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ORIGINAL ARTICLE

A new section for *Alternaria helianthiinficiens* found on sunflower and new asteraceous hosts in Russia

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Abstract
Alternaria helianthiinficiens previously has been found as a pathogen of sunflower and cosmos in northern hemisphere. This fungus comprises a monotypic lineage which obviously is a separate group that has not been formally described as a section. Information about morphology, distribution, and pathogenic characters of this species is very limited. During this study, two taxonomic novelties were entered. A new section, *A.* sect. *Helianthiinficientes*, was described. *Alternaria simmonsii* was acknowledged to be a synonym of *A. helianthiinficiens*. The present work allowed definition of *Arctium* sp. and *Sonchus* sp. (both are family *Asteraceae*) as new hosts for *A. helianthiinficiens*. Isolates of this plant pathogenic fungus were obtained from several new places in the Southern European part of Russia. Six strains were tested on nine asteraceous plants and supported pathogenicity of all strains and susceptibility of all hosts. All strains were more aggressive for *Helianthus annuus* and *Xanthium sibiricum* than for other plants regardless on host from which they were isolated. Moderate aggressiveness was detected for *Cirsium arvense* and *H. tuberosus* while expansion of lesions on *Arctium tomentosum*, *Artemisia vulgaris*, *Sonchus arvensis*, *Tanacetum vulgare*, and *Taraxacum officinale* was sufficiently slower.

Keywords *Alternaria* section *Helianthiinficientes* · *Alternaria simmonsii* · *Helianthus annuus* · Molecular phylogeny · Sunflower · Taxonomy

Introduction

Alternaria Nees is a large, morphologically diverse genus. Recently, a number of molecular phylogenetic studies have attempted to better resolve *Alternaria* phylogeny (Lawrence et al. 2012, 2013, 2014; Woudenberg et al. 2013, 2014; Al Ghafri et al. 2019). Morphological assessment and phylogenetic analysis allow the conclusion that up to date the genus consists of about 360 species (Wijayawardene et al. 2020). In total, currently, 28 sections were described in the genus *Alternaria* (Lawrence et al. 2016; Al Ghafri et al. 2019). There are a few monotypic lineages that have not been assigned a status of section. The species *A. helianthiinficiens* E.G. Simmons, Walcz & R.G. Roberts comprises one of such clade which obviously is a separate group, but formally must be still treated as a member of *A.* sect. *Alternaria*.

Alternaria helianthiinficiens was found as a pathogen of sunflower and cosmos in a few locations in North America, Europe, and Asia (Simmons 1986; Aćimović and Lačok 1991; Cho and Yu 2000; Luo et al. 2017). To our knowledge, there are only a few reports about solitary strains of this species isolated from sunflower in Russia (Gannibal 2011; Ivebor et al. 2013, 2014). Morphologically, *A. helianthiinficiens* resembles a number of *A.* sect. *Porri* species which are widely distributed on many asteraceous plants. Likely, this fungus could be misidentified with some of them. Thereby, in general information about its morphology, distribution and pathogenic characters are very limited.

The aims of this study were to clarify *A. helianthiinficiens* taxonomy, geography, and host specialization.

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Materials and methods

Alternaria strains

The collection of micromycetes of the Laboratory of Mycology and Phytopathology of the All-Russian Institute of Plant Protection contained six strains isolated from *Asteraceae* plants in different geographical locations of European Russia and morphologically similar to *A. helianthiinficiens*, including two strains of *A. simmonsii* Gannibal. One representative *A. helianthiinficiens* strain was used in this work. Information on strains is summarized in the Table 1.

DNA isolation, PCR, and sequencing

Mycelium (10–50 mg per strain) was obtained from cultures incubated on V4 agar medium for 7 days. DNA was extracted with Genomic DNA Purification Kit (Thermo Fisher Scientific) according to a manufacturer protocol.

The primers EF1-728f/EF1-986r (Carbone and Kohn 1999), gpd1/gpd2 (Berbee et al. 1999), and CALDF1/CALDR1 (Lawrence et al. 2013) were used to amplify parts of the gene for translation elongation factor 1-α *TEF*, the gene for glycerol-3-phosphate dehydrogenase *GPD*, and the gene for calmodulin *CALD*, respectively. Amplicons were sequenced by Sanger's method on ABIPrism 3500 (Applied Biosystems–Hitachi, Japan), with the Big Dye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, USA), according to the manufacturer's instructions. All sequences were deposited in the GenBank (Table 1).

