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INFLUENCE OF MACRO- AND MICRO-ELEMENT CONTENT ON MYCELIAL GROWTH OF *PHOMA* SP.1

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Phoma blight of conifer seedlings is poorly studied. The main causal agent in Belarus differs in DNA sequence from other described Phoma *species, and has not been fully taxonomically described, and therefore has been temporarily designated* Phoma *sp. 1. The basic morphological and growth culture characteristics of* Phoma *sp. 1 have recently been published. This report provides additional data about the influence of macro- and micro-elements on the growth of* Phoma *sp. 1* in vitro *to complement the description of growth culture and morphological features. It was found that addition of tricalcium phosphate and potassium chloride to the media at concentrations of 1 g/l and 2 g/l slows the mycelial growth by an average of 30%. The micro-elements copper sulphate (at 50 mg/l and 100 mg/l) and zinc sulphate (at 100 mg/l) decreased growth by 50%, 63%, and 32%, respectively. Urea completely inhibited* Ph*. sp.1 growth at 0.5 g/l. Summarising previously published information,* Phoma *sp. 1 is a mesophilic acidophile, capable of forming chlamydospores in culture. Pycnidia form deep in the media only after prolonged low-temperature storage. The optimal pH for growth is 4.3–4.9. Constant medium aeration favours the formation of typical mycelium, proportional growth and stable biomass accumulation.*

Keywords: *pathogenic fungi, forest nurseries, mineral nutrients,* in vitro *growth conditions, phytopathology.*

INTRODUCTION

Although in other countries cases of Phoma blight disease date back to the 1980s (Kliejunas *et al*., 1985; James, 1986; Hansen and Hamm, 1988; Srago *et al*., 1989; James, 1998), in Belarus this disease is considered to be new, yet already widespread (Baranov *et al*., 2012; Yarmolovich *et al*., 2013). The first symptoms of infection in plants appear in early May, when the lower needles of seedlings and saplings become golden brown, and then turn brown and die. The pathogen spreads along the young stem, causing the side shoots of the current year and the apical bud to die. As a result, plant growth is inhibited and the plant eventually dies. Phoma blight of agricultural plants has been known for a long time (causal pathogens are fungi from the genus *Phoma* Sacc.) (Smith *et al*., 1992; Fitt *et al*., 2006), yet studies of the causative agents of Phoma blight of forest trees only initiated in the 1980s.

The fungal species of the genus *Phoma* are numerous, and are difficult to systematise due to the absence of reproductive organs in most species, and they are characterised by high morphological variability in natural conditions and polyphilia (Boerema *et al*., 2004; Aveskamp, 2014).

The collection of fungal material by sampling tissues of infected plants was carried out in 36 Belarusian forest nurseries. Morphological identification of isolated pure cultures revealed that members of the *Phoma* genus were the most common fungi in the affected tissues of coniferous tree species (*Pinus sylvestris* L. and *Picea abies* L.). Sequencing of the intergenic transcribed spacer (ITS) region of ribosomal RNA genes using ITS1-F and ITS4-B primers (Gardes and Bruns, 1993) and comparison of the obtained data with NCBI database (www.ncbi.nlm.nih.gov) entries showed that the analysed *Phoma* isolates were not identical to any other *Phoma* species regarding the ITS region sequence (Baranov

et al., 2015). Full genome sequencing of one of the isolates was performed, revealing further genetic differences from other *Phoma* species (Panteleev *et al*., 2015). In this context it was concluded that these *Phoma* isolates represent a new species, which was given the temporary name *Phoma* sp. 1. *Ph.* sp.1 isolates were compared to each other by use of retrotransposon-based iPBS assays, which revealed the high level of their genetic similarity and differences from isolates of *Phoma herbarum* Westend and *Phoma glomerata* (Corda) Wollenw. & Hochapfel (Šķipars *et al.,* 2018), supporting the previous conclusion.

One of the stages of the inclusion of a new fungal species in the taxonomic database and assigning it a name is the description of its vegetative and reproductive structures *in vivo* and *in vitro*, as well as description of the conditions of mycelium growth and spore formation (Seifert and Rossman, 2010). A number of *in vitro* characteristics, like growth rate, optimal temperature, chlamydospore formation, colony appearance, optimal pH and aeration preference were described by Seredich (2016). With this report on the influence of micro- and macro- elements on *in vitro* growth of this microorganism, we complete the required descriptions of *Ph.* sp.1 concerning its vegetative and reproductive structures *in vitro*.

