



## First Record of *Phytophthium vexans* causing root rot on Mandarin (*Citrus reticulata* L. cv. Sainampueng) in Thailand

Noireung P, Intaparn P, Maumoon R, Wongwan T and To-anun C\*

Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand

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### Abstract

A survey was conducted during 2017–2019 to identify the causal agents of citrus root rot in Thailand. The causal agent was isolated from the roots and rhizosphere soil of *Citrus reticulata* plants, which shows symptoms of root rot. The taxonomy and phylogeny of the pathogen was studied. The molecular phylogeny was studied based on Cytochrome c oxidase I (Cox1). The taxonomy and phylogeny revealed the pathogen to be *Phytophthium vexans*. The pathogenicity of the isolate was tested by inoculating detached *C. reticulata* leaves with mycelial plug and zoospore suspension on root. It was found that *Phytophthium vexans* PS85 causes the brown rot on *C. reticulata* leaves and root rot. This is the first record of *Phytophthium vexans* causing root rot disease on Mandarin (*Citrus reticulata*) in Thailand.

**Key words** – morphology – pathogenicity – phylogenetic – root rot

### Introduction

*Phytophthium* are cosmopolitan soil saprophytes and important plant pathogens (Matsumoto et al. 1999, Mostowfizadeh–Ghalamfarsa & Banihashemi 2005, Ho 2009). *Phytophthium* was reported from stems of the citrus trees affected by gummosis (Benfradj et al. 2017). *Phytophthium* in Pythiaceae (Peronosporales) was introduced by Bala et al. (2010b) and typified with *Phytophthium sindhum*. The zoospore differentiation and releasing mechanism in *Phytophthium* is similar to *Pythium* (Bala et al. 2010b). The globose to ovoid, papillate sporangia, often with internal proliferations in *Phytophthium* are more similar to *Phytophthora*.

Mandarin (*Citrus reticulata* L. cv. Sainampueng) is one of the major economic crops in Thailand. The prominent root rot disease in citrus is caused by *Phytophthora* and *Pythium* (Maseko & Coutinho 2002, Benfradj et al. 2017). Gummosis and root rot disease in citrus is caused by *Phytophthora nicotianae* (syn. = *P. parasitica*). There are several diseases that can cause wilt in citrus and the most prominent citrus black spot disease in Florida was caused by *Guignardia citricarpa* (Katherine et al. 2013). In Thailand, the root rot disease in Tangerine (*Citrus reticulata*) is caused by *Pythium* spp. (Udompongsuk et al. 2017) and the root rot in Pomelo (*Citrus maxima*) is caused by *Phytophthora palmivora* (Hung et al. 2015). Most of the fungal pathogens are prevalent in soil that has poor drainage or gardens that has been over-watered. In the present study, our objectives were to identify the causal agent of root rot on Mandarin (*Citrus reticulata* L. cv.

Sainampueng) in Thailand.

## Materials & Methods

### Survey, collection, isolation and morphological analysis

Roots, crowns and rhizosphere soil were collected from diseased *Citrus reticulata* showing symptoms of root rot from different locations. All the samples were kept individually in separate plastic bags, and taken to the laboratory for the isolation of pathogens. Infected roots were washed under running tap water, and excess water was removed with filter paper. The roots were cut into 5 mm segments and disinfected with 1% (v/v) sodium hypochlorite for 1 min, then rinsed three times with sterile distilled water. Root segments were dried separately on sterilized filter paper and then placed in petri plates containing water agar (WA; 15 g agar/L distilled water).

For the baiting technique, the soil samples were mixed with sterile distilled water (1/4 w/v) and for the baits mature and healthy leaves of *Citrus reticulata*. Leaves of *C. reticulata* were used. The leaves cut in to 1 x 1 cm pieces and surface sterilized with 10% sodium hypochlorite solution for 1 min, rinsed twice with sterile distilled water, and floated on the soil solution. After 1–2 day of incubation at room temperature (25–28°C), discoloured baits were blotted on sterile paper towels to remove excess water and plated onto water (WA). The baiting plates were incubated at room temperature for 2 to 3 days. Hyphal tips growing from the baits were cut under a stereo microscope (Zeiss Stemi 305) and transferred to potato dextrose agar (PDA) for 5 days to confirm successful pure culture. Morphological characteristics of these structures (sporangia, oospore, oogonia and antheridia) (Waterhouse 1967) were examined by light microscopic (Axiovision Zeiss Scope–A1) taken and measurements.

