RESEARCH ARTICLE

Morphology, Molecular Phylogeny, and Fungicide Sensitivity of *Phytophthora nagaii* and *P. tentaculata* in Korea

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ABSTRACT

Phytophthora species, classified under Oomycota, cause significant damage to various crops and trees. The present study introduced *Phytophthora* species, *P. nagaii* and *P. tentaculata*, new to Korea, which pose notable risks to their respective host plants. Our research provided a comprehensive description of these species taking into account their cultural features, morphological characteristics, and molecular phylogenetic analysis using the internal transcribed spacer rDNA region and cytochrome c oxidase subunit mtDNA genes (*cox*1 and *cox*2) sequences. In addition, this study first evaluated the sensitivity of *P. nagaii* and *P. tentaculata* to five anti-oomycete fungicides, finding both species most responsive to picarbutrazox and *P. tentaculata* resistant to fluazinam. The data can guide targeted treatment strategies and offer insights into effective control methods. The findings expand our understanding of the diversity, distribution, and management of *Phytophthora* species in Korea.

1. Introduction

The genus *Phytophthora*, infamously called the "plant destroyer," is a significant group of the phylum Oomycota. It was previously classified under the kingdom Fungi but is now recognized as a fungal-like member of the Kingdom Chromista. This genus is remarkably diverse, with over 200 known species [1,2]. This significant increase from an earlier estimate of around 120 species [3] is attributed to the advent of molecular phylogenetic analysis.

Phytophthora species are renowned for causing critical diseases in a broad spectrum of agriculturally and ornamentally valuable crops and forest trees, with frequent reports from nurseries [4,5]. In addition, numerous species are featuring prominently in lists of global emergent threats to natural ecosystems [6–11].

In Korea, considerable research efforts have been invested in *Phytophthora* species due to their profound impact on agricultural and ecological settings. As it stands, 22 *Phytophthora* species, including *P. capsici*, *P. infestans*, *P. nicotianae*, *P. palmivora*, and *P. sojae*, have been recognized [12,13], most species of which have been reported as disease-causing agents in diverse plants [14–23]. The pioneering work in *Phytophthora* species identification in Korea began with the cataloguing and morphological examination of various species [24], providing an in-depth account of disease etiology, epidemiology, and management, but also greatly enhancing our understanding of *Phytophthora* diseases in Korean agriculture. In recent years, the scope of studies has broadened to encompass molecular identification and phylogenetic analysis. Seo et al. [25] conducted a molecular phylogenetic analysis for *Phytophthora* species in Korea, enriching our understanding of their genetic diversity and relationships. Moreover, a next-generation sequencing (NGS) investigation demonstrated that conventional farms employing chemical fertilizers and pesticides displayed a significantly higher abundance of plant pathogens, such as *Phytophthora* species, compared to organic farms [26]. In addition, *Phytophthora* species were frequently found in freshwater environments, displaying a distinct preference for plant debris [27].

In 2019, the Korean government implemented the Positive List System (PLS) to regulate the improper use of pesticides. This system specifies which pesticides suit particular plants and their corresponding diseases. As a result, this requires reevaluating how plant pathogen strains react to the range of fungicides used within the nation, including tests to gauge their resistance or sensitivity. Given this context, it is essential to examine how oomycete plant pathogens, such as *Phytophthora* species, react

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to commonly employed anti-oomycete fungicides. Currently, five fungicides, including metalaxyl, ethaboxam, dimethomorph, fluazinam, and picarbutrazox, are extensively applied in both national and international agricultural systems to control oomycete-related diseases [28–36].

Metalaxyl suppresses the activity of ribosomal RNA synthetase in oomycete pathogens [37,38]. Ethaboxam targets foliar diseases caused by *Phytophthora infestans* on pepper and potato and works by disrupting the assembly of microtubulin [31,32,39]. Dimethomorph hinders the enzyme responsible for cellulose synthesis, whereas Fluazinam, a member of the 2,6-dinitroanilines group, is broadly effective against various pathogens, including *P. infestans* [40–42]. Picarbutrazox specializes in oomycetes but has an as-yet unidentified mechanism of action. Despite their effectiveness, these compounds can lead to the development of resistance in some cases. For instance, resistance risks have been documented with metalaxyl [43,44] and dimethomorph [45]. As a result, ongoing monitoring and research are essential to ensure that these treatments remain effective in managing diseases caused by the target pathogens.

In the current research, *Phytophthora* isolates were identified by morphological and molecular phylogenetic methods and assessed their activities against five anti-oomycete fungicides. We also discussed their potential risks in domestic agriculture.