MATERIAL AND METHODS

The object of this study, the pure culture of the fungus *Ph.* sp.1, was isolated from an infected individual of *P. abies* L. with typical symptoms of Phoma blight, collected in the permanent forest nursery of the forest enterprise "Ivatsevichy Leskhoz". It was determined that the isolate does not belong to any described species by analysis of the DNA sequences 18S rRNA gene, 5.8S rRNA gene, 26S rRNA gene, intergenic transcribed spacer regions ITS1 and ITS2 of ribosomal RNA genes, conducted at the Forest Institute of the National Academy of Sciences of Belarus (Siaredzich, 2017; Baranov, unpublished). Comparison of the nucleotide sequence of this isolate with data of the NCBI database for the previously mentioned DNA regions showed that the most closely related species was *Ph. glomerata* (97% level of similarity of genetic material) (Siaredzich, 2017; Baranov, unpublished).

Evaluation of the influence of the presence of basic mineral nutrients on the growth rate of mycelium of *Ph.* sp.1 was carried out by introducing various macro- and microelements into starvation agar in different concentrations. The microelements tested included copper, zinc, manganese and boron as copper(II) sulfate, zinc sulfate, manganese(II) sulphate and boric acid, respectively. The micro-element concentrations tested were: 10 mg/l, 50 mg/l, and 100 mg/l. These are the concentrations that were also used by Zavertkina, 2007. The impact of the macro-elements nitrogen (urea, N content at least 46.2%), phosphorus (tricalcium phosphate, P content at least 26.2%), potassium (potassium chloride, K content at least 52%) and complex fertiliser (N, P_2O_5 and K_2O content not less than 16% each) were tested at three concentrations: 0.5 g/l, 1 g/l and 2 g/l. These are the concentrations that are recommended for use in Belarusian forest tree nurseries (Jakimov, 2007). In addition, the impact of dolomite ($CaMg(CO_3)_2$) flour with at least 85% carbonate content on mycelial growth was tested. Dolomite flour is often used to decrease soil acidity to hamper the growth and development of many types of fungi that prefer a weakly acidic or neutral environment. All experiments were performed in 3–5 replicates for each variant of the experiment.

RESULTS

The greatest influence on the growth of *Ph.* sp1 was exerted by urea, which completely suppressed the growth of mycelium in all tested concentrations. Dolomite flour, which is usually applied to the soil to reduce acidity, inhibited the growth of mycelium by 60% at a concentration of 2 g/l in the medium (Table 1). Thus, a sufficiently strong alkalisation of the medium leads to a fungistatic effect in respect to *Ph.* sp1.

Table 1. Mycelial growth rate of *Phoma sp.* 1 at various macro-element concentrations in starvation medium

Source of macro-element	Growth rate, ńm^2/24 h		
	2 g/l	1 g/l	0.5 g/l
Control (starvation agar)		4.0 ± 0.5	
Urea	–	–	–
Tricalcium phosphate	2.6 ± 0.7	2.8 ± 0.6	3.1 ± 0.8
Potassium chloride	2.9 ± 0.6	3.0 ± 0.6	3.5 ± 0.7
Complex fertiliser (N, P, K)	2.5 ± 0.8	2.5 ± 0.7	2.1 ± 0.4
Dolomite flour	1.5 ± 0.1	3.4 ± 0.7	3.5 ± 0.7

The use of tricalcium phosphate and potassium chloride at concentrations of 1 g/l and 2 g/l slowed the growth of mycelium by an average of 30%, compared to the control. A significant difference in comparison to the control was also observed in all variants of the experiment with the use of complex nitrogen-phosphorus-potassium fertiliser. When used, the growth rate of mycelium decreased on average by 40% and the mycelium changed its structure to feathery, became light-coloured and friable in the center of the colony (Fig. 1).

Evaluation of the effect of micro-elements on the growth of *Ph.* sp1 mycelium showed a significant difference, compared to the control, for copper sulphate at concentrations of 50 mg/l and 100 mg/l; and for zinc sulphate at a concentration of 100 mg/l. The growth rate decreased by 50%, 63%, and 32%, respectively, in these conditions (Fig. 2, Table 2).

DISCUSSION

Providing a comprehensive description of the *in vitro* properties of a new fungal species is essential for the inclusion of this species in the taxonomic database and assigning it a name. The current report on the influence of macro- and

