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Occurrence and characterization of *Alternaria* species associated with leaf spot disease in rapeseed in Serbia

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Abstract

The global demand for rapeseed makes it one of the fastest growing markets in crop production, with a need for increasing growing area and productivity, both of which depend on effective pathogen control strategies. *Alternaria* pathogens cause serious losses of brassica crops and occur in most rapeseed-growing regions around the world. In this study, morphological, molecular, and pathogenic analyses of 113 isolates collected from nine important rapeseed-growing areas in Serbia identified four pathogens: *Alternaria brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata*, causing leaf spot disease. Molecular analyses of ITS, *GAPDH*, *Alt a1*, and *ATP* sequences revealed one multilocus haplotype for *A. brassicae* and *A. japonica* isolates, whereas for *A. brassicicola* and *A. alternata* three and five haplotypes were distinguished, respectively. Pathogenicity tests showed that *A. brassicicola* was the most virulent while *A. brassicae* and *A. japonica* exhibited the same level of pathogenicity. The *A. alternata* population was generally weakly pathogenic with one nonpathogenic, genetically separated but closely related group of isolates, suggesting that pathogenicity is more unstable in this phylogenetic lineage. The data recorded on rate of growth and sporulation of isolates at 0, 5, 10, 15, 20, 25, 30, 35, and 40 °C revealed significant differences in evolutionary strategies among species, as *A. alternata* had the widest optimum range and the fastest growth rate, *A. brassicicola* showed the highest sporulation intensity, and *A. brassicae* expressed lower optimum temperatures for sporulation compared to other groups. All species indicated the potential for cross-infection of cabbage, and some haplotypes of *A. brassicicola* were previously isolated from horseradish in Serbia, suggesting the presence of one persistent *Alternaria* population on multiple brassica hosts in the region. This report describes the first detailed study of *Alternaria* spp. in rapeseed in Serbia.

KEYWORDS

A. alternata, *A. brassicae*, *A. brassicicola*, *A. japonica*, rapeseed

1 | INTRODUCTION

Rapeseed (oilseed rape, rape) is the second most important oilseed crop in the world with a production of over 36 × 10^6 ha hectares and 75 × 10^6 t in 2017 (USDA, 2020). Most of the global rapeseed crop refers to different forms, subspecies and varieties of two species of the Brassicaceae family – *Brassica campestris* (syn. *B. rapa*, also known as toria, sarson, field mustard, keblock, and Polish rape) and *Brassica napus* (Argentine rape, swede rape, rape kale, swede, Hanover-salad, and rape kale), while the term canola implies

rapeseed varieties low in erucic acid and glucosinolates suitable for the production of edible oils (Gulden *et al*., 2003). Due to the high percentage of oil in the seed (over 40%) and high protein content (20%–40%) of rapeseed, this crop is mostly used in the production of edible vegetable oils, biodiesel, animal feed, as a cover crop for improving soil structure and properties, and as one of the best honey plants (Raymer, 2002). Medicinal benefits and antimicrobial activity of rapeseed oil are often described, due to the high amount of unsaturated fatty acids, sterols, antioxidants, and vitamins.

Rapeseed agriculture is restricted by many factors, of which the particularly important ones are fungal-related diseases, such as clubroot (caused by *Plasmodiophora brassicae*), downy mildew (caused by *Hyaloperonospora parasitica*), alternaria blight (caused by *Alternaria brassicae* and *A. brassicicola*), blackleg (caused by *Leptosphaeria maculans* and *L. biglobosa*), which cause different yield losses in different cultivation systems (Söchting and Verreet, 2004).

Of particular economic importance for brassica crops are *Alternaria* species, described as prominent plant pathogens, saprobes or endophytes in more than 4,000 *Alternaria*–host associations, most of which are globally important agricultural species (Lawrence *et al*., 2013). Diseases known by different names, such as alternaria leaf blight, leaf spot, storage rot of vegetables, sooty spot, pod spot, and black mould, whose symptoms occur on rapeseed, cabbage, cauliflower, Chinese cabbage, kale, broccoli, and many others, are caused by two main pathogens: *A. brassicae* and *A. brassicicola* (Nowicki *et al*., 2012). In oilseed brassicas, symptoms start as small dark brown to grey spots with or without chlorotic halo and concentric rings on the surface on different parts of the plant. As spots spread and coalesce, the photosynthetic surface shrinks, while siliques wither, and the chemical composition of seeds changes, becoming unviable with loss or splitting of pods (Thomma, 2003). The disease can be seedborne, causing the death of seedlings or substantial loss in yield, both qualitative and quantitative. Heavy infections can decrease the number of seeds by up to 36% and a decrease of oil by up to 10%, and the average yield loss was documented to be 20% to 60% (Saharan *et al*., 2016). Studies describing the occurrence of *Alternaria* species causing leaf spot disease of rapeseed are limited and mostly focus on *A. brassicae*, although reports of the presence of the disease around the world are numerous. In Europe, *A. brassicicola* was detected in the UK, France, and Poland, and *A. ethzedia* was reported in Switzerland (Farr and Rossman, 2019). Recently, *A. arborescens*, *A. hordeicola*, *A. infectoria*, *A. japonica*, *A. malvae*, *A. metachromatica*, and *A. tenuissima* on rapeseed were reported in Australia for the first time (Al-Lami *et al*., 2019). The occurrence and incidence of the disease varied in a given production system, depending on environmental factors, regional climate conditions, and seasonal weather conditions (Al-Lami *et al*., 2019).

