



## First report of *Pythium deliense* causing root and crown rot on *Catharanthus roseus* in Thailand

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### Abstract

*Pythium* sp. was isolated from the roots, crowns and rhizosphere soil of *Catharanthus roseus* (L.) G. Don. (common name; vinca) showing symptoms of root and crown rot disease in plantation areas in Chiang Mai Thailand. The pathogen was studied by morphological and molecular analysis based on internal transcribed spacer (ITS) sequence. The *Pythium* isolate was confirmed as *Pythium deliense*. The pathogenicity of the isolate was tested by inoculating with an agar disc containing mycelium of the fungus on detached *C. roseus* leaves compared with an agar disc alone as the control. It was found that *P. deliense* infected the vinca leaves causing a brown rot. Moreover, *P. deliense* infected and caused rot symptoms on plant crowns 5 d after inoculation with a mycelial suspension. This is the first report of *P. deliense* as pathogenic to *C. roseus* in Thailand.

**Key words** – Pythiaceae – Phylogeny – Soil betting – Vinca

### Introduction

*Catharanthus roseus* (L.) G. Don (Apocyanaceae) also known as Madagascar periwinkle, bright eyes, cape periwinkle, graveyard plant, old maid, pink periwinkle, rose periwinkle or vinca, has been cultivated as a major colour crop for landscapes and as an herbal medicine. This plant performs well in dry, warm locations with full sun or partial shade (Verpoorte et al. 1997, Van der Heijden et al. 2004).

*Pythium* species are fungal-like organisms and are the common causes of seed rot, damping-off and root rot which are some of the most destructive and economically important agricultural problems worldwide in nursery and greenhouse crops (Alhussaen et al. 2011). Some species of *Pythium* cause significant diseases on several important, mostly herbaceous, crops. (Agrios 2005). Furthermore, some species require, moist and cool conditions (such as *P. irregulare* and *P. ultimum*) while others are most severe at higher temperatures (such as *P. aphanidermatum* and *P. myriotylum*) Plant diseases caused by *Pythium* are divided into two types: diseases that affect plant parts in contact with the soil (roots, lower stems, seeds, and tubers) and diseases that affect above ground parts (leaves, young stems, and fleshy fruits) (West et al. 2003, Agrios 2005). On *C. roseus*,

*Pythium* caused a waning of popularity of the plant by causing *Pythium* aerial blight and severe browning of stems and crowns. A few reports indicated that *P. ultimum* and *P. aphanidermatum* cause damping-off, root and crown rot disease in *C. roseus* (Burns & Benson 2000). Morphologic identification of *Pythium* species most often is based on the size and appearance of sexual reproductive structures (oogonia and antheridia) (Van der Plaats-Niterink 1981). Certain techniques which provide faster and more accurate identification include molecular tools such as PCR and sequencing (Baldauf 2008, Crous et al. 2003). The internal transcribed region (ITS) of the ribosomal nuclear DNA and the nucleotide sequence of this region is commonly used for identification of *Pythium* species (Nechwatal et al. 2005). Therefore, the objectives of this study were to isolate, and identify the *Pythium* species causing root and crown rot diseases of *C. roseus* in Thailand.

## **Materials & Methods**

### **Isolates and morphology**

Roots, crowns and rhizosphere soil were collected from diseased *C. roseus* plants showing symptoms of wilt and root rot in a greenhouse in Chiang Mai, at the beginning of November, 2018. All the samples were kept in individual new plastic bags, and brought to the laboratory at the Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand for isolation of the pathogen. 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 water (1/4 w/v) and baited with mature and healthy leaves of *C. roseus*. The leaves were washed with tap water, soaked in 70% ethanol, rinsed in sterile water and cut into 1x1 cm pieces. The pieces of leaves were floated on the soil solution. After 1-2 d of incubation at room temperature, discoloured baits were blotted on sterile paper towels to remove excess water and plated onto water (WA). Two days later, hyphal tips growing from the baits were cut under a stereo microscope and transferred to potato dextrose agar (PDA) for pure culture isolation. Cultures were stored on PDA slants under sterile paraffin oil for further identification and maintenance.

