Journal of Agricultural Technology 2015 Vol. 11(8): 1897-1910
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Identifications of *Phytophthora* spp. causing citrus root rots in Thailand

Phung Manh Hung[1, 2,*], Pongnak Wattanachai[1], Soytong Kasem[1] and Supattra Poeaim[3]

[1]Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand
[2]Northern Mountainous Agriculture and Forestry Science Institute (NOMAFSI), Phutho 293840, Vietnam
[3]Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok 10520, Thailand

Phung M.H., Pongnak W., Soytong K. and Poeaim S. (2015). Identifications of *Phytophthora* spp. causing citrus root rots in Thailand. Journal of Agricultural Technology 11(8):1897-1910.

Thailand is one of the largest citrus producers in Southeast Asia. Pathogenic infection by *Phytophthora*, however, has become one of major impediments to production. In this research, 3 slow-growth and 3 fast-growth isolates of *Phytophthora* spp. were obtained from samples in Chancheng Sao province and Bangkok, Thailand, respectively. Based on morphological characteristics and ITS ribosomal DNA sequence analysis, the slow-growth isolates were identified as *Phytophthora palmivora*. Meanwhile, the fast-growth isolates were identified as *Phytophthora nicotianae*. All obtained isolates showed high virulence for pomelo seedlings in pathogenicity test. The appearances and virulence of these *Phytophthora* spp. suggested they were causal agents of pomelo root rots in Chancheng Sao and Bangkok in Thailand.

Keywords: *Phytophthora*, citrus root rots

Introduction

Thailand currently, is one of the largest citrus producers in Southeast Asia, with the harvested area in 2013 is estimated at 0.1 million ha, resulting in production of 1.2 million tons of fruit (FAOSTAT 2013). With the prevalence of wet climatic conditions in Thailand, however, infection with *Phytophthora* has become a major problem for the citrus industry, causing yield losses of approximately 6~12% and economic losses of at least 37 million USD/yr (Drenth and Guest 2004).

***Coressponding Author:** Phung Manh Hung, **Email:** manhhungnomafsi@gmail.com

Although *Phytophthora* spp. are responsible for nearly 90 % of collar rots and 70% of all fine root diseases of woody plants, they often are not detected, leading to wrong diagnoses (Tsao 1990). Disease symptoms that caused by *Phytophthora* species often are confused with damages from other pathogens and abiotic agents. Therefore, the heavy losses of citrus and other crops due to *Phytophthora* are often the results of delays in recognition of the organisms as causal agents of the diseases under investigation. Isolation and identification of *Phytophthora* species are the only accurate method of early detection the pathogens, although that are always difficult (Erwin and Ribiero 1996).

This paper presents the isolation and identifications of *Phytophthora* spp. causing root rots pomelo (*Citrus maxima*) in Thailand.

Materials and Methods

Isolations of Phytophthora spp.

Phytophthora spp. were isolated from newly infected roots that taken from pomelo orchards having serious root rot problems, in Thailand. Transplanting methods being described below and selective medium were used to isolate the pathogens (Drenth and Sendall. 2001).

Newly infected roots were washed under running water to remove soil and other debris. The areas contained both healthy and diseased tissue on the roots then were aseptically cut into small pieces (approximately 2 × 2 mm). The root pieces then were transferred to selective medium PARPH. After incubating in the dark, at room temperature (25 – 30°C) for 2-3 days, materials from the margin of colonies that developed from the root pieces were transferred to plates containing a thin layer of WA medium. Pure cultures using for further studies then were obtained from hyphal tips developed from the colonies grown in the WA medium.

Pathogenicity test

Pathogenicity of each *Phytophthora* isolate was proved by artificial inoculation into roots of pomelo seedlings in order to satisfy Koch's postulate.

Twelve-week-old pomelo seedlings were thoroughly washed to be free of potting mix and then planted in plastic pots (10 × 15 cm) containing infested soil (with 5 propagules of *Phytophthora* sp. per cubic centimeter). Controls were prepared by planting the seedlings in same size pots, containing the sterilized potting medium.

All pots including the controls were maintained in the green house at temperature of about 25-30°C and flooded with water for 24 hr each week.