In Serbia, because of the suitable climate and soil conditions, rapeseed production increased almost threefold in 2018 with a production area of 45,628 ha, an average yield of 3 t/ha, and a total annual yield of rapeseed of 135,422 t (Statistical Office of the Republic of Serbia). The first heavy infestation of rapeseed plants by *A. brassicae* in Serbia was reported in 1938 in the municipality of Vrbas (Banat district), and since then, this pathogen has been commonly observed across the main production regions (Grujičić and Tomašević, 1956), causing minor or more severe annual yield losses on rapeseed crops. Field observations showed that under agroenvironmental conditions in Serbia, the optimal sowing time for winter rapeseed crop is late August and early September when average monthly temperatures reach 14–18 °C. In December when temperatures reach around 2 °C, young plants with several rosette leaves and stems up to 1 cm high go into a resting phase through January and February when annual monthly temperatures are around 5 °C and 1 °C, respectively. In the spring, when average monthly temperatures reach 5 °C in March and 15 °C in April, growth and development of the plant continues and buds start to form. Flowering and fertilization phases start during the end of April and through May, when monthly temperatures rise to 20 °C. The highest precipitation is during the winter months and spring, May and June (Republic Hydrometeorological Service of Serbia, http://www.hidmet.gov.rs/eng/meteorologija/klimatologija_produkti.php). To date, alternaria blight symptoms observed in rapeseed in Serbia were usually attributed to *A. brassicae*. Bearing in mind that different species under different conditions may respond to fungicides differently, a lack of epidemiological data and proper taxonomic identification could affect disease-control practices. In Serbia, alternaria leaf spot disease is controlled with fungicides applied to the foliage up to five times per growing season. Several fungicides are registered for use on rapeseed, including protective materials such as mancozeb, propineb, and chlorothalonil, systemics such as difenoconazole, and more recently the strobilurins azoxystrobin and pyraclostrobin.

The identification of *Alternaria* spp. has been conducted based on morphological and physiological characteristics; hence, delineation of groups due to morphological homoplasy, numerous *Alternaria*–host associations, and lack of an active sexual phase has been difficult and subject to constant change (Thomma, 2003). Molecular markers like the ITS region (internal transcribed spacers 1 and 2 and intervening 5.8S rDNA), the mitochondrial ribosomal large subunit (mtLSU) and the mitochondrial small subunit (mtSSU) have been routinely used, but revealed a strong relation only to some phylogenetic groups of the *Alternaria* genus, mostly the large-spored species and *A. infectoria* among small-spored species (Woudenberg *et al*., 2013). The advancement of different molecular methods and multigene studies based on sequences of protein-coding genes and some anonymous regions have enabled some of the recent revisions and more stable taxonomic classification of the genus (Andersen *et al*., 2009; Woudenberg *et al*., 2013; 2015; Lawrence *et al*., 2013; 2016; Ozkilinc *et al*., 2018; Ozkilinc and Sevinc, 2018). Information provided by these phylogenetic analyses depended upon loci were used and the group of small-spored *Alternaria* still remain the most challenging to distinguish. Lawrence *et al*. (2013) indicated that glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), plasma membrane ATPase (*ATP*), the major allergen precursor (*Alt a1*), and calmodulin gene (*Cal*) regions were suitable for delineating many species from section *Alternaria*, and that *ATP* and *Cal* genes were most suitable for

resolving the group of small-spored *Alternaria*. Woudenberg *et al.* (2015) used whole-genome sequencing, transcriptome analysis and multigene phylogeny using the ITS, mtSSU, mtLSU, *GAPDH* gene, RNA polymerase II subunit (*RPB2*), translation elongation factor 1-α (*TEF1*), *Alt a1*, endopolygalacturonase (*endoPG*), and an anonymous gene (*OPA10-2*) to delineate the main phylogenetic groups in section *Alternaria* but failed to clearly resolve the *A. arborescens* species complex and concluded that about 35 morphospecies, among which were *A. tenuissima* and *A. alternata*, should be considered together as one species, *A. alternata*. Stewart *et al.* (2014) and Ozkilinc *et al.* (2018) demonstrated the resolving power of *endoPG*, *ATP*, *Alt a1*, and some anonymous markers on morphologically indistinguishable small-spored pathogens causing alternaria citrus brown spot and alternaria blight of pistachio, respectively. Landschoot *et al.* (2017), investigating population diversity of *Alternaria* species in naturally occurring potato plants, concluded that the *GAPDH* gene was informative for distinguishing both large-spored and small-spored *Alternaria* isolates, *Cal* and *RPB2* genes for large-spored species, while *Alt a1* and *histone h3* genes provided resolution of small-spored isolates identified as *A. alternata*, *A. tenuissima*, and *A. arborescens*.

