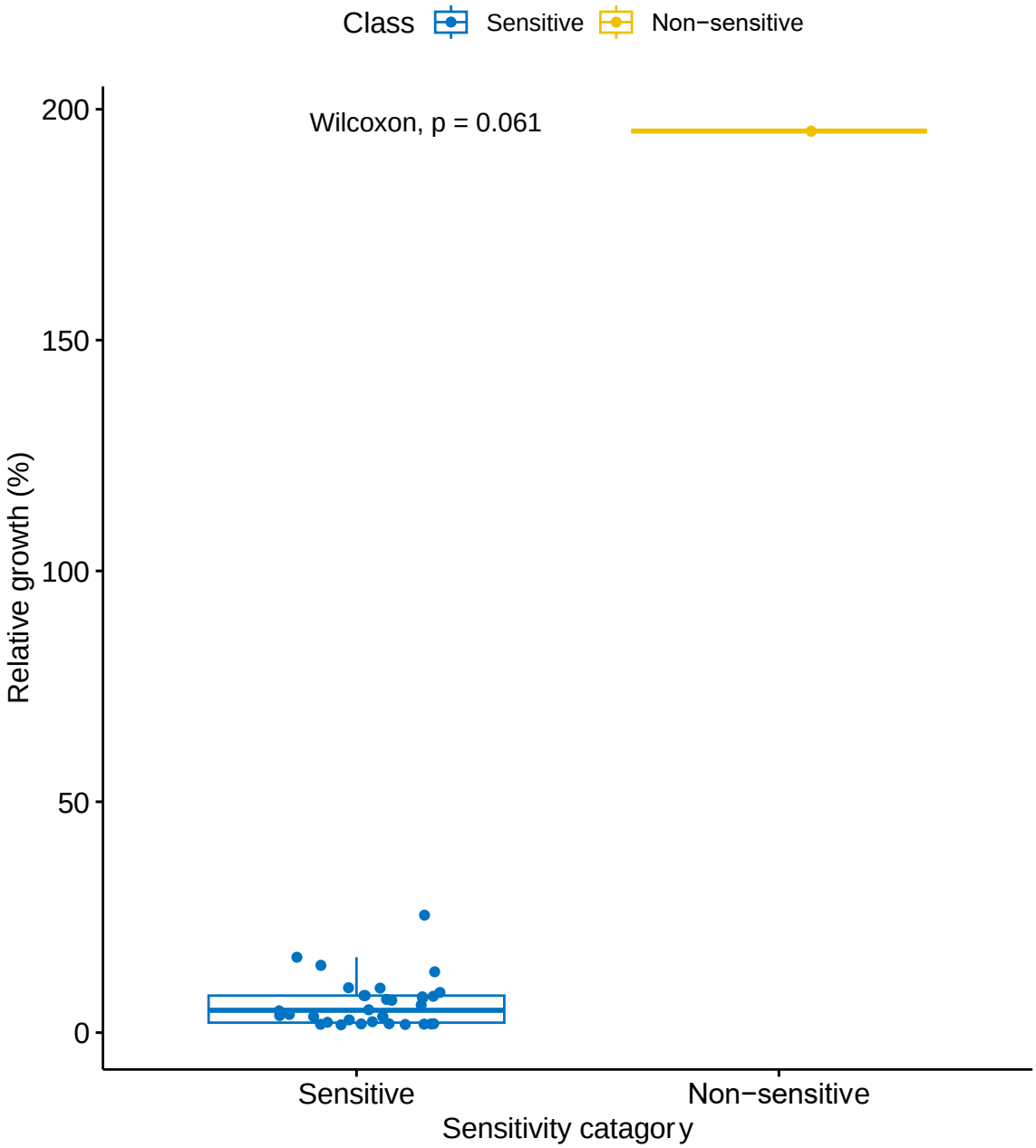

Figure 3. Boxplot of the relative growth (RG_{100}) of the sensitive and non-sensitive isolates of *Phytophthora nicotianae*. The single non-sensitive isolate hindered accurate statistical analysis; however, the boxplot indicates a difference between the RG_{100} of the two sensitivity groups.