After 6 wk, the plants were carefully removed from plastic tubes and washed free of potting mix. The root systems were then evaluated on following scales: 0 = all roots healthy; 1 = rotted roots apparent; 2 = obvious root rot, root system small; 3 = severe root rot, taproot necrotic, few new roots; 4 = no healthy root, stem girdled. Evaluations were independently made by two observers and the average rating presented. Additionally, up to 100 root tips of each seedling were rated as rotted or healthy and the data expressed as root rot percentage. The pathogens then were re-isolated from newly infected root symptoms and morphological characteristics were compared with the inoculated isolate.

Morphological characterization

All obtained *Phytophthora* isolates were cultured on PDA, V8 agar, and CMA for morphological study. Growth rates of isolates on PDA medium were recorded. Sporangia of isolates were produced by floating some mycelial discs (obtained from margins of a 3-day-old culture on V8 agar) in 10 mL of double distilled water. The discs were then incubated under fluorescent light, at temperature of 25-28°C for 3-4 days. To determine the caducity of sporangia, the floating mycelial discs (bearing sporangia) were raised in a drop of distilled water several times, and the length of pedicels was measured under a light microscope. Sporangia caducity was determined based on the uniformity of the pedicel length (Erwin and Ribeiro. 1996). The sporangia and other structures were observed and measured by a camera with associated software attached to an Olympus light microscope (CH40; Olympus Optical Co. Ltd., Tokyo, Japan). At least 50 spores of each spore type were measured for each isolate then mean and standard deviation were reported.

Identification of pathogens based on DNA sequences

DNA extraction and polymerase chain reaction (PCR)

Mycelium of isolates was collected from purified colonies grown in PDA, and separately ground with mortar and pestle in liquid nitrogen to fine powders. DNA of isolates then was extracted by CTAB method with some modifications (Prabha *et al*., 2013).

Either couple of universal primers ITS6/ ITS4 or ITS5/ ITS4 was used to amplify internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal DNA fragments by polymerase chain reaction (PCR) under previously described conditions (White *et al.* 1990; Cooke *et al.* 2000).

Sequencing and phylologeny analysis

To identify *Phytophthora* isolates into species level, the sequencing of cloned fragments (PCR products) of isolates then were performed at First Base Laboratory (Selangnor, Malaysia), using the same primers. The full-length determined ITS nucleotide sequences of isolates then were used as queries for BLAST searches in GenBank of National Center for Biotechnology Information (NCBI; http://www.ncbi/blast/) or Phytophthora Database (http://www.phytophthoradb.org). Subsequently, the sequences of isolates and related taxa (obtained from GenBank databases) were aligned and analyzed to construct a phylogenetic tree using software MEGA ver. 5.2 (Tamura *et al.* 2011).

Results

Morphological characteristics and pathogenicity of Phytophthora spp. from pomelo

Total 06 isolates of *Phytophthora* spp., dividing into 02 groups, were isolated from soil and root samples that collected from affected-pomelo orchards.

The first group, which comprised three isolates PHY01; PHY02; and PHY03, was isolated from samples collected in Chang chen Sao province. All these isolates have similar morphological characteristics. They were slow growth organisms, with colony diameters after 7 days grown on PDA were less than 4 cm (Fig. 1). Colonies of these isolates showed stellate pattern with aerial mycelia when grown on PDA. Hyphae are lumpy-branching with hyphal swellings. Sporangia produced readily and abundantly on agar surfaces of PDA and V8A after 3-5 days, occurred in groups on sympodium or irregularly. Sporangia were papillate and caducous with short pedicels (mean 3.3 µ long). Sporangial shape varied from ellipsoid, ovoid, pyriform, obpyriform to near spherical, with a length to breadth ratio of 1.6 – 1.7 : 1. Zoospores were directly released from sporangia when flooded in distilled water. Most of chlamydospores were globose in shape, produced abundantly from mycelia on agar surfaces of PDA and CMA. No sexual organ was observed in cultures of these isolates, thus, they were a heterothallic species. Morphological characteristics of isolate PHY02, the typical isolate of this group, are showing in Fig. 2. The identity in morphological characteristics of PHY01, PHY02 and PHY03 suggested they were same species.

