# PATHOGENICITY OF *Pythium deliense* ISOLATED FROM THE RHIZOSPHERE SOIL OF ORANGE IN VIETNAM

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## ABSTRACT

Orange (*Citrus sinensis*), a major crop in Vietnam, has been suffering from a disease with symptoms of leaf yellowing, gummosis, and root rot. This study identified the associated microorganism of the disease and evaluated its pathogenicity to citrus species. In 2021, two *Pythium* isolates, assigned as VN-Oo16 and VN-Oo29, were isolated from the rhizosphere soil of symptomatic orange plants in Tuyen Quang province in northern Vietnam, and were identified as *Pythium deliense* based on morphological and molecular characteristics. The two isolates formed numerous inflated, lobulate, and toruloid sporangia, smooth oogonia, and aplerotic, thick-walled oospores. In the phylogenetic trees based on the Internal Transcribed Spacers (ITS) and cytochrome c oxidase subunit 1 (*cox*1) sequences, these two isolates and other GenBank *P. deliense* isolates formed a distinct species cluster. Pathogenicity assays of *P. deliense* (VN-Oo29 isolate) were conducted by inoculation of mycelium plugs or zoospore suspension on the detached fruits, stems of grafted seedlings, and roots of seedlings grown from seeds. In pomelo and orange, *P. deliense* produced typical brown rot on wounded fruits and gummosis on wounded stems. *P. deliense* was not able to infect the roots of pomelo, orange, and lime seedlings. To our knowledge, this is the first report of *P. deliense* in association with citrus in the world as well as in Vietnam.

**Key words:** citrus, internal transcribed spacer, cytochrome oxidase subunit I, gummosis, brown rot

## INTRODUCTION

Species of the genus *Pythium* are currently classified in the family *Pythiaceae*, the order *Peronosporales*, the phylum *Oomycota* of the kingdom *Stramenopila* (*Chromista*) (Mostowfizadeh-Ghalamfarsa and Salmaninezhad 2020). *Pythium* and *Phytophthora* that is another member of the family *Pythiaceae* share many morphological and reproductive characteristics in common. However, one asexual feature that differentiates the two genera is the formation and release of zoospores. In *Pythium*, the differentiation of zoospores occurs within a vesicle that connects to sporangium through a short tube, and mature zoospores are released upon the rupture of the vesicle. In contrast, in

*Phytophthora*, zoospores are differentiated within the sporangium and released through the sporangial apex (Ho 2018).

In 2022, *Pythium* contains 376 species (www.mycobank.org), however, many of them are either synonymous or invalidated. Historically, in the 1980s, less than 90 species of *Pythium* were recognized, mostly based on morphological characters as described in the well-known comprehensive monograph written by Van der Plaats-Niterink (1981) that contains the identification key to 85 species. Since applying molecular approaches that are based on some housekeeping genes such as the Internal transcribed spacer (ITS) region of rDNA, β-tubulin, cytochrome c oxidase subunit 1 (*cox*1) and 2 (*cox*2), the identification and classification of *Pythium* species have become more accurate (Levesque and De Cock 2004; Robideau et al. 2011; Villa et al. 2006), and up to now, 157 species of *Pythium* are validated at the molecular level (Jayawardena et al. 2020). It is worth noting that several *Pythium* species, for example, clade K members (Levesque and De Cock 2004), that have intermediate characteristics between *Pythium* and *Phytophthora*, morphologically and phylogenetically, have been reassigned as the new genus *Phytopythium* (Bala 2010).

*Pythium* species are soil and water inhabitants and have worldwide distribution. They can live saprophytically but in certain conditions, they can become very pathogenic and cause rot of fruits, roots, or stems, pre- or post-emergence damping-off of seeds and seedlings of a wide range of plants (Ho 2018; Rai et al. 2020; Van der Plaats-Niterink 1981). Like other oomycetes, the major inoculum of *Pythium* is zoospores. Recently, many aspects of the interaction between *Pythium* and plants have been clarified at the molecular level, which could explain the necrotrophic lifestyle of *Pythium* (Judelson and Ah-Fong 2019).

