



## First record of *Phytophthium* sp. causing root and stem rot on *Catharanthus roseus* in Thailand

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### Abstract

During August to October 2019, *Catharanthus roseus* plants with root and stem rot symptoms were collected from a greenhouse which belongs to farmers in Chiang Mai, Thailand. The causal agent was isolated from the roots and rhizosphere soil of *C. roseus* showing symptoms of root and stem rot disease in plantation areas in Chiang Mai, Thailand. The pathogen was studied by morphological characteristics and molecular analysis based on the internal transcribed spacer (ITS) region it was determined to be *Phytophthium* sp. The pathogenicity of the isolate was tested by inoculating detached *C. roseus* leaves with an agar disc containing mycelium of the fungus compared with a sterile agar disc as the control. It was found that *Phytophthium* sp. isolate MCRC-Pp021 infected the *C. roseus* leaves causing a brown rot. Moreover, *Phytophthium* sp. MCRC-Pp021 infected and caused rot symptoms on plant crowns in 14 d after inoculation with a mycelial suspension. This research finding is the first record of *Phytophthium* sp. MCRC-Pp021 causing root and stem rot of *C. roseus* in Thailand. In addition, in a fungicide test, it was shown that dimethomorph and phosphorous acid were less effective against *Phytophthium* sp. MCRC-Pp021 at the recommended rate than mancozeb, propineb, thiram, fosetyl-aluminium, tridemorph and metalaxyl which exhibited high efficacy.

**Key words** – morphological characters – pathogenicity – phylogeny – poison food assay – vinca

### Introduction

The genus *Phytophthium* (Pp) in the family Pythiaceae, order Peronosporales is a recently described genus comprising the species formerly classified in *Pythium* phylogenetic clade K (Bala et al. 2010, de Cock et al. 2015). The common features of this genus are phytophthora-like ovoid to globose sporangia with papillae, internal proliferation, and pythium-like zoospore discharge, which differentiates this genus from the closely related genera *Pythium* and *Phytophthora* (Bala et al. 2010).

*Phytophthium* is an important genus in agricultural production and several species, such as *Phytophthium* sp. was reported to cause diseases on Cassava in Brazil (Boari et al. 2018). *Pp. helicoides* and *Pp. vexans* which is most frequent in tropical and subtropical regions, is a well-

known plant pathogen that has been reported to cause leaf, stem, root and crown rot, damping off and leaf blight in several host plants worldwide (Kageyama et al. 2002, Chellemi et al. 2000, Guha & Hong 2008, Tsukiboshi et al. 2007, Watanabe et al. 2007, Yang et al. 2013, Hong & Moorman 2005, Tao et al. 2011, Kageyama 2014).

*Catharanthus roseus*, also known as Vinca or Madagascar periwinkle, is a flowering potted plant that has become very popular. Growing 25 to 41 cm tall, with dark green leaves and five-petal blossoms in pink, mauve or white, these plants were rapidly adopted especially in the nursery trade. There are several diseases that can cause vincas to wilt, including *Phytophthora* stem blight and root rot, *Pythium* root rot, *Rhizoctonia* stem (*Rhizoctonia solani*) and root rot, gray mold and the tomato spotted wilt. *Phytophthora parasitica*, a soilborne fungus, causes serious losses and death in periwinkle with reports from India and the United States. Symptoms of these diseases include dark colored lesions on the stems and branches, yellowing leaves with stunted growth, spots on the foliage, gray mold on the plant or black concentric rings on the leaves (McMillan & Garofalo 2004). Moreover, *Fusarium* root rot has been reported from Taiwan (Chung et al. 1998). Blight caused by *P. nicotianae* (syn. = *P. parasitica*) is one of the most damaging diseases of plants in Florida. Other known blights include twig blight caused by *Colletotrichum dematium* in Florida, foliar blight brought about by *P. tropicalis* in Virginia (Hao et al. 2010), and gray mold blight caused by *Botrytis cinerea* in the United States, Italy, and Taiwan (Garibaldi et al. 2009). In addition, previous research in Thailand it was found that *P. deliense* causing root and crown rot on *Catharanthus roseus* (Intaparn et al. 2019). Many of these diseases and fungal pathogens are prevalent in soil that has poor drainage or gardens that have been over-watered. Chemical compounds are commonly used to control plant diseases, but their incorrect use has favored the development of pathogens resistant to fungicides, and resulted in non-target effects. In the present study, our objectives were to identify the causal agent of root and stem rot in *C. roseus* and pathogen sensitivity to fungicides