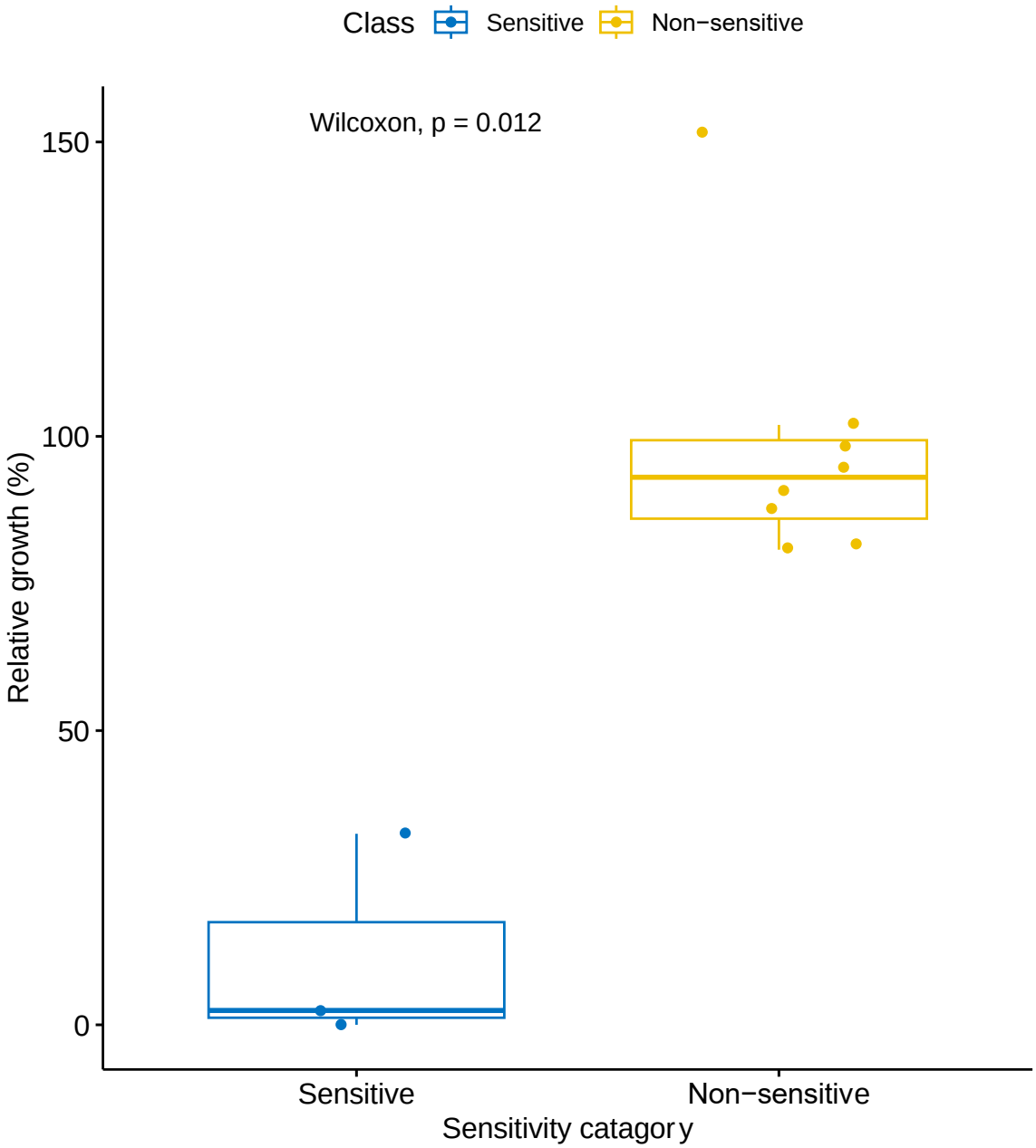

Figure 4. Boxplot of the relative growth (RG_{60}) of the sensitive and non-sensitive isolates of *Phytophthora citrophthora*. The boxplot indicates a statistical ($P \leq 0.05$) difference between the RG_{60} of the two sensitivity groups ($P = 0.012$).