Citrus, particularly orange (*Citrus sinensis*) and pomelo (*C. grandis*), is a major crop in Vietnam. In 2020, the area and production of citrus in Vietnam were 140,841 ha and 1,983,299 tonnes, respectively (FAO 2022). However, one of the major constraints to citrus production is a disease with the symptoms of leaf yellowing, gummosis, and root rot, resulting in plant death and a significant yield loss (Dang et al. 2004). Various species of *Phytophthora* and *Pythium* have been shown to be associated with the disease worldwide and in Vietnam (Dang et al. 2004; Maseko and Coutinho 2002; Puglisi et al. 2017). During an investigation to identify the causal agents involved in the disease in orange orchards grown in northern Vietnam, we isolated several *Phytophthora* and *Pythium* species from the rhizosphere soils and roots of orange trees that suffered from disease symptoms. Hence, this study aimed to identify a *Pythium* species isolated from the rhizosphere soil of the diseased orange in Vietnam and to elucidate its pathogenicity to various citrus species.

## MATERIALS AND METHODS

**Isolation of *Pythium*.** Rhizosphere soils were collected in diseased orange plants showing symptoms of leaf yellowing, stem gummosis, and root rot in orchards at Ham Yen (22°04'48.0"N, 105°02'07.5"E) and Yen Son (21°53'40.8"N 105°08'11.7"E) in Tuyen Quang province in northern Vietnam in 2021. Disease incidence in these two orchards is approximately 20 and 25%, respectively. *Pythium* was isolated using a baiting method. Approximately 20 grams of soil per sample were suspended in 100 ml tap water in a plastic cup, and 5 rose petals were then floated on the surface to bait zoospores in the soil sample. After 3 days, Pythium isolates were recovered by placing the surface-sterilized symptomatic petal tissues onto water agar (WA) medium supplemented with streptomycin sulfate (100 $mg.L^{-1}$). Pythium isolates were then subcultured by transferring the hyphal tips to potato dextrose agar (PDA) or V8 agar media.

**Morphological studies.** Two *Pythium* isolates, assigned as VN-Oo16 (from Ham Yen) and VN-Oo29 (from Yen Son), were isolated and used for morphological studies. The assessment of the growth rates of these isolates was conducted on both PDA and V8 in 90-mm Petri dishes incubated in an incubator

set at 28°C. Hyphal growth was recorded every day until the dishes were completely covered by mycelium. The morphological investigation was based on agar plugs which were cut from the edge of actively growing colonies on PDA. The agar plugs were initially immersed in V8 liquid medium in a 60-mm Petri dish and incubated for 24 hours. V8 liquid was replaced with sterile distilled water and 5-6 young rice leaf fragments were floated on the surface to stimulate the reproduction (Vafa et al. 2021). The dish was incubated for 24 hours for observation of sporangia and zoospores, and further 48 hours for observation of sexual structures. All steps were conducted at room temperature in light. Morphological identification of isolates was achieved using taxonomic keys for *Pythium* (Ho 2011; Van der Plaats-Niterink 1981).

**Molecular characterization.** The two above isolates, VN-Oo16 and VN-Oo29, were used for molecular characterization.

***DNA extraction***. DNAs were extracted using a NaOH method which was originally adopted for plant tissue (Wang et al. 1993). A few milligrams of mycelium mat from seven-day-old PDA culture were transferred into a 0.5 ml Eppendorf tube containing 5 µl of 0.5 M NaOH and homogenized using a pipette tip. The homogenate was diluted 20 times with 100 mM Tris pH 8.0. The extract was used immediately or stored at –20 °C until use.