Phylogenetic analysis

Sequences were assembled, edited, and aligned using Vector NTI advance 10 (Thermo Fisher Scientific) and MEGA X 10.1 software. Sequences of representative strains and type species were obtained from GenBank (Table 1). To implement phylogenetic analysis, two different datasets were made due to sequences of the same gene and species were represented in the GenBank by different strains. The first set was based on combined *gpd* and *TEF* sequences, whereas the second set included *cald* sequences. *Alternaria solani* strains were used as an outgroup in both sets. Phylogenetic analysis consisted of maximum likelihood (ML) and maximum parsimony (MP) was performed with MEGA X 10.1 (Kumar et al. 2018). Bootstrap support values with 1000 replications were calculated. Bayesian analyses and Bayesian probability calculation were carried out by Mr. Bayes v. 3.2.1. in Armadillo v. 1.1 (Lord et al. 2012).

Alignment and phylogenetic tree were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S28125).

Morphology characterization

For examination of micromorphological structures, the strains were grown on PCA (potato carrot agar) (Simmons 2007) and V-4 (Mikhailova et al. 2002) that is analogue of V-8 media using for description of large-spored and other *Alternaria* species (Simmons 2007). Strains were incubated for 14 days at 23 ± 1 °C without expose under light (for cultural study) or under an alternating light/dark cycle consisting of 12 h of cool-white fluorescent daylight. Species identification was performed with the *Alternaria* identification manual (Simmons 2007).

Pathogenicity test

Young wild plants (*Arctium tomentosum*, *Artemisia vulgaris*, *Cirsium arvense*, *Sonchus arvensis*, *Tanacetum vulgare*, *Taraxacum officinale*) with no visible symptoms were taken from the experimental field of the All-Russian Institute of Plant Protection (St. Petersburg, Russia). *Helianthus annuus*, *H. tuberosus*, and *Xanthium sibiricum* were grown in a greenhouse in pots. Plants with 4–6 pairs of true leaves were used. Leaf disks 10 mm in diameter or similar pieces of leaves were used for inoculation.

Strains were incubated in 250-mL flasks with 50-mL liquid soybean media (per 1 L: KH_2PO_4 2 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4$ 1 g, glucose 20 g, soybean flour 10 g; pH 6) for 3 days at room temperature with permanent shaking. Mycelium was filtered with a fabric, briefly dried, grinded with a pestle, and diluted with sterile water to get a suspension 50 mg/mL. A drop (10 mL) of suspension was placed on a reverse leaf disk (piece) side (4 disks per test, 3 replicates). Inoculated leaf disks were incubated on wet filter paper in Petri dishes at 24 °C under an alternating light/dark 12/12 h cycle. After 2 days, leaf disks were turned over. Diameter of necrosis was measured 2, 3, 4, 5, 9 dpi. All tests were repeated twice. The fungus was isolated from the blights and subjected to microscopic analysis to support identity of the pathogen and to abide Koch's postulates.

Results

Molecular phylogeny

The adjusted and aligned sequences in the phylogenetic analysis had the following lengths: *TEF*, 236 bp; *gpd*, 580 bp; and *cald*, 784 bp. The number of parsimony-informative sites per genome locus was 38 (15.7%), 40 (6.9%), and 129 (16.5%), respectively.

All six analyzed strains formed a compact clade with high bootstrap support containing all reference *A. helianthiinficiens* strains (Figs. 1 and 2). The topology of trees build by different methods was identical. Also, it was concordant with that reconstructed previously (Woudenberg et al. 2013).

Table 1 List of studied *Alternaria* strains.