The second group, which consisted of three isolates KA1; KA2; and KA3, was isolated from samples that collected near Bangkok. Unlike the first group, all these isolates of this group were faster-growth organisms, with

colony diameters after 7 days grown on PDA were from 83 – 87 mm. Colonies of these isolates showed dense-aerial, arachnoid, and branched mycelia with hyphal swelling when grown on PDA and V8A. However, their mycelia were spare when grown on CMA. No isolates of this group produced any spore type on agar surfaces of tested media. The isolates formed sporangia only when were flooded with distilled water. All the three isolates produced papillate, caducous sporangia with very short pedicels (mean length: 3.1 µm). The sporangial shape was predominantly subspherical and turbinate, with an average length-to-breadth ratio of 1.3:1. Chlamydospores of these isolates, which are globose in shape, formed abundantly when the method of Tsao (1971) was applied. Additionally, no sexual organs were observed in single cultures of these isolates. Morphological characteristics of KA1, the typical isolate of this group, are showing in Fig. 3. Because the isolates KA1, KA2 and KA3 had identical morphological characteristics, they probably belonged to one species.

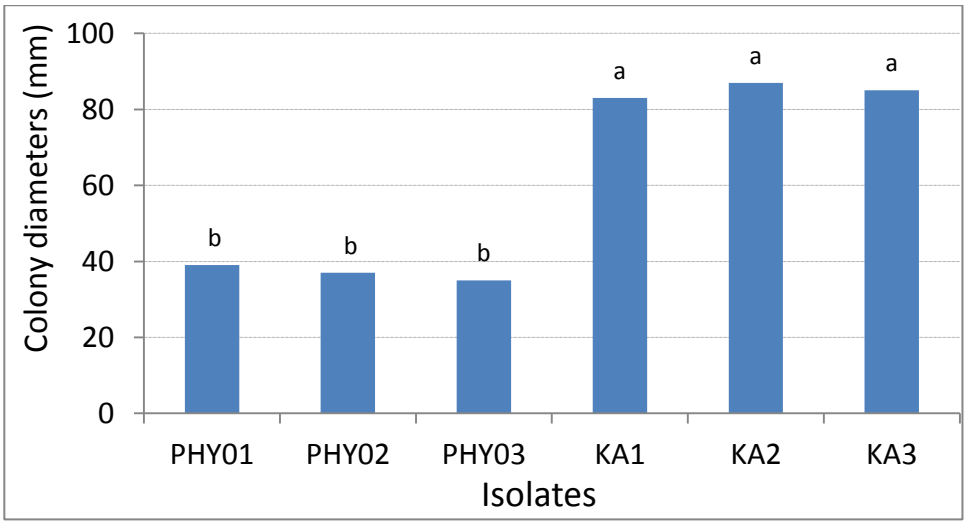

Fig. 1. Colony diameter of isolates of *Phytophthora* after 7 days grown on PDA (the same letter are not significantly different among isolates base on DMRT at $p = 0.05$)

Pathogenicity of isolates

As shown in table 4.2, all the six isolates were pathogenic to pomelo seedlings. Pomelo seedlings (12-week-old), which inoculated with 5 propagules of the isolates per cubic centimeter of soil, exhibited the root rot rates of 43.6 –

47.6%, with disease rating ranging from 2.5 – 2.9. These compared with no root rot and disease rating of 0.0 in the un-inoculated seedlings.

According to DMRT, there were no significant differences among the root rot percentages produced by isolates except for the slight difference between isolates PHY02 and KA3. All the inoculated seedlings produced very few new roots and leaves. Root tips of infected roots were soft and discolored. Root cortices of inoculated seedlings were turned soft and sloughed, leaving only the white stele (Fig. 4). These are typical symptoms of *Phytophthora* root rot in citrus, which was similar to symptoms observed in the affected orchards in Chang chen Sao and near Bangkok. In addition, all the isolates were re-isolated from newly infected roots of inoculated seedlings. Meanwhile, the Un-inoculated seedlings remain normally with abundant new roots and no symptom of rots. The results suggested that the obtained organisms were the causal agents of root rots in pomelo orchards in Chang chen Sao and near Bangkok.