***Polymerase chain reaction (PCR)***. Two widely accepted DNA barcodes for oomycetes, ITS and *cox*1 (Robideau et al. 2011), were selected for molecular identification. The ITS region was amplified using ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGAAAAGTCGTAACAAGG3') primers (White et al. 1990). The *cox*1 was amplified using OomCoxILevup (5'TCAWCWMGATGGCTTTTTTCAAC3') and Fm85mod (5'RRHWACKTGACTDATRATACCAAA3') primers (Robideau et al. 2011). PCRs were performed in a final reaction volume of 25 µl using MyTaq HS Mix (Meridian Bioscience). Amplification was carried out using a thermocycler programmed as follows: 1) preliminarily denaturation step at 94°C for 5 min, 2) 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 1 min, and 3) a final extension step at 72°C for 5 min.

***Sequencing***. The PCR products were purified from agarose gel using an Expin™ Gel SV Kit (GeneAll Biotechnology), estimated for the DNA concentration using agarose gel electrophoresis. Purified amplicons were directedly sequenced using the PCR primers at the Institute of Biotechnology in Hanoi, Vietnam.

***Sequence analyses***. The sequences were initially compared to GenBank ITS and *cox*1 sequences using the BLAST program available at the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Two data sets for each *Pythium* gene containing all GenBank isolates of Clade A, the ex-type isolates of the Clades B1, B2, C, D, E1, E2, G, H, I, and J (Levesque and De Cock 2004; Robideau et al. 2011) were used for phylogenetic analyses. The ex-type isolate CBS 124518 of *Phytopythium sindhum* (Bala 2010) was used as the out-group. The sequences were aligned using an online MAFFT program (Katoh and Standley 2013) available at https://mafft.cbrc.jp/alignment/server/. MAFFT alignment of ITS and *cox*1 was performed with the L-INS-i and default algorithms, respectively. The phylogenetic analyses were conducted by MEGA11 (Tamura et al. 2021). The maximum likelihood (ML) trees were constructed by using the best nucleotide substitution model (TN93+G) for ITS and (GTR + G) for *cox*1. All indels were kept which resulted in a total of 1262 and 679 positions in the final ITS and *cox*1 datasets, respectively. Supports for nodes were obtained using 1000 bootstrap replicates. Trees were rooted to the out-group.

**Pathogenicity test.** The representative isolate VN-Oo29 grown on V8 medium was used for all inoculation experiments. Each inoculation was replicated on three fruits or plants. All inoculated and control fruits or plants were kept at room temperature. The infection was evaluated by the appearance of the symptom and confirmed by re-isolation of *Pythium* from lesions. Three pathogenicity assays were performed.

In detached fruit inoculation, mature fruits of pomelo (*C. grandis* cv. Doan Hung and cv. Soi Ha) at 3 days after harvesting, and orange (*C. sinensis* cv. Vinh) purchased from the supermarket were surface sterilized using 70% ethanol and airdried. The fruits were wounded by making a shallow hole (5 mm in diameter and 0.5 mm in depth) at the peduncle or rind. The wounded and non-wounded fruits were inoculated by placing a 5-mm mycelium plug or dropping 200 µl of $1x10^6$ zoospore $ml^{-1}$ in the peduncle or rind. The inoculated sites were wrapped with sticky tape and fruits were incubated in food bags to maintain moisture. For the control, V8 agar plugs or water were used.

In stem inoculation, the grafted young plants of pomelo (*C. grandis* cv. Soi Ha) and orange (*C. sinensis* cv. Vinh) with a base stem of 1.5 - 2 cm were used for the experiment. The bark (1 cm wide x 2 cm length) in the middle stem was removed. A 3-mm mycelium plug was placed centrally in the wound, with the mycelium facing inward. The wound was then covered with the bark piece and wrapped by a saran membrane. The inoculation on non-wounded plants was also performed in a similar manner. For the control, V8 agar plugs were used.