## Materials & Methods

### Isolation and Identification of Pathogens

Isolation of pathogen was carried out by using a baiting technique. The soil samples were put in sterilized Petri dishes and sterile distilled water (SDW) was poured over the soil to a level of 4-5 mm. Thereafter, fleshy leaves of *C. roseus* were cut in 5 x 5 mm pieces, then surface disinfected by immersing in 10% sodium hypochlorite (Clorox), and rinsed with SDW. Ten leaf discs were then put into each Petri dish containing soil samples. The baiting plates were incubated at room temperature (27-30°C) for 2 to 3 d. The mycelium was picked out and transferred to plates containing water agar (WA) which were incubated at room temperature. Finally, the hyphal tips were transferred to potato dextrose agar (PDA) to get pure cultures. The colony growth was also periodically examined under a stereomicroscope and fungal structures, e.g. sporangia and oospores, were examined with a compound microscope. The assessment of growth rates for the isolates was conducted on PDA, V8 agar (V8A) and Potato-Carrot agar (PCA) (Van der Plaats-Niterink 1981) in 90 mm petri dishes incubated at room temperature (25-28°C). Hyphal growth was recorded every 24 hr for 3 d. Moreover, the growing fungus on PDA was also cut into small piece and put into SDW in Petri dishes and incubated overnight at room temperature to observe sporangia and zoospores. Pure cultures were kept in PDA slants for stock cultures and for further experiments. Isolates were identified by comparing colony growth patterns and morphological features: sporangia, oogonia, antheridia, hyphae and zoospores using genus and species descriptions reported in the literature (Erwin & Ribeiro 1996).

### Molecular Studies

#### DNA Extraction and Sequencing

Mycelia of the *Phytophthium* isolates harvested from liquid culture were used and DNA

extraction was done by the CTAB method modified by Weising et al. (2000). The ITS region was amplified using universal primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). Amplification was done with the following temperature cycling parameters: denaturation at 95°C for 3 min for the first cycle and 30 sec each for subsequent cycles, annealing for 1 min at 55°C, and elongation for 1 min at 72°C. The purified PCR products were sent for sequencing by Biogenomed Co., Ltd. ITS rDNA sequences of the pathogenic fungi were aligned with reference sequences of *Phytophthium* sp. and the dataset was analysed by MEGA7 using Neighbour-joining (NJ) parameters and bootstrap values with 1,000 replicates (Saitou & Nei 1987).

### **Pathogenicity Test**

The pathogenicity test was carried out by using Koch's postulates to confirm the pathogenic isolates (Agrios 1997). The tests were carried out with the detached leaf method.

Pathogenicity test was carried out as described by Soyong et al. (2005) with some modifications. One-month-old *C. roseus* leaves were used for the assessment of pathogenicity. For each isolate, healthy *C. roseus* leaves of approximately the same age (the same position on the plant) were collected, clipped at the base and apex, and surface-sterilized by soaking in 10% Clorox for 3 min and rinsed 3 times with SDW, and then each leaf was wounded with a syringe needle (5 stabs in each wound site). Then, agar culture discs (0.5 cm diameter) containing mycelium of the growing pathogen were cut by cork borer, and placed onto the wounded leaf surface and incubated in a moist chamber at room temperature (27-30 C) for 3 d. 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 3 d of incubation.

Root inoculation was involved for a secondary pathogenicity test. 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 2d and sterilized glass spreader was used to wipe the culture surface to release the sporangia into sterile water. Finally, the inoculum suspension was adjusted at  $1 \times 10^6$  zoospores/ml. The bare roots were prepared by taking out the whole healthy plants of *C. roseus* from soils. The roots were washed with tap water and dipped in the suspension. Then the test plants were replanted in plastic nursery bags containing sterile soil and maintained in greenhouse until the disease occurs. Then, the whole plants were taken out again. Four replicates were observed and compared with non-inoculated controls.

The Disease Index (DI) was determined as follows: Level 1 = healthy, green leaves (normal and non-aggressive), Level 2 = pale green, Level 3 = yellow leaves, Level 4 = pale brown (moderately aggressive), Level 5 = dark brown (highly aggressive). The virulence of isolates was determined according to disease index (DI).