2. Materials and methods

2.1. Oomycete isolates

Four *Phytophthora* isolates used in this study were obtained from the Korean agricultural culture collection (KACC; Jeonju, Korea) (Table 1). They were cultured on V8 agar (V8A), composed of 200 mL clarified V8 juice, 10 g CaCO₃, 15 g agar, 800 mL deionized water, and incubated at 25 °C for a week.

2.2. Cultural and morphological analysis

To examine the colony growth patterns on three different media, a 4-mm agar plug was placed on

plates of potato dextrose agar (PDA; Difco, Detroit, MI, USA), 20% V8A, and corn meal agar (CMA; Difco, Detroit, MI, USA). For observing the sexual organs of *Phytophthora* isolates (KACC 45737, 40909, 40912, and 40913), soil extract was utilized. The soil was moistened, set aside overnight, and subsequently filtered through Whatman No. 1 filter paper. The filtered soil extracts were then equally divided, and one portion passed through a 0.22 μm Millipore membrane. One-day-old mycelial plugs of *Phytophthora* isolates, grown in V8 liquid media, were rinsed with sterile deionized water and placed in a petri dish containing 10 mL of the soil extract. These plates were kept at room temperature (25 °C) and inspected at 2, 5, and 7 days.

The morphological traits of sporangia of KACC 40909, 40912, and 40913 were observed in the petri dish containing soil extract, as previously outlined. To stimulate the formation of sporangia in KACC 45737, an autoclaved leaf blade of *Zoysia japonica* was placed on a V8A medium, pre-inoculated with the KACC 45737 three days beforehand [46]. After three days, the colonized leaf was moved to a new Petri dish filled with distilled water [47]. All microscopic structures were examined using a Zeiss Axio Imager A2 microscope (Carl Zeiss, Oberkochen, Germany) and captured using a Dhyana 400DC camera (Tucsen, Fuzhou, China) attached to the microscope.

2.3. Molecular phylogenetic analysis

Genomic DNA was extracted with the MagListo 5 M Plant Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). The internal transcribed spacer (ITS) rDNA regions were amplified using the primer pairs ITS1/ITS4 [48]. In addition, the cytochrome c oxidase subunit I (*cox*1) and cytochrome c oxidase subunit II (*cox*2) mtDNA of oomycete strains were amplified using OomCox1-levup/ OomCox1-levlo [49] and cox2-F [50]/cox2-RC4 [51], respectively. The PCR amplicons were purified using an AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea) and then sequenced by Macrogen Inc. (Seoul, Korea). Editing was carried

Table 1. Information of *Phytophthora* isolates used in this study.

Species	Isolate (KACC)	Host plant	Date	Geographic origin	GenBank accession number (ITS / cox1 / cox2)
Phytophthora nagaii	45737	*Rosa hybrida*	Oct. 2010	Pyeongtaek, Gyeonggi, Korea	OR431879 / OR462170 / OR462166
Phytophthora tentaculata	40909	*Pleurospermum camtschaticum*	Jun. 2000	Pyeongchang, Gangwon, Korea	OR431879 / OR462171 / OR462167
Phytophthora tentaculata	40912	*Solidago virgaurea* subsp. *asiatica*	Aug. 2000	Ulleung Island, Gyeongbuk, Korea	OR431879 / OR462172 / OR462168
Phytophthora tentaculata	40913	*Cnidium officinale*	Aug. 2000	Ulleung Island, Gyeongbuk, Korea	OR431879 / OR462173 / OR462169

out with DNAStar software package 5.05 (DNAStar, Inc., Madison, WI), followed by BLASTn search against the NCBI GenBank database.

Phylogenetic analysis was undertaken based on a concatenated dataset of ITS, cox1, and cox2 sequences, including reference sequences retrieved from NCBI GenBank, and aligned by the G-INS-i algorithm of MAFFT 7 [52]. Phylogenetic trees were constructed using MEGA X with maximum likelihood (ML) and minimum evolution (ME) inferences, applying the Tamura-Nei model and bootstrapped with 1000 replicates.

