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FIRST REPORT OF ROOT ROT CAUSED BY *PYTHIUM APHANIDERMATUM* ON INDUSTRIAL HEMP (*CANNABIS SATIVA* L.) IN BULGARIA

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Abstract

Worldwide, thegrowers suffer serious losses from mycoses caused by *Pythium*, *Rhizoctonia*, and *Fusarium*. Due to the root rot disease, the whole plant dies, regardless of the phenophase at the moment of infection. The increase in *Cannabis sativa* acreage in Bulgaria and the accompanying problems associated with the severity and incidence of various diseases necessitated this study. The study aimed to survey the root rot symptoms on field and greenhouse plantations and monitoring the plant`s health status of cannabis-planted areas in Bulgaria during 2021-2022. The main problem was darkening at the base of the stem, yellowing, dieback, and wilting of whole plants from the seedling stage to flowering. Following the Koch's postulates and morphological characteristics by microscopic observation, all collected samples corresponded to genus *Pythium*. For complete identification, a molecular diagnostic was performed with primers ITS1 and ITS4 using four isolates from different regions of the country, which confirmed *Pythium aphanidermatum* as the causative agent. Based on the molecular analyses, this is the first report of root rot caused by *Pythium aphanidermatum* on cannabis in Bulgaria.
Keywords: *Pythium aphanidermatum*, root rot, Industrial hemp, *Cannabis sativa*

INTRODUCTION

Cannabis sativa L. belongs to the Order *Rosalesorder*, family *Cannabinaceae* with three species *C. sativa*, *C. indica*, and *C. ruderalis* (Small & Cronquist, 1976). The production of industrial cannabis with 0.3% tetrahydrocannabinol has been legalized in Bulgaria since 2018. The crop is used in many industries such as medicine, food, construction, etc. In the Netherlands and Canada the crop is often grown under artificial light, in hydroponic solution or other artificial substrate, which ensures the year-round production. In Bulgaria, this method is not popular and the climate conditions during the summer allow the outdoors cultivation. The cannabis vegetation period lasts, on average about, 130-160 days (Popov et. al., 1966).

The production of cannabis for medical and food industry requires a plant health monitoring that prevention of development of phytopathogens. The first problems related to root rot disease arise as soon as the seedlings germinate, which requires timely measures to limit the infestation (Punja, 2021). The symptom development was observed 13 days after sowing at temperatures between 25 to 30°C andthe affected plants have chlorotic leaves, were stunted and often withered (Beckerman et al., 2017). The disease has been described with symptoms of yellowing and browning of leaves, sudden wilting, and death of industrial hemp plants.

A stress caused by the high temperatures in combination with high humidity and waterlogging also influenced the development of plant damages (Chen et al. 2022). Necrotic lesions were observed at the base of the affected plants, the outer cortex was completely rotted and, when cut, the inner stem tissues were darkened. A white mycelium can also be

observed on the surface of infected plants (Hu & Masson, 2021). Characteristics of *Pythium aphanidermatum* when cultured on medium is the formation of rapidly growing white fluffy filamentous aseptate hyphae 3 to 7 μm wide, barrel-shaped anthers, globose oospores of 15 to 21 μm in globose oogonia of 25 to 31 μm in diameter (Watanabe, 2002).

The study aimed to investigate the root rot symptoms in industrial hemp grown under field and greenhouse conditions.

MATERIALS AND METHODS

A sampling of hemp stem and root

During the survey and monitoring which were done in early June until the end of July 2021 on hemp areas in Northeast (Dobrich), Central (Plovdiv, Yambol), and South Bulgaria (Haskovo) were collected seventy-two plant samples with symptoms of stunting, chlorotic, yellowing of leaves, wilting, necrosis at the base of the stem, and often withered. In waterlogged plants, white plaque on the stem was also observed.

Isolation

Root samples were placed under running water for 10 minutes to wash the soil particles off them thoroughly. Afterward, they were cut into 3mm pieces, and each piece was immersed for 20 seconds in 70% ethyl alcohol for surface disinfection, washed three times in sterile water, dried on sterile paper, and placed on potato dextrose agar (PDA) supplemented with vancomycin -1000mg/L. The Petri dishes were incubated at room temperature of 23-25℃ for 5-7 days. The colonies were transferred to another Petri dish for further identification. The genus level identification was based onpathogen e cultural characteristics after cultivation on different culture media - V8-Agar, Corn Meal Agar, Hemp Seed Agar, Potato Dextrose Agar, Oat Aga & Agar agar (Van der Plaats-Niterink, 1981, Leslie & Summerell, 2006).