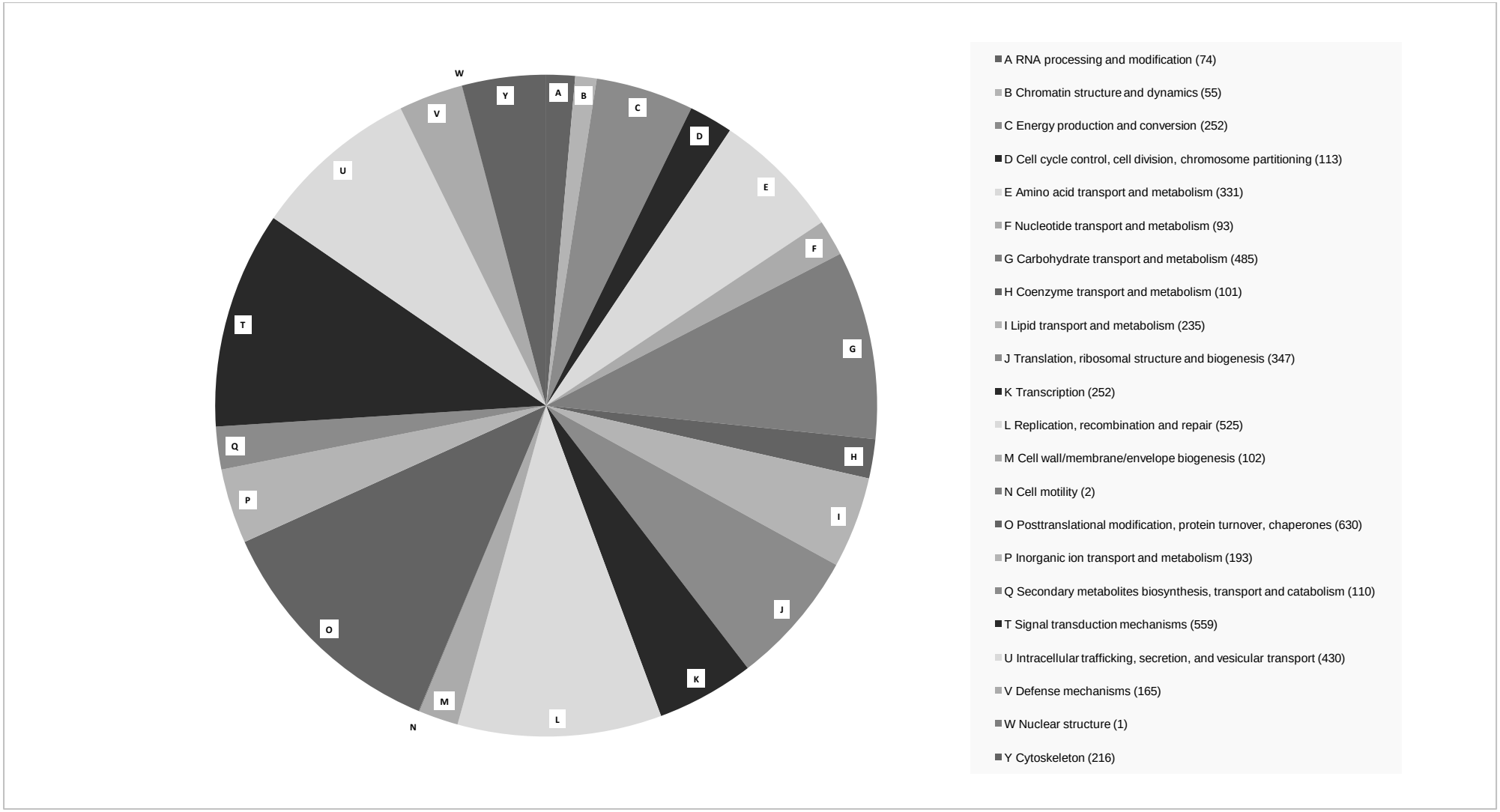

Figure 5. Functional protein classifications of *STE-U-9442* determined with EggNOG. Protein groups are indicated with A–Y and the number of genes in each group is indicated in brackets in the key.

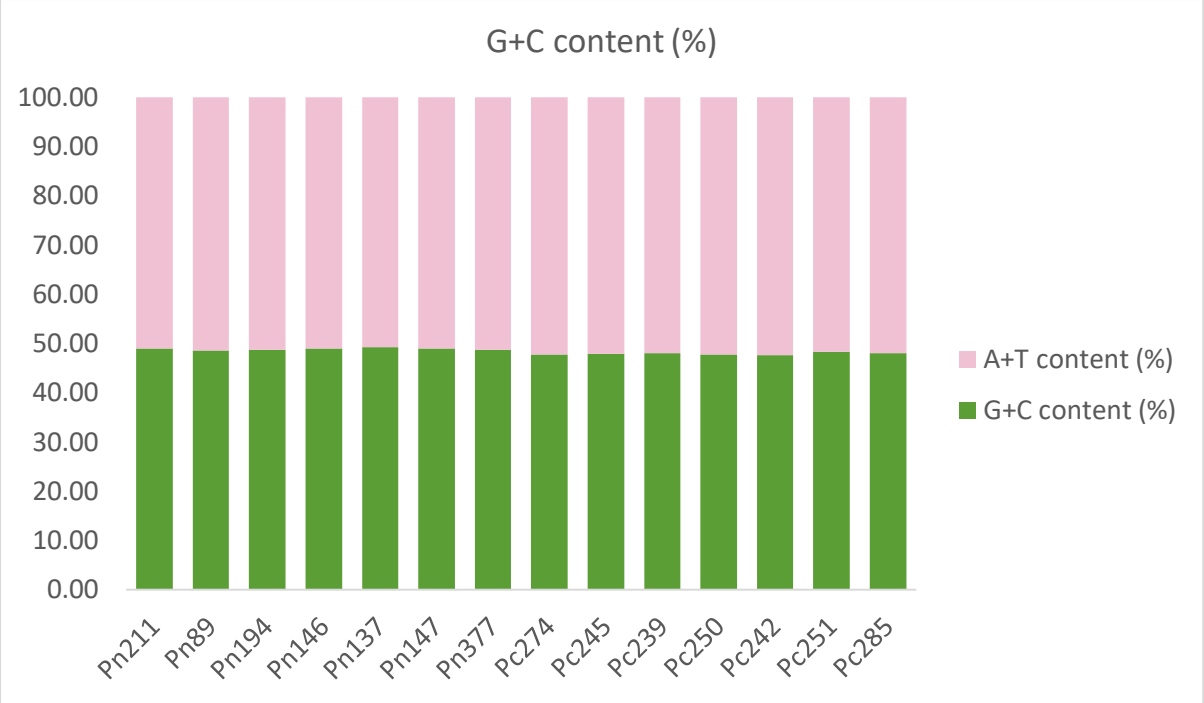

Figure 6. Genome G+C content (%) of isolates submitted for whole genome sequencing on the Illumina platform.