### **Fungicide Testing**

The Poison Food Technique (Nene & Thapliyal 1979) was used to test the in vitro efficacy of different concentrations of five systemic fungicides and three contact fungicides (0, 100, 500, 1,000, 1,500 and 2,000 ppm). Different quantities of fungicides were mixed with PDA. Based on active ingredient, the requisite quantity of the test fungicide was calculated, thoroughly mixed separately, autoclaved and cooled (40°C), and mixed with PDA in conical flasks to obtain the desired concentrations. This PDA medium amended separately with the test fungicides was then poured (15 ml / plate) aseptically into Petri plates (90 mm dia.) and allowed to solidify at room temperature. A Completely Randomized Design (CRD) was used for the experiment, with three replications. Uniform inoculum size of pathogen was placed in the centre of petri plates containing the poison PDA. Petri plates filled with plain PDA (without any fungicide) were inoculated with a pure culture disc of *Phytophthium* sp. MCRC-Pp021 and maintained as the non-treated control. The petri plates were kept at room temperature and mycelial growth was monitored. Radial mycelial growth/colony diameter were recorded at an interval of 24 h and continued till the non-treated control plates were fully covered. Mycelial growth inhibition of the test pathogen was calculated by

applying the formula of Vincent (1947) (see below). Data were recorded by measuring colony diameter (mm) after incubation.

**Table 1** Fungicides tested to control mycelial growth of *Phytophthium* sp. causing root and stem rot on *Catharanthus roseus*.

No.	Active ingredient	Trade/ product name	Fungicide group	Form	Type
1	mancozeb	Mancozeb	Dithiocarbamates	Wettable powder	Contact
2	propineb	Antracol	Dithiocarbamates	Wettable powder	Contact
3	thiram	Thianozan	Dithiocarbamates	Wettable granule	Contact
4	dimethomorph	Forum	dinnamic acid amides	Wettable powder	Systemic
5	fosetyl- aluminium	Aliette	ethylphosphonates	Wettable granule	Systemic
6	phosphorous acid	Foli- r- fos	Phosphonates	Emulsifiable concentrate	Systemic
7	tridemorph	Calixin	carboxylic acid amide	Emulsifiable concentrate	Systemic
8	metalaxyl	Metalaxyl	Acylalanines	Wettable powder	Systemic

Radial growths of the pathogen was used for calculating percent growth inhibition according to the formula:

$$\text{Percent inhibition of radial growth (\%PIRG)} = \frac{R1 - R2}{R1} \times 100$$

Where

R1 = radial growth of control (mm)

R2 = radial growth of treatments (mm)

## Results

### Isolation and Morphological Identification

Symptoms of diseased plants included yellowing and scorching of leaves, poor growth and stunting of plants, dark brown to black lesions on stems and branches, wilting and death. Plants with root rot had reduced root systems and the cortex of individual roots tended to slough off leaving the inner stele behind. (Fig. 1).

Colony characteristics of *Phytophthium* sp. MCRC-Pp021 included profuse white cottony growth on PDA in a mixed chrysanthemum and rosette pattern, a chrysanthemum pattern on PCA and no special pattern on V8A (Fig. 2).

Mycelium was hyaline, well branched, and the main hyphae up to 5 µm wide, dendroid, and often with short side-branches. Simple sympodia (Fig. 3A) were produced rarely in grass leaf blade cultures. Sporangia were ovoid to globose, terminal, papillate, proliferating internally, and 8.8–14.1 × 8.9–14.8 µm (Fig. 3B). Papillae may develop into discharge tubes (Fig. 3C-F). The zoospore discharge is *Pythium*-like: the cytoplasm flows out of the sporangium through a discharge tube to form a cytoplasm-filled vesicle at the tip (Fig. 3G). The vesicles generally contained ~25 zoospores and the zoospores were 2.5–3 µm in diameter (Figure 3H). The sporangia proliferated internally

(Fig. 3I, J). The isolates were tentatively identified as *Phytophthium* sp. based on the morphological characteristics descriptions in Van der Plaats-Niterink (1981) and Bala et al. (2010).

## Phylogenetic Analyses

### Amplification and characterization of the rDNA ITS sequences

PCR amplification of the rDNA ITS region of *Phytophthium* isolates using the ITS5-ITS4 primer pairs was successful and gave PCR products of 871 base pairs (bp). The isolate MCRC-Pp021 was identified as *Phytophthium* sp. based on molecular analysis, corroborating the above morphological identification, BLAST analysis of this isolate revealed 100% identity with reference sequences of *Phytophthium* sp. D37 in the GenBank.

