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Association of Two Unknown *Pythium* spp. without Forming Sexual Structures on Root Rot of Hydroponically Grown Welsh onion

S. Shimizu [a,b] and M. Tojo [b*]

[a] Hiroshima Prefectural Technology Research Institute, Agriculture Research Center, 6869 Hara Hachihonmatsu-cho, Higashihiroshima, Hiroshima 739-0151, Japan.
[b] Graduate School of Agriculture, Osaka Metropolitan University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Aim of this study was to elucidate association of *Pythium* isolates without forming sexual structures on root rot of hydroponically grown Welsh onion, as well as to clarify their taxonomic positions.
Place and Duration of Study: Commercial hydroponic greenhouses in Hiroshima Prefecture, Hiroshima Prefectural Technology Research Institute, and Osaka Metropolitan University, between March 2020 and March 2021.
Methodology: Total 2,966 isolates of *Pythium*-like organs were isolated from the plant roots during the survey period. All the isolates didn't form any sexual organs, and were separated into three groups based on colony patterns on agar media such as cornmeal agar. Among of them, 236 isolates were equally selected from each three groups. Since two of the three groups were thought to be first records on Welsh onion, 17 representative isolates from the two groups were examined on morphology, hyphal growth response to temperatures and sequences of the ribosomal internal transcribed spacer (ITS) regions and mitochondrial *COI*. Inoculation tests were performed on Welsh onion seedlings under a hydroponic condition by using one representative isolate from each group.

**Corresponding author: E-mail: c21968d@omu.ac.jp, tojo@omu.ac.jp;*

Results: The three groups were identified as *Pythium* Cluster B2a sp., B1d sp. and Clade A sp. on sequences of ITS and *COI*, respectively. *Pythium* isolates belonged to *Pythium* Cluster B1d and Clade A showed similar morphology and hyphal growth response to temperatures each other. Inoculation tests confirmed that both representative isolates from two groups cause roots rot of hydroponically grown Welsh onion 14 days after their inoculation. *Pythium* Cluster B1d showed stronger pathogenicity than *Pythium* Clade A.
Conclusion: The present study firstly demonstrated that *Pythium* Cluster B1d sp. and Clade A sp. which don't form any sexual structures associate root rot of hydroponically grown Welsh onion.

Keywords: Welsh onion; root rot; hydroponic culture; pythium cluster B1d, pythium clade A.

1. INTRODUCTION

Welsh onion (*Allium fistulosum* L.) is commonly grown hydroponically in Japan as well as other countries [1]. Hiroshima Prefecture is one of the largest production areas of hydroponically grown Welsh onion in the country [2]. Since the cultivation began in 1988 in Hiroshima Prefecture, root rot followed by leaf browning has been problem causing significant loss of harvest (Fig. 1). One of the causal agents has been identified as *Pythium* Cluster B2a species [3]. During a survey of hydroponic greenhouses in the same area from March 2020 to March 2021, we newly isolated two types of *Pythium*-like organisms showing different colony patterns from *P*. Cluster B2a on agar media including cornmeal agar (CMA, Nissui, Tokyo, Japan), potato dextrose agar (PDA, Nissui, Tokyo, Japan) and V8 juice agar (V8A, [4]) (Fig. 2A, B). They didn't form any of sexual organs as well as *P*. Cluster B2a on all the media and in Welsh onion roots. The plant symptoms were indistinguishable from those observed in *P*. Cluster B2a [3]. Therefore, we consider that at least two different species of *Pythium* other than *P*. Cluster B2a also associate with root rot of hydroponically grown Welsh onion. Aim of this study was to clarify association of these two unknown *Pythium* spp. without forming sexual structures on root rot of hydroponically grown Welsh onion, as well as to clarify their taxonomic positions.