Species	Strain ID[a]	Host plant	Origin	GenBank accession numbers			References
				GPD	*TEF*	*CALD*	
A. helianthiificiens	MF P024011 (VKM F-4110)[b]	*Sonchus arvensis*, leaf	Russia, Voronezh region	**MW240966**	**MW240960**	**MW240972**	**This study**
A. helianthiificiens	MF P024021	*Sonchus arvensis*, leaf	Russia, Voronezh region	**MW240967**	**MW240961**	**MW240973**	**This study**
A. helianthiificiens	MF P061021	*Helianthus annuus*, leaf	Russia, Krasnodar region	**MW240968**	**MW240962**	**MW240974**	**This study**
A. helianthiificiens	MF P283031	*Arctium tomentosum*, leaf	Russia, Republic of Dagestan	**MW240969**	**MW240963**	**MW240975**	**This study**
A. helianthiificiens	MF P283041	*Arctium tomentosum*, leaf	Russia, Republic of Dagestan	**MW240970**	**MW240964**	**MW240976**	**This study**
A. helianthiificiens	MF P437021	*Helianthus annuus*, leaf	Russia, Krasnodar region	**MW240971**	**MW240965**	**MW240977**	**This study**
A. helianthiificiens	CBS 208.86[c]	*Helianthus annuus*, seed	USA	KC584120	EU130548	**MW240978**	Woudenberg et al. 2013 **This study**
A. helianthiificiens	CBS 117370 (EGS 50.174)[d]	*Helianthus annuus*	UK	KC584119	KC584661		Woudenberg et al. 2013
A. helianthiificiens	YZU 161169	*Cosmos bipinnatus*	China	MF414167	MF414169		Luo et al. 2017
A. helianthiificiens	YZU 161170	*Cosmos bipinnatus*	China	MF414168	MF414170		Luo et al. 2017
A. brassicae	CBS 116528 (EGS 38.032)	*Brassica oleracea*	USA	KC584102	KC584641	JQ646181	Woudenberg et al. 2013; Lawrence et al. 2013
A. cinerariae	CBS 116495 (EGS 49.102)	*Ligularia sp.*	USA	KC584109	KC584648		Woudenberg et al. 2013
A. cinerariae	EGS 33-169	*Cineraria maritima*	USA			JQ646182	Lawrence et al. 2013
A. solani	CBS 116651 (EGS 45.020)	*Solanum tuberosum*	USA	KC584139	KC584688		Woudenberg et al. 2013
A. solani	EGS 44098	*Solanum tuberosum*	USA			KJ397981	Gannibal et al., 2014
A. sonchi	CBS 119675 (EGS 43.131)	*Sonchus asper*	Canada	KC584142	KC584691		Woudenberg et al. 2013
A. sonchi	EGS 46-051	*Sonchus asper*				JQ646183	Lawrence et al. 2013

[a] Strain numbers with acronym MF means pure culture collection of All-Russian Institute of Plant Protection (VIZR, Laboratory of Mycology and Phytopathology), St. Petersburg, Russia. *VKM*—All-Russian Collection of Microorganisms, Moscow, Russia; *EGS*—personal collection of Dr. E. G. Simmons, Crawfordsville, IN, USA; *CBS*—culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; *YZU*—pure cultures were deposited in the culture collection of Yangtze University, Jingzhou, Hubei, China; [b] Ex-type strain of *A. simmonsii*; [c] Ex-type strain of *A. helianthiificiens*; [d] Representative strain of *A. helianthiificiens*. Underlined numbers indicate strains used for pathogenicity study. GenBank accession numbers highlighted in bold indicate sequences obtained during this study.

