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MORPHOLOGICAL CHARACTERIZATION AND PATHOGENICITY TO ORNAMENTAL PLANTS OF A BULGARIAN ISOLATE *PHYTOPHTHORA PLURIVORA*

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Abstract

Phytophthora plurivora, isolate RZlPan2016/25a, was derived from Zlatna Panega River at the time of a large-scale examination of *Phytophthora* distribution in aquatic ecosystems in Bulgaria. The species identification was performed using DNA sequence analysis of the ITS region and morphological characterization. The isolate RZlPan2016/25a demonstrated strong pathogenicity to oleander and azalea in a detached leaf bioassay. An application of fungicides Funguran OH 50 WP, Dithane® M-45, Acanto® and Acanto® Plus led to *in vitro* inhibition of *P. plurivora* growth. The potential threat for the wide range of host species, including ornamental plants by *P. plurivora* is discussed as well as the necessity of effective control to prevent the spread of the pathogen worldwide.

Keywords: *Phytophthora*, disease, azalea, oleander.

INTRODUCTION

Phytophthora plurivora is a well known soil-borne plant pathogen that is worldwide spread and is characterized by a high aggressiveness to a wide range of host plants. The species occurs abundantly in forests, semi-natural ecosystems and nurseries in Europe where it causes disease symptoms on at least 41 woody host species (Jung and Burgess 2009), as well as many ornamentals (Lilja et al., 2011; Mrázková et al., 2011; Mrázková et al., 2013; Prospero et al., 2013; Knaus et al., 2015). *P. plurivora* is frequently isolated from different ornamentals with disease symptoms in European nurseries (Lilja et al., 2011; Mrázková et al., 2011; Prospero et al., 2013). *P. plurivora* was identified on *Rhododendron* spp. and *Syringa vulgaris* in Finland (Lilja et al., 2007; Lilja et al., 2011). Investigation of ornamental gardens and nurseries in the Czech Republic reviled that *P. plurivora* is the main cause of an infection on ericaceous plants *Azalea* sp., *Rhododendron* sp., *Pieris floribunda* and *Vaccinium* sp. (Mrázková et al., 2011; Mrázková et al., 2013). This taxon is the second most common pathogen after *P. ramorum* isolated from ornamental nurseries in Swiss (Prospero et al., 2013). In the USA, the species was detected at most of the rhododendron nursery gardens investigated in Oregon (Knaus et al., 2015).

P. plurivora is one of the four species of the former *P. citricola* complex (Jung and Burgess 2009). It is considered to be a homothallic species, characterized by the production of sexual spores by self-fertilization. A distribution assessment of *P. plurivora* population showed that it most likely was introduced from Europe into the USA and spread around the world through transfer of diseased plant material (Schoebel et al., 2014).

Here, we describe the morphological characterization and pathogenicity analyses of a Bulgarian isolate *P. plurivora*, RZlPan2016/25a. The isolate was derived by Zlatna Panega River and was determined as *P. plurivora* using sequencing with ITS5 and ITS4 primers, and BLAST search. Mycelium growth characteristic in different culture media, as well as morphological structures, were described. Pathogenicity of *P. plurivora,* isolate RZlPan2016/25a, to azalea (*Azalea indica*) and oleander (*Nerium oleander* L.) using detached leaf method was assessed.

MATERIALS AND METHODS

Phytophthora isolation

P. plurivora, isolate RZlPan2016/25a, was derived from Zlatna Panega River using a baiting method via *Rhododendron* leaves (Jung et al., 2011). The isolation of *Phytophthora* was performed by cultivation on selective PARNHB media (carrot agar supplemented with 10 mg Pimaricin, 250 mg Ampicilin, 10 mg Rifampicin, 50 mg Nistatin, 1.3 ml Tahigaren и 15 mg Benomyl/1l). Plates were incubated at 23-25ºC and were observed for mycelium growth for 3-5 days. A mycelium plug was transferred on water agar to

growth for about 3-4 days and then hyphal tips were taken with a needle under a microscope and were transferred to a fresh vegetable media, depending on future analyses (Werres, 2015).

DNA extraction, amplification and sequencing

DNA was isolated from fresh mycelium using DNeasy Plant Mini Kit (QIAGEN GmbH). The PCR was performed by PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare Life Sciences), according to the manufacturer's instructions with primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') under the following PCR program: 96ºC – 2 min, followed by 35 cycles of 96ºC – 1 min, 55ºC – 1 min, 72ºC – 2 min and final elongation at 72ºC – 10 min. The PCR products were purified using Sephedex and send for sequencing to GATC Biotech AG (Germany). A data analysis was performed using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database.