Based on the pathogenicity and morphological characteristics of isolates in each group, we chose the isolates PHY02 and KA1 for further studies.

Table. 1. Morphological characteristics of *Phytophthora* isolates obtained from pomelo soil and roots in Thailand

Structures	**Mean of size**					
	PHY01	PHY02	PHY03	KA1	KA2	KA3
Sporangia[1]						
Length (μm)	54 ± 10.1[2]	54 ± 9.8	56 ± 9.5	49 ± 8.6	52 ± 7.7	49 ± 8.2
Breadth (μm)	34 ± 3.7	33 ± 4.4	34 ± 4.5	37 ± 4.7	39 ± 4.3	37 ± 4.6
Length/Breadth ratio	1.6 ± 0.3	1.6 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2
Papilla length (μm)	6.6 ± 1.6	6.4 ± 1.6	6.3 ± 1.6	4.8 ± 1.0	4.9 ± 1.0	5.0 ± 1.0
Pedicel length[3] (μm)	3.1 ± 1.1	3.2 ± 1.1	3.2 ± 1.1	3.1 ± 1.0	3.2 ± 1.0	3.0 ± 1.0
Chlamydospore[4] *diameter* (μm)	37 ± 6.4	37 ± 6.0	37 ± 6.6	37 ± 4.6	37 ± 6.4	36 ± 6.2

[1]Data collected from at least 100 separate sporangia; [2]Mean ± standard deviation; [3]Data collected from 85 separate detached sporangia; [4]Data collected from 50 separate chlamydospores

Table 2. Effect of different *Phytophthora* isolates on root rots of 12-week-old pomelo seedlings after six weeks of inoculation

Isolate	Source	Root rot (%)	Disease severity
PHY01	Roots	45.7 ab[1]	2.8[2]
PHY02	Roots	47.6 a	2.9
PHY03	Soils	45.1 ab	2.6
KA1	Roots	45.3 ab	2.8
KA2	Soil s	44.7 ab	2.6
KA3	Soil s	43.6 b	2.5
Uninoculated seelings		0.0 c	0.0

[1] Mean four replicates, the same letter represents no significant difference among treatments base on the Duncan's multiple range test at $p = 0.05$
[2]Rate on a scale of 0 = all roots healthy to 4 = no healthy root, stem girdled.

Identification and phylogeny of Phytophthora spp. caused root rots of pomelo in Thailand

Primers ITS5 and ITS4 were used to amplify internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal DNA fragments of isolates PHY02. Meanwhile, primers ITS6 and ITS4 were use for amplifying the same regions of isolates KA1. Both PCR products of isolates were about 900 kb. Nucleotide sequences of the ITS ribosomal DNA fragments of isolates PHY02 and KA1 were then determined and deposited in the GenBank under accession number KT175509 and KT175508, respectively. The DNA sequences of the two isolates were used as queries to search GenBank (NCBI) using the BLAST function.

The BLAST analysis showed that the nucleotide sequences of PHY02 shared 99.75% (809/811) with those of *Phytophthora palmivora* accession numbers PD00627, PD01515 and PD00491; and 99.87% (782/783) identity with those of PD02505. Phylogenetic analysis confirmed the relationships between PHY02 and these related taxa (Fig. 5). The isolate PHY02 was identified as *Phytophthora palmivora* (Butl.), based on its morphology and the molecular analysis.

For the isolate KA1, the analysis showed that its nucleotide sequences shared 100% identity with those of *Phytophthora nicotianae* accession Nos. GU111681 and GU111670 from *Citrus* spp. in Taiwan; JF792541 and JF792530 from citrus soils in India; and many other isolates existing in the GenBank database. Phylogenetic analysis confirmed the relationships between KA1 and the related taxa (Fig. 5). Thus the pathogenic isolate KA1 was identified as *Phytophthora nicotianae* (Breda de Haan).