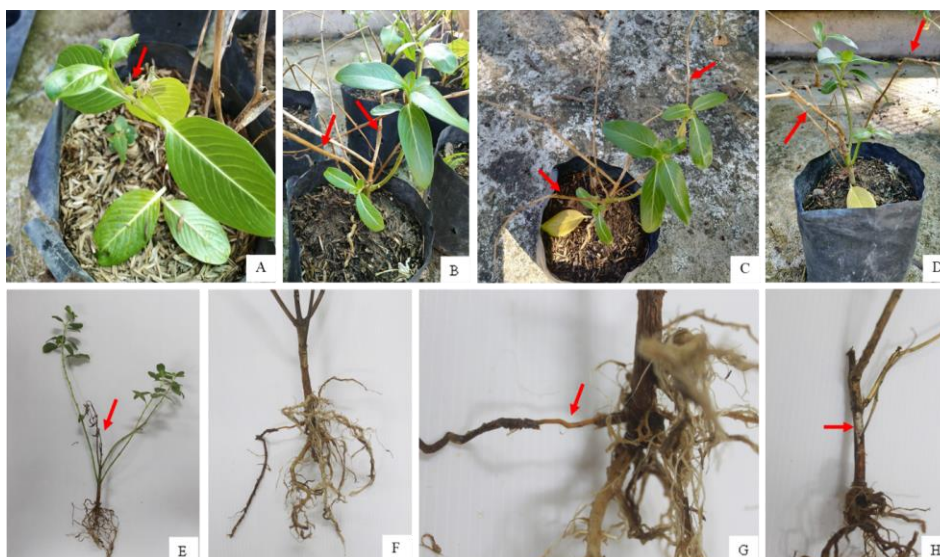
The DNA sequences were analyzed for phylogenetic relationships using MEGA 7 software. Bootstrap analysis was performed with 1,000 replications to determine the support for each group. The phylogenetic tree shows that the isolate MCRC-Pp021 was well embedded within comparative *Phytophthium* sp. isolates with string support at 100%. (Fig. 4)

### Pathogenicity Tests

In the detached leaf pathogenicity test, *Phytophthium* sp. isolate MCRC-Pp021 caused brown lesions (mean of 15.5 mm) on *C. roseus* leaves after 33 hr. Control leaves remained healthy and showed no symptoms. Moreover, the plants inoculated with the mycelial suspension of *Phytophthium* sp. showed dark brown lesions on stems and branches, and wilt symptoms in 7 d. Symptoms of root rot included yellowing and scorching of leaves, poor growth and stunting of plants, wilting and death in 14 d. Moreover, plants with root rot had reduced root systems and the cortex of individual roots tended to slough off leaving the inner stele behind. (Fig. 5)

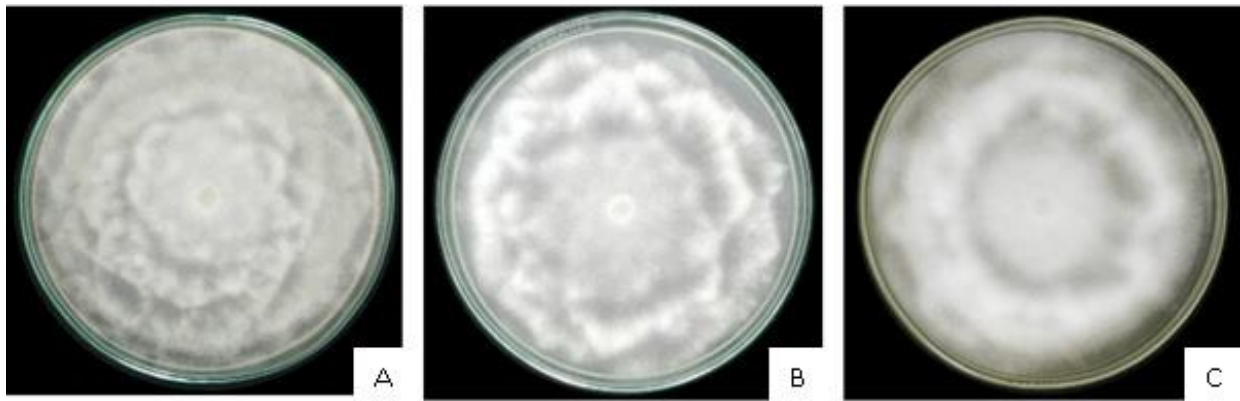
### Fungicide Testing

In poison food assays, mancozeb, propineb, thiram and tridemorph were the most effective in inhibiting mycelial growth of *Phytophthium* sp. isolate MCRC-Pp021 (Table 2). Mancozeb, propineb, thiram and tridemorph caused 100% mycelial growth inhibition at all concentrations tested (100, 500, 1000, 1500 and 2000 ppm).