Morphological characteristics

Different culture media were used for morphological characterization of the isolate RZlPan2016/25a, as follow: V8A (vegetable agar: 16 g agar, 3 g $CaCO_3$, 100 ml V8 juice/1l), CA (carrot agar; 16 g agar, 3 g CaCO3, 100 ml carrot juice/1l), PDA (Potato Dextrose Agar, Difco), CMA (Corn Meal Agar, TM Media) and HSA (50 g hemp seeds, 16 g plant agar/1l). Sporangia were produced by the flooding of V8 agar plugs of fresh *P. plurivora* culture as described by Jung et al. (2003). Observations were performed on a microscope Zeiss Axio Imager equipped with AxioVision 4.8.2 software.

Pathogenicity analyses

A detached leaf bioassay was performed to assess the pathogenicity of *P. plurivora*, isolate RZlPan2016/25a, to oleander and azalea. A mycelium plugs (0.5/0.5 cm) of 7-days old culture of the isolate were placed on leaves after slight wounding of the surface. Leaves of both plant species supplemented agar plugs without *Phytophthora* were prepared as a control. The inoculated leaves were incubated onto a wet filter paper in Petri dishes to maintain high relative humidity at 24°C/22°C (16 h light/8h night). Disease symptoms were evaluated 6 days after inoculation (dpi).

Fungicide treatment

The effect of five fungicides on the growth of *P. plurivora* was analyzed using *in vitro* experiments. The list of the tested fungicides is as follow: Funguran OH 50 WP (Copper hydroxide, 77%), Topsin® M70 (Thiophanate-methyl, 70.0%), Dithane® M-45 (Mancozeb, 800 g/kg), Acanto® (Picoxystrobin, 250 g/l) and Acanto® Plus (Picoxystrobin, 200g/l and Cyproconazole, 80g/l).

P. plurivora, isolate RZlPan2016/25a, was cultivated on V8A for 24 h at 25°C in dark. Two spots (20 µl) of each fungicide (0.2% water solution) was applied on both sides of the growing mycelium plug. Two replicats for each variant was prepeared, as well as a control Petri dishes with *P. plurivora* without a fungicide. All cultures were incubated at 25°C in the dark for 5 days. Radial growth rate was measured along a line from the centre of the mycelium to each of both fungicide spots. The fungicidal effect is expressed as the mean value ± standard error of the mean (SEM). The statistical significance of the differences in mycelium growth between control and tested fungicides was assessed by two-tailed t-test at the probability levels of $P<0.001$ (***) and $P<0.01$ (**).

RESULTS AND DISCUSSION

Isolation, identification and morphological characteristics of *P. plurivora*

P. plurivora, isolate RZlPan2016/25a, was derived from Zlatna Panega River at the time of the assessment of *Phytophthora* distribution in aquatic ecosystems in Bulgaria. It belongs to clade 2 whose representatives are the second most widespread *Phytophthora* group after clade 1 in this study.

The isolate RZlPan2016/25a was referred as *P. plurivora* based on the results from the BLAST search and the high homology with sequences of this taxon in the GenBank database at the NCBI. It shares 99% homology with the sequences of the ITS region belonging to several *P. plurivora* species as isolate TW117 (GenBank: KU682581.1), isolate 250498 (GenBank: KT383056.1), isolate 19/4 (GenBank: KF963049.1), strain IBL704 (GenBank: KT306889.1) etc.

Colony growth patterns of the *P. plurivora* RZPan2016/25a on V8A, CA, CMA PDA and HSA are shown in Figure 1. The isolate forms a narrow petaloid chrysanthemum-like submerged colonies on V8A and CMA, to stellate on CA and HSA, and rosaceous on PDA. Aerial mycelium was most abundant on PDA.