Discussions

Phytophthora spp. are one of the major pathogens of many horticultural crops causing incalculable losses. The pathogens are decimating citrus industry worldwide (Naqvi, 2004). However, due to the difficulties in their isolations, early and accurate diagnosis of *Phytophthora* diseases in plant often is very difficult not only for grower but also for plant pathologists (Erwin and Ribeiro, 1996). In this research, six isolates of *Phytophthora* spp., dividing into two different groups, were isolated from samples collected in the affected pomelo orchards.

The first group contained slow-growth isolates that had papilate-caducous sporangia with short pedicels, and occurring in groups of 5 – 15 on one sympodium. These are typical characters of *P. palmivora* (Butl.) according to Erwin and Ribeiro (1996), which described the occurrences of groups (up to 20) conspicuous papillate sporangia with short pedicels on a sympodium as the distinguished characteristics of this species. More importantly, the analysis of their ITS sequences confirmed these isolates were *P. palmivora* (Butl.). The appearance of only *P. palmivora* in the samples from affected orchards in Chang chen Sao and its high virulence for roots of pomelo suggest that this species is the causal agent of the disease appearing there. This finding is in correspondent with others of previous authors. Zitko *et al.* (1991) demonstrated that *P. palmivora* often is more aggressive and damages even larger root than *P. parasitica* on citrus. Serious root rot disease of citrus caused by *P. palmivora* has been recorded in America, India (Zitko and Timmer, 1994; Naqvi, 2004). Additionally, *P. palmivora* are known as a prominent plant pathogen with a wide host range, which infects various important crops such as black pepper, durian, citrus, rubber...*etc*, in Southeast Asia (Drenth and Guest, 2004).

All morphological characteristics of isolates from the second group were similar to those of *P. nicotianae* Breda de Haan (syn. *P. parasitica*) (Erwin and Ribeiro, 1996), except the production of caducous sporangia. Despite the unusual characteristics of the sporangia, the ITS sequences of this isolate were identical to those of many isolates of *P. nicotianae* found in

GenBank. The Phylogenetic analysis also strongly confirmed the relationships between the isolate KA1and the related taxa. Caducity of sporangia is an important and useful character for morphological study and identification of *Phytophthora* species. According to the extensive reviews by Erwin and Ribeiro (1996), *P. nicotianae* does not produce caducous sporangia, but produces persistence sporangia on the long stalks. However, Cacciola *et al.* (1994) found that isolates identified as *P. nicotianae* obtained from affected *Forsythia* plants had caducous sporangia with a very short pedicels (less than 5 μm). From lavender (*Lavandula angustifolia* Mill.), Álvarez *et al*. (2007) also obtained 05 isolates identified as *P. nicotianae* that had caducous sporangia with short pedicels (2.1 – 3.8 μm). Their descriptions are consistent with our observations for isolates of *P. nicotianae* obtained from pomelo. The appearance and high virulence of isolates of *P. nicotianae* in pomelo, as shown in this study, supported the conclusion that "*P. nicotianae* is the main causal agent of root rot in all types of citrus worldwide" (Erwin and Ribeiro,1996; Naqvi, 2004). The species is well known to distribute widely and cause root rots of citrus plants in citrus-growing areas America. It is estimated that 20 – 80% citrus orchards in Florida are infested by *P. nicotianae* (Graham and Timmer, 2008). Recently, *P. nicotianae* is report to be the most predominant pathogens of citrus root rots, foot rots and gummosis in Egypt (Ahmed *et al.*, 2012), South Africa (Meitz-Hopkins, 2014). In Thailand and other countries in Southeast Asia, *P. nicotianae* not only infects various citrus types, causing root rots and foot rots, but also cause root rots of many other crops such as papaya, tobacco, durian, black pepper, pineapple … *etc* (Drenth and Guest, 2004). Therefore, it is unsurprised when the virulence pathogen infects and causes root rots of pomelo, which is widely grown in Thailand and considered to be susceptible to *Phythopthora* (Naqvi, 2004).

