



Biological properties of *Phoma macrostoma* related to non-dermatophyte onychomycosis

Elena Kukhar^a, Ainura Smagulova^b, Vladimir Kiyan^{b,*}

^a Department of Microbiology and Biotechnology, S. Seifullin Kazakh Agrotechnical University, Nur-Sultan, Kazakhstan

^b Research Platform of Agricultural Biotechnology, S. Seifullin Kazakh Agrotechnical University, Nur-Sultan, Kazakhstan



ARTICLE INFO

Keywords:

Onychomycosis
Opportunistic invasive pathogen
Mycelium
Pycnidia
Conidia

ABSTRACT

We report a rare case of non-dermatophytic onychomycosis of the toenail caused by *Phoma macrostoma*. Was studied the biological properties of the strain isolated in Kazakhstan. *P. macrostoma* forms pink colonies, the reverzum is pink-orange. The mycelium is colorless, septate. The appearance of growth tubes from pycnidiospores occurs within 12 hours, mycelial growth and branching after 18 hours, the appearance of pycnidia is 48 hours. The saccharolytic and urease activity of the strain is low.

1. Introduction

Onychomycosis is the most common nail disease. Most cases are caused by dermatomycetes *Trichophyton rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum* [1]. In chronic paronychia and onycholysis, the disease causes by *Candida albicans* [2]. Mycelial mold fungi of the genera *Aspergillus spp.*, *Fusarium spp.*, *Acremonium spp.*, *Scopulariopsis brevicaulis*, *Scytalidium spp.* can be also cause nail damage. Mixed infections are often encountered, caused simultaneously by dermatophytes, mold and/or yeast [3–5]. It is believed that fungi have weaker keratinase and proteinase activity compared with dermatomycetes, which determines their secondary importance in the etiology of onychomycosis [6].

Phoma spp. (synonym *Didymella macrostoma*) [7] is a diverse group of organisms that are ubiquitous; commonly found in soil, organic matter, plants and water sources, are phytopathogens. Diseases caused by fungi of the genus *Phoma spp.* called fomes. The most famous is *P. exigua*, the causative agent of potato fomes, *P. rostrupii* - causative agent of carrot fomes, and *P. betae* is the causative agent of beet fomes. *Phoma spp.* is contaminated seeds, nuts, soy, potatoes, bananas, sorghum, corn, kiwi, lemons, tomatoes, eggplant and pomegranates [8–12]. There are reports of pathologies caused by fungi of the genus *Phoma spp.* in patients with immunosuppression [13]. *Phoma spp.* can transform from opportunistic to pathogenic organisms upon contact with the corresponding host [14]. It was reported that this species is an opportunistic invasive pathogen in humans and animals. The number of infections caused by *Phoma spp.* increases with the development of medicine, primarily due to an increase in the number of patients at risk

of immunodeficiency [15]. The incidence of diseases caused by *Phoma spp.* is constantly increasing, and the representative of *Phoma herbarum* is among the five most common types of *coelomycetes* [7,13].

For the first time in the Republic of Kazakhstan, we have established a case of onychomycosis caused by the opportunistic invasive pathogen *Phoma spp.* isolated from pathological material. The basic biological properties of the opportunistic pathogen of onychomycosis were studied and its species affiliation was established.

2. Case

A 32-year-old male patient, during primary sanitization in the department of narcology, an extensive lesion of the left 1, 2, 3, 4, 5 fingernail and right first fingernail was revealed in the form of opacification and thickening; signs of foot mycosis. Fingernails have a lesion that was 1/3 of the surface at the 2 to 5 toes on the left foot and 2/3 of the surface at the first toe of both feet. Fingernails are dirty-gray color, dull, thickened due to subungual hyperkeratosis, crumbling at the free end. The skin of the feet is thickened due to hyperkeratosis, in the interdigital spaces noticeable flaking and peeling skin, the presence of vesicles with serous-purulent contents, an unpleasant odor. The patient does not work anywhere, has been abusing alcohol about 15 years, and has never been treated for mycosis of the feet or onychomycosis.

Nail clippings from different parts of the affected toenail were collected on day 0 after proper sterilization of the affected area with 70% alcohol and transported to the mycology research laboratory, Microbiology and Biotechnology Department at S.Seifullin Kazakh Agrotechnical University, for examination by culture method.

* Corresponding author.

E-mail address: vskiy@gmail.com (V. Kiyan).