### **Morphological studies**

The assessment of growth rates for the isolates of *Pythium* spp. was conducted on PDA, 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. The morphology of sporangia including shape, size and presence or absence of papillae were recorded and photographed (Drenth & Sendall 2001). Investigation of sporangial development was based on agar discs which were cut from the edge of actively growing colonies on PDA, and floated in sterile distilled water for 24 hr at room temperature for production of sporangia, zoospores, and sexual structures. Identification up to the species level was based on the criteria of Ho (2011).

### **Molecular studies**

#### **DNA extraction**

DNA extraction was performed using the CTAB protocols as modified by Weising et al. (2000). Mycelia of the *Pythium* isolates were grown in 50 ml test tubes containing 20 ml PDB (Potato dextrose broth) at 28°C on an orbital shaker (180 rpm) for 3 d. The mycelium was harvested by filtration. Excess water was removed from the mycelium by pressing in a sterile paper towel. A mycelial mat was placed in a prechilled mortar, frozen with liquid nitrogen, and ground to a fine powder. Mycelial powder 0.05 g was suspended in 500 µl CTAB buffer (cetyltri-methyl-

ammonium) bromide), vortexed and incubated at 65°C for 30 min, and 250 µl phenol and chloroform isoamyl alcohol (CIA) was added. The samples were then centrifuged at 13,000 rpm for 10 min. The aqueous phase was transferred to a new microtube and repeat the CIA extraction was repeated. After the second CIA, twofold ethanol was added, the samples were incubated at -20°C for 1 hr. centrifuged at 13,000 rpm for 10 min and washed with 50-60 µl 70% ethanol and centrifuged twice at 13,000 rpm for 10 min. DNA pellets were dried in an incubator at 37°C before dissolving with TE buffer and storing at -20°C.

### **ITS rDNA sequencing and analysis**

Molecular identification was performed by sequencing the ITS region. Amplification of the ITS regions was done using 40 µl PCR reaction each containing 5 µl genomic DNA 50 ng, PCR buffer 1x, dNTP 0.2 mM, 10 pmole of each primer, MgCl<sub>2</sub> 1 mM and *Taq* polymerase 1 unit, 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. To assess the efficiency of the amplification, 5 µl PCR products were electrophoresed in a 1% agarose gel in 1X TAE buffer. The remaining volumes of the PCR amplicons were purified and sequenced by Biogenomed Co., Ltd

ITS rDNA sequencing of the pathogenic fungi was aligned with reference sequences of *Pythium* spp. obtained from the GenBank database. The dataset was analysed by using MEGA7. Neighbour-joining (NJ) phylogenetic analysis was conducted and clade stability was assessed in a bootstrap analysis with 1,000 replicates.

### **Pathogenicity test**

One pathogenicity test of isolates used the detached leaf method (Vawdrey et al. 2005). *Catharanthus roseus* leaves were rinsed with sterile water and surface sterilized with 70% ethanol. Wounds were created by puncturing the leaves in one spot five times with a sterile needle. Four detached *C. roseus* leaves were immediately wounded two times. Before inoculation, isolates were cultured on PDA at 25°C for 7 d. Then, the PDA containing mycelia was cut into 0.5 mm diameter discs with a sterilized cork borer and were inoculated on the *C. roseus* leaves. Leaf discs inoculated with PDA alone served as controls. After that, each leaf was incubated in a moist chamber box at room temperature for 24 h. The diameter of the brown rot around wounds was measured and photographed.