2.4. Fungicide sensitivities

Anti-oomycete fungicides used in this study include metalaxyl (ai 25% WP), ethaboxam (ai 15% WP), dimethomorph (ai 50% WP), fluazinam (ai 25% WP), and picarbutrazox (ai 10% WP) (Table 2). The sensitivity of mycelial growth of Phytophthora isolates to the fungicides was tested using an agar dilution method. Each fungicide was dissolved in sterilized distilled water and then to amended to 20% V8A to obtain final concentrations of 0 (control), 0.01, 0.1, 1, 10, 100, and 1000 μg/mL. Rifampicin was added at a concentration of 15 μg/mL to avoid medium contamination. After culturing the pathogen on V8A for one week at 25 °C, an agar plug with a diameter of 4 mm was made at the end of the hyphae and subsequently inoculated on a V8A with each of five different fungicides. Each triplicated culture, inoculated with each isolate, was incubated in the dark at 25 °C, and mycelial growth was measured daily when the diameter of the untreated control group reached 60 mm. The percentage of mycelial growth inhibition for each isolate was measured in relation to the control. The concentrations required to inhibit 50% of the mycelial growth (EC50 values) were calculated as described previously [53].

3. Results and discussion

3.1. Cultural and morphological identification

The present study presented a detailed description of the cultural and morphological characteristics of four Korean isolates of Phytophthora (KACC 45737, 40909, 40912, and 40913) (Figure 1A–F). These fungal-like organisms exhibit filamentous, highly branched hyphae that are generally nonsegmented and contain multiple nuclei. The shape and size of sporangia differ among the isolates (Figure 1G,I), giving rise to mobile zoospores. Thick-walled oospores were produced in soil extract (Figure 1H,J).

Colonies of the isolates KACC 45737 grew colorlessly in a radiate pattern with few aerial mycelia on three media, PDA, V8A, and CMA. The shape and features of the sporangia, sporangiophores, oogonia, oospores, and antheridia closely mirrored those typically found in Phytophthora nagaii [47]. On host plant leaf cultures, this isolate mainly produces papillated sporangia (Figure 1G), although non-papillate forms were also noticed.

In the KACC 40909, 40912, and 40913, colonies exhibited a similar pattern as P. nagaii (KACC 45737) but displayed notable morphological distinctions. The colonies were smaller in diameter, and these isolates predominantly generated sporangia with a papilla when grown in soil extract, exhibiting diverse shapes. The morphological aspects of their sporangiophores, oogonia, and antheridia matched those of Phytophthora tentaculata [54] but differed slightly in size and shape.

These detailed descriptions of P. nagaii (KACC 45737) and P. tentaculata (KACC 40909, 40912, and 40913) provide valuable insights into their morphology and growth characteristics. The information is crucial for accurately identifying and distinguishing these species, whether in the field or in a laboratory. These findings can enhance our understanding of Phytophthora biology and help in species differentiation and identification, potentially aiding in plant disease management and control.

Table 2. Sensitivity ranges and mean values of effective concentrations to inhibit mycelial growth of Phytophthora nagaii and P. tentaculata isolates by EC50 values for five anti-oomycete fungicides, metalaxyl, ethaboxam, fluazinam, dimethomorph, and picarbutrazox.