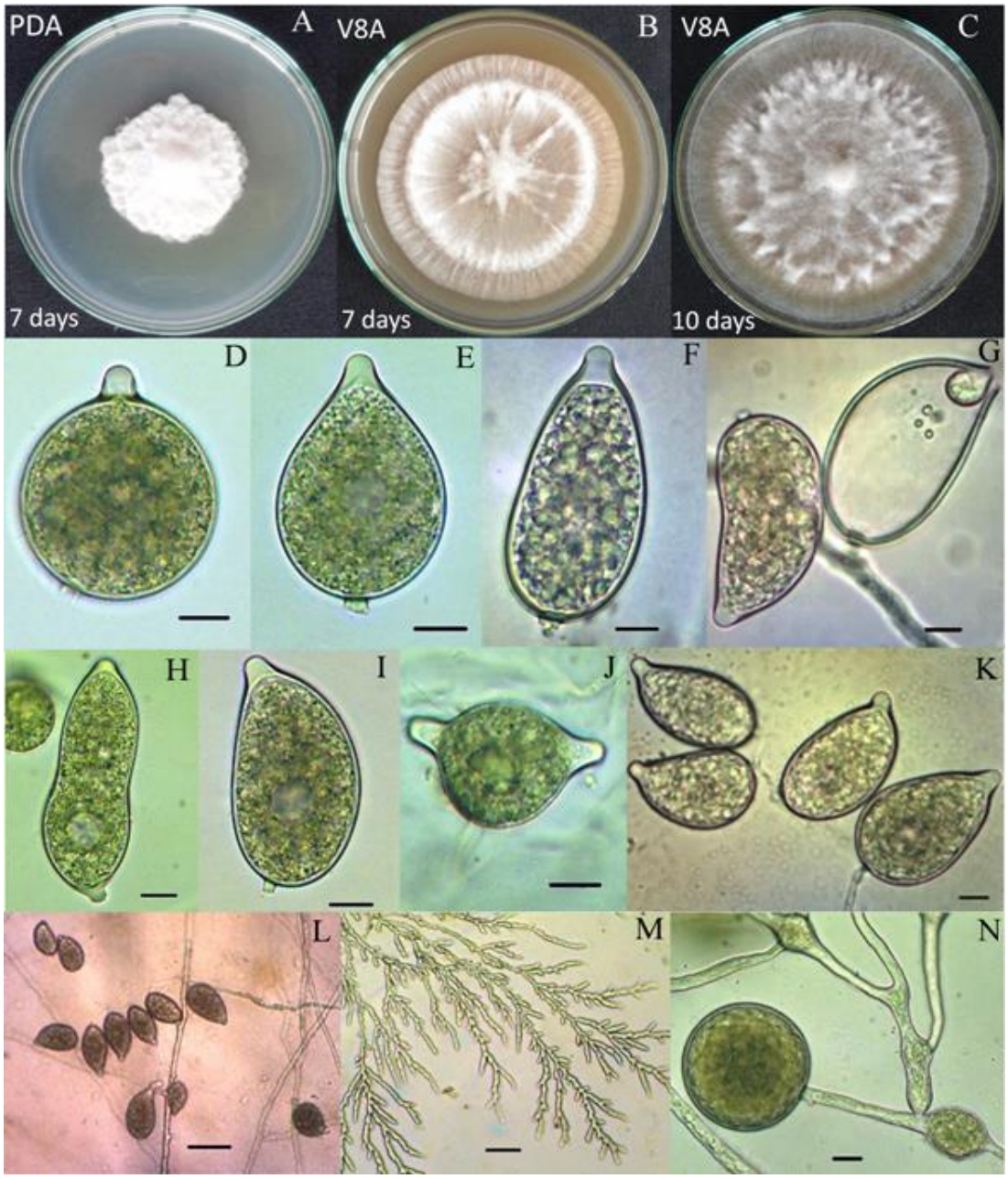

Fig. 2. Morphological characteristics of isolate PHY02. A-C, Colony types of PHY02 at 7 days grown on different media; D-J, Zoosporangia; K and L, Occurrence of sporangia on sympodium; M, Lumpy-branching mycelia; J, Chlamydospore and swelling hyphae (scale bars: D-G, N = 10 μm, L, M = 50 μm).