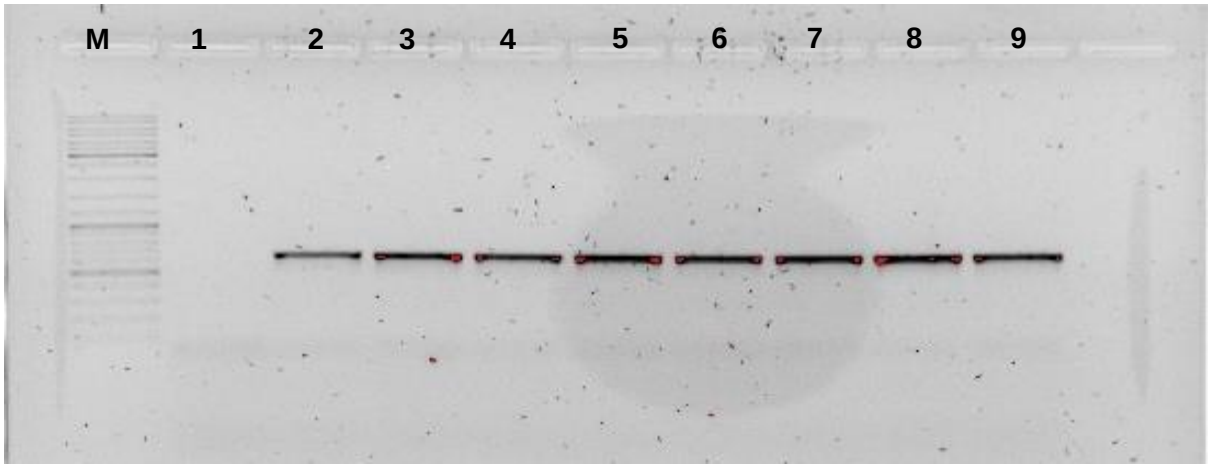

Figure 7. An amplification product of 632 bp from the *polr2a* gene region in *Phytophthora nicotianae* isolates using primer set *PN2*. Lane M represents a 100 bp DNA ladder. Lane 1 contained the non-template control (PCR-grade water). Lane 2–9 represents the PCR products from *Phytophthora nicotianae* isolates.

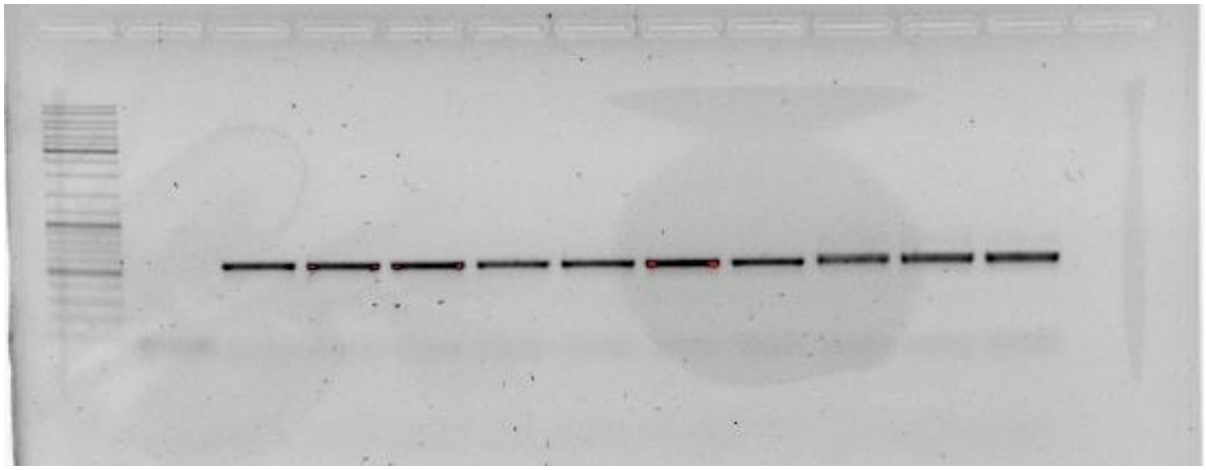

Figure 8. An amplification product of 558 bp from the *polr3a* gene region in *Phytophthora citrophthora* isolates using primer set *PC3*. Lane M represents a 100 bp DNA ladder. Lane 1 contained the non-template control (PCR-grade water). Lane 2–11 represents the PCR products from *Phytophthora citrophthora* isolates.