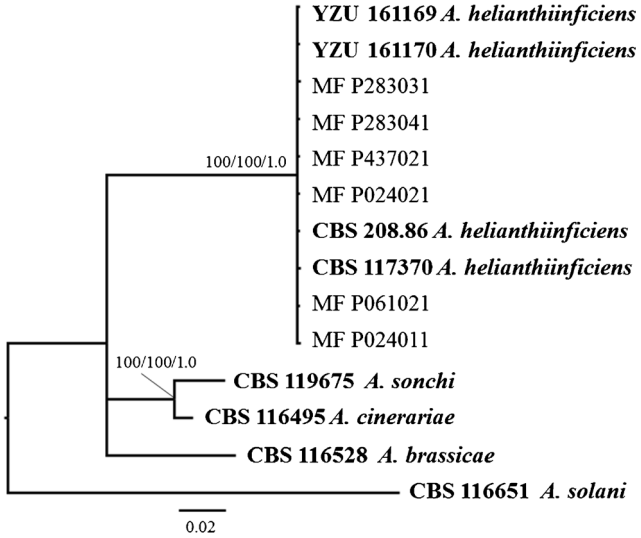

Fig. 1 Bayes phylogenetic tree for *Alternaria helianthiinficiens* and allied species inferred from combined *gpd* and *TEF* gene sequences. Bootstrap percentages from maximum likelihood/maximum parsimony (>70%) and Bayesian posterior probabilities (>0.95%) are given at the nodes.

Morphology

Strain MF P283031 was characterized by the best sporulation. It formed moderate sporulation on V-4 and it was only one strain with abundant sporulation on PCA after 5–7 days of incubation. Therefore, it was used for morphological description of *A. helianthiinficiens*.

On PCA, conidiophores are solitary 20–80 × 4–6 μm. Conidia are mainly solitary but sometimes in chains of two due to apical or lateral secondary conidiophores (Fig. 3). Mature conidia have narrow- to broad-ovoid or ellipsoid body, reaching a size of ca. 35–65(75) ×16–20 μm with 7–9 transverse septa and 1–3 longitudinal septa in 4–7 transverse segments. Conidia are not or slightly constricted by most of transverse septa. Many conidia have narrow or wide conical apical cell. Some conidia produce short or long filiform beak (up to 300 × 2–4 μm or longer). Solitary conidia have apical secondary conidiophore 5–30 × 3–6 μm. Rarely, conidia produce one or two short lateral secondary conidiophores.

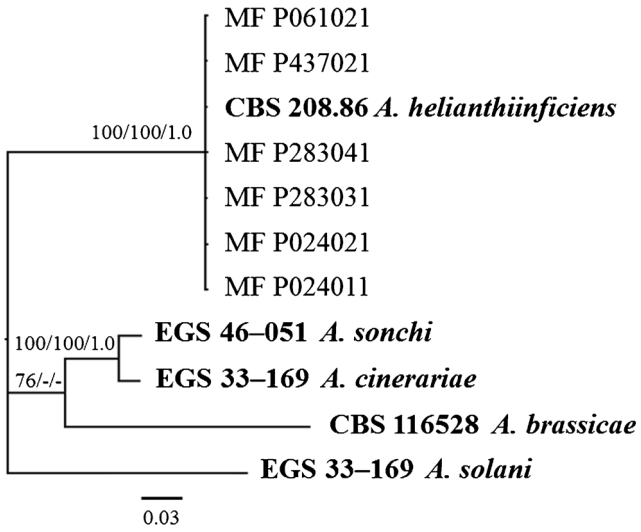

Fig. 2 Bayes phylogenetic tree for *Alternaria helianthiinficiens* and allied species inferred from *cald* gene sequences. Bootstrap percentages from maximum likelihood/maximum parsimony (>70%) and Bayesian posterior probabilities (>0.95%) are given at the nodes.

On V-4, conidiophores are solitary or in small groups, simple or slightly branched 50–90(200) × 5–6 μm with 1–2(3) conidiogenous loci. Conidia are solitary or sometimes in chains of 2(3). Basal conidia in a chain can form one apical and 1–4 lateral secondary conidiophores 5–30 × 4–6 μm long; each of them bear 1–2(3) conidiogenous loci. Due to formation of secondary conidiophores with several conidiogenous loci, 2–3-week-old cultures contain "bushes" of 10–30 conidia. Conidia body on V-4 is slightly wider than that produced on PCA, mainly 30–60 × 14–22(30) μm ovoid to broad-ovoid. Some conidia have broad-ovoid or saccular body 50–80(105) × 18–23(29) μm and filiform apical beak 20–150 × 2–4 μm. Conidial body has 4–7(9) transverse and 4–11 longitudinal or oblique septa, i.e., 1–3 in several of the broadest transverse segments. Conidia are slightly or moderately constricted by most of transepta.