2. MATERIAL AND METHODS

2.1 Isolation and Identification

Isolation and identification of *Pythium*-like organisms were performed from March 2020 to March 2021 by slightly modified method at the same location reported elsewhere [3]. It is briefly described as follows: The symptomatic tissue of roots of the hydroponically grown Welsh onion were examined for existing nonseptate hyphae by a microscopic observation every month during the survey period. The tissue samples were surface sterilized by immersing them in sodium chlorate solution (0.2% effective chlorine concentration) for a few minutes and placed on *Pythium* selective NARF medium [5]. The samples on the medium were incubated at 25°C for 2 days. When *Pythium*-like mycelia were developed on the medium, their single hyphal tips were transferred to CMA and maintained at 25°C in darkness until use. Morphological features of the single-hyphal isolates were examined on CMA, V8A, a grass blade culture and potato carrot agar (PCA) [6]. Hyphal growth rates from 1–46°C were measured by culturing on PCA. Total 2,966 isolates of *Pythium*-like organs were isolated from the plant roots during the survey period (Table 1), and were separated into three groups based on colony patterns on CMA, PDA and V8A. Among of them, 236 single-hyphal isolates were equally selected from each three groups. The ribosomal internal transcribed spacer (ITS) regions were amplified and sequenced for the 236 isolates according to the method reported elsewhere [7]. Since two of the three groups were thought to be first records on Welsh onion, 17 representative isolates from the two groups were further examined on morphology, hyphal growth response to temperatures and sequences of the ITS regions and mitochondrial *COI*.

2.2 Inoculation Test

The laboratory inoculation test by using Welsh onion seedlings was performed on the 236 isolates of *Pythium* by the same method described previously [3]. The greenhouse inoculation test was performed on the representative isolates P108 and P149 as follows. Each isolate was cultured on V8A at 25°C for 17 days. Welsh onion (cv. Koutou) were grown at a density of six plants on rock wool cubes and grown at greenhouse for 2 weeks. They were placed on precultured P108 and P149 on V8A for 24h at room temperature and transplanted to 90L container filled with receiving nutrition solution prepared at EC1.3 by Otsuka A

Fig. 1. Root rot followed by leaf browning of hydroponically grown Welsh onion caused by unknown *Pythium* spp. without forming sexual structures in Hiroshima Prefecture in June 2020

Table 1. Number of isolates of unknown *Pythium* spp. without forming sexual structures in Hiroshima Prefecture from roots of hydroponically grown Welsh onion from March 2020 to March 2021

Taxonomic group of *Pythium*	Number of *Pythium* isolates obtained*													
	2020										2021			Total
	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	
P. Cluster B2a	28	145	210	142	350	0	37	103	73	51	77	60	60	1,336
P. Cluster B1d	84	85	61	274	153	376	215	116	8	61	50	29	30	1,542
P. Clade A	0	0	0	0	0	0	15	65	0	2	0	6	0	88
Total	112	230	271	416	503	376	267	284	81	114	127	95	90	2,966

**The number of isolates of Pythium spp. obtained from 60 pieces of 3-cm long Welsh onion roots on Pythium selective NARF medium in each month*

prescription (Total N:130 ppm, P_2O_5:60 ppm, K_2O:203 ppm, CaO:115 ppm, MgO:30 ppm, OAT Agrio Co., Ltd.) and adjusted to pH 5.5. The inoculated and noninoculated plants were grown at greenhouse for 48 days. The experiment was repeated twice using 36 cubes (6 plants in a cube) per replication.

3. RESULTS AND DISCUSSION

Total 2,966 isolates of *Pythium*-like organs didn't form any sexual organs on CMA, PDA, V8A and in Welsh onion roots. They were divided into three groups based on the media. A total 236 isolates equally selected from the three groups were belonged either of *Pythium* cluster B2a [3], Cluster B1d [8] and Clade A [9]. Among of them, 156 isolates caused obvious weak growth and root rot 14 days after their inoculation to Welsh onion seedlings. No disease was observed in the non-inoculated plants. Among of the 156 isolates, a total 17 isolates other than *Pythium* cluster B2a were used for the phylogenetic analysis of the ITS and *COI* sequences. Eight isolates of the 17 isolates were formed single clade within Cluster B1d [8] (Fig. 3). They were isolates P85, P89, P93, P104, P108 (MAFF 247702, LC710645 for ITS, LC710650 for *COI*), P110 (MAFF 247704, LC710647 for ITS, LC710652 for *COI*), P193 and P194. The other nine isolates of the 17 isolates were formed single clade within Clade A [9] based on the same sequences (Fig. 3). They were isolates P97, P98, P100, P117 (MAFF 247705, LC710648 for ITS, LC710653 for *CO1*), P118, P137, P138, P149 (MAFF 247706, LC710649 for ITS, LC710654 for *CO1*) and P150.