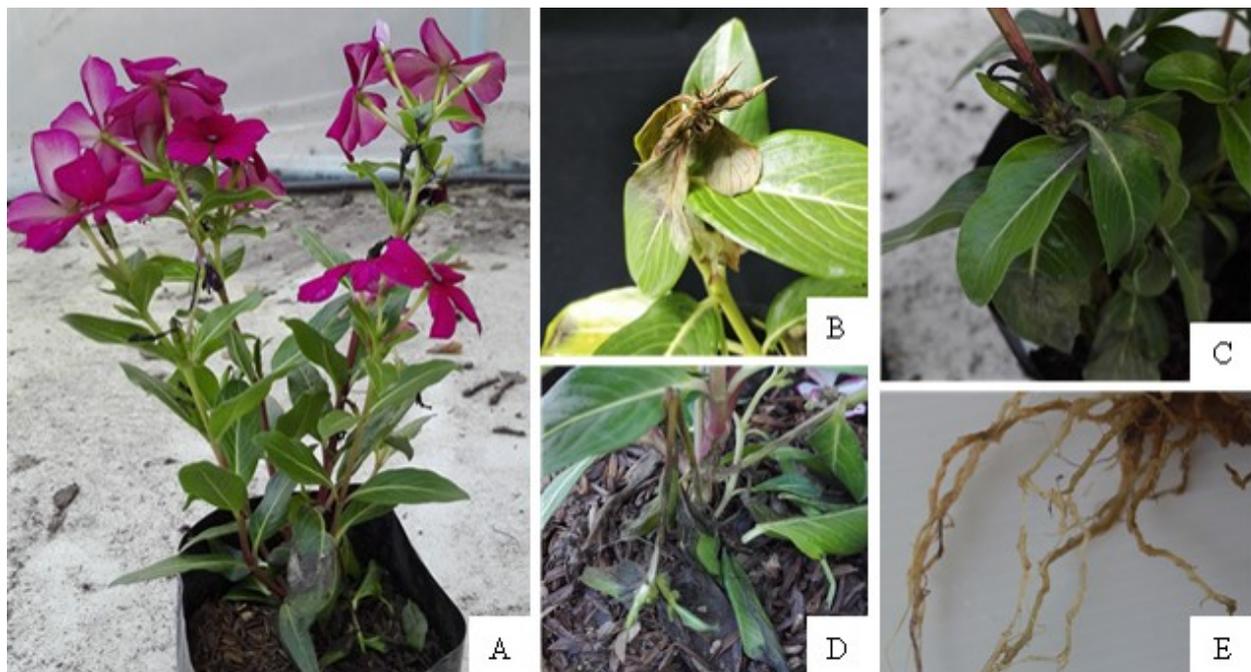
A second pathogenicity test involved root inoculation. The inoculum was prepared by the modified method of Shang et al. (1999). The seven-day-old culture was flooded with 20 ml sterile distilled water for 48 hr. and aflame sterilized glass spreader was used to rub the colony surface to dislodge the sporangia into sterile distilled water. Then, the inoculum was adjusted at 1×10<sup>6</sup> zoospores/mL (chern et al. 1998). Whole plants of *C. roseus* 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 then 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**

### **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)

The colony morphology of isolates on PDA was obviously different from that on PCA and V8A, for which the edges were irregular. They produced an indistinct chrysanthemum pattern with radial mycelium on PDA. Colonies on PCA formed a radiate pattern with very little loose aerial mycelium, and on V8A formed thick cottony aerial mycelium. Main hyphae were up to 10  $\mu\text{m}$  wide; sporangia mostly terminal, sometimes intercalary and consisted of inflated structures, and lacked papillae. Oogonia were terminal, globose, smooth, 19.8.0–23.4  $\mu\text{m}$  in diameter, oogonial stalks were mostly curved towards the antheridia; antheridia often intercalary; oospores aplerotic, 16.1–18.5  $\mu\text{m}$  in diameter, oospore wall up to 2.0  $\mu\text{m}$  thick (Fig. 2); and zoospores formed at 25–30°C.



**Fig.1** – Symptoms observed on affected *C. roseus*. A Wilt and sudden death of plants. B, D stem rot, crown rot and falling leaves. E root rot in plant showing orange discoloration of the crown area and rotted roots and root tips.

