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New or Unusual Disease Reports

First report of Pythium root rot of hydroponic lettuce (*Lactuca sativa*) in Greece, caused by *Pythium* Cluster B2a sp.

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Summary. Pythium root rot has been reported in several countries, but in Greece this disease was first detected in 2021, causing severe yield losses in a hydroponic lettuce crop. Isolations, morphological and molecular characterization, as well as pathogenicity assays identified a *Pythium* Cluster B2a species causing the disease in hydroponically grown lettuce. This is the first report of *Pythium* Cluster B2a sp. causing lettuce root rot in Greece.

Keywords. Minor root pathogens, molecular characterization, pathogenicity assays.

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INTRODUCTION

Hydroponic and soilless culture cropping systems occupy approx. 5% of the greenhouse area in Greece, with a tendency for further expansion, especially in new high-tech greenhouse facilities. The increasing interest in soilless production systems has led to development of specialized decision support systems, which provide full support in managing the nutrition of these crops through balanced nutrient solutions (Savvas *et al*., 2023). Lettuce and other leafy vegetables are mainly produced in open fields and high tunnel houses in Greece, but there is an increasing tendency for year-round production in modern greenhouses using hydroponic technologies such as the Nutrient Film Technique (NFT) Barbosa *et al*., 2015). The lack of contact with soil in hydroponic lettuce production considerably reduces infections by soil-borne pathogens, although hydroponics cannot fully eliminate this risk.

Pythium root rot has been a major concern for hydroponic lettuce growers, and has been reported to cause important yield losses in Italy, Cyprus, and Connecticut, United States of America, especially when warm temperatures occur (Garibaldi *et al*., 2017; Pantelides *et al*., 2017; McGehee *et al*., 2018; Cacciola and Gullino, 2019). The disease has also been reported in hydroponi-

cally grown Welsh onions in Japan (Shimizu and Tojo, 2021). Currently, there are no approved fungicides for the control of the disease, but research has been conducted on effects of some biological control agents and chemicals to control the disease (Utkhede *et al.*, 2000).

MATERIALS AND METHODS

Sampling and isolation procedures

During September 2021, 30% of young 'Jokary' lettuce plants, grown hydroponically in an NFT system located in Viotia region, Greece, exhibited dark brown necrotic lesions scattered throughout the root system at approx. 2 weeks post transplantation (Figure 1A). In mature plants, the whole root systems were necrotic, with reduced biomass compared to apparently healthy plants (Figure 1B). In both cases, older leaves on the affected plants were chlorotic and wilted. Isolations were conducted onto V8-PAR medium, selective for oomycetes (Jeffers, 1986). For these, diseased roots were removed, washed with running tap water for 30 min and surface sterilized by immersion in 10% sodium hypochlorite for 2 min, 70% ethanol for 3 min, and then rinsing twice with sterile double distilled water. Small fragments of sterilized roots were placed onto V8-PAR medium and incubated at 25ºC with a 12 h photoperiod.

DNA extraction and PCR amplification

Total genomic DNA was extracted from selected isolates according to Zelaya-Molina *et al.* (2011). An initial PCR assay was carried out using the universal primer set ITS5 and ITS4 (White *et al.*, 1990) targeting the internal transcribed spacer region 1 and 2 containing the 5.8S region (Table 1). PCR products were precipitated using ammonium acetate and subjected to sequencing. The derived consensus sequences were edited using Benchling software (https://www.benchling.com/), and were compared to GenBank database sequences by BLASTn analysis. Due to the incapability of ITS region sequencing for identifying the isolated oomycete to the species level, additional molecular markers were employed. Cytochrome b oxidase subunits 1 and 2 (COI1, COI2), which are accepted for oomycete barcoding, were amplified by PCR using appropriate primers (Table 1).

PCR products were purified and sequenced as described above. Sequences of ITS and cytochrome b oxidase subunits 1 and 2 were deposited in GenBank under the respective accession numbers OQ657948, OQ686764 and OQ686765.