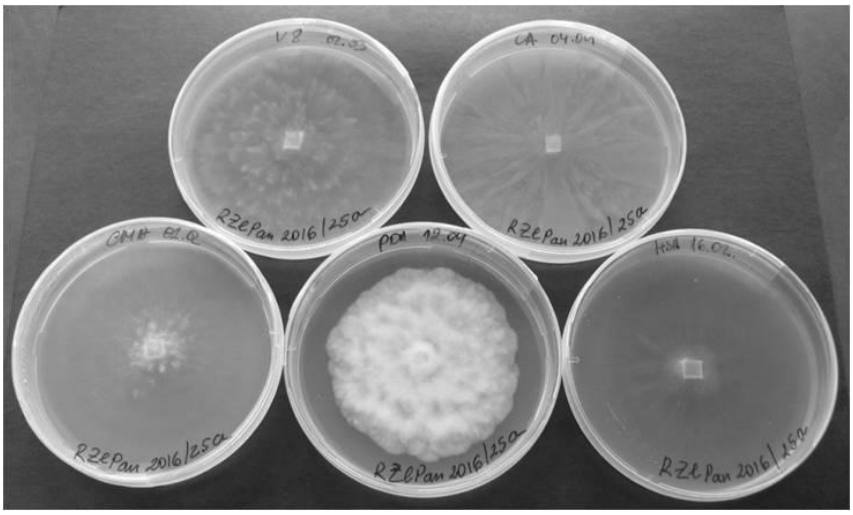

Fig. 1. *Mycelium growth of P. plurivora, isolate RZlPan2016/25a, on V8A, CA, CMA, PDA and HSA media for 8 days (from up to down and from left to right)*

Sporangia of the isolate *P. plurivora* RZPan2016/25a were produced abundantly in spring water under laboratory conditions. They were persistent semi-papillate, predominantly ovoid (Fig. 2 a), obpyriform (Fig. 2 b, c), sometimes bipapillate (Fig. 2 d) or unilaterally constricted (Fig. 2 e). After the discharge of zoospores, the empty sporangia remains with narrow exit pore (Fig. 2 f). Average dimensions of sporangia were 50.75 µm ± 5.12 x 36.62 µm ± 3.8 with length/breadth ratio of 1.39±0.13. Our isolate *P. plurivora* RZPan2016/25a was homothallic, readily producing an abundant quantity of oospores on V8A. Oogonia were globose with paraginous antheridia. Oospores were plerotic (Fig. 2 g) and aplerotic (Fig. 2 h, i). The average diameter of the oogonia was 32.48 µm ± 2.42, and of oospore 29.04 µm ± 1.65. Antheridia were paragynous with length 12.05 µm ± 2.36. Average oogonial wall diameter was 1.72 µm ± 0.46. The cultural and morphological parameters of our isolate correspond with the described in the literature for *P. plurivora* (Jung and Burgess, 2009).

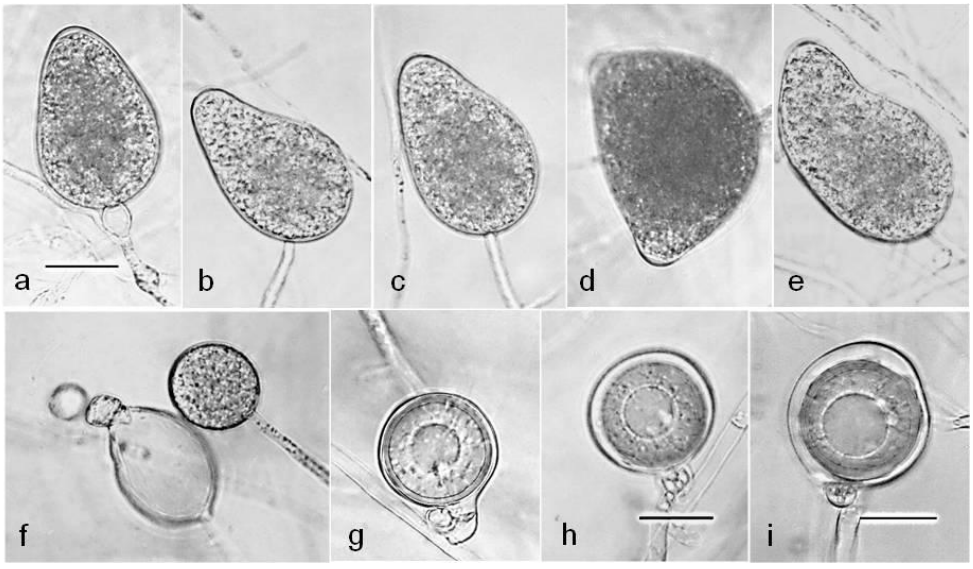

Fig. 2. *Different shapes of sporangia and oospores of P. plurivora, isolate RZPan2016/25a: a - ovoid sporangium; b, c – semi papillate intercalary obpiriform sporangia; d - bipapillate sporangium; e - semi papillate unilaterally constricted ellipsoid sporangium; f - hyphal swelling and sporangium with medium exit pore releasing zoospores; g – paraginous plerotic oospore; h - amphiginous aplerotic oospore; i – aplerotic oospore; Bar = 20 µm*