In root inoculation, seeds of pomelo (*C. grandis* cv. Doan Hung, cv. Soi Ha and cv. Duong), orange (*C. sinensis* cv. Vinh, cv. Sanh, cv. Duong and cv Duong Canh) and lime (*C. aurantifolia* cv. Tu Quy) were sowed on sterile sand in plastic pots (7 x 9 cm) for three months. At this time, seedlings had 3-4 (for orange and lime), and 5-6 (for pomelo) true leaves. The nutrient solution was supplied every week. Two methods of root inoculation were performed. In the first method, the pots were flooded several times with water to remove extra nutrient salts that may affect zoospores before inoculation. The mycelium plugs were submerged in water for the production of zoospores. Five plugs and 100 ml of a zoospore suspension ($10^4$ zoospores $ml^{-1}$) were applied to the base region of seedlings. To stimulate the further formation of zoospores, the water saturation stature of sand was maintained for two days after inoculation. In the second method, the seedlings were gently removed from the sand, and the roots were washed with water. The seedlings were hung on 300 ml water in a plastic cup using clip support. Then, the mycelium plugs and zoospore suspension were applied to the cup as for the first method. To verify the activity of zoospores, three rose petals were also floated on the water of each cup. The infection was evaluated by the lesion formation on the roots until one month after inoculation.

## RESULTS AND DISCUSSION

**Identification of *Pythium* isolates.** Two *Pythium* isolates, VN-Oo16 and VN-Oo29, were isolated from the rhizosphere soils of orange plants showing symptoms of yellowing, gummosis, and root rot. These two isolates showed identical morphological characteristics. They grew fast and completely colonized PDA or V8 agar plates after 4 days. Colonies on PDA formed cottony aerial mycelia and have a chrysanthemum pattern with radial mycelium. On V8 agar, the aerial mycelia were thicker and had less distinct chrysanthemum patterns compared with those on PDA (Fig. 1 a, b). They did not reproduce on agar media. On the agar plugs immersed in water, they formed numerous sporangia that were inflated, lobulate, and toruloid, forming swollen side branches sometimes in clusters, mostly terminal, occasionally intercalary (Fig. 1 c). Both isolates had smooth globose oogonia, 18.9-24.6 (average 21.3) µm in diameter, mostly terminal, with oogonial stalks bending towards the monoclinous antheridia (Fig. 1 d). Their oospores were aplerotic, 18.1-22.2 (average 19.5) µm in diameter, and had a thick wall with thicknes of 1.7-2.8 (average 2.4) µm (Fig. 1 e, f).