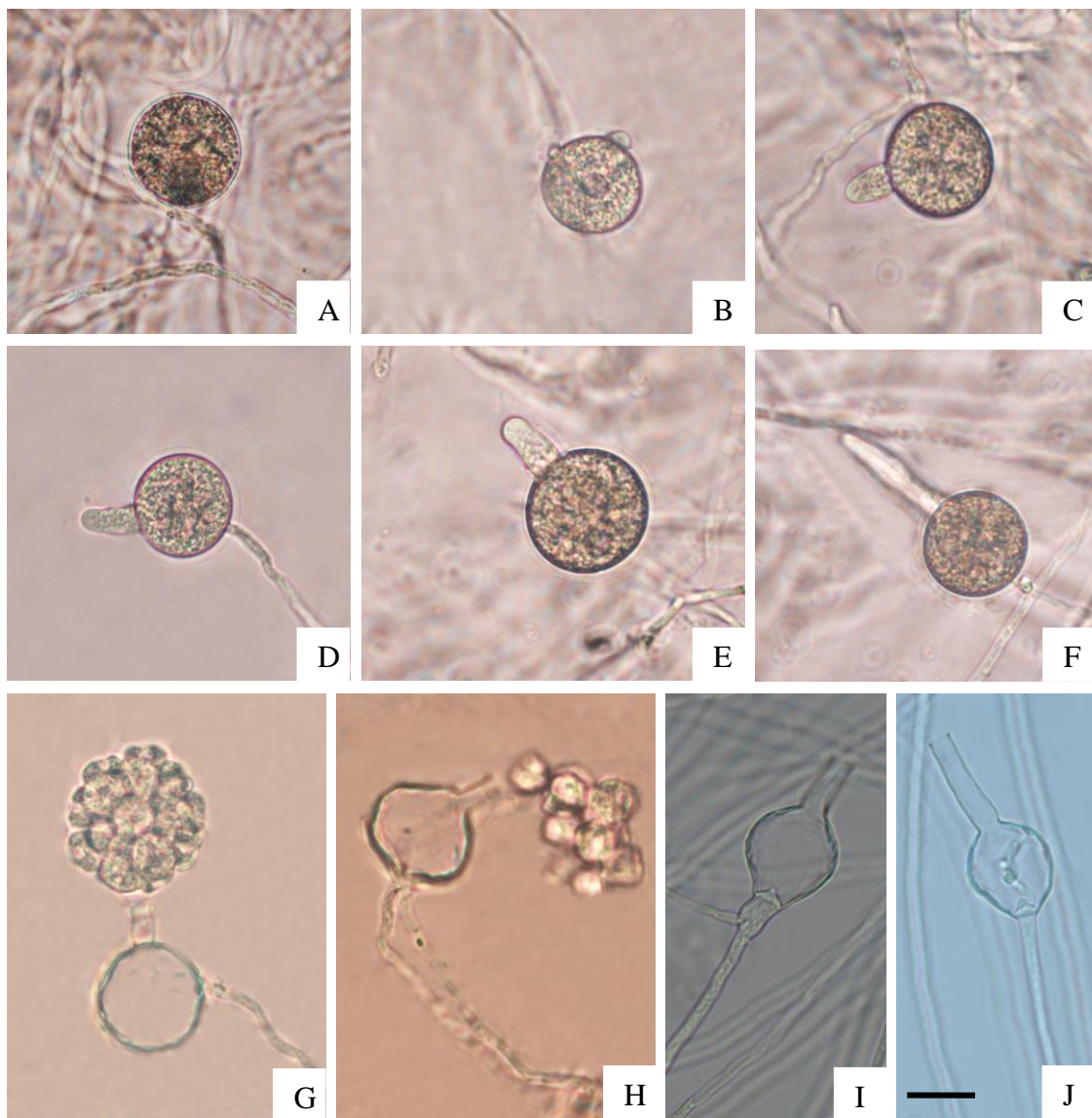


**Fig. 1** – Symptoms of root and stem rot associated with *Pp. helicoides* on *Catharanthus roseus* seedlings in the nursery. (A-B) stem rot, crown rot and falling leaves, (C) progressively dying scions on living rootstocks, (D-E) dead plants in a later stage, (F-G) root rot in plant showing orange discoloration of the crown area and rotted roots and root tips (H) aerial mycelia on the diseased stems. Arrows indicated lesions (A, B), diseased plants (C-F) or aerial mycelia (H)