Pathogenicity of *P. plurivora* to oleander and azalea

Pathogenicity analyses of the isolate RZPan2016/25a using detached leaf bioassay showed aggressiveness to both tested plant species oleander and azalea (Fig. 3). Fast growing necrosis around the infected area was observed within the period of 3–6 days after inoculation. No disease symptoms on control leaves were detected (Fig. 3). These data confirmed the ability of *P. plurivora* to infect ornamental species that was previously reported by other authors (Knaus et al., 2015; Lilja et al., 2007; Lilja et al., 2011; Mrázková et al., 2011; Mrázková et al., 2013; Prospero et al., 2013). It was also reported to be pathogenic to numerous woody hosts including coniferous and deciduous plants from different genus as *Abies, Tsuga*, *Alnus*, *Acer*, *Aesculus*, *Fagus*, *Fraxinus*, *Quercus*, *Carpinus*, *Tilia*, *Syringae*, etc. (Jung and Burgess, 2009).

Undoubtedly, *P. plurivora* is a potential threat for plants in nurseries, city parks and natural ecosystems where the host species are distributed. Therefore, an active plant protection strategy to control the pathogen is needed.

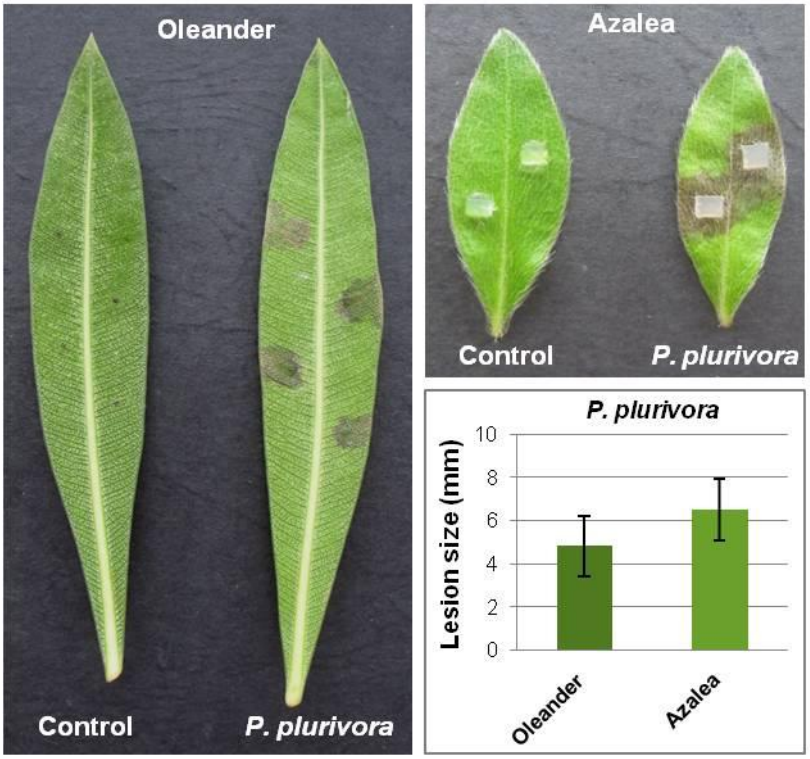

Fig. 3. *Pathogenicity of P. plurivora, isolate RZPan2016/25a, to oleander and azalea leaves at 6 dpi*

Inhibition of *P. plurivora* by fungicides

The results from the *in vitro* tests for the effect of five different fungicides on *P. plurivora*, isolate RZPan2016/25a, showed inhibition of mycelium growth by Funguran OH 50 WP, Dithane® M-45, Acanto® and Acanto® Plus (Fig. 4). The most effective fungicide against the isolate RZPan2016/25a was a treatment with Acanto® and Acanto® Plus (Fig. 4 e, f). They arrest colony growth overall probably by diffusion of the active ingredient Picoxystrobin in the media. The effect of Dithane® M-45 was also significant, but most evident at the application spots (Fig. 4 d). Less active to *P. plurivora* was Funguran OH 50 WP which application in the media leads to partial inhibition of mycelium growth (Fig. 4 b). No difference in the growth of *P. plurivora* between the control plate and the application of Topsin® M70 was observed (Fig. 4 a, c).