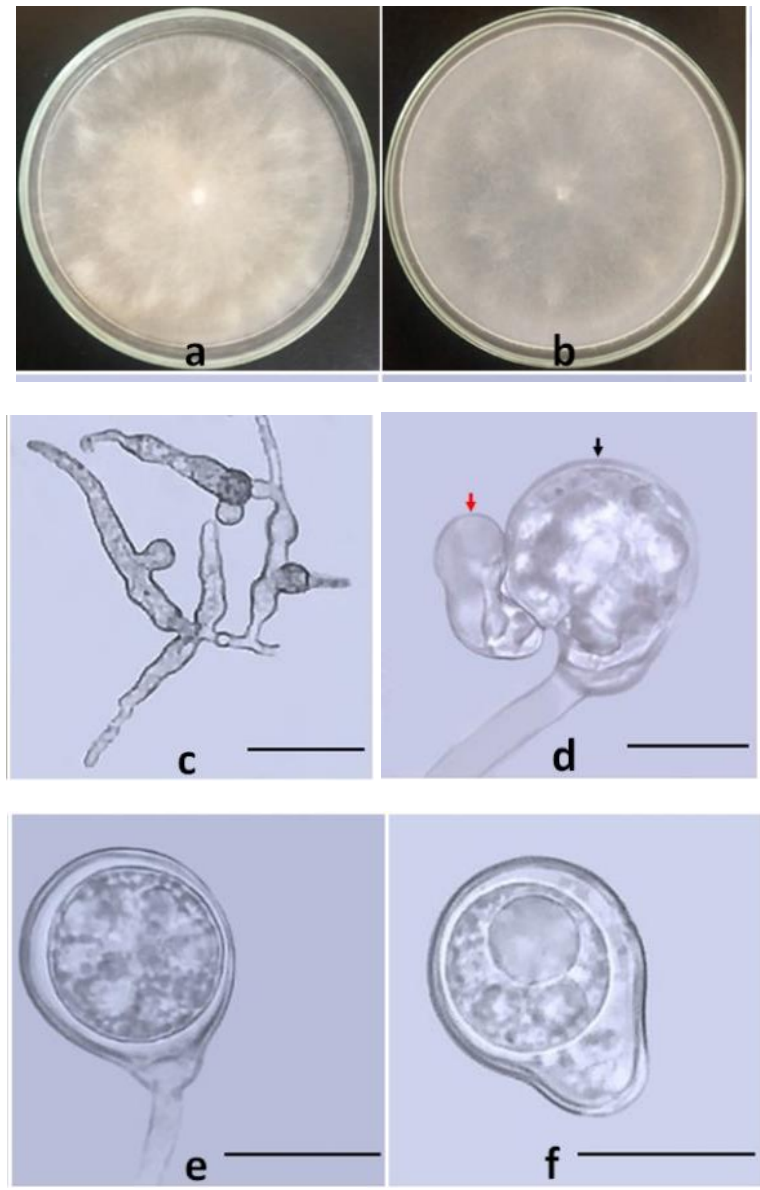


**Fig. 1.** Cultural and morphological characteristics of *P. deliense* – isolate VN-Oo29. Colonies on potato dextrose agar (a) and V8 media (b) after 5 days. Sporangia (c); an antheridium (red arrow) mating an oogonium (black arrow) (d); an oospore (e and f). Scale bars: 50 µm (c) and 15 µm (d, e, f).

The ITS and *cox*1 genes of the two *Pythium* isolates were successfully amplified and sequenced. The sequences were deposited in GenBank under accession numbers ON573330 (ITS, VN-Oo16), ON573331 (ITS, VN-Oo29), ON563235 (*cox*1, VN-Oo16), and ON563236 (*cox*1, VN-Oo29). The ITS and *cox*1 sequences of the two isolates were initially searched for the homologous sequences in GeneBank. The Blast searches evidenced that both *Pythium* isolates from Vietnam were most closely related to *P. deliense* isolates. In the ML phylogenetic ITS and *cox*1 trees, both *Pythium* isolates from Vietnam and other GenBank *P. deliense* isolates formed a distinct species cluster that was well bootstrap supported (99% for both markers). In the two trees, this *P. deliense* species cluster also grouped well and consistently with the *P. aphanidermatum* species cluster within the clade A of *Pythium* (Fig. 2).