### DNA extraction, PCR amplification and sequencing

Seven days old fungal mycelia were scraped and DNA were extracted following the methodology described in Robideau et al. (2011). The DNA amplifications was performed by polymerase chain reaction (PCR) based on the relevant to the genes, the Cytochrome c oxidase I (Cox1). The primers used for each region are listed in Table 1. The PCR reaction conditions for the Cox1 consisted of an initial denaturation at 94°C for 5 min, 32 cycles of 94°C for 30 s. annealing at 50°C for 30 s and extension at 72°C for 90 s, with a final extension step at 72°C for 7 min.

The quality of PCR amplification was confirmed on 1% agarose gels electrophoresis and by viewing under ultra-violet light. Sizes of amplicons were determined against a HyperLadder™ I molecular marker (BIOLINE). Further purification of PCR product was performed using NucleoSpin® Gel and PCR Clean-up (MACHEREY–NAGEL GmbH and Co., KG Düren Germany) following the manufacturer's protocol. DNA concentrations were determined by a NanoDrop quantification as outlined by the manufacturer. The purified PCR fragments were sent to a commercial sequencing provider (Kembangan, Malaysia). The nucleotide sequence data acquired were deposited in GenBank.

**Table 1** Primers used for amplification and sequencing of The Cox1 region.

Region	Primer name	Primer sequence (5'–3')	Reference
Cox1	OomCox1–Levup	TCAWCWGMGATGGCTTTTTTCAAC	Bala et al. (2010b)
	FM85mod	RRH WAC KTG ACT DAT RAT ACC AAA	Bala et al. (2010b)

### Phylogenetic analysis

Phylogenetic analyses were conducted based on Cox1 sequence data. The sequence data of *Phytophthium* were downloaded from GenBank. *Phytophthora infestans* (CBS 36651) and *Phytophthora ramorum* (CBS 101553) were selected as the outgroup. The Cox1 gene dataset was initially aligned by using MEGA (Molecular Evolutionary Genetics Analysis) version 7.0 (Tamura & Nei 1993, Kumar et al. 2016). The final alignment of the Cox1 sequence dataset was analyzed

and inferred the phylogenetic tree based on maximum likelihood (ML) using the RAxML–HPC2 on XSEDE (v. 8.2.8) (Stamatakis et al. 2008) via the CIPRES Science Gateway platform (Miller et al. 2010) with the GTRGAMMA + I model of nucleotide substitution.

### **Pathogenicity test**

Pathogenicity test was carried out as described by Soyong et al. (2005) with some modifications. Two-month-old citrus leaves were used for the assessment of the pathogenicity. Citrus leaves of approximately the same age (the same position on the plant) and with healthy conditions were collected. The selected leaves were clipped at the base and apex. The leaves were surface-sterilized by soaking in 10% Clorox for 3 min and rinsed 3 times with sterile distilled water. Each citrus leaf was wounded with syringe needle (5 punches in each wound). Then, mycelial plugs of the growing pathogen were cut by cork-borer and placed on the wounds on the leaves and the treated leaves were incubated in a moist chamber at room temperature (25–28°C) for 7 days. The non-inoculated leaves were treated with sterile agar discs and served as controls. The lesion diameter was recorded and confirmed by re-isolation after 5 days of incubation.

A second pathogenicity test involved root inoculation. The inoculum was prepared following the method of Shang et al. (1999). The seven-day-old culture of the pathogen was flooded with sterile distilled water for two days and sterilized glass spreader was used to wipe the culture surface to release the sporangia into sterile water. Then, the inoculum was adjusted at  $1 \times 10^6$  zoospores/mL. Whole plants of *C. reticulata* were taken out of non-inoculated soils and the roots were washed with tap water. The cleaned roots were dipped in 100 ml of a zoospore suspension. The inoculated plants were individually replanted in nursery bags containing sterile soil. All tested plants were maintained indoor until root and crown rot occurs. Then, the roots were removed from nursery bags. Four replicates were observed and compared with non-inoculated controls.