Descriptions of representative isolates P108 (MAFF 247702) and P149 (MAFF 247706) from *Pythium* Cluster B1d and *Pythium* Clade A are provided here. The widths of hypha were up to 2.4 and 2.3 µm wide for isolates P108 and P149, respectively. Appressoria were knob-like terminations, and sporangia were filamentous, inflated and toruloid in both isolates (Fig. 1D, E). Zoospores were formed at 20–25°C and 20–30°C for isolates P108 and P149, respectively.

The diameters of the encysted zoospores ranged from 9.6–12.4 (av. 10.4) and 9.6–11.5 (av. 9.8) µm for isolates P108 and P149, respectively. The cardinal temperatures for hyphal growth on PCA were 4°C minimum, 31°C optimal, and 34°C maximum, and 10°C minimum, 34 °C optimal, and 40 °C maximum for isolates P108 and P149, respectively. The daily growth rate at 25 °C was 4.6 mm and 1.6 mm per day for isolates P108 and P149, respectively.

Pathogenicity of *Pythium* isolates P108 and P149 were confirmed by the same method described above. The Welsh onion seedlings inoculated with the isolates showed weak growth and root rot 14 days after their inoculation (Fig. 2C). The isolates belonged to either of *Pythium* Cluster B1d or Clade A were consistently re-isolated from the diseased plants, thus fulfilling Koch's postulates.