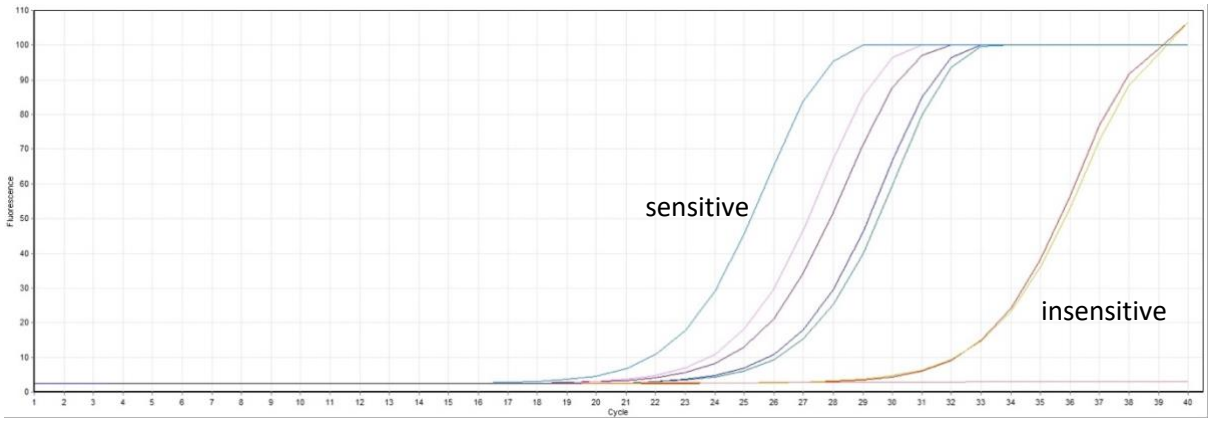

Figure 9*.* Fluorescence curve of the qPCR assay testing primer set *QPN4* on a small cohort of *Phytophthora nicotianae* isolates. The insensitive isolates can be differentiated from sensitive isolates.

		5'		3'
a)	Isolate *Pc274* (Res)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGGTAGATACTTG	-----	GACCACTGGATACAAGAAGGCGCGTG
	Isolate *Pc245* (Res)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGGTAGATACTTG	-----	GACCACTGGATACAAGAAGGCGCGTG
	Isolate *Pc239* (Res)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGGTAGATACTTG	-----	GACCACTGGATACAAGAAGGCGCGTG
	Isolate *Pc250* (Res)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGGTAGATACTTG	-----	GACCACTGGATACAAGAAGGCGCGTG
	Isolate *Pc242* (Res)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGGTAGATACTTG	-----	GACCACTGGATACAAGAAGGCGCGTG
	Isolate *Pc251* (Sen)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGATAGATACTTG	-----	GACCAGTGGATACAAGAAGGCGCGTG
	Isolate *Pc285* (Sen)	GACATTCTCAAGACACACAAGCAAATGGGAAGGGATAGATACTTG	-----	GACCAGTGGATACAAGAAGGCGCGTG
b)		*QPC2F* AAGCAAATGGGAAGGG**A**TAG		*QPC2R* CA**G**TGGATACAAGAAGGCGC

Figure 10. Excerpt of the alignment of the *polr3a* gene sequences **(a)** obtained from whole genome sequencing (Res: Resistant; Sen: Sensitive). **(b)** The binding positions of primer set *QPC2* is indicated with SNPs indicated in bold.

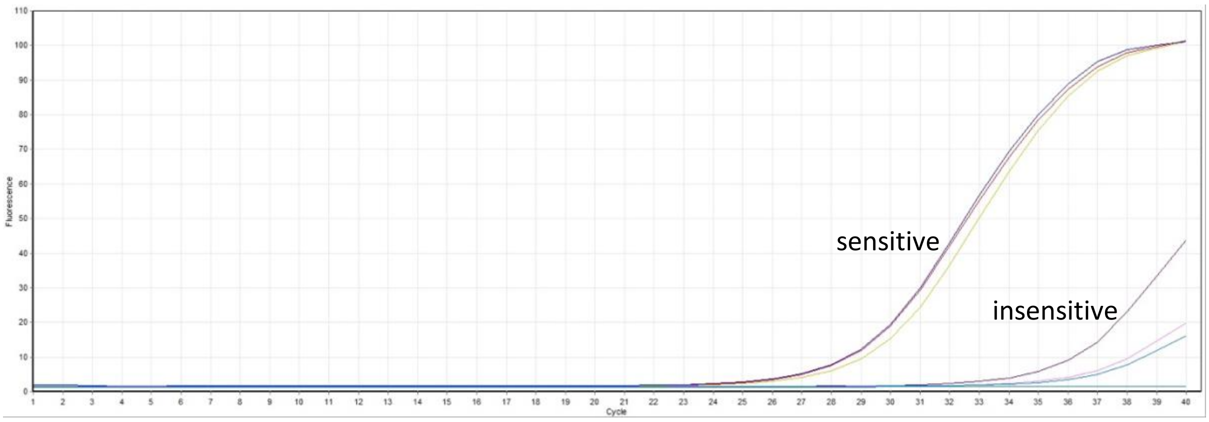

Figure 11. Fluorescence curve of the qPCR assay testing primer set *QPC1* on a small cohort of *Phytophthora citrophthora* isolates. The insensitive isolates amplify from 33 cycles and onwards and can therefore be differentiated from sensitive isolates.