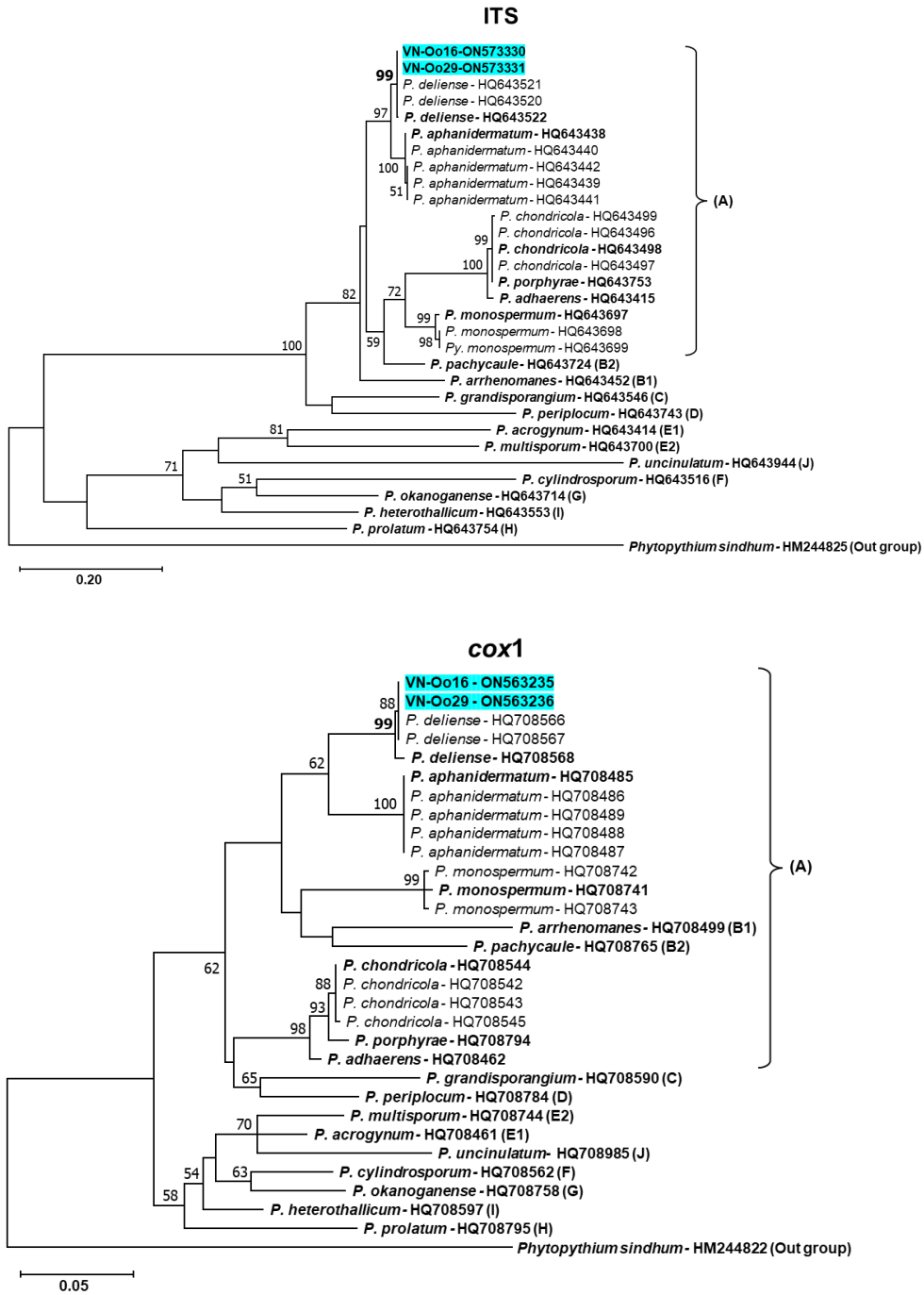


**Fig. 2.** Maximum likelihood trees of *Pythium* isolates based on the ITS and *cox*1 sequences. The isolates from Vietnam are shown in bold and shaded. The ex-type isolates of *Pythium* species representative for the clades A, B1, B2, C, D, E1, E2, G, H, I, and J (Levesque & De Cock, 2004; Robideau et al., 2011) are shown in bold and parentheses. The numbers at nodes represent percentage bootstrap support values calculated from 1000 replicates. Only bootstrap support values greater than 50% are shown. The scale bars indicate the number of substitutions per site.

Based on morphological and molecular analyses, the two *Pythium* isolates were identified as *P. deliense* species and as such, this species was detected for the first time in Vietnam. *P. deliense* and other 5 species, *P. adhaerens, P. porphyrae, P. chondricola, P. monospermum* and *P. aphanidermatum* are members of clade A of *Pythium* (Levesque and De Cock 2004; Robideau et al. 2011). In this clade, *P. deliense* and *P. aphanidermatum* are very morphologically similar having inflated sporangia, fast growth, and monoclinous antheridia, whereas the remaining members have filamentous non-inflated sporangia, slow growth, and diclinous antheridia (Levesque and De Cock 2004). *P. deliense* was first isolated and described from the tobacco plant in Indonesia in 1933 and then reported to cause diseases in a wide range of dicot and monocot plants in warmer regions of the world (Van der Plaats-Niterink 1981).

