

Association of two unknown *Pythium* spp. without forming sexual structures on root rot of hydroponically grown Welsh onion

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 January 2021.

Methodology: The representative 17 isolates selected from total 236 isolates of *Pythium* without forming sexual structures were used. They were examined on sequences of the ribosomal internal transcribed spacer (ITS) regions and mitochondrial *COI*. Since the 17 isolates were divided into two phylogenetic groups based on the ITS and *COI* sequences, one representative isolate from each group was examined on morphology and hyphal growth response to temperatures. Inoculation tests were performed on Welsh onion seedlings under a hydroponic condition by using one representative isolate from each group.

Results: The sequence analysis on the ITS and *COI* sequences demonstrated that the 17 *Pythium* isolates belonged to either of *Pythium* Cluster B1d or Clade A. The one representative isolate from each group showed similar morphology and hyphal growth response to temperatures each other. Inoculation tests confirmed that both representative isolates from two phylogenetic groups cause roots rot of hydroponically grown Welsh onion 48 days after their inoculation.

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.

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. Hiroshima Prefecture is one of the largest production areas of hydroponically grown Welsh onion in the country. 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 [1]. During a survey of hydroponic greenhouses in the same area from March 2020 to January 2021, we 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, [2]) (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 [1]. 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.

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 2021.

2. MATERIAL AND METHODS

2.1 Isolation and identification

Isolation and identification of *Pythium*-like organisms were performed from March 2020 to January 2021 by slightly modified method at the same location reported elsewhere [1]. It is briefly describes as follows: The symptomatic tissue of roots of the hydroponically grown Welsh onion were examined for existing nonseptate hyphae by a microscopic observation. 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 [3]. The samples on the medium were incubated at 25°C for 3 to 7 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) [4]. Hyphal growth rates from 1–46°C were measured by culturing on PCA. The ribosomal internal transcribed spacer (ITS) regions and mitochondrial *COI* of the isolates were amplified and sequenced according to the method reported elsewhere [5].

2.2 Inoculation test

Inoculation tests were performed on the 236 single-hyphal isolates of *Pythium* by the same method described previously [1] with slight modifications. They were 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 prescription (Total N:130ppm, P₂O₅:60ppm, K₂O:203ppm, CaO:115ppm, MgO:30ppm, OAT Agrico 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

A total of 236 *Pythium*-like organisms were obtained in this study. All the isolates caused weak growth and root rot 48 days after their inoculation. No disease was observed in the non-inoculated plants. The 236 isolates were divided into three groups based on colony patterns on CMA, PDA and V8A. All the 236 isolates produced asexual structures but did not form any sexual structures on the three media and in Welsh onion roots. One of the three groups were identified as *Pythium* cluster B2a [1] based on the colony copatterns and the ITS and *COI* sequences. A total 17 isolates other than *Pythium* cluster B2a were used for the phylogenetic analysis of the ITS and *COI* sequences. Among the 17 isolates used, eight isolates were formed single clade within Cluster B1d [6] (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 were formed single clade within Clade A [7] based on the same sequences (Fig. 3). They were isolates P97, P98, P100, P117 (MAFF 247705, LC710648 for ITS, LC710653 for *COI*), P118, P137, P138, P149 (MAFF 247706, LC710649 for ITS, LC710654 for *COI*) 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 width of hyphae 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 48 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.

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 [4,6]. To the best of our knowledge, this is the first record of *Pythium* Cluster B1d and Clade A which don't form sexual structures on Welsh onions. Since *Pythium* Cluster B1d sp. and Clade A sp. can cause significant damage on the plant as well as *Pythium* Cluster B2a sp. [1], management practices should also be developed to reduce them.

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 *P. 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, *P. 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 *P. Clade A* sp. isolate P149 (E). Scale bars, 20 μ m.

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 Cluster B1d [6]. The other nine isolates P97, P98, P100, P117 (MAFF 247705), P118, P137, P138, P149 (MAFF 247706) and P150 formed single clade within Clade A [7]. *Phytophythium helicoides* was used as outgroups. Numbers along nodes indicate bootstrap confidence levels (gaining more than 50% support) for NJ.

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.

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