Although alternaria leaf spot disease on brassicas is widespread with high economic impact under conducive weather conditions, there is no specific data on the number of causal species or detailed study of the main aspects of the disease on rapeseed crops in Serbia. Therefore, studies were undertaken to gain insight into the *Alternaria* population present on rapeseed plants in Serbia. Isolation, morphological and molecular characterization, and identification of the *Alternaria* isolates from rapeseed and their population diversity, are necessary steps to improve control measures against alternaria disease. Additionally, the study attempts to determine the cross-infection potential of these species on cruciferous crops most commonly grown in close proximity and to evaluate the effects of temperature on growth rate and sporulation.

2 | MATERIALS AND METHODS

2.1 | Sample collection and fungal isolation

In 2015 and 2016, a total of 22 fields of rapeseed were selected and surveyed at nine localities of eight administrative districts of Vojvodina, the major production area of rapeseed crop in Serbia (Figure 1). Individual plants showing *Alternaria*-induced dark leaf spot lesions were collected on the production plots in two diagonal transects, and random plants were evaluated for disease incidence estimation. The number of samples varied according to the size of the crop area, resulting in 10 to 25 plants being sampled at equal distances, which were then placed in separate paper bags and stored at 5 °C in a field refrigerator until they were sent to the laboratory. For fungal isolations, 2-cm-long fragments were cut from a margin of the lesions on leaves, stems or siliques. The surface of the sample was disinfected in 1% NaOCl for 1 min, rinsed in sterile distilled water, and placed onto potato dextrose agar (PDA, Difco) amended with 100 mg/L of streptomycin sulphate (Sigma-Aldrich) and incubated at 23 °C for 72 hr. Mycelial

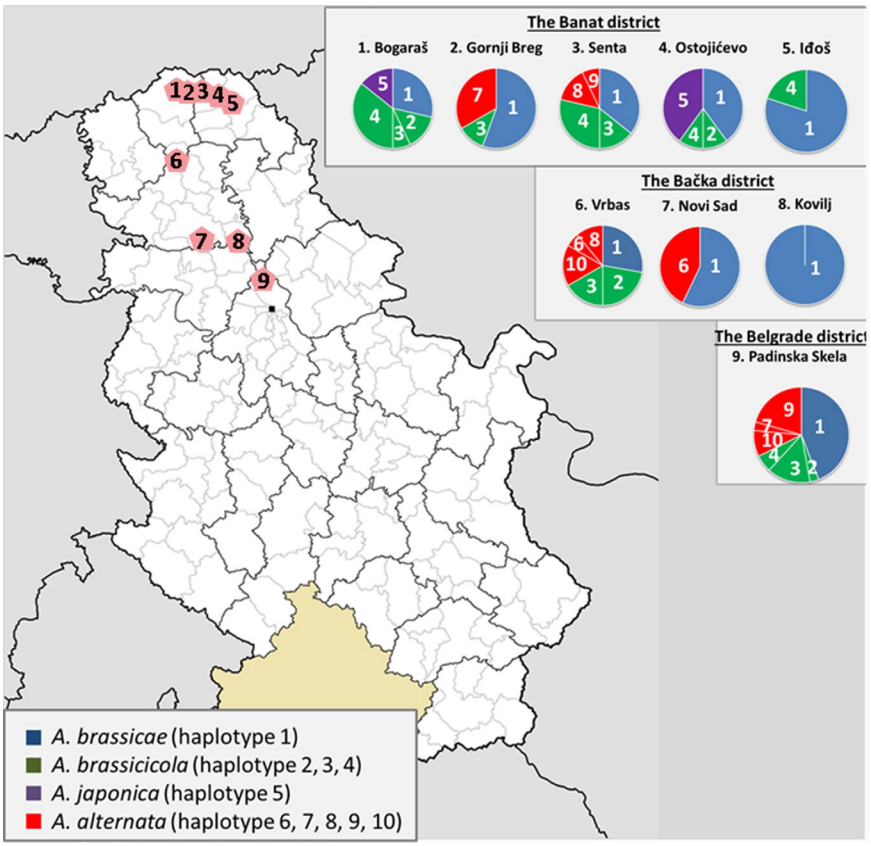

FIGURE 1 Map showing nine localities in Serbia, the main growing areas of rapeseed in which *Alternaria* spp. were detected [Colour figure can be viewed at wileyonlinelibrary.com]

fragments were taken from the growing colony margin under a stereomicroscope and placed on V8 agar (200 ml Campbell V8 juice, 3 g $CaCO_3$, 15 g agar, and 800 ml sterile distilled water) followed by incubation at 23 °C for 5 days. Purification of the isolated fungi was done by transferring single spores or hyphal tips from V8 agar onto fresh PDA at 23 °C. After adequate growth, pure cultures were maintained at 4 °C in the culture collection of the Institute for Plant Protection and Environment, Belgrade, Serbia. The reference strains *A. brassicicola* CBS 118699 and *A. brassicae* CBS 116528 were used as positive controls.