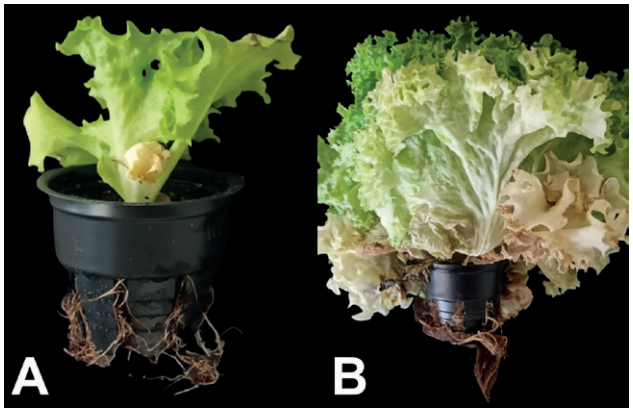

Figure 1. A) 2-week-old hydroponically grown lettuce plant with dark brown lesions in the roots and wilting of the lower leaves. B) mature plant showing chlorosis, yellowing, and wilting symptoms of the lower leaves, while the whole root system is necrotic.

Phylogenetic analysis

Phylogenetic analyses were conducted using the MEGA-X Software (Kumar *et al.*, 2018). Phylogenies were inferred using the p-distance substitution model for the three loci using the Neighbor-Joining (NJ) statistical method and 1,000 bootstrap replications. Analyses were also run using the concatenated sequences of ITS, COI1 and COI2, utilizing the p-distance substitution model to construct phylogenetic trees using the NJ statistical method with 1,000 bootstrap replications.

Pathogenicity assays

Pathogenicity tests were conducted on 'Jokary' lettuce plants to investigate the role of the isolated oomycete species in disease development. Eight 2-week-old lettuce plants were grown in 60 × 39 × 20 cm (l/w/h) tanks (one with eight inoculated plants, and the other with eight non-inoculated plants), each containing a standard nutrient solution for commercial crops (pH = 5.6, EC = 2.63 dS m^{-1}, K^+ 10.20 mmol L^{-1}, Ca^{2+} 4.86 mmol L^{-1}, Mg^{2+} 1.20 mmol L^{-1}, NH_4^+ 1.81 mmolL^{-1}, SO_4^{2-} 1.45 mmolL^{-1}, NO_3^- 16.78 mmolL^{-1}, $H_2PO_4^-$ 1.36 mmolL^{-1}, Fe 62.27 mmolL^{-1}, Mn^{2+} 5 mmolL^{-1}, Zn^{2+} 4 mmolL^{-1}, Cu^{2+} 0.71 mmolL^{-1}, B 43.8 mmolL^{-1}, Mo 0.70 mmolL^{-1}, Cl^- 2.75 mmolL^{-1}, Na^+ 0.20 mmolL^{-1} and HCO_3^- 1.03 mmolL^{-1}). For oxygen flow and nutrient circulation, two air pumps were used in each tank to provide enough oxygen for the plants. For pathogen inoculum, two intact cultures of a *Pythium* Cluster B2a sp. isolate from

Table 1. Primer sets used for phylogenetic analysis of oomycete species isolated from diseased lettuce plants.

Primer	Strand	Target	Sequence (5' -> 3')	Reference
ITS5	Forward	ITS	GGAGTAAAAGTCGTAACAAGG	White *et al.*, 1990
ITS4	Reverse		TCCTCCGCTTATTGATATGC	
OomCoxILevup	Forward	Cytochrome oxidase subunit 1	TCAWCWMGATGGCTTTTTTCAAC	Robideau *et al.*, 2011
OomCoxI-Levlo	Reverse		CYTCHGGRTGWCCRAAAAACCAAA	
Cox2F	Forward	Cytochrome oxidase subunit 2	GGCAAATGGGTTTTCAAGATCC	Choi *et al.*, 2015
Cox2RC4	Reverse		TGATTWAYNCCACAAATTTCRCTACATTG	

90 mm Petri dishes were mixed with 250 mL of nutrient solution and homogenized using a mixer. The mix containing the pathogen and nutrient solution was then poured into a tank (total volume of 3.5 L), while pure nutrient solution was added into a tank containing the non-inoculated control plants. The plants were then kept in a greenhouse at 25ºC for 4 weeks.