**Pathogenicity assay.** In the pomelo and orange fruit inoculation experiment, only wounded fruits inoculated with either mycelium plug or zoospore suspension developed the symptom (Table 1). The lesion appeared 2 days after inoculation and enlarged gradually to form the typical fruit brown rot symptom (Fig. 3). *Pythium* isolates recovered from the infected tissues of the inoculated fruits had morphological features identical to those of the VN-Oo29 isolate. The fruit assay in this study suggested *P. deliense* appear to be virulent to fruits of pomelo and orange producing typical fruit brown rot; on the contrary, *P. irregulare, P. aphanidermatum, P. paroecandrum* and *P. ultimum* produced small brown necrotic lesions on the wounded rind surface of citrus fruit (Maseko and Coutinho 2002).

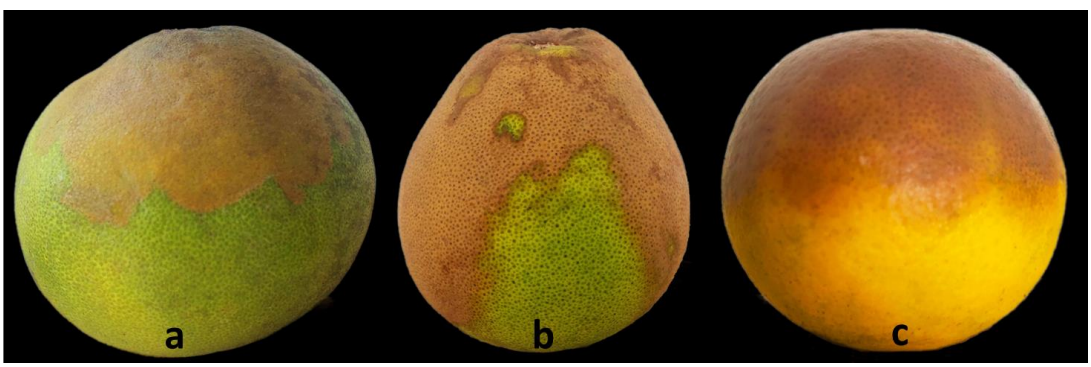


**Fig. 3.** Fruit rot of pomelo cv. Soi Ha (a), pomelo cv. Doan Hung (b) and orange cv. Vinh (c) inoculated with mycelium plug of *P. deliense* (isolate VN-Oo29) at wounded peduncle. Symptom was pictured at 7 days after inoculation.

It is widely known that fruit brown rot of citrus is caused by multiple species of Phytophthora when conditions are cool and wet (Graham and Feichtenberger 2015; Naqvi 2003). To our knowledge, this is the first report of *P. deliense* associated with brown rot of citrus fruit. Further, our fruit assay also indicated that *P. deliense* was not able to infect intact fruit rind or non-injured peduncle. The formation of a cuticle layer on the rind and lignification of cells adjacent to the abscission zone of the peduncle during ripening (Merelo et al. 2017; Wang et al. 2016) could prevent citrus fruits from direct penetration of *Pythium*.

In stem inoculation, the infection occurred on both pomelo and orange seedlings resulting in the gummosis and browning of the necrotic area at the inoculated sites (Table 1, Fig. 4). The stem assay in this study indicated that *P. deliense* is pathogenic to the wounded stem of pomelo and orange, inducing the formation of gummosis and vascular rot. *Pythium* isolates recovered from the infected tissues of the inoculated stem had morphological features identical to those of the VN-Oo29 isolate.

Once again, stem infection of *P. deliense* was different from that of other *Pythium* species. Maseko and Coutinho (2002) when inoculating 16 isolates of *P. irregulare, P. aphanidermatum, P. paroecandrum, P. vexans, P. rostratum, P. ultimum,* and *Pythium* group G & F concluded that only isolates of *P. irregulare, P. ultimum,* and *P. paroecandrum* were weakly pathogenic producing superficial lesions without gum exudation on the wounded stems of citrus rootstocks.