| Fungicide	Frac code	Target site	EC50 (μg/mL)	
			P. nagaii (KACC 45737)	P. tentaculata (KACC 40912)
Metalaxyl	4	RNA polymerase	0.05868 (0.02468–0.08478)	0.28566 (0.16966–0.39068)
Ethaboxam	22	Tubulin polymerization	0.02065 (0.01782–0.02356)	0.07452 (0.06527–0.08145)
Fluazinam	29	Uncouplers of oxidative phosphorylation	0.48973 (0.42886–0.55275)	9.11459 (4.74457–16.8783)
Dimethomorph	40	Cellulose synthase	0.68215 (0.6478–0.7202)	0.57110 (0.49248–0.63706)
Picarbutrazox	U17	Unknown	0.00014 (0.00007–0.00021)	0.00375 (0.00264–0.00536)
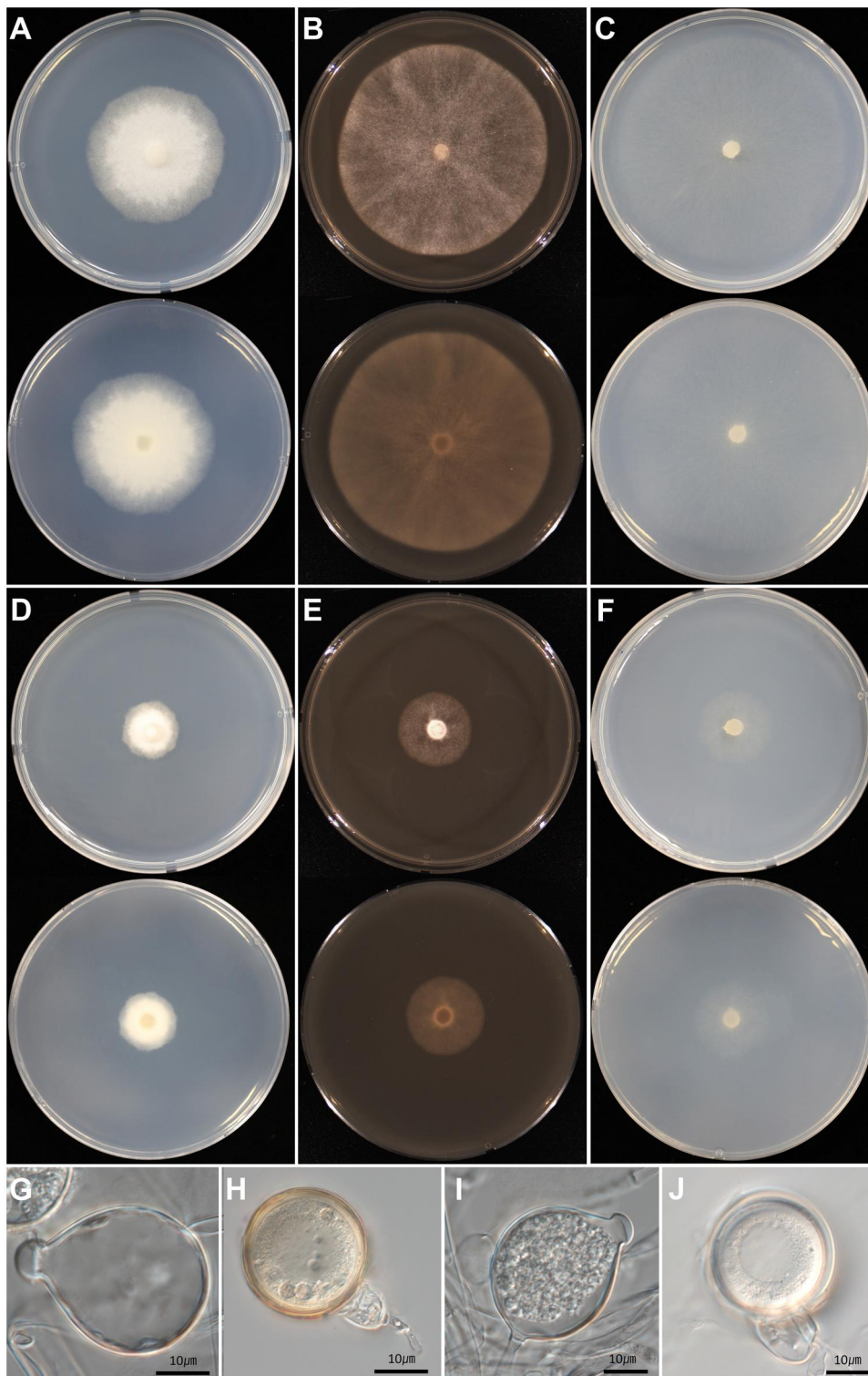
Figure 1. Cultural and morphological characters of *Phytophthora nagaii* (A–C and G–H) and *Phytophthora tentaculata* (D–F and I–J). (A–C) Colonies of *P. nagaii* observed after 6 days of inoculation on potato dextrose agar (A), V8 agar (B), and corn-meal agar (C); (E–F) Colonies of *P. tentaculata* on potato dextrose agar (D), V8 agar (E), and cornmeal agar (F); (G) Subspherical sporangium of *P. nagaii*; (H) Oospore of *P. nagaii*; (I) Spherical, papillate sporangium of *P. tentaculata*; (J) Oogonium of *P. tentaculata*. Sources: KACC 45737 for *P. nagaii* and KACC 40912 for *P. tentaculata*.