2.2 | Morphological characterization

To examine morphological characteristics, mycelial plugs (diameter of 5 mm) were cut from the growing edges of the 5-day-old cultures and placed onto V8 agar 40 cm below cool white fluorescent bulbs and incubated at 23 °C for 7 days with an 8 hr/16 hr photoperiod. Culture characteristics, such as colony colour, size, texture, and radial growth rate, were assessed after 7 days, and conidial morphology (length and width of the conidial body and the beak of 50 randomly chosen conidia per isolate), and sporulation patterns were observed using an Olympus BX51 microscope (400×) and EU instrument dissection scope (90×), respectively. Colony diameter was calculated daily for 7 days along two perpendicular axes. The two measures were averaged, and the data were converted to radial growth in mm/day. The protocol recommended by Simmons (2007) was used for morphological identification of *Alternaria* species. Three replicates per isolate were used, and the experiment was repeated twice.

2.3 | Pathogenicity test

The pathogenicity test was conducted on 8-week-old rapeseed (*B. napus* var. *napus* 'Jovana') and cabbage (*Brassica oleracea* var. *capitata* 'Futoški') plants. Healthy plants were chosen with a minimum of 10 completely formed leaves. A suspension of spores was prepared from 7-day-old pure V8 agar cultures grown at 23 °C. The cultures were suspended in 5 ml sterile distilled water (SDW), gently scraped with a glass stick, and filtered through two layers of cheesecloth. The number of conidia was adjusted to 10^6 conidia/ml using a haemocytometer. Two inoculation methods were tested, first with the surface of the leaf left intact (unwounded), and secondly with the leaf lamina injured by pricking with a sterile needle, after which 20 µl of inoculum was placed onto six symmetric locations of both the intact and injured leaf surfaces. Similarly, intact and injured leaves of plants assessed as negative controls were inoculated with SDW. The test was repeated twice, four leaves per plant and two plants of rapeseed and cabbage were used for each isolate. After inoculation, plants were placed in a growing chamber with relative humidity between 95% and 100% for 72 hr at 17 °C/23 °C and an 8 hr/16 hr photoperiod. Plants were examined after 7 days, and the lesion area of each leaf was estimated using the program ImageJ (Schneider *et al.*, 2012). Fragments of lesion area were cut, and surfaces were sterilized, before placing onto V8 agar. The morphological characteristics of these isolates were compared with original isolates to fulfil Koch's postulates.

2.4 | Temperature effects on growth and sporulation

Mycelium plugs (5 mm in diameter) were cut from the edge of 4-day-old PDA cultures incubated at 23 °C and placed in the centre of new PDA plates, which were then incubated in the growth chamber at 0, 5, 10, 15, 20, 25, 30, 35, and 40 °C with an 8 hr/16 hr light/dark photoperiod. Three replicate plates were used for each isolate and incubation temperature. The radial colony diameter was measured along two perpendicular axes for 7 days, and the average growth rate was calculated as mm/day. Incubated PDA plates were flooded with 2.5 ml SDW with 0.01% Tween 80, and mycelia with spores were scraped with a sterile glass stick and placed in microtubes, while muslin cloth was used to filter the spore suspension. If necessary, stock solutions were diluted with SDW to enable the spores to be counted with a haemocytometer.

2.5 | Data analyses

The collected data from mycelium growth analyses, pathogenicity tests, and temperature effect experiment were analysed using analysis of variance (ANOVA) in the statistical software package SPSS v. 20.0 (SPSS Inc.). Experiments were conducted for all isolates, but for statistical estimation, representative isolates of *A. brassicae* (nine isolates sampled in different regions), *A. brassicicola* (eight isolates: two isolates of every haplotype, one isolate from Banat district, and one isolate from Bačka district), *A. alternata* (10 isolates: two isolates of every haplotype), *A. japonica* (all six isolates), and the reference strains of *A. brassicae* and *A. brassicicola* were used, if not stated otherwise. Data between runs of an experiment, as well as between values for each isolate of *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata*, were pooled after checking for homogeneity of the experimental error variances (Levene's test). Differences between treatment means were compared by Tukey's honestly significance difference test if not stated otherwise. Principal component analysis (PCA) was used to summarize temperature variation within a data set.