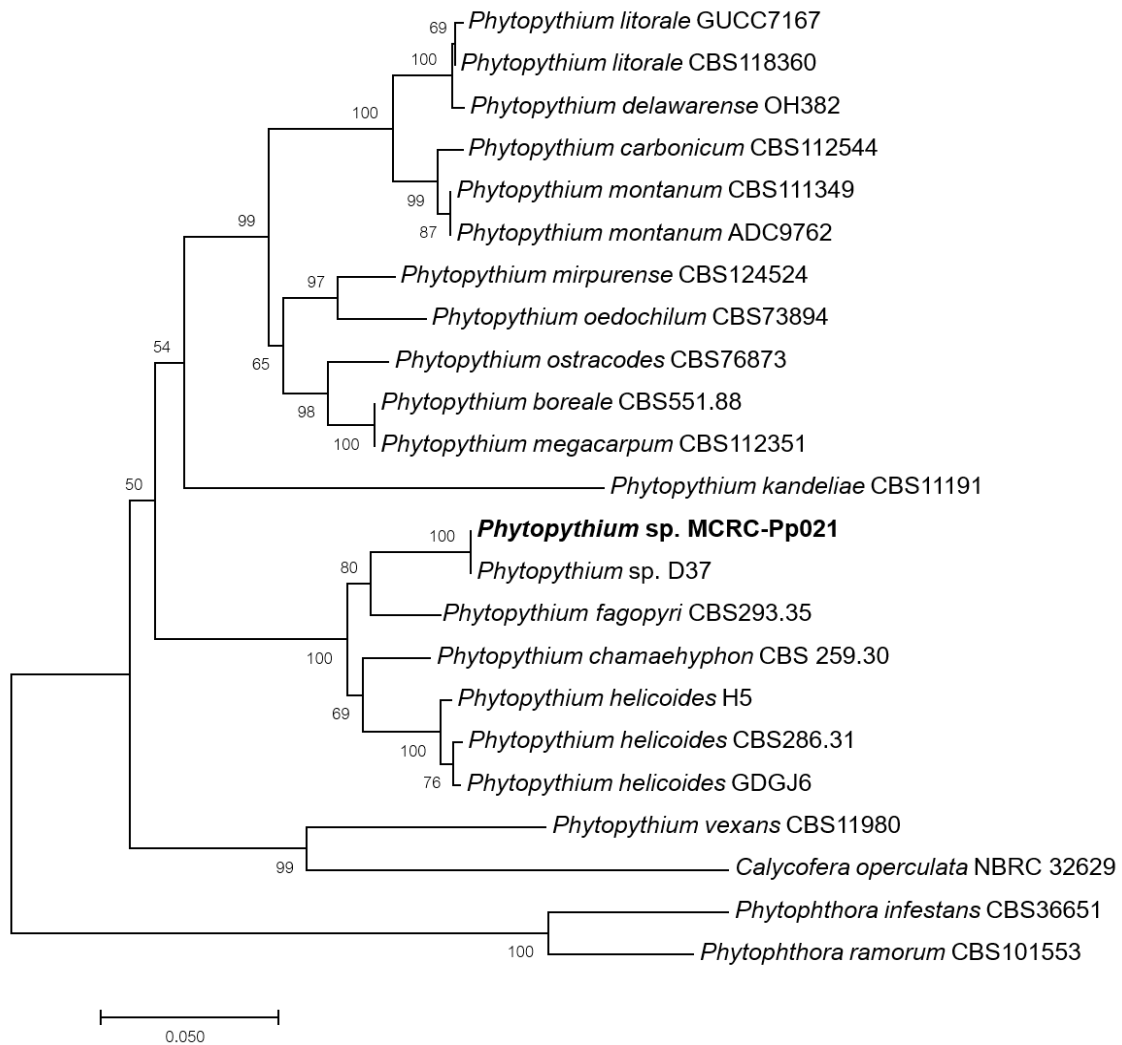




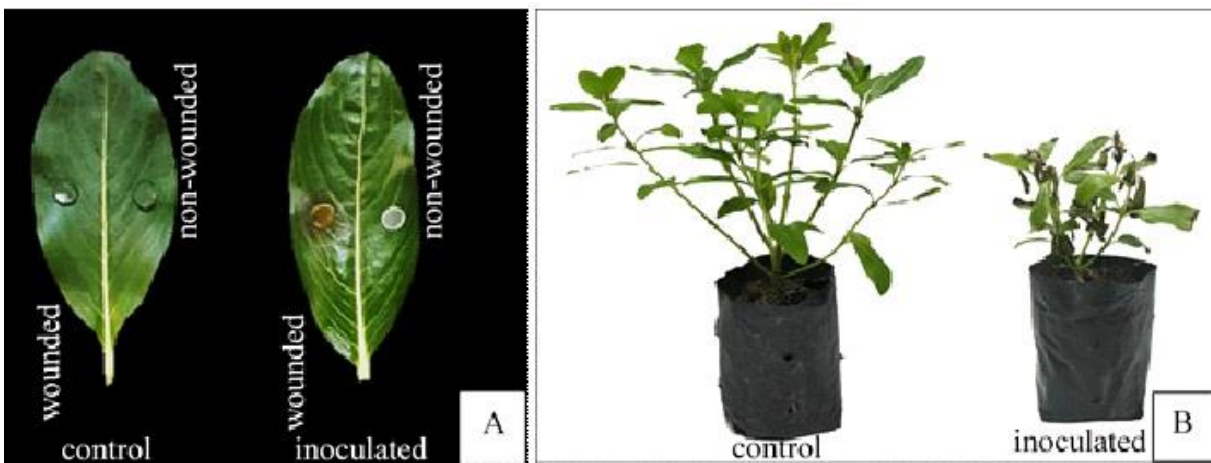
**Fig. 2** – Colony morphology of *Phytopythium* sp. isolate MCRC-Pp021 on (A) PDA, (B) PCA, and (C) V8A



**Fig. 3** – Morphological characteristics of *Phytopythium* sp. Asexual structures of *Phytopythium* sp. MCRC-Pp021 in water: A Simple sympodia. B Sporangium with two papillae. C, D Pyriform sporangia. E, F Outgrowing papilla. G Sporangium vesicle with zoospores. H Zoospore releasing from vesicle. I, J Empty sporangia with internal proliferation. Scale bar = 10  $\mu$ m.



**Fig. 4** – Neighbor-joining (NJ) consensus phylogenetic tree obtained from ITS rDNA sequences of *Phytophthora* sp. isolates using the MEGA 7.0 program. Bootstrap values are indicated on the branches (1000 replications)



**Fig. 5** – A The lesion on detached leaves 24 hr after inoculation. B stem rot, crown rot and falling leaves

At concentrations tested, dimethomorph was less