3.2. Molecular phylogenetic identification

Sequence analysis was performed on the ribosomal ITS region and mitochondrial *cox*1 and *cox*2 gene sequences. Through a BLASTn search, KACC 40909 isolate showed high sequence similarities with the reference strain of *Phytophthora tentaculata* ex-type CPHST BL29 across all three markers: ITS sequence at 830/831 bp (MG865591; 99.88%), *cox*1 at 681/682 bp (MH136983; 99.85%), and *cox*2 at 476/478 bp (JF771611; 99.58%). Similarly, isolate KACC 40912 matched the ex-type of *P. tentaculata* (CPHST BL29) in ITS sequences at 830/831 bp (MG865591; 99.88%),
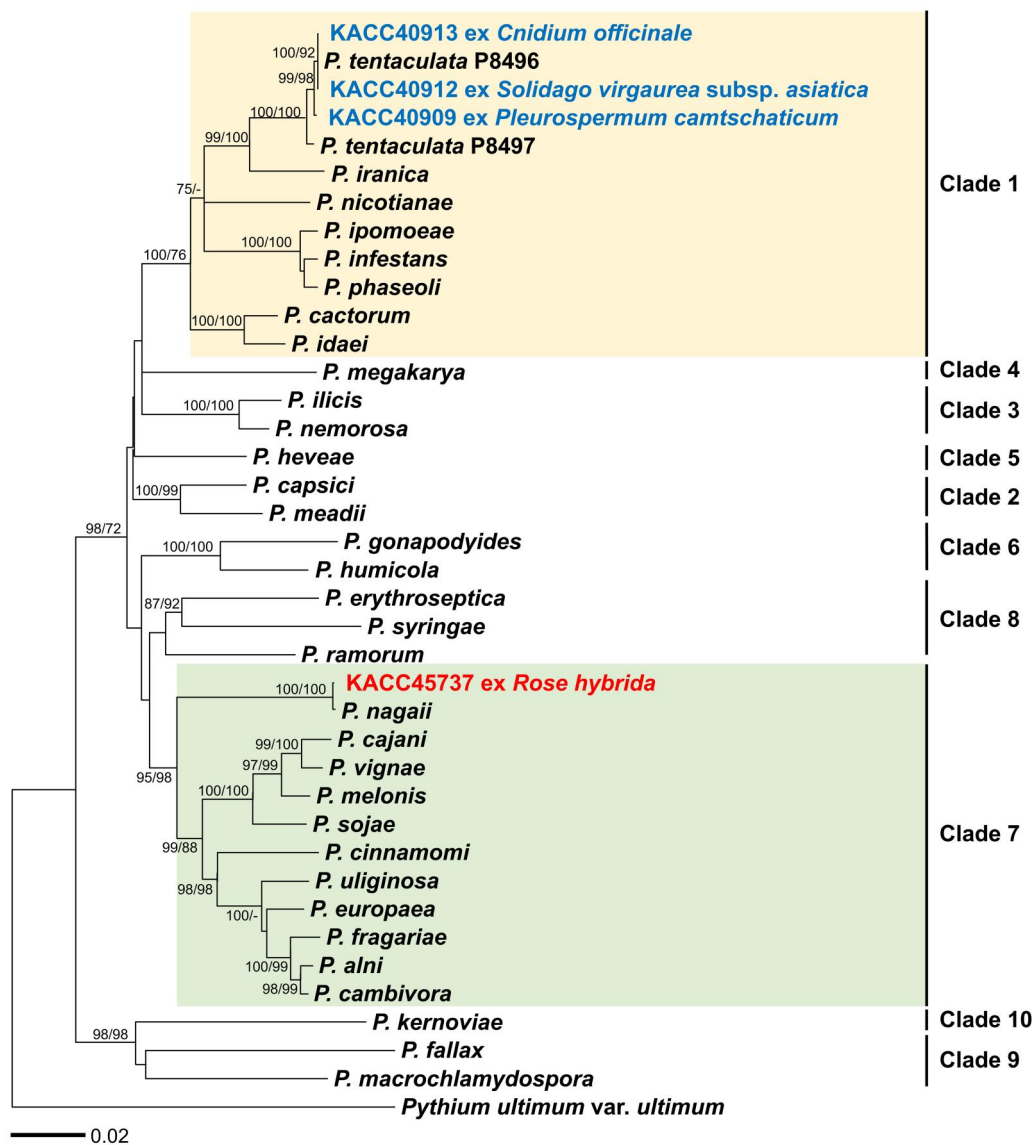
Figure 2. Minimum evolution of *Phytophthora* species based on a concatenated alignment of the ITS rDNA, *cox*1, and *cox*2 mtDNA sequences. Bootstrapping values (minimum evolution/maximum likelihood) higher than 70% were shown above/below the branches (1000 replicates). A yellow box presents Clade 1, containing *Phytophthora tentaculata*, whereas a green box means Clade 7, containing *Phytophthora nagaii*.