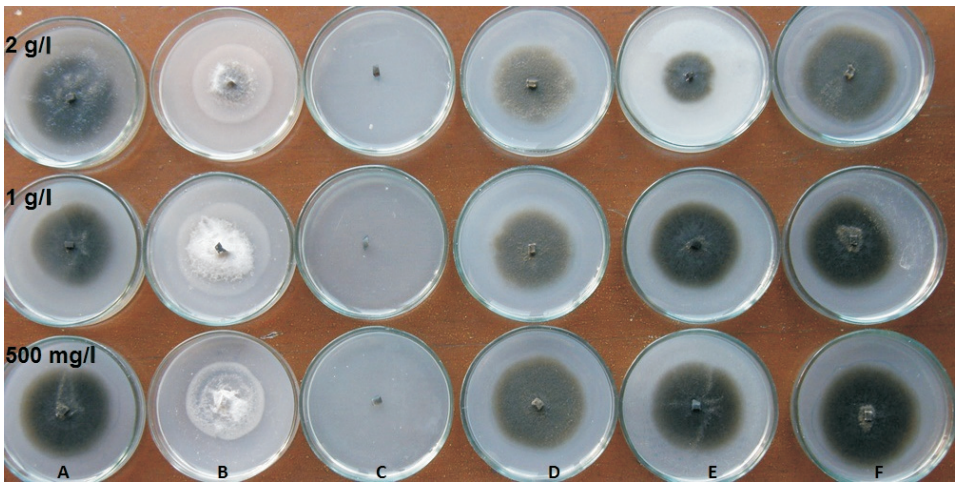

Fig. 1. Effect of macro-elements on mycelial growth of *Phoma* sp. 1. A, tricalcium phosphate; B, complex fertiliser; C, urea; D, potassium chloride; E, dolomite flour; F, control (starvation agar).

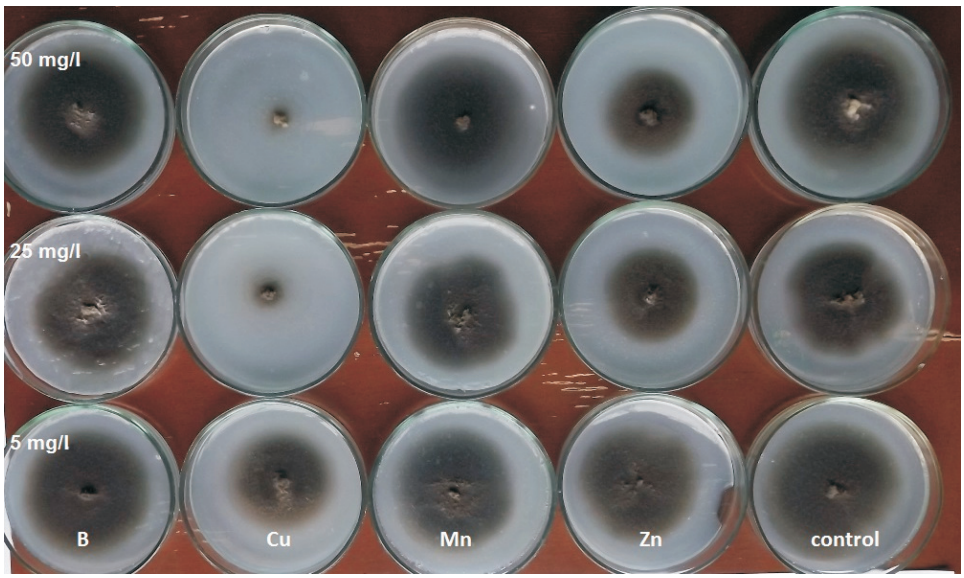

Fig. 2. Effect of micro-elements on growth of *Phoma* sp. 1 *in vitro.*

Table 2. The effect of micro-elements on the growth of *Phoma* sp. 1 *in vitro*

Micro-element	Concentration (mg/l)	Colony growth rate, cm^2/24 h
Copper	10	3.19 ± 0.51
	50	2.03 ± 0.32
	100	1.51 ± 0.15
Zinc	10	4.02 ± 0.65
	50	3.49 ± 0.61
	100	2.77 ± 0.48
Manganese	10	3.83 ± 0.45
	50	3.58 ± 0.58
	100	3.30 ± 0.46
Boron	10	3.90 ± 0.35
	50	3.42 ± 0.49
	100	3.83 ± 0.46
Control		4.07 ± 0.69

micro-elements on mycelial growth and culture morphology complements and refines the description of *Ph.* sp.1 concerning its vegetative and reproductive structures *in vitro*.

Presence of urea, increased content of nitrogen, copper sulphate and zinc in culture media inhibits *Ph.* sp.1 growth *in vitro*. The introduction of dolomite flour into the medium also retarded mycelial growth, probably due to the increased sensitivity of the pathogen to alkalisation of the medium. The mentioned effects provide information that is potentially useful for mitigating the economic losses from *Ph.* sp.1 in tree nurseries.

As this report represents the final step in characterisation of *Ph* sp.1 in *in vitro* conditions, we offer a short comparison of some biological features of the growth of the Belarusian isolate *Phoma* sp. 1 with the morphological description of *Ph. herbarum* and *Ph. glomerata* cited by Boerema *et al*. (2004) (Table 3).