RESULTS AND DISCUSSION

Forty-eight hours after isolation, oomycete-like colonies were observed on isolation plates, and these were transferred to new V8-PAR or PDA plates. The isolates produced white, flat, rosette-like mycelium on PDA (Figure 2), while on V8-PAR the mycelium was white and flat. Under microscopic observation, the colonies produced filamentous sporangia that formed dendroid structures and intercalary oogonia with straight oogonial stalks, of about 21 μm diameter. Antheridia were monoclinous and more than one antheridium per oogonium was observed. Oospores, while rarely developed, were plerotic or almost plerotic and uncoloured. Based on morphology, identification of the isolates to the species level was not possible, so molecular identification methods were used.

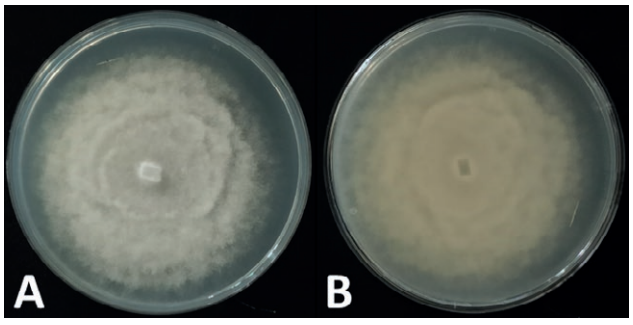

Figure 2. *Pythium* Cluster B2a sp. colony in PDA. A) front side and B) rear side of *Pythium* Cluster B2a sp. isolated from the roots of diseased lettuce plants.

BLASTn analysis against GenBank database sequences revealed 100% identity of the isolates obtained with the *Pythium* species *P. dissotocum*, *P. diclinum*, *P. coloratum and P. lutarium*. According to Robideau *et al.* (2011), the above species belong to a group indicated as *Pythium* Cluster B2a, and these organisms are indistinguishable based only on their ITS regions. Phylogenetic analysis carried out with the three genetic loci separately or in concatenation produced similar topologies (Figure 3A and 3B). Based on the evolutionary distances computed with the p-distance method for ITS locus and the concatenated sequences, the present study isolate belongs to the *Pythium* Cluster B2a species complex, and is closely related to *P. diclinum* with an evolutionary p-distance of 0 to 1,49 × 10^{-3}, respectively (Supplementary Table 1 and 2).

Approximately 10 days post-inoculation, plants inoculated with *Pythium* Cluster B2a sp. isolate showed chlorosis, yellowing and wilting symptoms of the lower leaves near the crowns, while their roots became brownish and eventually necrotic (Figure 4). Two weeks post-inoculation, almost all the inoculated plants collapsed, while control plants showed no symptoms. To fulfill Koch's postulates, isolations were carried out from the inoculated and control plants into corn meal agar (CMA) containing tetracycline hydrochloride 0.01% v/v). Isolations from the inoculated plants yielded pure cultures identical to *Pythium* Cluster B2a sp. while no microbial growth was observed from the isolations conducted from the control plants, thus fulfilling Koch's postulates.

In some cases, *Pythium* species are considered as "minor root pathogens" (Stanghellini, 1986), but favourable conditions for the pathogen may lead to significant yield losses. Although prevalence of Pythium root rots in hydroponically grown lettuce is limited in Greece, it is important that the pathogen biology and the conditions leading to disease outbreaks are understood.

This is the first report of *Pythium* sp. belonging to *Pythium* Cluster B2a causing root rot in hydroponically grown lettuce in Greece.