*cox*1 at 694/694 bp (MH136983; 100%), and *cox*2 at 482/483 bp (JF771611; 99.79%). Also, KACC 40913 aligned with the identical reference of *P. tentaculata* in ITS at 828/829 bp (MG865591; 99.88%) but showed 100% identity for *cox*1 (MH136983) and *cox*2 (JF771611) sequences. Lastly, isolate KACC 45737 matched the same reference of *P. tentaculata* in the ITS at 854/855 bp (MG865547, 99.88%) and *cox*2 at 545/546 bp (LC596024, 99.82%), while showing a 100% identity in *cox*1 sequence (MH136940). Notably, KACC45737 was identical to *Phytophthora nagaii* in *cox*1 but exhibited a single nucleotide difference in ITS and *cox*2 sequences.

For the molecular phylogenetic identification of these four KACC isolates, representative species from clades 1–10 of the genus *Phytophthora* were selected from the World Phytophthora Collection (WPC). The sequences of three markers (ITS, *cox*1, *cox*2)

were aligned to standardize all sequence lengths. This resulted in sequence lengths of 1002 bp for the ITS region, 654 bp for the *cox*1 gene, and 455 bp for the *cox*2 gene. Phylogenetic trees were constructed using both ML and ME methods based on the concatenated alignment of the three markers. In the phylogenetic tree (Figure 2), KACC 40909, 40912, and 40913 formed a well-supported group with the reference sequences of *P. tentaculata*. Interestingly, the Korean isolates formed two subgroups by three nucleotide differences in *cox*1 and *cox*2 sequences. Meanwhile, KACC 45737 grouped with *P. nagaii*, demonstrating a high bootstrapping value of 100%.

3.3. Activities to anti-oomycete fungicides

This study tested the sensitivity of two *Phytophthora* species against five different fungicides (metalaxyl,

ethaboxam, dimethomorph, fluazinam, and picarbu-trazox). Sensitivity was determined by observing if the colony growth on the fungicide media fell below an EC50 threshold of 1 μg/mL [34]. For *P. nagaii* KACC45737, the EC50 value range (mean) for each fungicide was 0.02468 to 0.08478 μg/mL (0.05868) for metalaxyl, 0.01782 to 0.02356 μg/mL (0.02065) for ethaboxam, 0.42886 to 0.55275 μg/mL (0.48973) for fluazinam, 0.6478 to 0.7202 μg/mL (0.68215) for dimethomorph, and 0.00007 to 0.00021 μg/mL (0.00014) μg/mL for picarbutrazox (Table 2). As a result, the isolate displayed a high level of susceptibility to the five fungicides, with picarbutrazox showing the highest sensitivity.

For of *P. tentaculata* KACC40912, the EC50 value range (mean) was 0.16966 to 0.39068 (0.28566) μg/mL for metalaxyl, 0.06527 to 0.08145 (0.07452) μg/mL for ethaboxam, 4.74457 to 16.8783 (9.11459) μg/mL for fluazinam, 0.49248 to 0.63706 (0.57110) μg/mL for dimethomorph, and 0.00264 to 0.00536 (0.00375) μg/mL for picarbutrazox (Table 2). Based on these results, *P. tentaculata* isolate showed sensitivity to met-alaxyl, ethaboxam, dimethomorph, and picarbutrazox but less sensitive to fluazinam.

The EC50 measurements for inhibiting the mycelial growth of *P. nagaii* and *P. tentaculata* with metalaxyl, ethaboxam, and dimethomorph were comparable to those observed for other *Phytophthora* species, such as *P. agathidicida*, *P. cactorum*, *P. citrophthora*, *P. capsici*, *P. parasitica*, and *P. sojae* [55–59]. Both species were most sensitive to picarbutrazox among all the fungi-cides tested. Previously, fluazinam exhibited an EC50 value ranging from 0.14 to 0.27 against *P. infestans* [60]. However, in the present study, *P. tentaculata* dis-played high mycelial growth on fluazinam-containing media, compared to other *Phytophthora* species, sug-gesting its resistance to this particular fungicide, unlike other *Phytophthora* species.

The effects of varying fungicide concentrations on the growth of the mycelia of both species were visu-ally depicted in Figure 3, providing further insight into their reactions to different levels of fungicides. This study provided crucial information for under-standing the sensitivity of two *Phytophthora* species to the most widely used anti-oomycete fungicides, which can guide targeted treatment strategies and help create more effective control methods. The dis-tinct resistance of *P. tentaculata* to fluazinam com-pared to *P. nagaii* is of particular interest, suggesting species-specific responses that may require custom-ized approaches in fungicide application. The notable sensitivity of both species to picarbutrazox under-scores its promise as an effective agent for controlling these pathogens. Future research might focus on the mechanisms behind these sensitivity patterns and explore the field efficacy of these fungicides.