The main differences of *Phoma* sp. 1 to these genetically close species are the formation of different types of chlamydospores (or presumed absence of their formation), as well as the morphology of the fungus on oatmeal agar. However, in terms of growth rate, *Ph*. sp. 1 is similar to *Ph. glomerata* and *Ph. herbarum*.

Summarising the conclusions from all studies performed on this proposed new species, the plant pathogenic fungus *Ph.* sp.1 cultures showed pronounced polymorphism in various growth and developmental conditions. *Phoma* sp. 1 does not require special selective media with complex composition

Table 3. Comparison of *in vitro* growth characteristics of *Ph*. sp.1 to *Ph. herbarum* and *Ph. glomerata* as described in the *Phoma* Identification Manual (Boerema *et al*., 2004) (citing the exact text referring to the subject)

Parameter of comparison	*Phoma* sp. 1	*Ph. herbarum*	*Ph. glomerata*
Type of chlamydospores	Chain of unicellular chlamydospores	No information about observations of *Ph. herbarum* producing chlamydospores is mentioned in the *Phoma* Identification Manual nor in any other source of information to our knowledge	"Chlamydospores highly variable in shape and dimensions, generally multicellular-dictyosporous, occasionally solitary-terminal, but usually in branched or unbranched chains of 2–20 or more elements, alternarioid, smooth at first, later roughened, dark brown to black"
Growth rate on oatmeal agar	4–6.65 cm	4–5 cm	3.5–7 cm
The morphology of fungal colonies on oatmeal agar	Light grey to dark grey	"… regular, mostly without any aerial mycelium and flesh coloured with greenish tinge by the conidial masses on abundant pycnidia; reverse greenish olivaceous to olivaceous; mycelial strains or sectors usually producing a yellowish brown to red diffusible pigment"	"Most variable in appearance, strains (sectors) with rather sparse aerial mycelium and abundant aerial mycelium, dense and woolly in places, olivaceous, greenish olivaceous, olivaceous buff or dull green; reverse dark olivaceous to blackish beneath sectors with dense mycelium, paler elsewhere"

for growth and development. The optimal nutrient medium for the cultivation of the fungus is a standard wort agar medium. Colonies of differing colour, texture and density form on various agarised media types. For rapid induction of vegetative spore (chlamydospore) formation, we recommend to grow the *Ph.* sp.1 culture on starvation agar at a temperature of approximately + 4 °C. For the formation and maturation of asexual spores (pycnidia with conidia), specific cultivation conditions are required, which still have to be clarified. *Phoma* sp. 1 prefers weakly acidic media (pH 4.3–4.9) for its development, yet it can remain active in acidic and strongly alkaline media. Mycelium of *Ph*. sp.1 can be stored for a long time (a year or more) on the same medium without loss of viability.

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FOMOZI IZRAISOŠĀS SĒNES *PHOMA* SP.1 KULTŪRAS UN MORFOLOĢISKAIS RAKSTUROJUMS

Skujkoku stādu fomoze ir maz pētīta. Baltkrievijā galvenā šīs slimības izraisītāja DNS sekvence atšķiras no citām aprakstītajām *Phoma* sugām, un tas vēl nav pilnībā taksonomiski aprakstīts, tāpēc pagaidu nosaukums ir *Phoma* sp. 1. Šeit aprakstītas galvenās *Phoma* sp. 1 morfoloģiskās un augšanas kultūras īpašības. *Phoma* sp. 1 ir mezofīls acidofīls, kas spēj veidot hlamidosporas kultūrā. Hlamidosporu veidošanās sākas ar protoplazmas fragmentu atdalīšanos, tad micēlijs sadalās ar starpsienu palīdzību un pārklājas ar biezu apvalku. Hlamidosporu veidošanās visātrāk — 7 dienu laikā, norisinājās +4 °C temperatūrā. Piknīdijas attīstījās dziļi barotnē tikai pēc ilgstošas uzglabāšanas zemā temperatūrā, tomēr sporas nenobrieda. Maksimālā biomasas produkcijas intensitāte novērojama pH diapazonā 4,3–4,9, bet sēnes augšana notiek pH diapazonā no 2,5 līdz 8,5, atkarībā no barotnes. Augot suboptimālas pH apstākļos, sēne izmaina barotnes pH, tuvinot to optimumam. Barotnes pH iespaido *Phoma* sp.1. šūnu un kolonijas morfoloģiju. Konstanta barotnes aerācija veicina tipiska micēlija veidošanos, proporcionālu augšanu un stabilu biomasas akumulāciju.