A treatment with fungicides Funguran OH 50 WP, Dithane® M-45, Acanto® and Acanto® Plus could be applied to azalea, oleander and other ornamental host plants to prevent the growth of *P. plurivora*. The efficacy of two other products, Micora (Mandipropamid) and Segovis (Oxathiapiprolin) to control *P. plurivora* infection of azalea and rhododendron was reported as a result of a large-scale investigation of *Phytophthora* disease management in ornamental horticulture (Palmer and Vea, 2016).

The additional data received in our study for active components against these pathogens will help to limit their spread and induced damage. Moreover, the application of suitable fungicides could be used to protect not only cultivated plants in nurseries but also natural flora in parks and gardens.

The control of *P. plurivora* is essential to restrict dissemination of the pathogen because of it's highly aggressiveness to a wide range of hosts. Global trade with ornamental plants, as well as other vegetation, leads to increasing in the prevalence of pathogens around the world and has been pointed as a main reason for the introduction of invasive *Phytophthora* species into new territories (Brasier, 2008; Westphal et al., 2008; Jung et al., 2013). Therefore the phytosanitary control and the treatment of a plant material in advance with appropriate plant protection compounds can reduce the spread of dangerous pathogens.

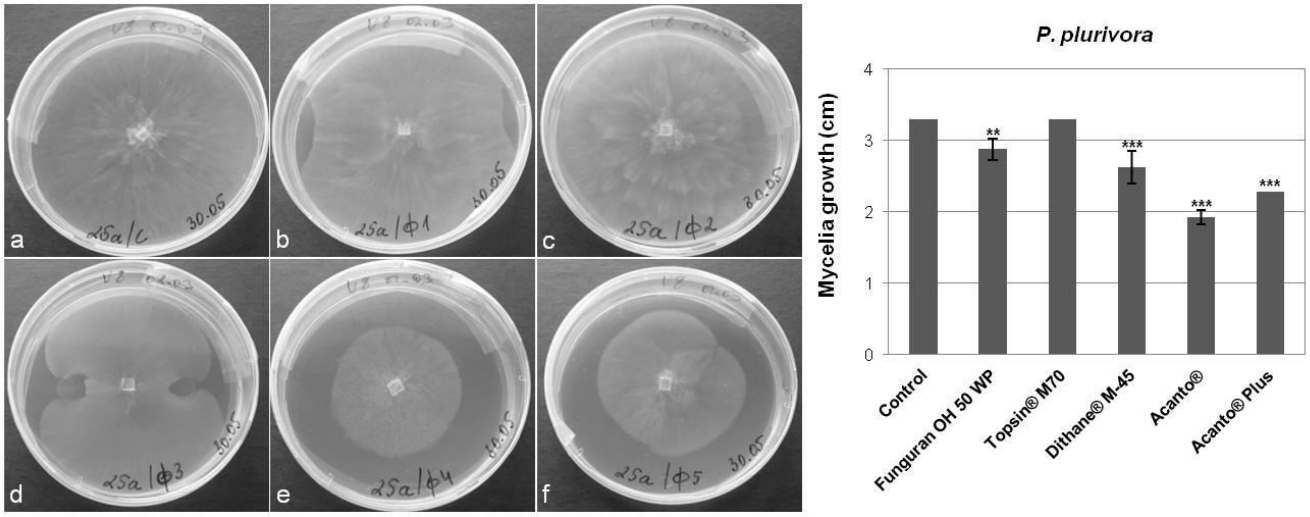

Fig. 4. *Inhibiton effect of different fungicides to the growth of P. plurivora isolate RZPan2016/25a after 5 days a – Control; b - Funguran OH 50 WP; c - Topsin® M70; d - Dithane® M-45; e - Acanto®, f - Acanto® Plus Asterisks ***, ** represent a significant difference at P<0.001 and P<0.01 respectively, according to two-tailed t-test*

CONCLUSSIONS

The isolation of *P. plurivora* from Zlatna Panega River is the first record for a distribution of this *Phytophthora* species in an aquatic ecosystem in Bulgaria, according to our best knowledge. The pathogen demonstrated strong aggressiveness to both tested ornamental plants azalea and oleander, but could be effectively controlled by some fungicides as Funguran OH 50 WP, Dithane® M-45, Acanto® and Acanto®.

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