2.6 | Molecular identification

Total genomic DNA of all isolates was extracted from pure cultures grown on PDA for 7 days at 23 °C using a DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Characterization of the four protein-coding genes was used for identification and

phylogenetic analysis of *Alternaria* species from Serbia. Primers ITS1 and ITS4 (White *et al*., 1990) were used to amplify the ITS region of the nuclear ribosomal DNA, including the 5.8S rDNA gene, following the amplification conditions of Woudenberg *et al*. (2013); amplification of the *Alt a1* allergen gene fragment was performed using the Alt-for/Alt-rev primer pair following the thermal protocol by Hong *et al*. (2005); the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene region was amplified using gpd1/gpd2 primers following Lawrence *et al*. (2013); while the plasma membrane ATPase gene was amplified with primers ATPDF1 and ATPDR1 as previously described by Lawrence *et al*. (2013) with some changes in parameters of annealing phase (Table 4). All amplifications were performed in a 25 μl volume containing 5 ng DNA, 1 × PCR buffer (20 mM Tris.HCl pH 8.4, 50 mM KCl), 1 μM of each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 1 U*Taq* DNA polymerase (Fermentas) in a Mastercycler Nexus GSX1 (Eppendorf). Amplicons were separated in 1% agarose gels in 0.5 × TBE buffer and visualized by ethidium bromide staining under UV illumination. The obtained products were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced with an automated DNA sequencer (ABI PRISM 3700, Macrogen Inc.). Sequences were edited and assembled using FinchTV v. 1.4.0 (Geospiza http://www.geospiza.com/finchtv) and aligned with ClustalW within the MEGA 6 software (Tamura *et al*., 2013). Sequences of fungal isolates from Serbia were compared, and haplotype representative sequences were deposited in the NCBI GenBank (Table 1).

2.7 | Construction of a phylogenetic tree and a haplotype network

Phylogenetic studies based on ITS, *Alt a1*, *GAPDH*, and *ATP* gene regions were performed using one representative isolate of every *Alternaria* species haplotype from rapeseed (Table 1) and the reference isolates (Table 2). For phylogenetic analyses, the partition homogeneity test implemented in PAUP* v. 4.0b10 (Swofford, 2003) was used for the ITS and *GAPDH*; *Alt a1* and *ATP*, and ITS, *Alt a1*, *GAPDH*, and *ATP* sequence alignments, to examine the suitability of a concatenated analysis. Data partitions were considered significantly different at $p < .05$. The phylogenetic analyses were based on corrections, alignments, and comparisons of the sequences performed using ClustalW integrated into MEGA 6 software (Tamura *et al*., 2013). All positions containing gaps were excluded from the analysis. The phylogenetic relationships among the *Alternaria* spp. from rapeseed were inferred by two methods: maximum-likelihood phylogeny and means of Bayesian phylogeny. For concatenated data, the Bayesian information criterion (BIC) indicated the best-fitting nucleotide substitution model using the software jModeltest v. 2.1.7 (Darriba *et al*., 2012). Maximum-likelihood phylogeny was reconstructed using MEGA 6 software, applying 1,000 bootstrap replicates and pairwise deletion (Tamura *et al*., 2013). Bayesian-based phylogenetic analysis was performed in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The analyses were carried out using two simultaneous Markov chain Monte Carlo (MCMC) runs of 1,000,000 generations each, and trees were sampled once every 100 generations. The first 25 trees were discarded as burn-in samples, and the standard deviation of the split frequencies was checked at the end of each run until it reached a value below 0.01. The convergence of the MCMC chains and their stationarity were checked by using Tracer v. 1.5 and the phylogenetic tree was visualized using FigTree v. 1.4. To infer genetic relations and the level of genetic diversity and to associate haplotype groups with the possible geographic separation of *Alternaria* isolates from rapeseed, concatenated sequences of the ITS and *GAPDH* for *A. brassicae*, *A. brassicicola*, and *A. japonica*, and *Alt a1* and *ATP* gene sequences for *A. alternata*, were used in the haplotype network analyses in TCS v. 1.21 (Clement *et al*., 2000) using statistical parsimony with a confidence level of 95%. The sequences of *Stemphylium calistephi* served as outgroup taxa based on results from Lawrence *et al*., 2013.

3 | RESULTS

3.1 | Samples, disease symptoms and pathogen isolation

Leaves, stems, and siliques with grey to dark-brown circular lesions were collected from nine localities in the main rapeseed production regions of Serbia (Figure 1). During the phase of intensive growth of the rapeseed plants with branch and bud development, insect-induced lesions were noticed along with more intensive symptoms of alternaria leaf spots on older leaves. With flowering and develment of the seedpods, infection became more noticeable and severe. In May and June, during the phase of seed maturation, infection was intensive, covering all plant parts. Leaves near the ground were often almost decomposed as a result of the coalesced lesions and spore abundance. The lesion diameter ranged from 2 to 27 mm. In addition to *Alternaria* spp., which was the most commonly found (75%), *Leptosphaeria* sp. was also present (18.8%), while *Penicillium* sp. and *Sclerotinia* sp. were detected sporadically. The disease incidence of alternaria leaf spot was 17% to 43%, with minimum disease occurrence in the Banat district and maximum occurrence in the Belgrade district.