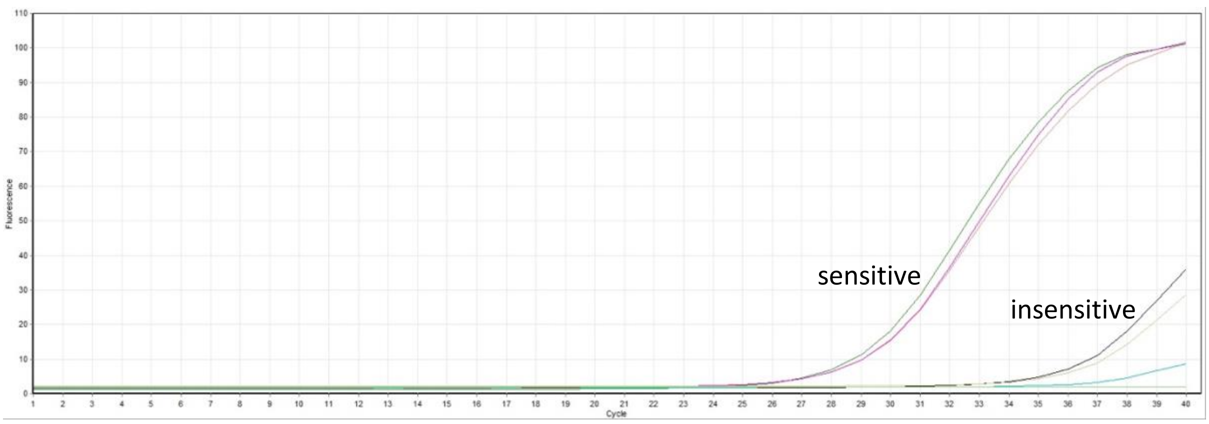

Figure 12. Fluorescence curve of the qPCR assay testing primer set *QPC2* on a small cohort of *Phytophthora citrophthora* isolates. The insensitive isolates amplify from 33 cycles and onwards and can therefore be differentiated from sensitive isolates.

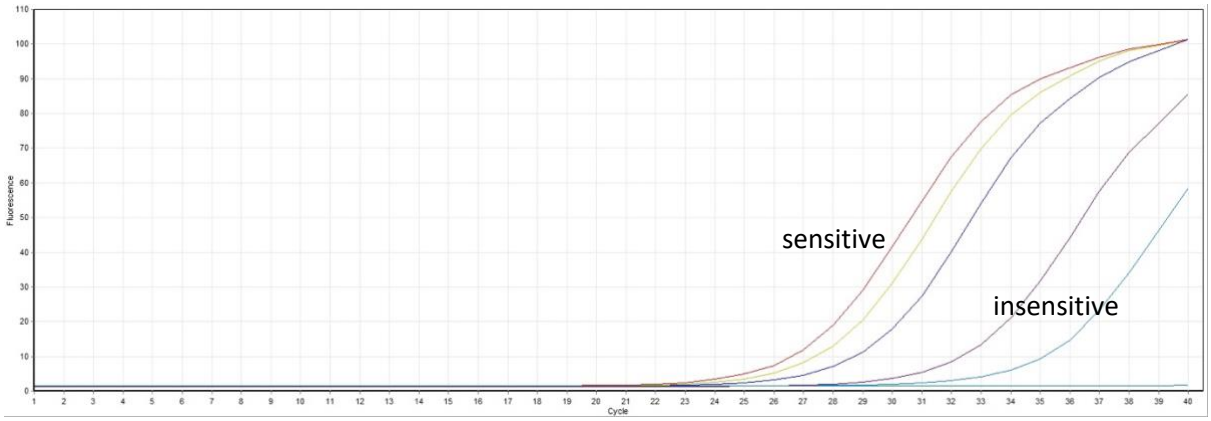

Figure 13. Fluorescence curve of the qPCR assay testing primer set *QPC4* on a small cohort of *Phytophthora citrophthora* isolates. The two insensitive isolates amplify from 30 cycles and onwards and can be differentiated from sensitive isolates.