4. Taxonomy

4.1. Phytophthora nagaii *M.Z. Rahman, S. Uematsu, Toru Takeuchi, K. Shirai & Kageyama, Journal of General Plant Pathology 80(4): 353 (2014) [MB#804991]*

4.1.1. Description

Colonies grow colorlessly with a radiate pattern and few aerial mycelia on PDA, V8A, and CMA at 25 °C, submerged growth on CMA, and measured 40–50 mm on PDA, 75–80 mm on V8A, and 75–85 mm in diameter on CMA after 72 h. The isolate produces mostly papillate but often non-papillate sporangia abundantly. Sporangia are terminal, single, ellipsoid often with tapering bases, and measured (25.2–) 26.6–32.7 (–34.4) × (18.4–) 20.2–24.9 (–26.2) (av. 29.6 × 22.5) um ($n = 50$). Sporangiophores are hyalin, simple sympodial and show eccentric basal attach-ment to sporangia. Oogonia are hyalin, mostly spher-ical and occasionally funnel-shaped with tapering bases and short stalks. Oospores are dark brown, aplerotic, mostly spherical, with a thick wall, and measured (35.2–) 36.1–39.1 (–40.1) (av. 37.6) um in diameter ($n = 50$). Antheridia are hyalin, predomin-antly paragynous and sometimes amphigynous.

4.1.2 Host plant

Rosa hybrida (Rosaceae)

4.1.3. Notes

Phytophthora nagaii isolates were previously classified under *Phytophthora megasperma* when discovered on roses in Japan [61]. However, a later, in-depth phylo-genetic analysis recognized it as a distinct species [47]. While the initial documentation described this species as generating non-papillate sporangia when grown on grass blades [47], our current research indi-cates that the species more frequently produces papil-late than non-papillate sporangia. The recent discovery of *P. nagaii* in Korea could have wide-rang-ing implications, particularly in agriculture and plant disease management, given its impact on economic-ally significant roses. This study contributes to our understanding, as its precise identification and mor-phological features may lead to improved treatment and control measures. The potential risk mandates ongoing investigation and monitoring.