effective, and inhibited mycelial growth of *Phytophthium* sp. isolate MCRC-Pp021 by 31.94–52.64%, while fosetyl- aluminium inhibited mycelial growth by 39.31–100%, phosphorous acid by 47.50-86.11%, and metalaxyl by 83.05-100% at 1000 ppm (Table 2).

**Table 2** Percent inhibition of mycelial growth of *Phytophthium* sp. isolate MCRC-Pp021 exposed to eight fungicides at different concentrations using the poison food assay.

Type	No.	recommended concentration (ppm)	Inhibition of mycelial growth (%)					
			Concentration (ppm)					
			0	100	500	1,000	1,500	2,000
contact fungicide	1	1,200	0.00	100.00	100.00	100.00	100.00	100.00
	2	1,750	0.00	100.00	100.00	100.00	100.00	100.00
	3	200	0.00	100.00	100.00	100.00	100.00	100.00
systemic fungicide	4	500	0.00	31.94	37.50	39.44	40.28	52.64
	5	2,000	0.00	39.31	66.39	76.67	88.89	100.00
	6	100	0.00	47.50	71.25	78.74	80.28	86.11
	7	187.5	0.00	100.00	100.00	100.00	100.00	100.00
	8	125	0.00	83.05	90.56	100.00	100.00	100.00