Other strains on PCA and V-4 formed weak or moderate sporulation. In comparison with MF-P283-031, conidia were in general slightly smaller (40–105 × 17–26(28) μm on V-4) and less percentage of conidia looks mature. Only comparatively short beaks 20–100(140) μm long were observed in some conidia even after 2 weeks of growth.

Taxonomy

Section ***Helianthiinficientes*** Gannibal, sect. nov.

MycoBank: MB839327.

Type species: *Alternaria helianthiinficiens* E.G. Simmons, Walcz & R.G. Roberts.

Diagnosis: Primary conidiophores are simple or branched, with one or a few conidiogenous loci. Conidia are solitary or in short chains. Body of mature conidia is moderately large, narrow- to broad-ovoid or ellipsoid, constricted near septa. Conidia have several transverse and longitudinal septa. Some conidia are non-beaked when other form apical secondary conidiophore or short to very long filiform beak. Conidia can produce one or a few lateral secondary conidiophores. Sexual morph is not known.

Notes: Molecular phylogenetic analyses performed by a number of authors (Woudenberg et al. 2013; Al Ghafri et al. 2019) based on three to six (*SSU+LSU+ITS+GPDH+TEF+RPB2*) genomic loci unambiguously demonstrated that *A. helianthiinficiens* formed an independent, well-separated lineage within the genus *Alternaria* and could not be assigned to any of the known sections. Its nearest neighbors were

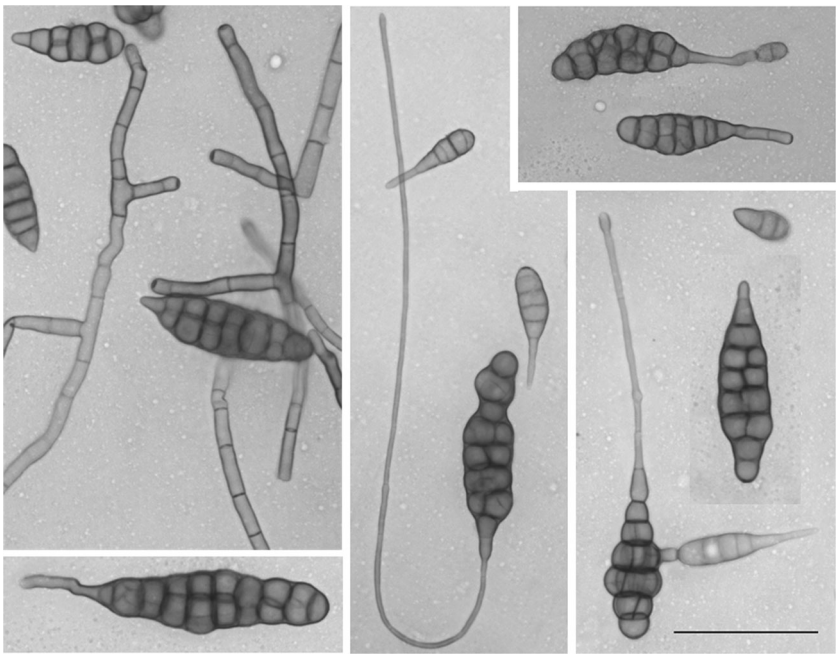

Fig. 3 Conidia and conidiophores of *Alternaria helianthiinficiens* strain MF P283031 after 5–7 days of incubation on potato carrot agar under an alternating light/dark cycle. Scale bar means 50 μm.

another monotypic lineage represented by *Alternaria brassicae* and sect. *Sonchi*.

Ex-type *A. simmonsii* strain MF P024011 (VKM F-4110) and similar strain MF P024021 obtained from the same place and date and all our other strains have *TEF*, *gpd*, and *cald* sequences identical to that of ex-type *A. helianthiinficiens* strain CBS 208.86. Strain MF P024011 and MF P024021 had insufficient morphological difference with *A. helianthiinficiens* strains. Thus we postulate that *Alternaria simmonsii* Gannibal (Gannibal 2010) [MB#518504] is a synonym of *Alternaria helianthiinficiens* E.G. Simmons, Walcz & R.G. Roberts (Simmons 1986) [MB#534400].