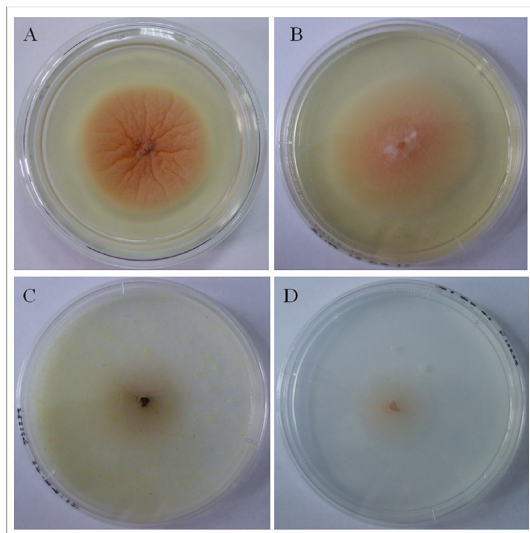


Fig. 1. Macroscopic appearance of cultured colony of *P. macrostoma* on Sabouraud agar (A), honey-agar (B), corn-agar (C) and Chapek's medium (D).

During the initial isolation of the pathogen (1 day) and upon obtaining a pure culture, the surface cultivation of the fungus was carried out at a temperature of 28 °C for at least 18 day until the formation of characteristic colonies. Mycological diagnosis was performed at +20 days. To study the cultural and morphological properties of the isolated *Phoma macrostoma* fungus strain were used agar media (Sabouraud's medium, Chapek's medium, and media based on honey and corn) (Fig. 1).

Colonies grow rapidly on Sabouraud agar, and are wrinkled, velvety, intensely pigmented, pink-orange, diameter 6.3×6.0 cm, and often largely submerged in the medium. From the front, colonies are initially white (0.5 cm) and later become brown. The center of the colony is unevenly raised. From the reverse, they are dark brown (Fig. 1A). Colonies on honey-agar are smooth, velvety-fluffy, diameter 6.8×5.8 cm, with pronounced zonation. From the front, colonies are initially unpigmented (0.8 cm) with a pronounced growth zone of colorless mycelium. The middle of the colony is velvety, pale pink; the central region is intensely pink with secondary foci of white mycelium growth. From the reverse, they are light pink (Fig. 1B). On honey-agar the colonies are small, shapeless and brownish, diameter 4.0×3.8 cm. The central region is dark brown (Fig. 1C). On Chapek's medium are formed small and pale pink colonies, diameter of 3.9×3.3 cm, more intensely colored in the center, almost colorless by half the diameter (Fig. 1D).

The growth characteristics of the mycelium strain were studied by agar block method. Phenotypic identification was performed using the determinant of microorganisms (Sutton D. et al., 2001) [16]. Microscopic structure of the *P. macrostoma* strain is characterized by developing of the septate, branching and colorless mycelium passing into conidiophores (Fig. 2.1). Conidiophores had different lengths (short and long), in the structure are more thickened than mycelial hyphae, multicellular (Fig. 2.2). Mature pycnidia are large, round to pyriform and deeply immersed in agar (Fig. 2.3). Pycnidia are dark in color and bear phialides at their inner lining, with one to several openings - ostioles (Fig. 2.4). Picospores come from mature pycnidia as mucoid droplet mass.

Genomic DNA was extracted from fungal strain using liquid nitrogen and phenol-chloroform extraction method, and the genomic DNA was analyzed by electrophoresis on 1% agarose gel. The *ITS* region on rDNA was amplified by using specific primers *ITS4* (5'-TCTCCGCTTATTGATATGC-3') and *ITS5* (5'-GGAAGTAAAAGTCGTAACAAGG-3') (Integrated DNA Technologies, Inc., USA) and received the PCR product with size of 526 bp. The PCR reaction was done in a SimpliAmp thermal cycler (Applied biosystems) under the following conditions: an initial

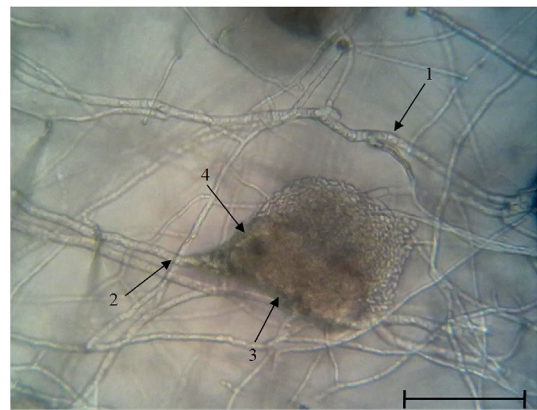


Fig. 2. Microscopic structures of *Phoma macrostoma* like the mycelium is septate, branching and colorless (1); conidiophores are long, thick and multicellular (2); pyriform pycnidia (3), ostiole (4), Scale bar = 50 µm.