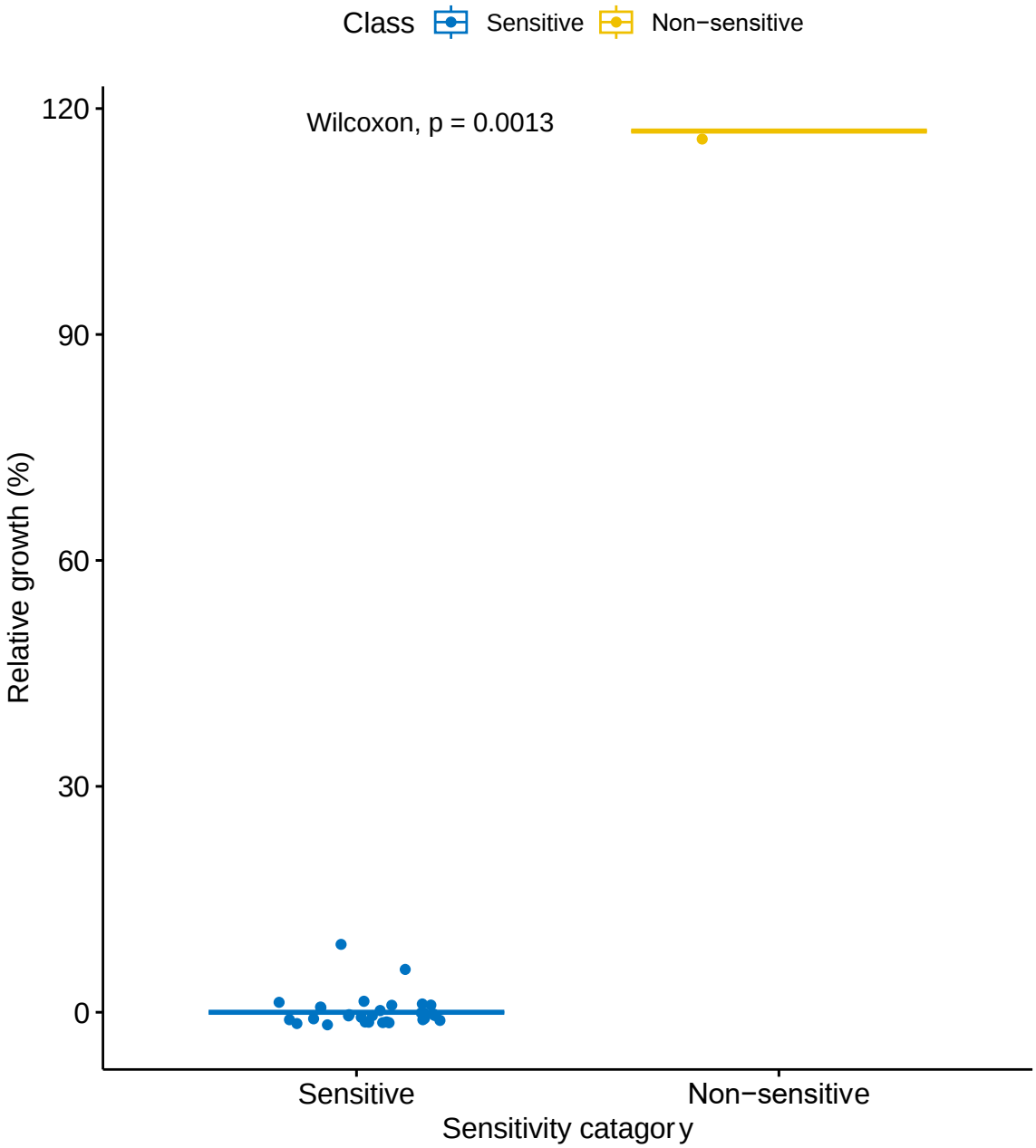

Figure 14. Boxplot of the relative growth (RG_{30}) of the sensitive and non-sensitive isolates of *Phytophthora nicotianae*. A single non-sensitive isolate hindered accurate statistical analysis, however, the boxplot indicates a difference between the RG_{30} of the two sensitivity groups.