## Molecular identification and sequence analysis

### Amplification and characterization of the rDNA ITS sequences

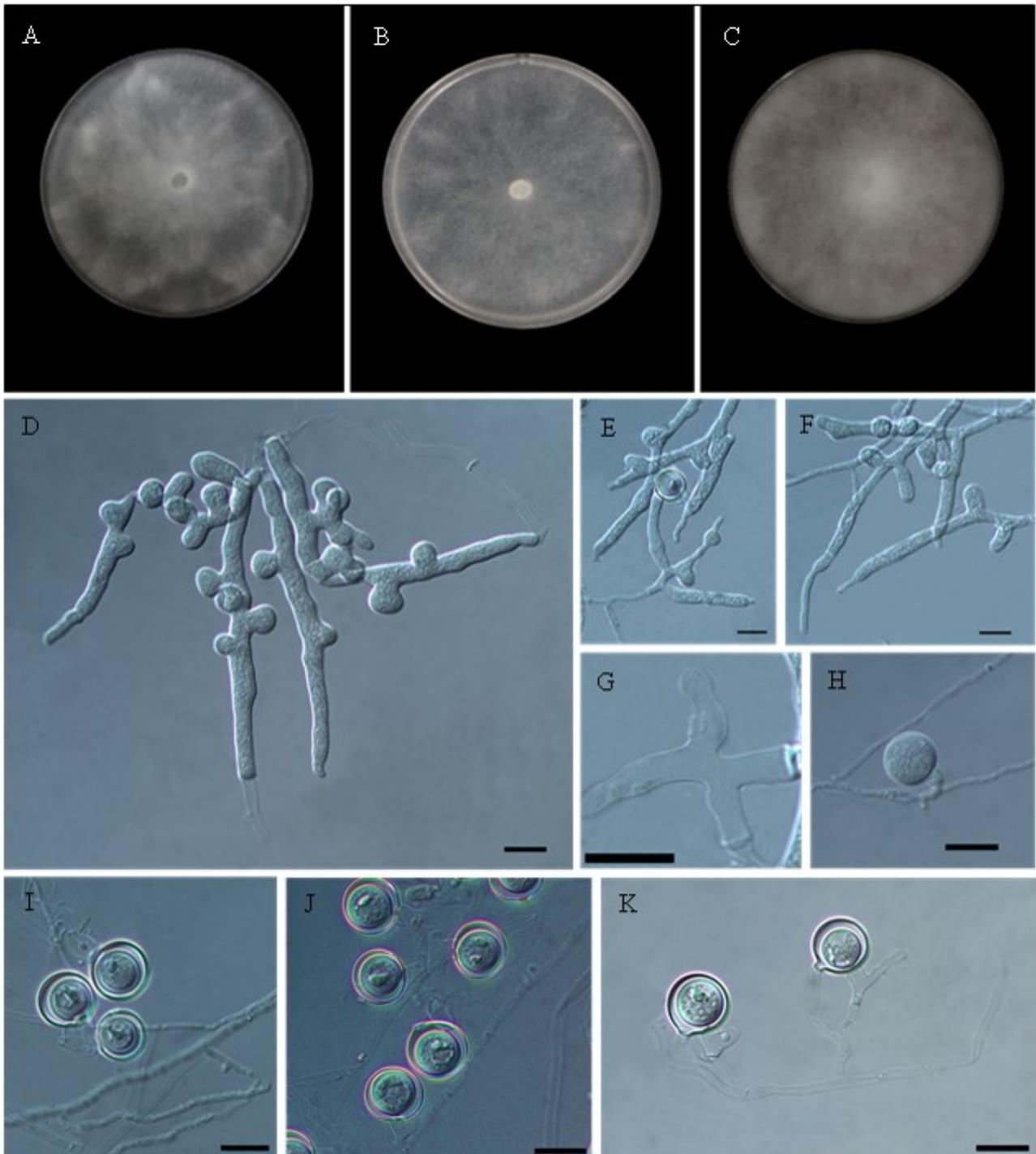
PCR amplification of the rDNA ITS region of *Pythium* isolates using the ITS5-ITS4 primer pairs was successful and gave PCR products of 871 base pairs (bp). The isolate MCRC-P005 was identified as *Pythium deliense* based on molecular analysis, corroborating the above morphological identification, BLAST analysis of this isolate revealed 100% identity with reference sequences of *P. deliense* 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-P005 was well embedded within comparative *P. delicense* and separated from *P. aphanidermatum* with strong support at 100%. (Fig. 3)

### Pathogenicity tests

In the detached leaf pathogenicity test *P. deliense* isolate MCRC-P005 caused brown lesions (mean of 17.5 mm) on *C. roseus* leaves after 24 hr. Control leaves remained healthy and showed no symptoms. Moreover, the plants inoculated with the mycelial suspension of *P. deliense* showed dark brown lesions on stems and branches, and wilt symptoms in 2 d. Symptoms of root rot included yellowing and scorching of leaves, poor growth and stunting of plants, wilting and death

in 5 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.4).