4.2. Phytophthora tentaculata *Kröber & Marwitz, Z. Pflanzenkrankh. Pflanzenschutz 100: 251 (1993) [MB#360186]*

4.2.1. Description

Colonies grow colorlessly with a radiate pattern and few aerial mycelia on PDA, V8A, and CMA at 25 °C, submerged growth on CMA, and measured 15–20 mm in diameter on PDA and V8A, and 20–
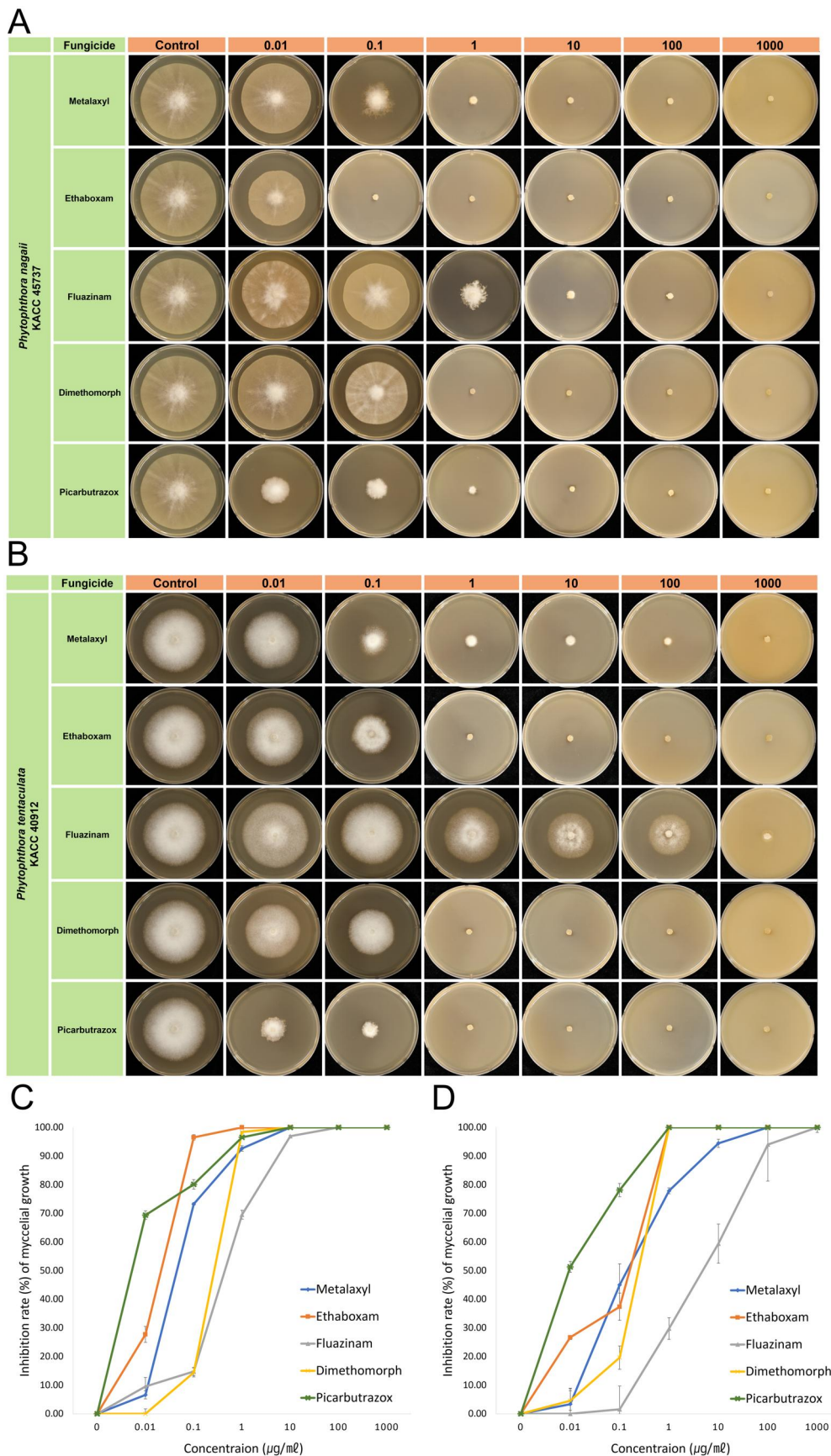
Figure 3. Mycelial growth of *Phytophthora nagaii* (A and C) and *Phytophthora tentaculata* (B and D) on V8 agar media, containing different concentrations of anti-oomycete fungicides (0, 0.01, 0.1, 1, 10, 100, and 1000 ug/mL). Agar plugs sourced from seven-days-old colonies were inoculated on V8A, with daily measurements of mycelial diameter until the control group reached a diameter of 60 mm.

25 mm on CMA after 72 h. The isolates produce mostly papillate sporangia abundantly in soil extract. Sporangia are mostly ellipsoidal but often ovoid or elongated and measured (26.2-) 28.4–35.8 (-39) × (21.3-) 23.8–29.1 (-30.4) (av. 32.1 × 26.4) um ($n = 50$). Sporangiophores are hyalin and have branching points. Oogonia are hyalin and spherical to subglobose. Oospores are hyalin, aplerotic, spherical, and measured (23-) 25.7 – 33.3 (-36.9) (av. 29.5) um in diameter ($n = 50$). Antheridia are hyalin, predominantly amphigynous.

4.2.2. Host plant

Cnidium officinale (Apiaceae), *Pleurospermum camtschaticum* (Apiaceae), and *Solidago virgaurea* subsp. *asiatica* (Asteraceae)

4.2.3. Notes

This plant pathogen *Phytophthora tentaculata*, known for causing root and stalk rot, was initially isolated in a German nursery in 1993, where it affected plants such as *Chrysanthemum*, *Verbena*, and *Delphinium* species [54]. Due to its ability to cause substantial economic harm to both the nursery industry and native plant species [62], it is considered one of the top five most concerning *Phytophthora* species in the United States [63,64]. Later, this pathogen was also discovered in Spain, affecting *Verbena* plants [65]. Recent studies have shown the host range of *P. tentaculata* to be broader, including *Santolina chamaecyparissus* (Lavender cotton) in Spain [66], *Origanum vulgare* (Oregano) in Italy [67], and *Aucklandia lappa* in China [68]. This study isolated the Korean strains from *Cnidium officinale* (Apiaceae), *Pleurospermum camtschaticum* (Apiaceae), and *Solidago virgaurea* subsp. *asiatica* (Asteraceae). These findings support the broad host range of this pathogen and suggest that it can cause significant damage on various plants, not just the hosts recorded in this study. Considering its potential economic impact in Korea, there is an urgent need for further studies to explore its host range and financial impact within the country.

Disclosure statement

No potential conflict of interest was reported by the authors.

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