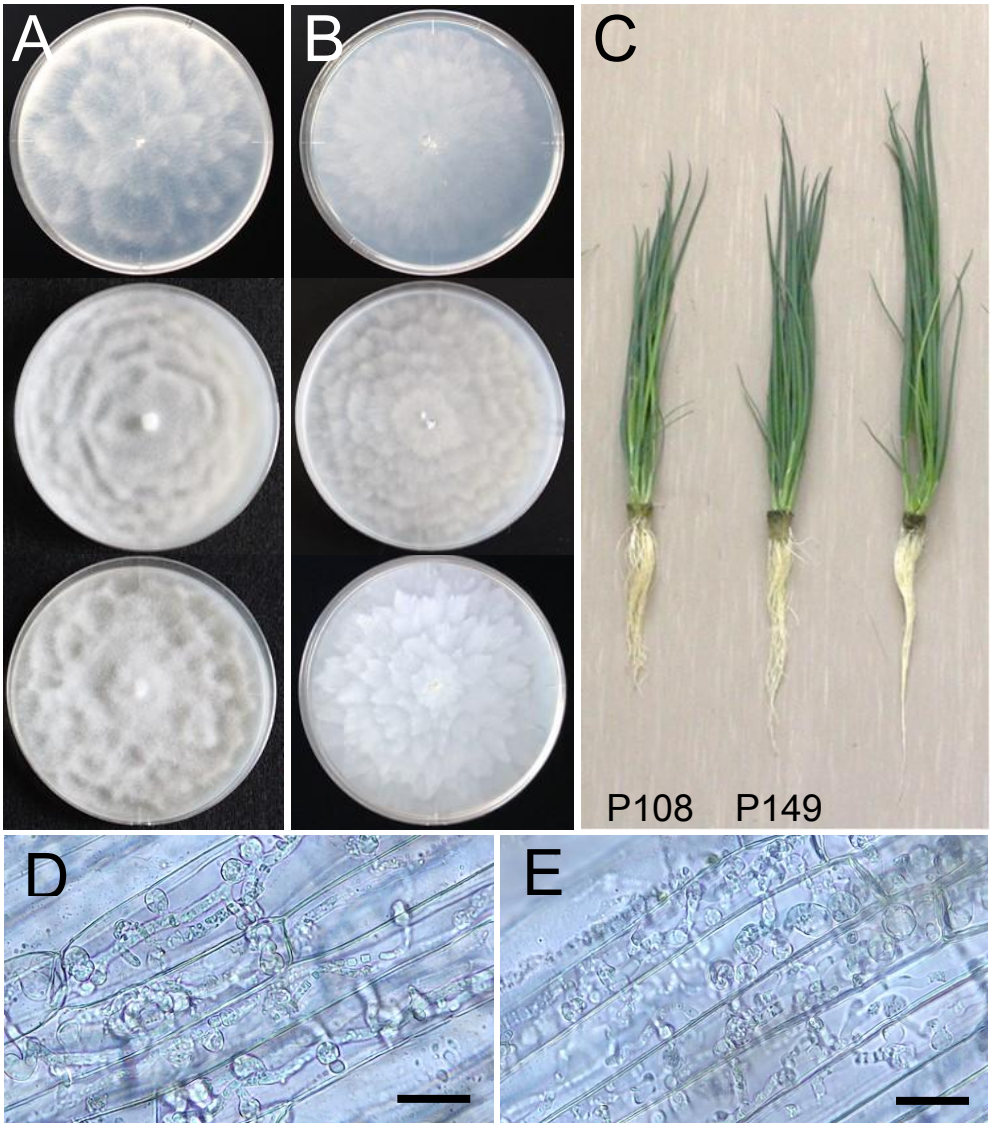

Fig. 2. Features of *Pythium* Cluster B1d isolate 108 and *Pythium* Clade A isolate P149 obtained from rotted roots of hydroponically grown Welsh onion in a commercial greenhouse in Hiroshima Prefecture, Japan

A, B: Colonies of Pythium Cluster B1d isolate P108 (A) and Pythium Clade A sp. isolate P149 (B) on CMA, PDA, and V8A (upper to lower)

C: Symptoms of root rot of hydroponically grown Welsh onion inoculated Pythium Cluster B1d isolate P108, Pythium Clade A sp. isolate P149 and non-inoculated

D, E: Nonseptate hyphae and inflated sporangia formed in diseased roots of hydroponically grown Welsh onion inoculated Pythium Cluster B1d isolate P108 (D), and Pythium Clade A sp. isolate P149 (E). Scale bars, 20 µm