3.2 | Morphological characterization

Based on morphological features of mycelia, conidia, and the sporulation pattern, all 113 isolates of *Alternaria* grown on V8 agar for 7 days were classified into four different groups that were identified as species (Figure 2; Table 1). The first group of 48 large-spored isolates was identified as *A. brassicae*, the second group of 33 small-spored isolates was *A. brassicicola*, the third group consisted of only six isolates of *A. japonica*, and the fourth group, morphologically the

TABLE 1 *Alternaria* spp. isolated from leaves of rapeseed with symptoms in Serbia

Species	District	Locality	Number of isolates	GenBank accession number				Haplotype
				ITS	*Alt a 1*	*GAPDH*	*ATP*	
A. brassicae	Banat	Bogaraš	4	–	–	–	–	1
		Gornji Breg	5	–	–	–	–	
		Senta	5	–	–	–	–	
		Ostojićevo	4	–	–	–	–	
		Iđoš	4	–	–	–	–	
	Bačka	Vrbas	5	–	–	–	–	
		Novi Sad	4	–	–	–	–	
		Kovilj	2	–	–	–	–	
	Belgrade	Padinska Skela	15	MN173822	MN173503	MN175513	MN175523	
A. brassicicola	Banat	Bogaraš	2	–	–	–	–	2
			1	–	–	–	–	3
			5	–	–	–	–	4
		Gornji Breg	1	–	–	–	–	3
		Senta	2	–	–	–	–	3
			4	–	–	–	–	4
		Ostojićevo	1	–	–	–	–	2
			1					4
		Iđoš	1	–	–	–	–	4
	Bačka	Vrbas	4	–	–	–	–	2
			3	–	–	–	–	3
			1	MN173823	MN173504	MN175514	MN175524	2
	Belgrade	Padinska Skela	5	MN173824	MN173505	MN175515	MN175525	3
			2	MN173825	MN173506	MN175516	MN175526	4
A. japonica	Banat	Bogaraš	2	MN173826	MN173507	MN175517	MN175527	5
		Ostojićevo	4	–	–	–	–	
A. alternata	Banat	Gornji Breg	3	–	–	–	–	7
		Senta	2	–	–	–	–	8
			1	–	–	–	–	9
	Bačka	Vrbas	3	–	–	–	–	10
			1	–	–	–	–	6
			2	MN173830	MN173511	MN175521	MN175531	8
		Novi Sad	3	MN173828	MN173509	MN175519	MN175529	6
	Belgrade	Padinska Skela	3	MN173827	MN173508	MN175518	MN175528	10
			1	MN173829	MN173510	MN175520	MN175530	7
			7	MN173831	MN173512	MN175522	MN175532	9

most variable one, consisted of 26 isolates of *A. alternata* according to Simmons (2007) (Table 3).

The colony colour was predominantly white or light brown for *A. brassicae*, black-brown for all *A. brassicicola* isolates, and similar shades of grey, brown, and green for *A. japonica* and *A. alternata*. When colony growth rates were compared among isolates within species groups, no significant differences were observed (p = .313, p = .569, p = .342, and p = .171 for *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* isolates, respectively). A significant difference was observed among groups (p = .000), *A. alternata* showed the highest growth rate (10.0 ± 1.12 mm/day), and *A. brassicicola* had a medium growth rate (5.9 ± 0.5 mm/day), while *A. japonica* (4.0 ± 0.5 mm/day) and *A. brassicae* (3.2 ± 0.7 mm/day) had the slowest growth rates. The sporulation pattern showed solitary or few large conidia rising from the straight conidiophores for *A. brassicae*; solitary or chains of up to four small conidia for *A. japonica*; branched conidiophores and conidial chains of up to 20 small conidia for *A. brassicicola* isolates; and secondary and occasionally tertiary chains branched from apical

TABLE 2 Species used for phylogenetic analyses in this study, their sources, and GenBank accession numbers

			GenBank accession numbers			
Species	Country	Source[a]	ITS	*Alt a1*	*GAPDH*	*PM-ATP*
Alternaria alternata	India	EGS 34-016	AF 347031	KP 275691	AY 278808	JQ 811979
Alternaria arborescens	USA	EGS 39-128	AF 347033	AY 563303	AY278810	JQ671880
Alternaria tenuissima	UK	EGS 34-015	AF 347032	KP 275690	AY 278809	JQ 881989
Alternaria brassicae	USA	EGS 38-032	JQ693663	AY563309	AY562414	JQ 671847
Alternaria brassicicola	USA	EEB 2232	AF 229652	AY 563311	AY 278813	JQ 671843
Alternaria japonica	Netherlands	EGS 50-099	AF229474	–	AY278814	–
Stemphylium callistephi	–	EEB 1055	AF 229482	AY563276	AY 278822	JQ 671769