Pathogenicity

Any or all analyzed *A. helianthiinficiens* strains demonstrated pathogenic properties in artificial inoculation of each tested plant species. All analyzed strains were highly aggressive to *Helianthus annuus* and *Xanthium sibiricum* (both belong to tribe *Heliantheae*), and caused 100% necrosis of leaf discs on 4 dpi (Fig. 4). The inoculation of *Helianthus tuberosus* leaf discs (also tribe *Heliantheae*) by the analyzed strains led to development of necrosis from 4.4 ± 1.1 to 8.7 ± 0.5 mm (on average 5.6 ± 1.3 mm). The exception was *A. helianthiinficiens* strain MF P024021 that was not pathogenic for *Helianthus tuberosus*. The analyzed

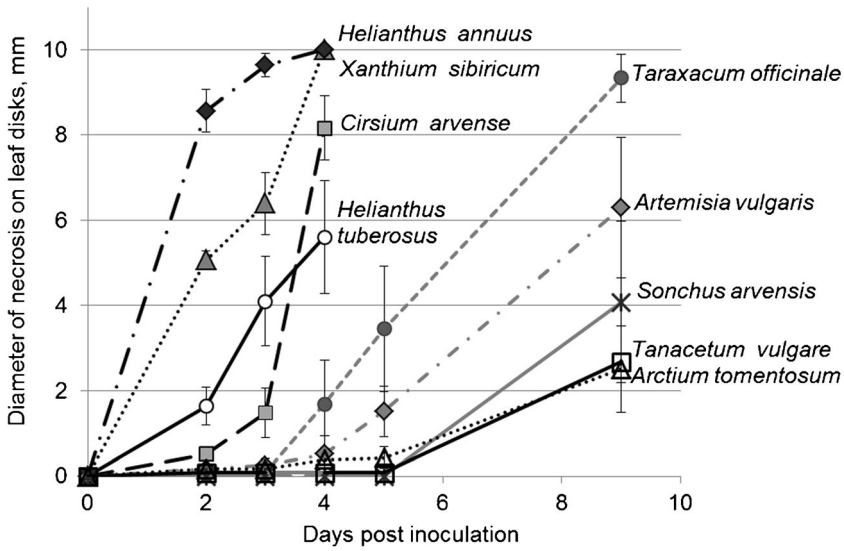

Fig. 4 Average expansion of lesions caused by six *Alternaria helianthiinficiens* strains on leaves of nine asteraceous plants. Vertical bars denote 95% confidence intervals for the mean of counts.

A. helianthiinficiens strains also turned out to be highly aggressive when infected the leaf discs of *Cirsium arvense* (tribe *Cynareae*) and caused necrosis from 5.5 ± 2.2 to 10.0 mm (on average 8.2 ± 0.7 mm) on 4 dpi.

The analyzed *A. helianthiinficiens* strains were, on average, weakly aggressive to other tested asteraceous plants. The size of necrosis did not exceed 1.7 mm on 4 dpi. On 9 dpi, all analyzed strains caused significant necrosis 6.5 ± 2.3–10 mm on leaf discs of *Taraxacum officinale* (tribe *Lactuceae*). Only three *A. helianthiinficiens* strains induced necrosis from 5.5 ± 2.2 to 10 mm on leaf discs of *Sonchus arvensis* (also tribe *Lactuceae*) on 9 dpi. Five *A. helianthiinficiens* strains of six were pathogenic to *Artemisia vulgaris* and four strains were pathogenic to *Tanacetum vulgare* (both belong to tribe *Anthemideae*) and caused necrosis 6.3 ± 1.7 and 2.7 ± 1.2 mm on 9 dpi, respectively. Four strains were weakly pathogenic to *Arctium tomentosum* (tribe *Cynareae*); the size of necrosis varied between 1.8 ± 1.2 and 6.5 ± 0.8 mm on 9 dpi.

Strains MF P283031 and MF P437021 isolated from *Arctium tomentosum* and *Helianthus annuus*, respectively, were the most aggressive in majority of the tests. No interrelation between host origin of strains and their pathogenicity to tested plants was revealed.

Discussion

Accurate identification of *A. helianthiinficiens* as well as many other *Alternaria* species is a bit troublesome. Careful adherence to standard protocols of strain cultivation is essential. However, even if the conditions are met, conidia size and shape can vary between strains and between different passages of the same strain.