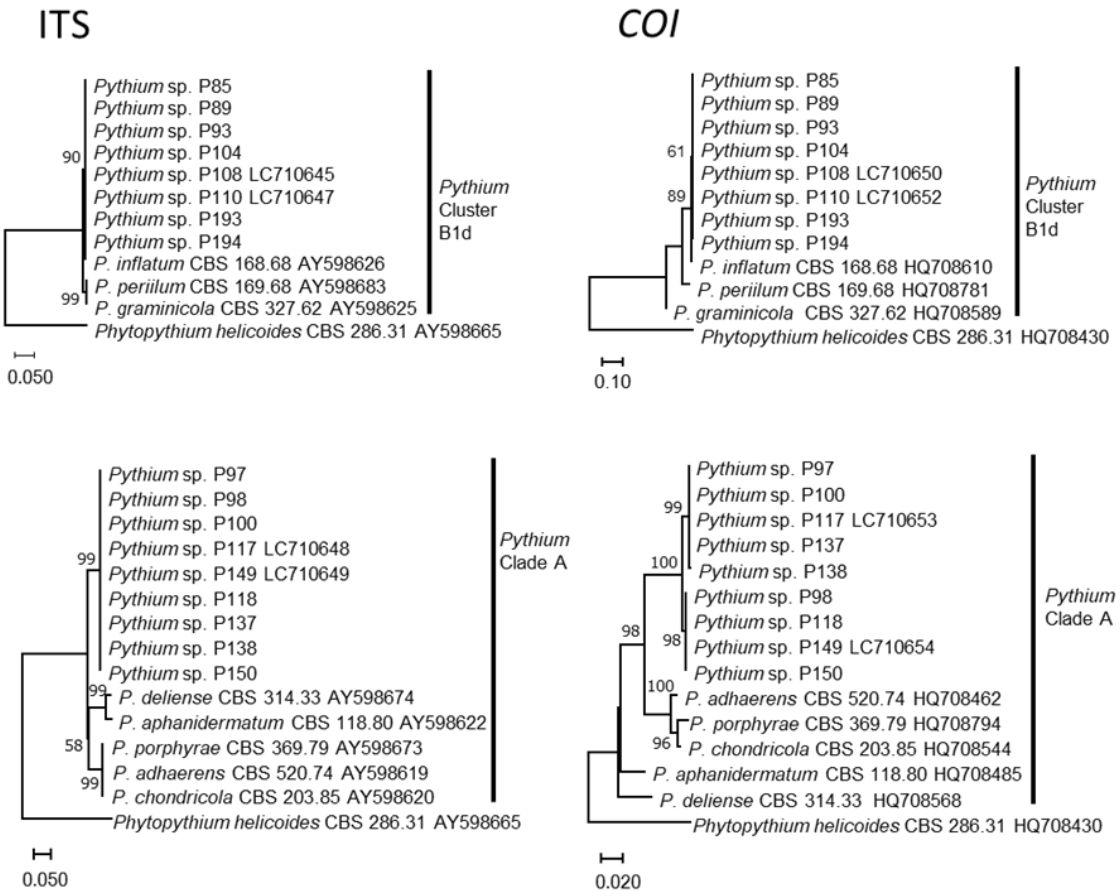

Fig. 3. Phylogenetic positions of 17 isolates of *Pythium* spp. without forming sexual structures from rotted roots of Welsh onion grown under a hydroponic system in Hiroshima Prefecture, Japan

Phylogenetic trees were built using the Neighbor-Joining (NJ) based on the rRNA gene-ITS and COI regions. Among the 17 isolates used, the eight isolates P85, P89, P93, P104, P108 (MAFF 247702), P110 (MAFF 247704), P193 and P194 formed single clade with Pythium inflatum isolate CBS 168.68 within Pythium Cluster B1d [8]. The other nine isolates P97, P98, P100, P117 (MAFF 247705), P118, P137, P138, P149 (MAFF 247706) and P150 formed single clade within Pythium Clade A [9]. Phytopythium helicoides was used as outgroups. Numbers along nodes indicate bootstrap confidence levels (gaining more than 50% support) for NJ

Pythium Cluster B1d and Clade A which don't form any sexual structures have never been recorded as plant pathogens in Welsh onions, including in hydroponic cultures, although they have been known as pathogens of other plants [6,8]. *P. aphanidermatum* of the Clade A has been commonly found in hydroponic cultures worldwide [10], but has not been recorded as a pathogen of Welsh onions. To the best of our knowledge, this is the first record of *Pythium* Cluster B1d and Clade A which don't form any sexual structures. Present study demonstrated that *Pythium* Cluster B1d sp. and Clade A sp. can cause significant damage on the plant as well as *Pythium* Cluster B2a sp. [3]. Therefore, management practices should be tailored in accordance with their pathological and epidemiological properties.

Pythium isolates which belong to Clusters B2a, B1d or Clade A were obtained through the experimental period (Table 1). The total numbers of the isolates were high from May to September when the root rot was frequently found [1]. *Pythium* Cluster B2a and B1d were showed high frequency comparing with *Pythium* Clade A. Most of the *Pythium* Clade A isolates were detected in September and October. *Pythium* Clade A showed weak pathogenicity comparing with *Pythium* Cluster B2a and B1d [3] (Fig. 2C). These results suggested that *Pythium* Cluster B2a and B1d are more important pathogens of root rot of Welsh onion than *Pythium* Clade A.

Pythium isolates of B2a, B1d and Clade produce zoospores, and don't form oospores on culture media and on Welsh onion roots. Zoospores are

the most important agent for the spreading of *Pythium* spp. in hydroponic cultures [10]. The addition of fungicides in hydroponic cultures might be effective to control zoosporic pathogens [10,11]. However, fungicides are usually not allowed to use in hydroponic cultures. Because fungicides should have a lag period between their application and harvest, and hydroponic vegetables are usually harvested every day [10]. There is also a potential risk of development of chemically resistant strains [10]. Zoosporulations of *Pythium* spp. are affected by several chemical factors such as mineral ion concentrations [12]. Our further study is ongoing to regulate zoospore spreading in nutrition of hydroponic culture of Welsh onion by controlling chemical factors of the nutrition.

4. CONCLUSION

The present study firstly demonstrated that *Pythium* Cluster B1d and Clade A which don't form any sexual structures associate root rot of hydroponically grown Welsh onion. *Pythium* Cluster B1d showed stronger pathogenicity than *Pythium* Clade A. Since the pathogens produce zoospores, control methods to regulate zoospore spreading in nutrition of hydroponic culture are needed to be established.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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