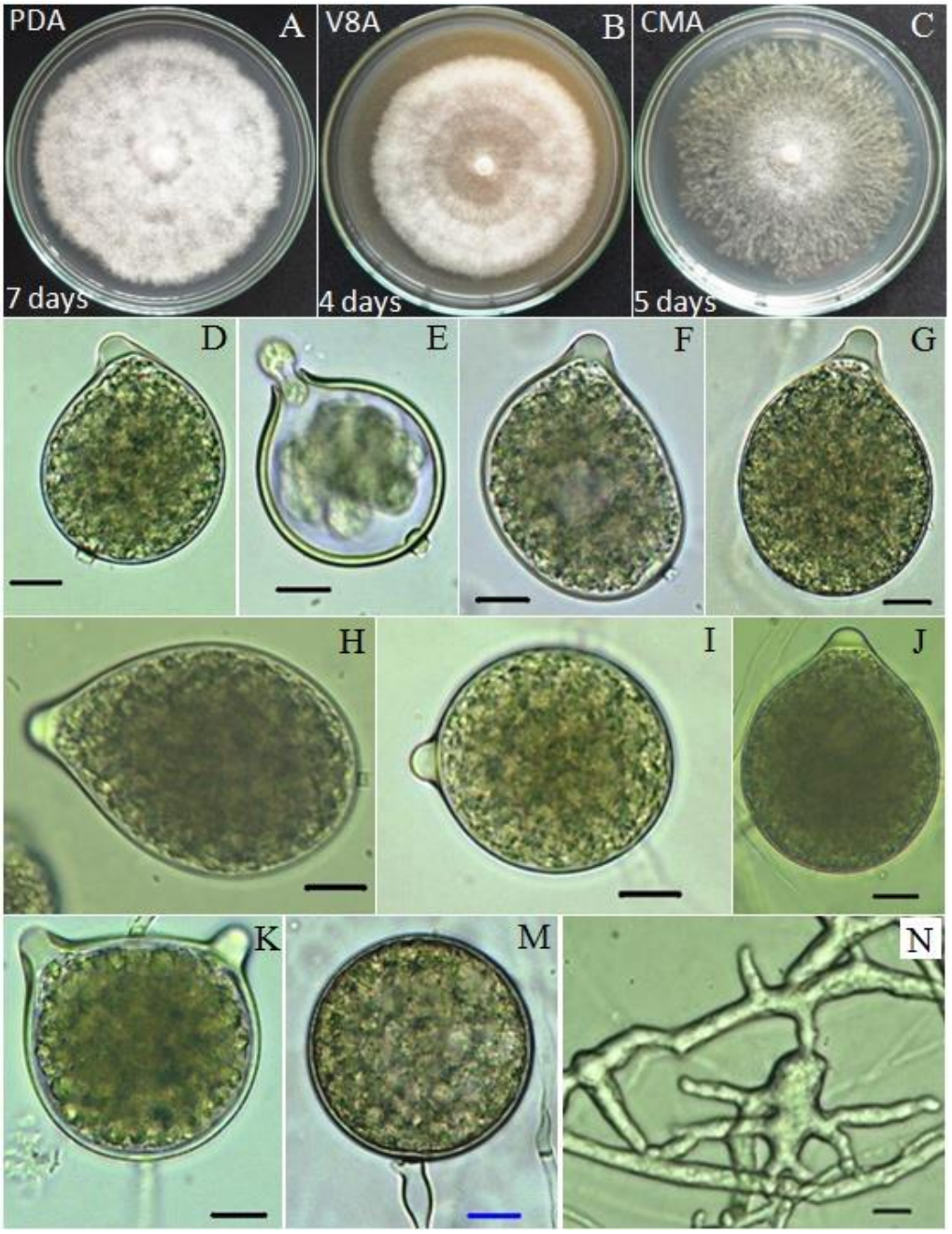

Fig. 3 a. Morphological characteristics of *Phytophthora* sp. KA1. A – C, cultures of KA1 on different media; D – K, Sporangia ; M, Chlamydospore; N, Hyphae swelling (scale bars: D – M = 10 µm; N = 50 µm).

Fig. 4. A and B – Seedlings and their root systems after six weeks inoculation with different *Phytophthora* isolates ; C – closed symptoms of root rots in an inoculated seedling.