denaturation set up at 94 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 52 °C for 40 sec and extension at 72 °C for 50 sec, with a final extension step of 72 °C for 7 min. The sequencing was done by using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the sequence was deposited in GenBank with accession no. MN701978.1 (*ITS*). These sequences were compared with other sequences in the GenBank by using the BLAST analysis. The phylogenetic analysis was carried out with MEGA 6 software.

At +21 days, oral lamisil (250 mg/day) in combination with topical terbinafine cream (1%), twice per day were given for a further 3 months. At +31 days, the patient visited the dermatology out-patient department; his fingernails were not completely cured, KOH microscopy of nails revealed fragments of mycelial filaments. After discharge, the patient did not visit the dermatology out-patient department of hospital after the last therapy, for that reason the patient medical condition cannot be confirmed.

3. Discussion

In the present study, we isolated a fungus strain from the toenail of a 32-year-old man that was assigned to the genus *Phoma* (synonym *Didymella*). We confirmed the identification of our isolate to species level as *Didymella macrostoma* (synonym *Phoma macrostoma*) (accession no. MN701978.1) by DNA sequence analysis. A nucleotide BLAST search with 582 bp showed a maximum homology of 96% with *Phoma* spp. of China origin (FJ176472.1), 95% with USA *D. macrostoma* strains (MH854608.1), 92% with New Zealand *P. macrostoma* strains (DQ474097.1) and with *P. macrostoma* from USA (DQ474072.1) (Fig. 3).

According to Bennett A. et al. fungi of the genus *Phoma* can be potentially pathogenic for plants, animals and people [17]. Indeed, in 2007, Faisal M. et al. described two cases of outbreaks of phaeohyphomycosis in the chinook salmon grown in the hatchery (*Oncorhynchus tshawytscha*) caused by *Phoma herbarum* [18]. Recently, an immunocompromised population increases, so do the reports of these infections. Human infections by coelomycetous fungi are becoming more frequent and range from superficial to systemic dissemination. The number of infections caused by *Phoma* spp. increases with the development of medicine, primarily due to the increase in the number of patients at risk of immunodeficiency conditions.

Medical advances have allowed for the increase the number of organ transplant operations, chemotherapies and other immunosuppressants to treat malignancies, which have resulted in a greater population at risk when exposed to diverse fungi including *Phoma* spp [17].

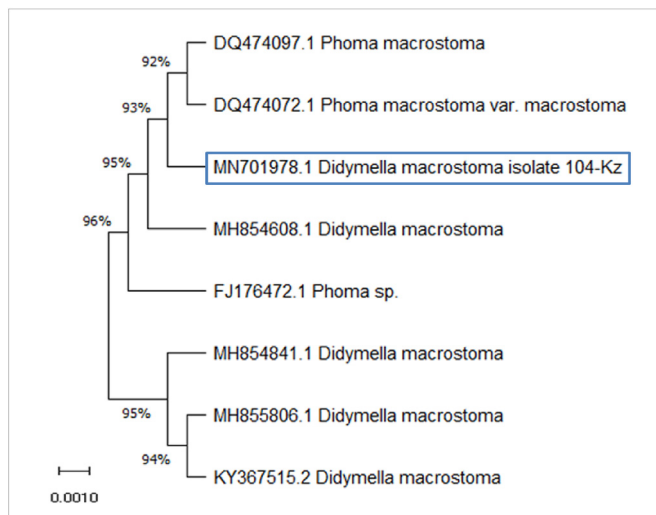


Fig. 3. Phylogenetic analysis (tree) generated with using ITS primer.

The first case of human infection caused by *Phoma* spp. was registered in 1973. Young N.A. et al. revealed the causative agent in a subcutaneous abscess in a patient after kidney transplantation and using mycological and histological methods confirmed his affiliation with *Phoma* spp [15]. In 1981, Bakerspigel A. et al. reported a case of isolation a strain of the soil-borne fungus *P. eupyrena* from the skin of an 18-month-old boy who had a crusting, erythematous, perioral eruption [19].