**Fig.** 4. Stem rot of pomelo cv. Soi Ha (a) and orange cv. Vinh (b) inoculated with mycelium plug of *P. deliense* (isolate VN-Oo29) at wounded site. Symptom was pictured at 14 days after inoculation.

In root inoculation, no difference in root systems between the inoculated and un-inoculated (control) seedlings of all cultivars was observed in both methods, suggesting this species could not infect the root (Table 1). Recent pathogenicity tests demonstrated *P. deliense* could infect underground parts of numerous plants causing pod rot in peanuts (Parkunan et al. 2014), root rot in melons (Cara et al. 2008), crown and root rot in walnut (Ghaderi and Banihashemi 2011) and periwinkle (Intaparn et al. 2019), and soft rot of ginger (Vafa et al. 2021). However, the root assays on seven citrus cultivars in this study indicated that *P. deliense* is unable to infect the root of citrus seedlings. Infection of roots from the inoculum involves pre- and post-penetration. The pre-penetration sequence of oomycetes zoospores includes zoospore movement, encystment, cyst adhesion, and germination (Deacon and Donaldson 1993) that involves multiple homing responses, including chemotaxis, electrotaxis, host-triggered encystment, and germ tube tropism (Judelson and Ah-Fong 2019). Among compounds mediating homing responses of oomycetes zoospore, $Ca^{2+}$ in the environment was proved to play a vital role (Addepalli and Fujita 2002; Deacon and Donaldson 1993). In our root inoculation experiments, the absence of $Ca^{2+}$ during inoculation might be a factor that affected the pre-penetration of the zoospore. Furthermore, *Pythium* is widely known to be necrotrophic oomycetes, which feed on nutrients from lysed cells and most members of this group are opportunistic root pathogens with broad host ranges (Judelson and Ah-Fong 2019). So, another possibility of no infection of *P. deliense* to the citrus root was the incompatible interaction between zoospore and citrus roots under the test condition. More work needs to be done to clarify the interaction between *P. deliense* and citrus. Likewise, it is necessary to undertake studies on management strategies against this pathogen.

**Table 1.** Pathogenicity test of *P. deliense* (isolate VN-Oo29) on different parts of citrus plants

| Plant | Cultivar | Detached fruit[1] | | | Root of seedling grown from seed | | Stem of grafted young plant | |
|---|---|---|---|---|---|---|---|---|
| | | Non-wounded, mycelium plug | Wounded, mycelium plug | Wounded, zoospore suspension | Grown in sand | Hung on water | Non-wounded, mycelium plug | Wounded, mycelium plug |
| Pommelo (*C. grandis*) | cv. Doan Hung | -[2] | +[3] | + | - | - | nt | nt |
| | cv. Soi Ha | - | + | + | nt | - | - | + |
| | cv. Duong | nt[4] | nt | nt | nt | - | nt | nt |
| Orange (*C. sinensis*) | cv. Vinh | - | + | + | - | - | - | + |
| | cv. Sanh | nt | nt | nt | nt | - | nt | nt |
| | cv. Duong Canh | nt | nt | nt | nt | - | nt | nt |
| Lime (*C. aurantiifolia*) | cv. Tu Qui | nt | nt | nt | - | - | nt | nt |

[1] Wounded and non-wounded were performed on both peduncle and rind;
[2] -: No symptoms
[3] +: With symptoms
[4] nt: not tested

## CONCLUSION

This is the first report of *P. deliense* isolated from the rhizosphere soil of orange plants with the symptoms of leaf yellowing, stem gummosis, and root rot in Vietnam. *P. deliense* is pathogenic to the wounded stem and fruit of pomelo and orange. *P. deliense* was not able to infect the roots of pomelo, orange, and lime seedlings under the test condition, implying the complicated interaction between the *P. deliense* zoospores and citrus roots.

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