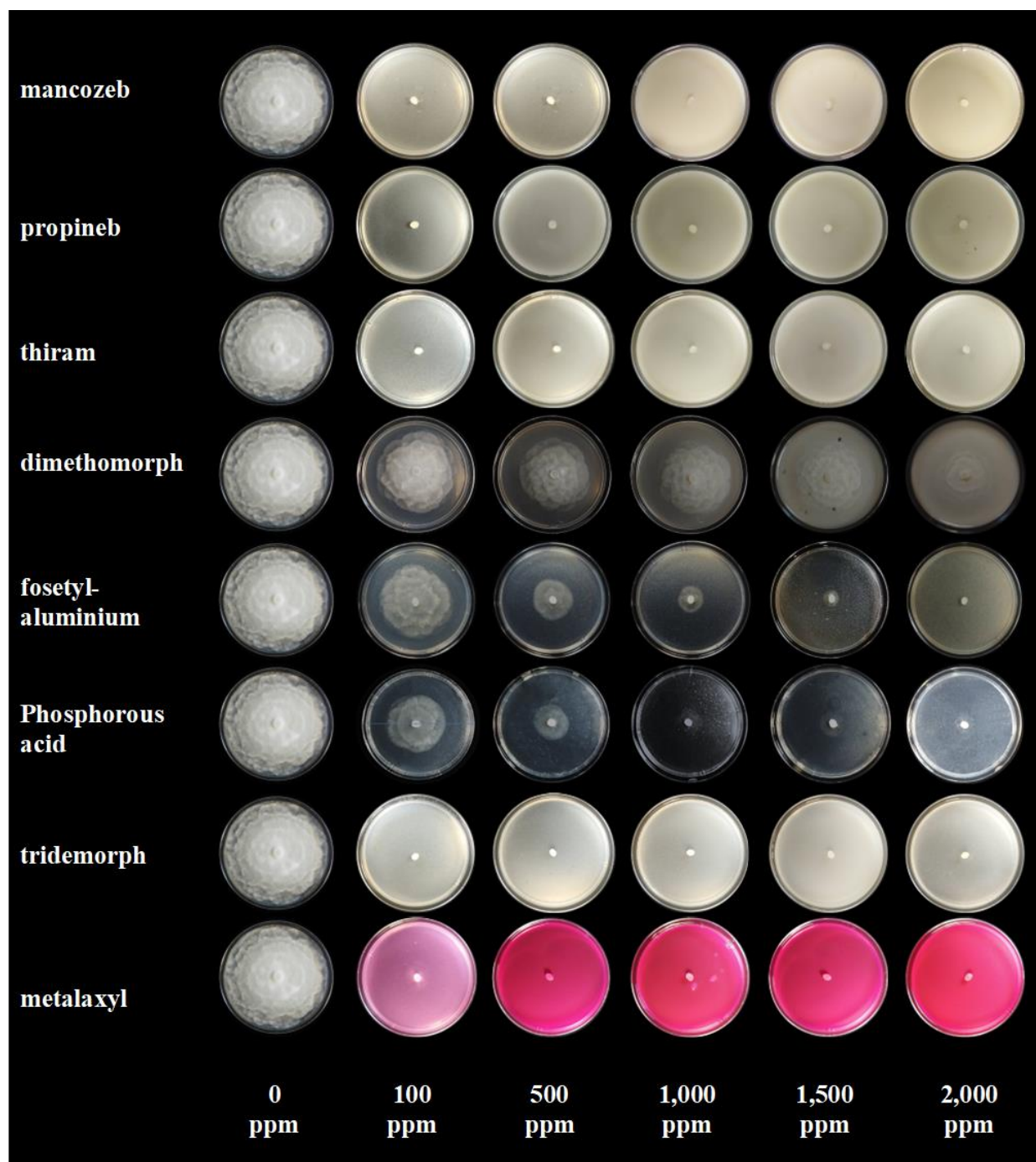
Note: 1 = mancozeb, 2 = propineb, 3 = thiram, 4 = dimethomorph, 5 = fosetyl- aluminium, 6 = phosphorous acid, 7 = tridemorph, 8 = metalaxyl

## Discussion

The pathogen was isolated from soil in the rhizosphere of *C. roseus* showing root and stem rot disease. Isolated fungi infected detached leaves of *C. roseus* as well as the roots and crowns. The fungi were identified based on morphology and confirmed to be *Phytophthium* sp. by ITS rDNA sequencing analysis comparing to the reference sequences it was showed that *Phytophthium* sp. MCRC-Pp021 was distinct from other known species but similar to *Phytophthium* sp. D37 isolated from sediment sample in Virginia (Yang & Hong 2016). It maybe conduced both of two strains are new species. However, more information on distinct characters of morphology are needed to study and sequence of another gene may be needed for molecular analysis. Moreover, it has never been reported on *C. roseus* in Thailand. To our knowledge, this present study represents the first report of *Phytophthium* sp. causing root and stem rot on *C. roseus* in Thailand.

Simultaneously, understanding the sensitivity of the pathogen to fungicides is necessary to provide farmers with a control strategy for root and stem rot of *C. roseus*. Phenylamides (PAs), carboxylic acid amides (CAA), phosphonates and dithiocarbamates are the major fungicide groups used to control the oomycete, fungus-like eukaryotic microorganisms (Gisi & Sierotzki 2015). This study showed that dimethomorph and phosphorous acid seem to be less effective against *Pp. helicoides* even used at recommended rates than mancozeb, propineb, thiram, fosetyl-aluminium, tridemorph and metalaxyl which exhibited high efficacy. This information might be useful to effectively control *C. roseus* root and stem rot in future.





**Fig. 6** – Percentage inhibition on of mycelial growth of *Phytophthium* sp. isolate MCRC-Pp021 exposed to eight fungicides at different concentrations using poison food.

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