Tullio V. et al. (2010) reported about three cases of foot dermatomycoses caused by filamentous fungi are described in immunocompetent subjects. Among the pathogens, which were identified under a light microscopy, processed in a 20% KOH solution, cultured in Mycobiotic agar and Sabouraud dextrose agar containing chloramphenicol was *P. herbarum*, *Chaetomium globosum* and *Microascus cinereus* [13].

Annually, there are reports of diseases caused by *Phoma* spp. Proof of this is the data of cultural-morphological and molecular genetic studies provided by various authors [17]. The results of determining the distribution of the coelomycetes in clinical samples by a phenotypic and molecular study of a large set of 230 isolates received from a U.S. reference mycological institution identified *P. herbarum* and *D. heteroderiae* as among the five most prevalent pathogens [7].

Our research has expanded the list of lesions in people caused by fungi of the genus *Phoma*. Onychomycosis of the fingernail plate identified as *D. macrostoma* (synonym *P. macrostoma*), was detected in a patient of a narcological clinic.

The culture of *P. macrostoma*, which caused damage to the patient's fingernail plate, was distinguished by characteristic features, not described earlier. During the first day, the surface cultivation on honey and Sabouraud agar was noted the formation of a colony with a delicate-pink tinge, gradually acquired a pronounced pinkish-orange color. The substrate is also painted in a delicate-pink color. On the corn-agar, the formation of velvety light to dark-brown colonies was noted. Similar colonies are described by Sutton D. et al. [16].

In the thickness of the agar, a colorless, septate, grayish mycelium growth was noted. On the third day of growth on the hyphae were formed "alternarioid" chlamydospores and spherical pycnidia with ostioles. It should be noted the presence of a lot of characteristic short and rare long-thick conidiophores, not characteristic of *Phoma* spp. The increasing volume and ripening, spherical pycnidia changed shape to pyriform, due to the allocation of oval pycnospores in the form of a mucous mass. The conidia accumulation on the pycnidia was in the form of droplets, which gradually distributed along the growth of mycelial filaments, into the space between the hyphae. Biochemical

analysis revealed low enzymatic activity of the strain: low saccharolytic activity and practical absence of urease activity.

Phoma spp. identification remains controversial and difficult, since the form of damage to the toenail plates is similar with onychomycosis caused by pathogens *T. rubrum*, *T. interdigitale* and *Candida* spp [20]. The identification is performed using morphological characteristics in vitro: colony color, size and shape of pycnidia, conidiophores and chlamydospores, rate and nature of the formation of structures on agar media. These signs must be considered when differentiating from other pathogens. The presence of unicellular or multicellular alternarioid chlamydospores arranged in the form of chains of dark brown color requires differentiation from *Alternaria* spp. or *Stemphylium* spp. Relatively reliable morphological criteria are the presence of pycnidia, their shape and the outlet on them. Also characteristic is the mucus-like accumulation of pycnidospores at the pycnidic membrane and between mycelial hyphae.

In conclusion, we report the case of onychomycosis of a toenail of a young addict male caused by *P. macrostoma*. Identification of the causal agent was confirmed by molecular methods. Drug addiction is likely to be a predisposing factor in our reported case.

Declaration of competing interest

There are none.

Acknowledgements

This investigation was financially supported by the Ministry of Education and Science of the Republic of Kazakhstan within the framework of the project No. 0112RK01358 for 2012–2014 and initiative research project 0118RKI0321for 2018–2022.

We are thankful to the staff of the Shared Laboratory of the National Center for Biotechnology of the Republic of Kazakhstan for their assistance in conducting molecular genetic studies.