[a]EEB, E. E. Butler, Department of Plant Pathology, University of California, Davis, CA 95616, USA; EGS, E. G. Simmons, Mycological Services, Crawfordsville, IN 47933, USA.

and intercalary cells of 8 to 20 small conidia of *A. alternata* isolates. The number of transverse septa was 3 to 15 for *A. brassicae* and similar (1 to 8) for other three species groups. The number of transverse septa could not differentiate the four species. Longitudinal septa were rarely observed for *A. brassicae* (10% of counted conidia) and *A. brassicicola* (7% of counted conidia); however, for *A. japonica* and *A. alternata*, conidia with longitudinal septe were often observed (76% and 44% of counted conidia, respectively). Round conidia without beaks were found in 37% of conidia of *A. japonica*.

3.3 | Pathogenicity assays

Four days after inoculation by the unwounded and wound inoculation methods, brown spots symptomatic of alternaria leaf spot disease appeared on rapeseed and cabbage leaves. After 7 days, spots were circular, ranging from 1 to 21 mm in size, similar to that observed in the rapeseed fields (Figure 3). Occasional variations in spot colour were observed for *A. brassicae*, *A. alternata*, and *A. japonica*, and darker brown was observed for *A. brassicicola*. Among the 26 *A. alternata* isolates, 8 were nonpathogenic for cabbage or rapeseed plants, and these were excluded from further statistical analysis of this group. Koch's postulates were fulfilled for all *A. brassicae*, *A. brassicicola*, and *A. japonica* isolates and for pathogenic *A. alternata* group by successful reisolation and morphological confirmation of original strains. After 14 days, spots enlarged and coalesced, leading to severe damage to the photosynthetic surface of the leaves, reaching 100%, and eventually causing defoliation. A three-way mixed ANOVA was run to understand the effects of species (*A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata*), host (rapeseed and cabbage), and inoculation method (unwounded and wounded leaf surface) on the percentage of necrotic leaf area. Although Levene's test for equality of variances showed that homogeneity of variance was not observed between populations, the ratio of the largest to the smallest group variance was approximately 1.7, and the test was continued. There was a statistically significant three-way interaction between host, inoculation method, and species ($F_{3,33}$ = 6.7, p = .001). The statistical significance of a simple two-way interaction and a simple main effect was accepted at a Bonferroni-adjusted α level of 0.025. There was a statistically significant simple two-way interaction between host and inoculation method for *A. brassicicola*, *A. japonica*, and *A. alternata* ($F_{1,9}$ = 8.43, p = .017; $F_{1,5}$ = 12.71, p = .016; and $F_{1,9}$ = 14,69, p = .004; respectively) but not for *A. brassicae* ($F_{1,10}$ = 3.78, p = .084). When the leaf surface was wounded, there was a statistically significant simple main effect of the host for *A. brassicicola* and *A. alternata* ($F_{1,9}$ = 9.29, p = .014; $F_{1,9}$ = 27.18, p = .001; respectively) but not for *A. japonica* ($F_{1,5}$ = 0.37, p = .569). In treatments where the leaf surface was intact, there was a statistically significant simple main effect of the host for *A. brassicicola* and *A. japonica* ($F_{1,9}$ = 129.77, p = .001; $F_{1,5}$ = 64.97, p = .000; respectively) but not for *A. alternata* ($F_{1,9}$ = 0.437, p = .066). Isolates of *A. brassicicola* were the most virulent, *A. brassicae* and *A. japonica* did not show significant differences in pathogenicity, while *A. alternata* expressed the weakest virulence (Figure 4). The injured leaf-stem of cabbage was more prone to infection. Cabbage plants were more severely damaged than rapeseed in all examined cases. No symptoms were observed on any noninoculated plants. Pathogenicity tests with intact leaf surfaces of rapeseed showed that *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* caused 15.6%, 39.4%, 13.1%, and 6.7% leaf necrosis, respectively. In intact cabbage leaves, leaf necrosis was 25.6%, 61.6%, 19.8%, and 7.4% for *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata*, respectively. Leaf necrosis for injured leaves was 27.1%, 61.5%, 29.2%, and 10.7% for rapeseed, and 36.1%, 73.7%, 30.1%, and 13.1% for cabbage for *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata*, respectively (Figure 4).