Pathogenicity test

Pathogenicity tests were performed on healthy cannabis leaves and stem cuttings. For this purpose, mycelial discs of d-8mm from the pure culture were used. The healthy plant parts were surface disinfected with 70% ethyl alcohol and three times washed out with sterile water. The tissue sections were made with a sterile needle with 2-3 pricks, and three mycelial discs were inserted. A sterile scalpel was used to make a 3-cm-long transverse incision in the hole, where a mycelial disc was placed over the cut tissue and then covered with parafilm. Controls were made in the same way except that the pure PDA disks were used on healthy leaves and stem cuttings. The leaves and stems were placed in a chamber with moistened sterile paper and covered with transparent film at room temperature - 23-25 ℃ for seven days. The experiment was set with three repetitions. Based on the Koch's postulates, after seven days, the re-isolation was done from the necrotic tissue to confirm the pathogenicity.

Molecular diagnostics

For molecular diagnostics, four samples were selected from different regions (Northeast, Central, and South Bulgaria). The selection was narrowed down to one isolate per region due to the same rate of development and aggressiveness to the crop. It has been taken into account the different cultivation methods and predecessors - in the Northeast, and South Bulgaria the plants were grown in a greenhouse with vegetable precursors. In Central Bulgaria, the field production has cereal-grain precursors.

The pure cultures were identified to species level in the Mycology Laboratory at Institute ILVO Belgium, where molecular analyses were performed by PCR confirming the causative agent *Pythium aphanidermatum.* The analyses used two primers - ITS1 and ITS4 (ITS1-FCTTGGTCA-TTTAGAGGAAGTAA and ITS4 TCCT-CCGCTTATTGATATATGC). The mycelium from the culture was cultivated for seven days

in a liquid medium of potato dextrose broth, harvested and identified using the QIAGEN DNeasy Plant kit.

For PCR were used 1µl containing 5-20µg of DNA and 25µl reaction mix comprised of 2.5µl 10×buffer, 0.5µl dNTP, 0.25 µl Taq DNA polymerase (QIAGEN), 10µl forward and reverse primer, and 20.25 µL DNAase-free and RNAase-free water (Invitrogen). All PCR amplifications were performed in a Bio-Rad T100 thermal cycler. The used program was as follows - 3 min at 94°C; 30 s at 94°C, 30 s at 60° C, 3 minutes at 72°C (35 cycles), and 7 minutes at 72°C. PCR products were separated on 1% agarose gels; the bands of the expected size (~700 bp) were cut and purified with the QIA quick Gel Extraction Kit and sent for sequencing.

RESULTS AND DISCUSSION

The symptoms of infected plants were yellowing of the leaves and necrosis at the base of the stem. After removal from the soil, necrosis of the root system, and lack of root hairs ware observed (Figure 1). Seventy-two plant samples, with the corresponding symptoms, were collected, and the causal agent of *Pythium* root rot was isolated from twenty-three of them.

In laboratory analyses, the culture characteristics of the pathogen were observed on different media (V8-Agar, Corn Meal Agar, Hemp Seed Agar, Potato Dextrose Agar, Oat Agar & Agar agar). On OA and HSA the mycelia were fast-growing, white, fluffy, cotton-like mats growing all over the substrate and exit the petri dish. On PDA is the mycelia was also fluffy and white, but not as bulky. On the CMA and Agar-Agar media the hairs were faint, with the plaque spreading from the disc along the substrate. On V8, the smear was more flattened, less fluffy, and whitish beige. The cultural characteristics of the isolates were similar to *Pythium* (Figure 2). The microscopic observation revealed aseptic hyphae forming zoosporangium and oospores (Figure 3).

Fig.1. Symptoms of *Pythium* in the plant samples with yellowing of leaves, wilting, necrosis at the base of the stem, and absence of root hairs were visible.