References

- [1] P. Veer, N.S. Patwardhan, A.S. Damle, Study of onychomycosis: prevailing fungi and pattern of infection, *Indian J. Med. Microbiol.* 25 (2007) 53–56.
- [2] G. Ge, Z. Yang, D. Li, G. Sybren de Hoog, D. Shi, Onychomycosis with greenish-black discolorations and recurrent onycholysis caused by *Candida parapsilosis*, *Med. Mycol. Case Rep.* 24 (2019) 48–50.
- [3] E. Haneke, Fungal infections of the nail, *Semin. Dermatol.* 10 (1) (1991) 41–53.
- [4] Debopriyo Samaddar, Reena Ray, Shiv Sekhar Chatterjee, Swagata Pal, A study of dermatophytic onychomycosis in patients attending dermatology department in a tertiary care hospital in eastern India, *J. Dent. Med. Sci.* 14 (2) (2015) 79–84.
- [5] J. Issakainen, H. Heikkilä, E. Vainio, P. Koukila-Kähkölä, M. Castren, O. Liimatainen, T. Ojanen, M. Koskela, O. Meurman, Occurrence of *Scopulariopsis* and *Scedosporium* in nails and keratinous skin. A 5-year retrospective multi-center study, *Med. Mycol.* 45 (3) (2007) 201–209.
- [6] T.V. Medvedeva, L.M. Leina, Onychomycosis: Current Ideas about Etiology, Epidemiology, Methods of Therapy, MAPO Research Institute of Medical Mycology, St. Petersburg State Pediatric Medical Academy, 2019.
- [7] N. Valenzuela-Lopez, D.A. Sutton, José F. Cano-Lira, Katihuska Paredes, Wiederhold Nathan, Josep Guarro, Alberto M. Stchigel, Coelomycetous fungi in the clinical setting: morphological convergence and cryptic diversity, *J. Clin. Microbiol.* 55 (2) (2017) 552–567.
- [8] G.J. Kovics, J. de Gruyter, H.A. van der Aa, *Phoma sojicola* comb. nov. and other hyaline-spored coelomycetes pathogenic on soybean, *Mycol. Res.* 103 (1999) 1065–1070.
- [9] R.C. Oliveira, S.S. Goncalves, M.S. Oliveira, P. Dilkin, C.A. Mallmann, R.S. Freitas, P. Bianchi, B. Correa, Natural occurrence of tenuazonic acid and *Phoma sorghina* in Brazilian sorghum grains at different maturity stages, *Food Chem.* 230 (2017) 491–496.
- [10] A.L. Do Amaral, M.L. de Carli, J.F.B. Neto, F.K. Dal Soglio, *Phoma sorghina*, a new pathogen associated with *Phaeosphaeria* leaf spot on maize in Brazil, *Plant Pathol. J.* 53 (2004) 259.
- [11] Q. Migheli, S.O. Cacciola, V. Balmas, A. Pane, D. Ezra, G. Magnano di San Lio, Mal secco disease caused by *Phoma tracheiphila*: a potential threat to lemon production worldwide, *Plant Dis.* 93 (2009) 852–867.
- [12] S. Palavouzis, S. Tzamos, E. Paplomatas, T. Thomidis, First report of *Phoma Aliena* causing fruit rots of pomegranates in northern Greece, *J. Plant Pathol.* 97 (2015) 215.
- [13] V. Tullio, G. Banche, V. Allizond, J. Roana, N. Mandras, D. Scalas, M. Panzone,

- O. Cervetti, S. Valle, N. Carlone, A.M. Cuffini, Non-dermatophyte moulds as skin and nail foot mycosis agents: *Phoma herbarum*, *Chaetomium globosum* and *Microascus cinereus*, *Fungal Biol.* 114 (4) (2010) 345–349.
- [14] M.M. Aveskamp, J. De Gruyter, P.W. Crous, Biology and recent developments in the systematics of *Phoma*, a complex genus of major quarantine significance, *Fungal Divers.* 31 (2008) 1–18.
- [15] N.A. Young, K.J. Kwon-chung, J. Freeman, Subcutaneous abscess caused by *Phoma* sp. resembling *Pyrenochaeta romeroi*: unique fungal infection occurring in immunosuppressed recipient of renal allograft, *Am. J. Clin. Pathol.* 59 (1973) 810–816.
- [16] D. Sutton, A. Fothergill, M. Rinaldi, The Determinant of Pathogenic and Conditionally Pathogenic Fungi, M: The World, 2001.
- [17] A. Bennett, M.M. Ponder, J. Garcia-Diaz, *Phoma* infections: classification, potential food sources, and its clinical impact, *Microorganisms* 6 (3) (2018) 58.
- [18] M. Faisal, E. Elsayed, S.D. Fitzgerald, V. Silva, L. Mendoza, Outbreaks of phaeohyphomycosis in the chinook salmon (*Oncorhynchus tshawytscha*) caused by *Phoma herbarum*, *Mycopathologia* 163 (1) (2007) 41–48.
- [19] A. Bakerspigel, D. Lowe, A. Rostas, The isolation of *Phoma eupyrena* from a human lesion, *Arch. Dermatol.* 117 (6) (1981) 362–363.
- [20] G. Tchernev, P.K. Penev, P. Nenoff, L.G. Zisova, J.C. Cardoso, T. Taneva, et al., Onychomycosis: modern diagnostic and treatment approaches, *Wien. Med. Wochenschr.* 163 (1–2) (2013) 1–12.