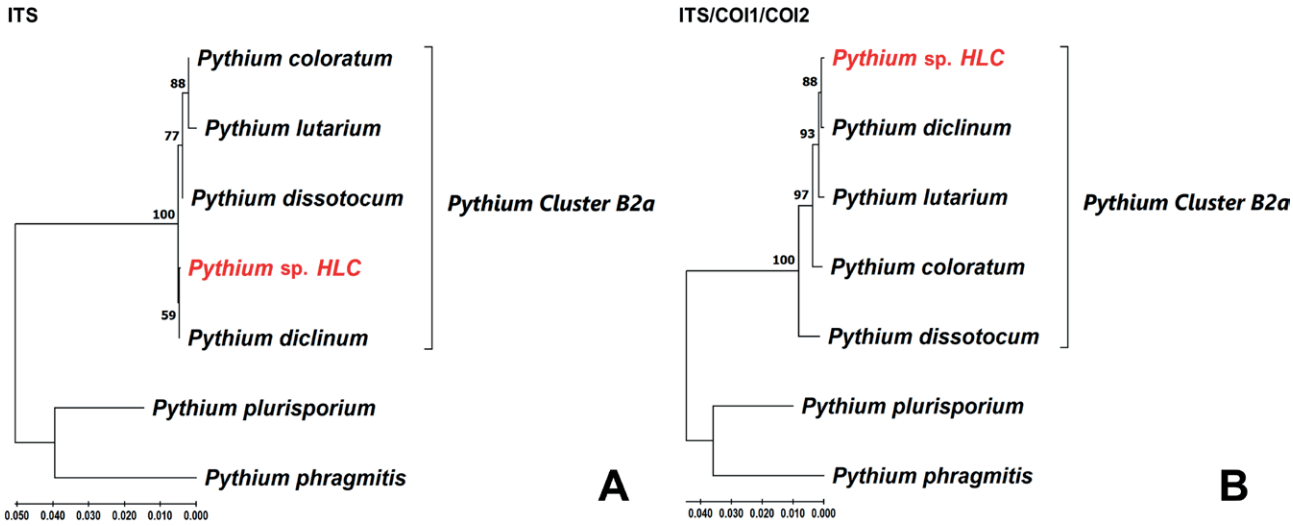

Figure 3. A) Phylogenetic tree of *Pythium* Cluster B2a sp. generated using the ITS sequences. B) phylogenetic tree of *Pythium* Cluster B2a sp. generated using the concatenated sequences. Phylogeny was inferred using the Neighbor-Joining method (NJ) with 1,000 bootstrap replications utilizing the p-distance substitution model. The optimal trees are shown. The phylogenetic analyses involved seven sequences with 894 final nucleotide positions for the ITS, and 2150 final nucleotide positions for the concatenated sequences. The OTU highlighted in red color represents the species isolated in this study. *Pythium plurisporum* and *Pythium phragmitis* were used as outgroups. The numbers on branches represent the bootstrap values.

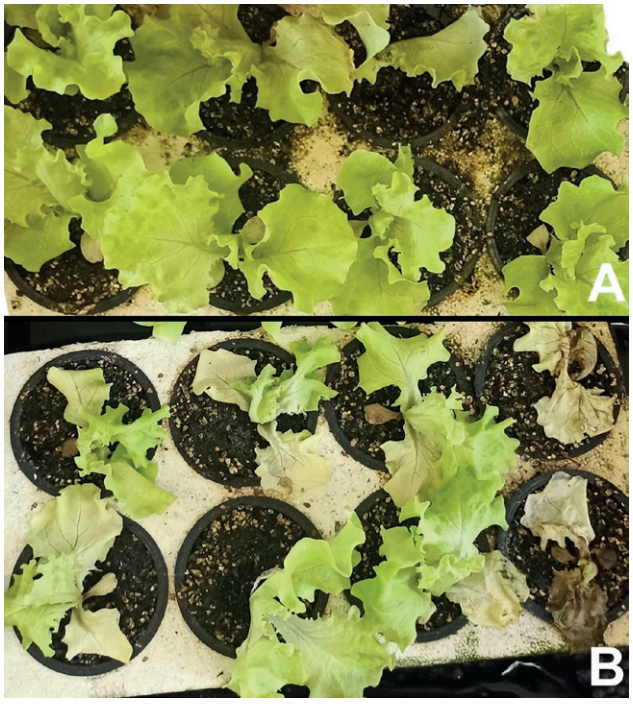

Figure 4. Pathogenicity assays with *Pythium* Cluster B2a sp. Images taken 2 weeks post inoculation with the pathogen. A) Control (non-inoculated) lettuce plants, and B) plants inoculated with *Pythium* Cluster B2a sp.

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