## **Results**

### **Survey, collection, isolation and morphological analysis**

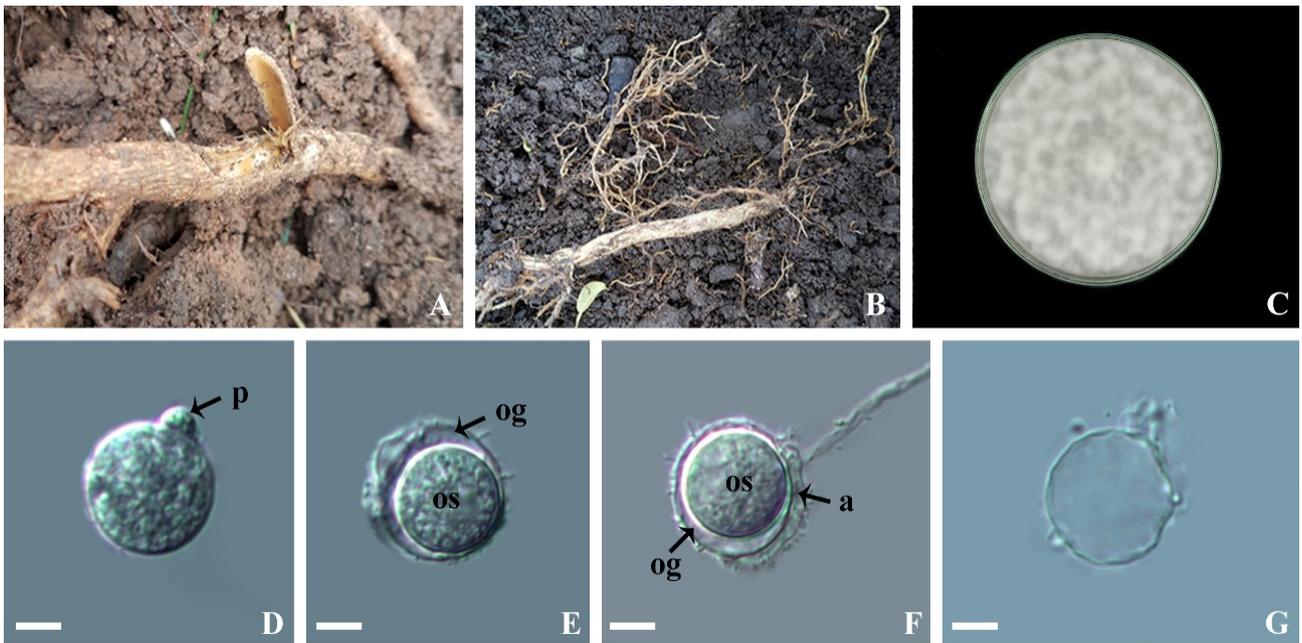
Symptoms of diseased plants included yellowing, rot and gummosis associated with die back and general wilting of the whole plant. Plants with root rot had reduced root systems and the cortex of individual roots disintegrate and leaving the inner stele behind (Fig. 1A–B). Colonies of isolate PS85 grown on PDA showed chrysanthemum and rosette pattern (Fig. 1C). The main hyphae measured 2.0–5.8  $\mu\text{m}$  wide. They were nonpapillate, spherical, ovoid or pyriform, terminal or intercalary,  $20.6 \pm 0.3 \times 17.3 \pm 0.5 \mu\text{m}$ , producing short protruberances (Fig. 1D). The sex organs were produced readily on PDA. The oogonia were spherical, terminal or occasionally intercalary,  $19.7 \pm 0.5 \mu\text{m}$ . Antheridia were irregular, monoclinal, terminal, 1 per oogonium (Fig. 1E–F).