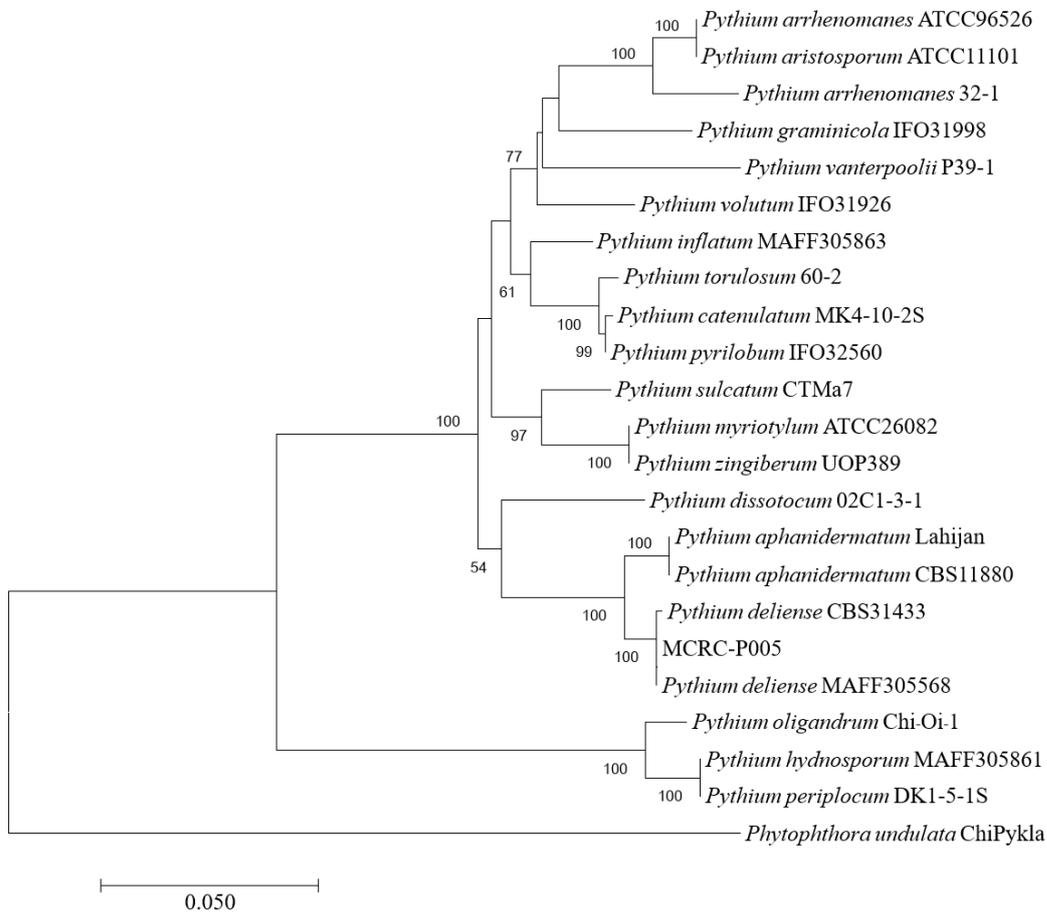


**Fig. 2** – Colony morphology of *Pythium deliense* on (A) PDA, (B) PCA, and (C) V8A. Asexual structures of *P. deliense* in water: (D-F) toruloid sporangia, (G) empty toruloid zoosporangia, (H) young oogonia, (I-K) oogonia with antheridia, (K) bending of oogonial stalks towards the antheridia. Scale bar: 20  $\mu$ m.