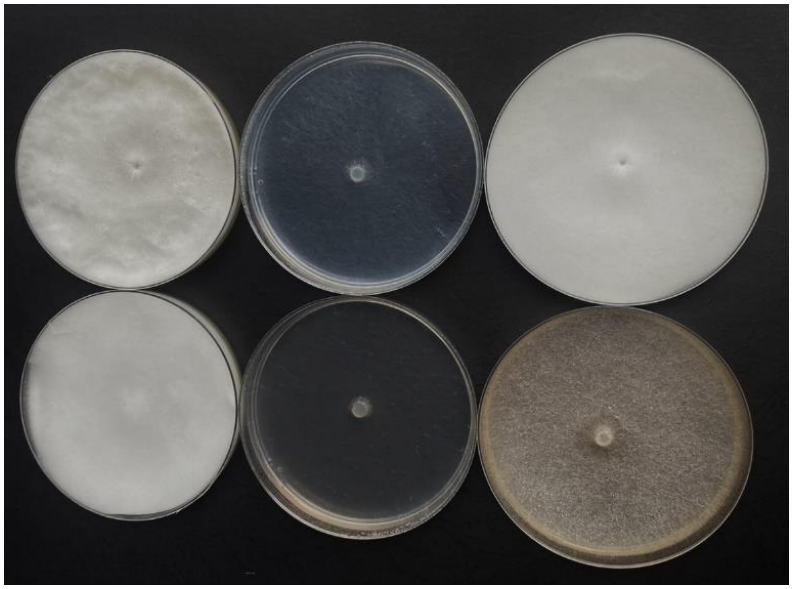

Fig.2. Cultural characteristic of *Pythium aphanidermatum* growing in different media
PDA, CMA, HAS (first row/left to right),
OA, Agar-agar, V8 (second row/left to right) after 10 days incubation.

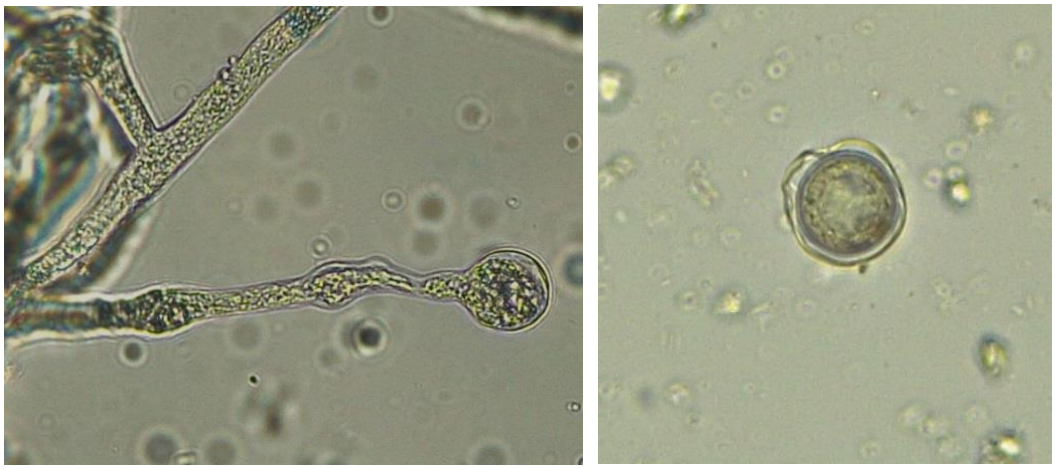

Fig.3. Sporangium with aseptate hyphae (on the left side), and oospores (on the right).

The pathogenicity was confirmed by the browning of the tissues, and the formation, less than three days after inoculation, of white mycelium on leaf and stem tissues. In the control, there was no change in the staining of the tissues, they appeared visibly healthy (Figure4).

The generated sequencing results were compared with results from the (NCBI) GenBank database, *Pythium aphanidermatum* strain UTHSCSA DI-14-349 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene (Farmer, A.R. et al. 2015). The partial sequence where a 100% gene match with those of *Pythium aphanidermatum* was confirmed for the chosen four isolates from different regions.

The species *Pythium aphanidermatum*, a pathogen that primarily attacks *Solanaceae,* was first reported in Bulgaria in 2016 by Vatchev et al. (2016) as a problem in hydroponic cultivation in pepper, but there were also reports for tomatoes (Gilardi et al., 2021) and lettuce (Stouvenakers et al., 2019).

Fig.4a. Control of pathogenicity test after 5 days with pure PDA discs.

Fig.4b. Pathogenicity test of samples of *Pythium* after 5 days

The characteristics of the species gathered from the available information, display their ability to develop very well in hydroponic cultivation, which was not observed during cannabis surveys in the current study and raises questions about seedling production. This is the first step in plant cultivation and would be a source of contamination.

Based on the characteristics and molecular diagnostics of cannabis symptoms, the presence of *Pythium aphanidermatum* was confirmed. Further analyses are forthcoming concerning the above question about the origin of infection in hemp plants. To our knowledge, this is the first report of *Pythium aphanidermatum* on cannabis in Bulgaria.

CONCLUSION

In the present study, four isolates from cannabis roots taken from different regions of the fast-growing oomycete were identified. The cultural, morphological characteristics, pathogenicity test, and molecular analysis were used and *Pythium aphanidermatum* species was confirmed as a causative agent of infection.

It is characterized by leaf yellowing, wilting, necrosis at the base of the stem, and root rot. The micelium was characterized by fluffy white growing rapidly in the petri dish, and the microscopy revealed aseptic hyphae, zoosporangium, and oospores. The soilborne pathogen poses risks to growers ranging from whole plant death as it can infect the crop from seedling to flowering.

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