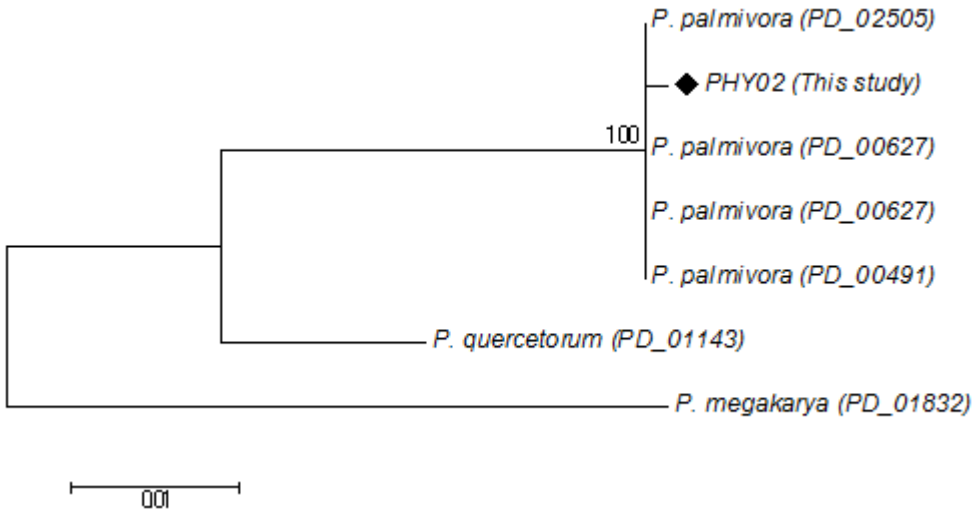

Fig. 4. Phylogenetic relationship between *Phytophthora palmivora* PHY02 and related taxa inferred using a neighbor joining method with internal transcribed spacer (ITS) rDNA sequences. Bootstrap value based on 1,000 replications is shown above the branch.

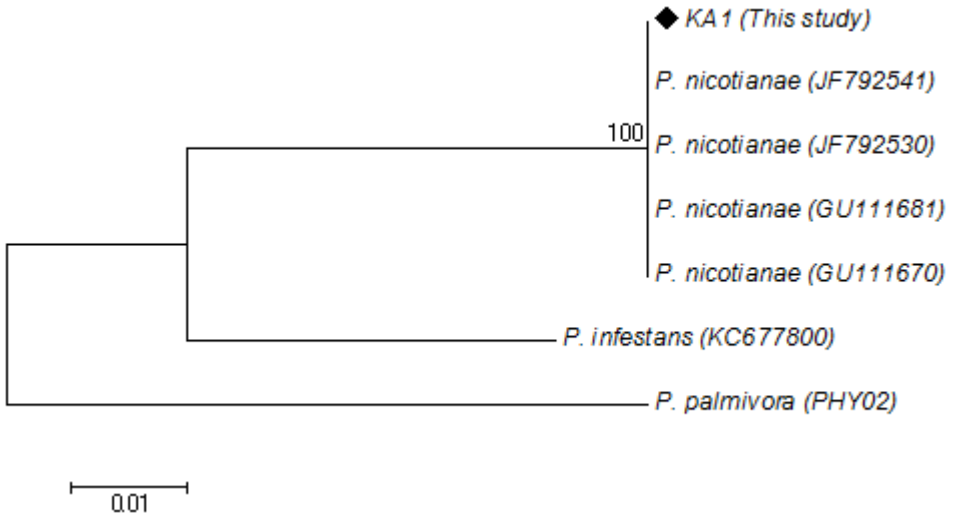

Fig.5. Phylogenetic tree showing relationship between *Phytophthora nicotianae* KA1 and related taxa base on the internal transcribed spacer ribosomal DNA sequences, using the neighbor-joining method with 5,000 bootstrap replicates. *P. infestans* (KC677800) (the species placed in the same clade 1 with *P. nicotianae*) was isolated from a potato in India, and *P. palmivora* PHY02 (KT1755

Acknowledgements

This work is supported by King Mongkut's Institute of Technology Ladkrabang Research Fund (Grant number: KREF125602).

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