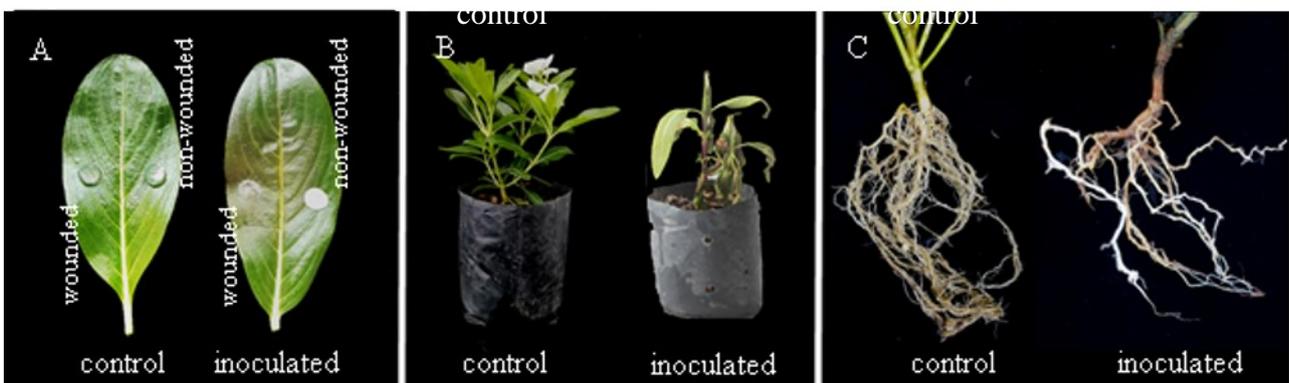
## Discussion

The genus *Pythium* has been classified in the Pythiaceae and Peronosporales (Uzuhashi et al. 2010). *Pythium* species are spread worldwide (Paul 2004). Morphologically, the sporangia are globose shaped without papillae, and produce zoospores in a vesicle. Zoospores in the sporangium

are released when the vesicle breaks (Burgess et al. 2008). There are two species of *Pythium* including *P. aphanidermatum* and *P. deliense* that are characterized by the presence of toruloid sporangia, aplerotic oospores and intercalary antheridia (Dick 1990). Isolate MCRC-P005 differed from *P. aphanidermatum* by its simple and less complicated sporangia as well as the bending of oogonial stalks towards the antheridium, which clearly designates our isolate as *P. deliense* (Ho 2011).



**Fig. 3** – Neighbor-joining (NJ) consensus phylogenetic tree obtained from ITS rDNA sequences of *P. deliense* isolates using the MEGA 7.0 program. Bootstrap values are indicated on the branches (1000 replication)



**Fig. 4** – (A) The lesion on detached leaves 24 hr after inoculation, (B) stem rot, crown rot and falling leaves (C) rot of small roots and rotted outer root layers which easily striped off after 5 d of inoculation

*P. deliense* is a common species and typically a plant pathogen of warm regions (Abdelzaher 1999). It was also recorded in Nicaragua and Papua-New Guinea from tobacco (Drechsler 1960, Stamps et al. 1972); in India from tomato, *Phaseolus aureus*, *Tephrosia vogelii* and ginger (Singh & Srivastava 1953, Ragunathan 1968, Pandotra et al. 1971, Haware & Joshi 1974); in Malaysia from *Carica papaya*, *Lactuca indica*, *Momordica charantia* and *Vigna sinensis* (Liu 1977); and in Pakistan from betelvine rhizosphere. In Oman, *P. deliense* has been isolated from muskmelon and has been reported to cause sudden collapse of whole crops (Deadman et al. 2007). In addition, previous research demonstrated the pathogenicity of *P. deliense*. It can cause diseases such as root rot and damping-off, stalk and rhizome rot, soft rot, fruit rot and pod rot of many plants all over the world including *Arachis hypogaea*, *Cucumis melo*, *Juglans regia*, and *Glycine max* (Parkunan et al. 2014, De Cara et al. 2008, Ghaderi & Banhashemi 2011, Sung 2003). Moreover, in Thailand, *P. deliense* is a devastating pathogen on many plants, especially on durian (*Durio zibethinus*) and sugar beet, and has been reported since 1935 (Suksiri et al. 2018). Additionally, *P. deliense* has been reported as a pathogen on durian and mungbean (*Vigna radiata*) in Thailand (Singburadom et al. 1994). However, it has never been reported on *C. roseus* in Thailand. To our knowledge, this present study represents the first report of *P. deliense* causing root and crown rot on *C. roseus* in Thailand. Species identification was confirmed by morphology using microscopic observation and molecular phylogeny based on ITS-rDNA sequences.

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