### **Phylogenetic analyse**

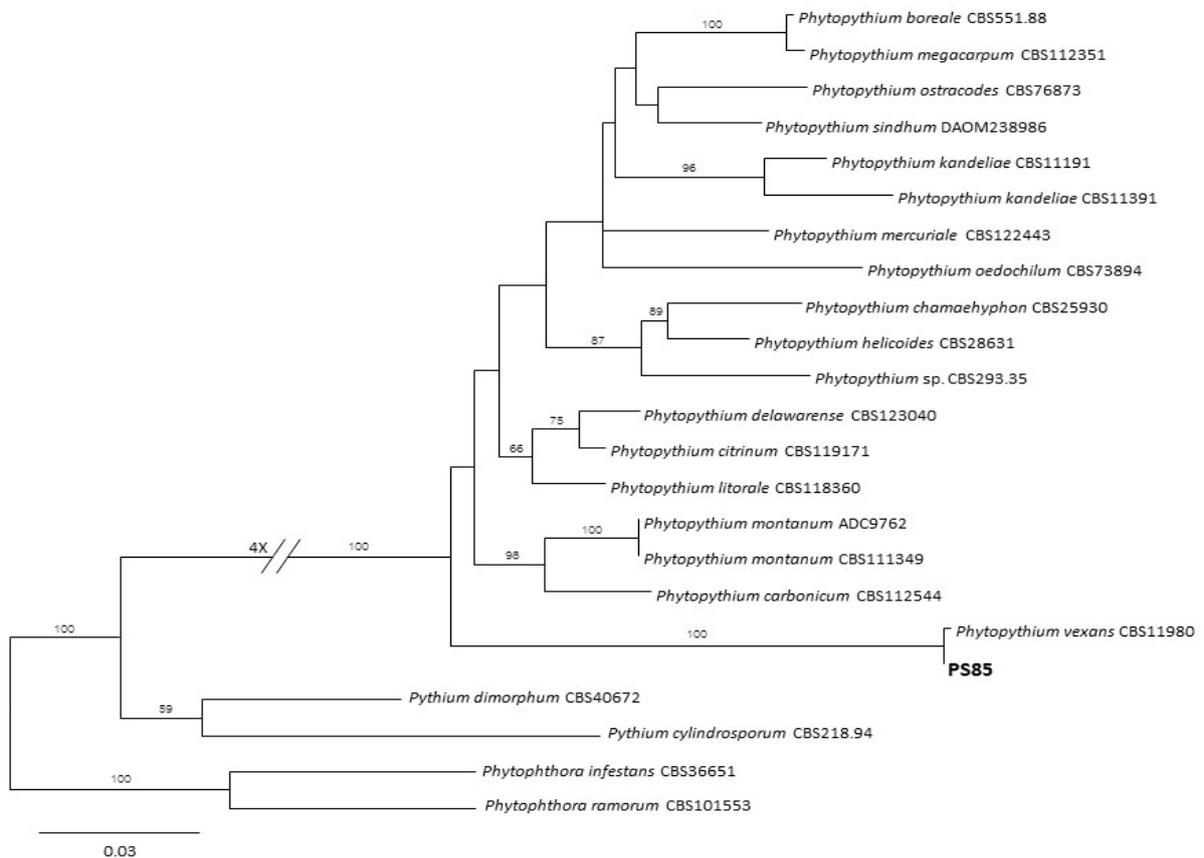
Phylogenetic tree was constructed with the Cox1 dataset comprised 23 strains including the strain from this study (isolate PS85) (Fig. 3). *Phytophthora infestans* (CBS 36651) and *Phytophthora ramorum* (CBS 101553) were used as out-group. In the phylogenetic tree, our strain formed a stable (RAxML 100) clade with *Phytophythium vexans* (Fig. 2).

### **Pathogenicity tests**

In the detached leaf pathogenicity test, *Phytophythium vexans* isolate PS85 showed water-soaked grayish brown lesion expanded around mycelial plug on *C. reticulata* leaves after 3 days. Control leaves remained healthy and showed no symptoms. The result showed the fungus caused disease symptoms on tangerine leaves that were similar to the report of Udompongsuk et al. 2017, which reported pathogenicity test on detached leaves of *C. reticulata* leaves. In addition, the plant with inoculated root system with the mycelial suspension of *Phytophythium vexans* isolate PS85 showed root rot and the cortex of individual roots tended to disintegrate and leaving the inner stele behind 14 days after inoculation.



**Fig. 1** – Symptoms of root and morphology of *Phytophthora vexans* isolate PS85. A–B Symptoms of root on *Citrus reticulata*. C Colony morphology of isolate PS85 on PDA. D Sporangia to show short protruberances (p). E–F Sexual structures to show oogonium (og), oospore (os) and antheridium (a). G Empty sporangia. Scale bars = 10  $\mu$ m.



**Fig. 2** – The ML consensus tree inferred from the Cox1 sequence alignments. The bootstrap support values higher than 50% from 1000 replicates are shown at the nodes. *Phytophthora infestans* (CBS 36651) and *Phytophthora ramorum* (CBS 101553) were used as out–group.



**Fig. 3** – A Inoculation on *C. reticulata* leaves (right), control (left). B, D Control. C, E Root rot and the cortex of individual roots tended to slough off leaving the inner stele behind.

### Discussion

In recent years, root rot disease has been observed in most of the citrus cultivation areas of Thailand and became a serious problem for citrus production (Hung et al. 2015). In this study, *Pythium*-like organism was isolated from the rhizosphere of the symptomatic infected *Citrus reticulata*. Isolated fungi were tested for the pathogenicity on detached leaves of *C. reticulata* as well as the roots. The pathogen was identified based on morphology (Fig. 1) and phylogeny based on Cox1 gene (Fig. 2). The pathogen was identified to be the *Phytopythium vexans*. The morphology of the *P. vexans* is similar to *Pythium vexans* causing patch canker of rubber trees on Hainan Island, China (Zeng et al. 2005). However, more collection is needed to understand the diversity and distribution of *Phytopythium* in Thailand. Furthermore, informative morphological identifications and sequence data for more gene regions are needed for the broad taxonomic and phylogenetic approaches. Hence our study is the first record of *Phytopythium vexans* causing root rot disease on Mandarin (*Citrus reticulata* L. cv. Sainampueng) in Thailand.

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