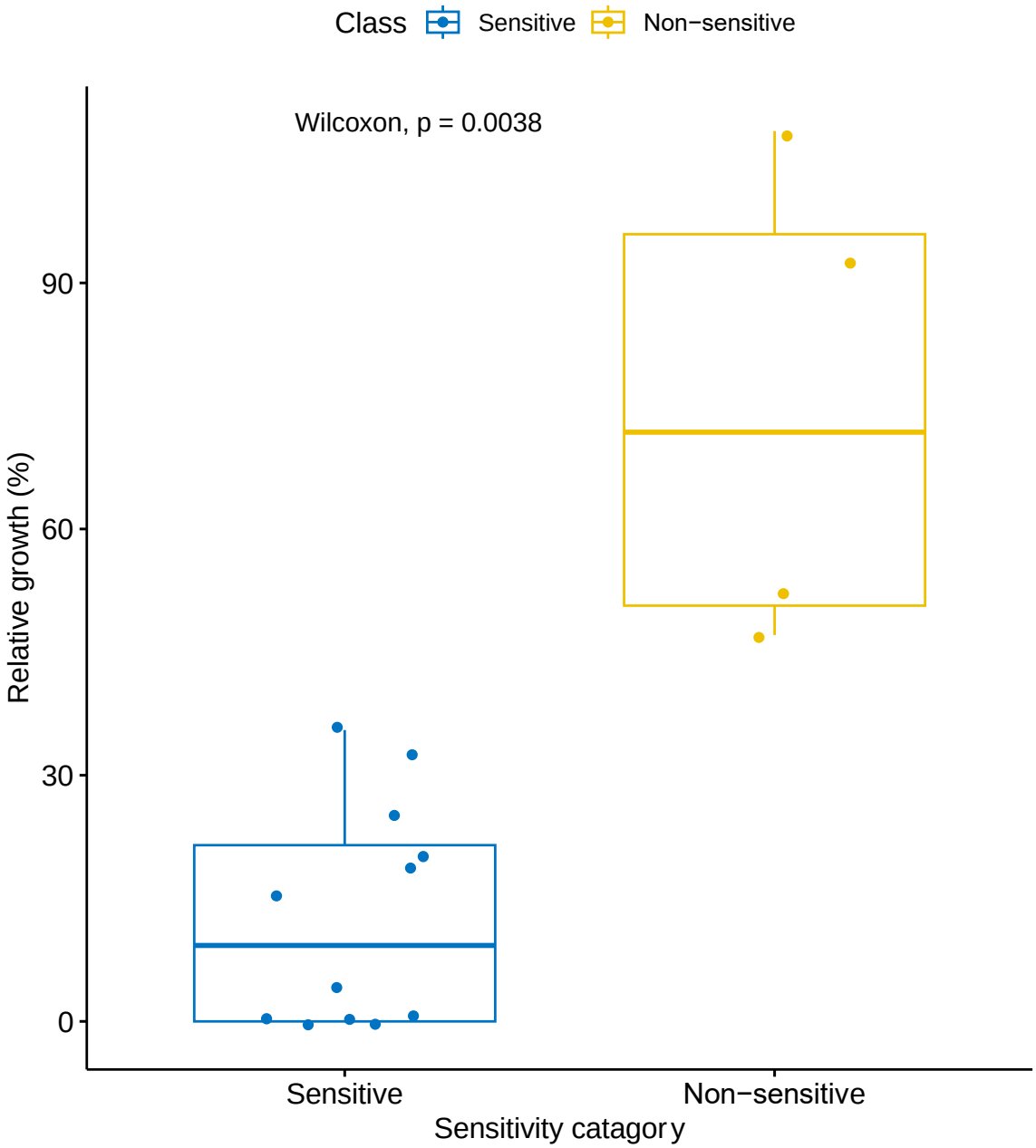

Figure 15. Boxplot of the relative growth (RG_{30}) of the sensitive and non-sensitive isolates of *Phytophthora citrophthora*. The boxplot indicates a statistical ($P \leq 0.05$) difference between the RG_{30} of the two sensitivity groups ($P = 0.0038$).

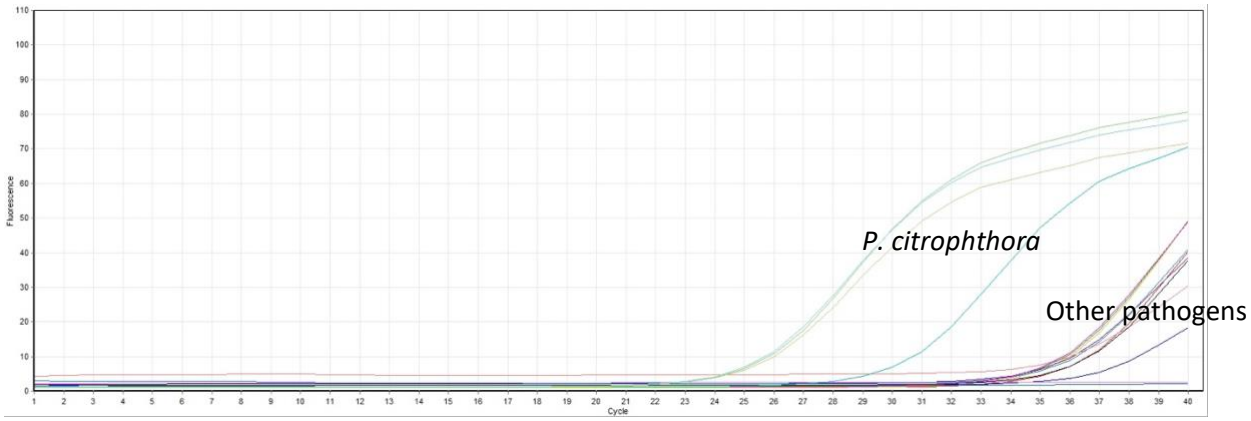

Figure 16. Fluorescence curve of the qPCR assay testing the specificity of primer set *QPC2* for *Phytophthora citrophthora* isolates. The primer set was tested using a range of negative control isolates (i.e. closely related species and other citrus pathogens), including *Phytophthora nicotianae*, *Phytophthora cactorum*, *Phytophthora infestans*, *Phytophthora capsici*, *Phytophthora citricola*, *Phytophthora mengei*, *Pythium irregulare s.s.*, *Neocosmospora solani*, *Neocosmospora citricola* and *Neocosmospora ferruginea*. Among these isolates, three *Phytophthora citrophthora* isolates exhibited amplification starting at cycle 23, while one began amplification at cycle 28. In contrast, all negative control isolates showed amplification at cycle 34 or later.

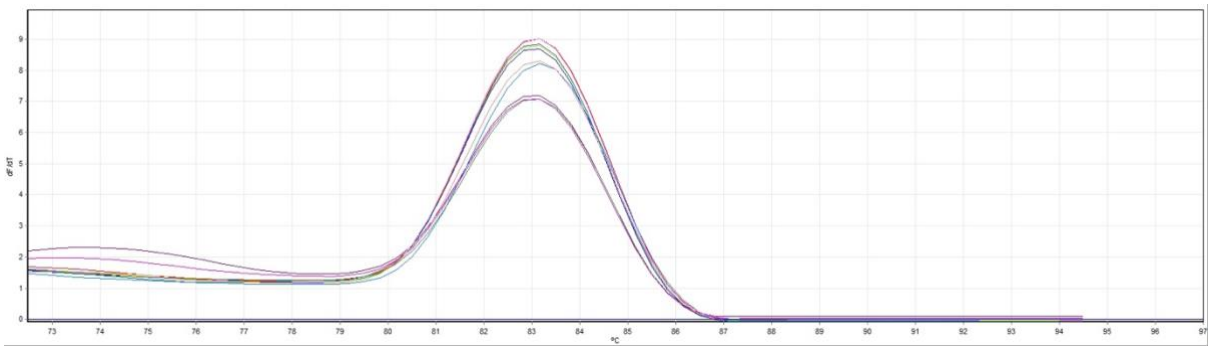

Figure 17. Melt curve of the multiplex qPCR assay using *Phytophthora citrophthora* species-specific primers and primer set *QPC2*. Both primer sets exhibit a melt curve peak at 83°C which does not afford the ability to differentiate between the species-specific primer set and *QPC2*.