Alternaria helianthiinficiens morphologically is very similar to many species of *Alternaria* sect. *Porri*. Strains of at least 21 *Alternaria* sect. *Porri* species were obtained from asteraceous plants (Woudenberg et al. 2014). There are two large-spored *Alternaria* species excepting *A. helianthinficiens* detected on sunflower—*Alternaria carthami* S. Chowdhury (*A. heliophytonis* E.G.Simmons) and *A. protenta* E.G.Simmons (Simmons 1986, 1997, 2007). Taxonomy of both species was supported by molecular phylogenetic approach (Woudenberg et al. 2014). The first species was found on *Helianthus* and *Carthamus* plants (both *Asteraceae*) while the second species was isolated worldwide from at least five species of four families. Neither *A. carthami* nor *A. protenta* were found on the territory of Russia.

Another large-spored species, *A. zinnia* M.B. Ellis, was repeatedly reported as sunflower-associated fungus (Neergaard 1945; McDonald and Martens 1963; Rao 1971; Carson 1987; Gulya et al. 1991; Prathuangwong et al. 1991). Most likely, all those cases were result of misidentification since those reports have been done primarily before *A. helianthiinficiens*, *A. carthami*, and *A. protenta* were described. The concept of the species *A. zinnia* was previously rather wide and resulted in combination of several morphologically similar species under this name (Simmons 1986, 1997).

Isolates of *Alternaria helianthiinficiens* were obtained from several locations in the South of European Part of Russia (Table 1). Recently, a few times, this fungus was isolated from sunflower leaves from other regions of European Russia (Lipetsk, Samara, and Kursk regions), as well as in Siberia (Altai Krai) (Gomzhina, Orina, unpubl.). Previously, *Alternaria helianthiinficiens* was also isolated from sunflower seeds grown in Altai Krai (Gannibal 2011) and Saratov region (Ivebor et al. 2014). Also *A. helianthiinficiens* was found on sunflower in North Dakota, USA (Simmons 1986), former Yugoslavia (Aćimović and Lačok 1991), South Korea (Cho and Yu 2000), and on cosmos in China (Luo et al. 2017). Obviously, this fungus has worldwide distribution.

Alternaria helianthiinficiens is a more aggressive pathogen for sunflower than another wider distributed Alternaria blight pathogen, *Alternariaster helianthi* (*Alternaria helianthi*), but it has a longer incubation period in the laboratory tests (2–3 days instead of 1–2 days for *Alternariaster helianthi*) (Cho and Yu 2000). During winter time, *A. helianthiinficiens* can survive as mycelium on plant residues and in seeds (Aćimović and Lačok 1991).

The present work adds *Arctium* sp. and *Sonchus* sp. to previously known hosts of *A. helianthiinficiens—Helianthus annuus* and *Cosmos bipinnatus*. However, it demonstrates different aggressiveness of pathogen to different hosts when sunflower can be affected in higher degree. It is interesting that *A. helianthiinficiens* strain MF P024021 isolated from *Sonchus* was nonpathogenic to its host plant but simultaneously was highly aggressive to *Helianthus annuus*. Similarly, *Arctium*-borne strains MF P283031 and MF P283041 very weakly infected their host plant, but induced a significant necrosis on sunflower leaf discs. These observations, as well as the analysis of the pathogenicity of strains to other tested plant species, suggest that *A. helianthiinficiens* may appear on some other plant species of the *Asteraceae* family. The ability to infect weeds made this fungus potentially common sunflower pathogen and complicates the control of disease caused by *A. helianthiinficiens* in the field.

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Author contribution All authors contributed to the study conception and design. Philipp B. Gannibal contributed in sample collection, fungi isolation, morphology study, and taxonomic conclusions. Phylogenetic analysis was carried out by Aleksandra S. Orina. Elena L. Gasich performed all experiments to study pathogenicity. The manuscript was written by Philipp B. Gannibal and all authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors read and approved the final manuscript.

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Data availability All new sequences are deposited in GenBank (https://www.ncbi.nlm.nih.gov) as specified in Table 1. Other data generated or analyzed during this study are included in this published article and its supplementary information file.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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