3.4 | Temperature effect

A PCA was based on the growth and sporulation rates of all isolates at temperatures of 0, 5, 10, 15, 20, 25, 30, 35, and 40 °C (Figure 5). The suitability of PCA was assessed by inspection of the correlation matrix, which showed that all variables had at least one correlation coefficient greater than 0.3. The overall Kaisser-Meyer-Olkin (KMO) measure was 0.82 with individual KMO measures all greater than 0.7. Bartlett's test of sphericity was statistically significant (p = .000). PCA revealed two components that had high eigenvalues explaining

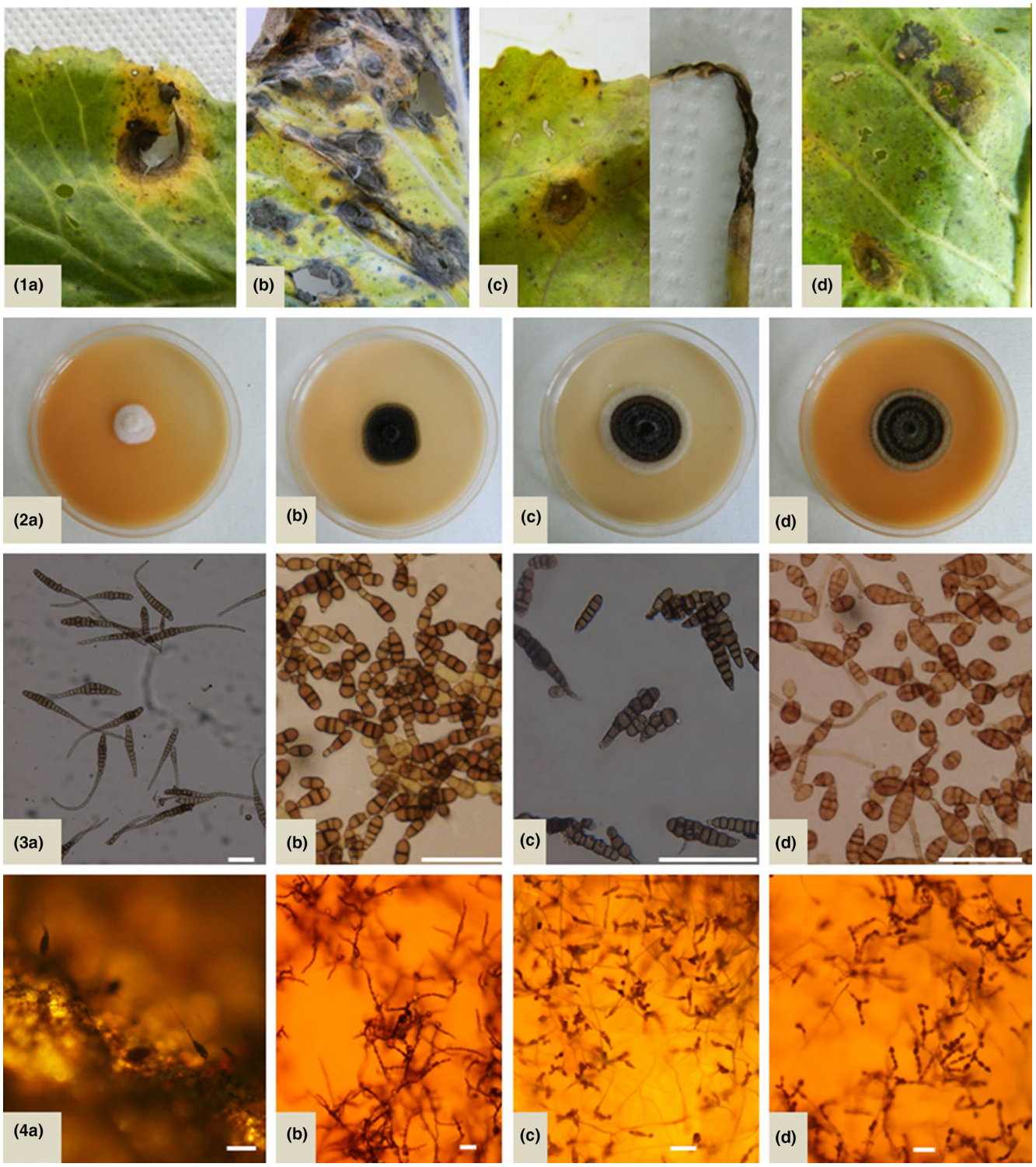

FIGURE 2 (1a–d) Symptoms of leaf spots on rapeseed with concentric rings on lesions; (2a–d) 7-day-old culture on V8 agar; (3a–d) conidia; (4a–d) sporulation pattern on V8 medium; a - *Alternaria brassicae*, b - *A. brassicicola*, c - *A. japonica*, d - *A. alternata*. Bars: 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]

59.8% and 23.4% of the total variance, respectively, which was also confirmed in the scree plot; therefore, two components were retained explaining 83.2% of the total variance. The main contributing variables for component 1 were sporulation rate at 25, 15, 20, 10, 30, and 5 °C, respectively, and for component 2, the strongest loadings were variables measuring growth rates at 20, 10, 5, 25, and 15 °C, respectively. The projection of *Alternaria* isolates on the plane of component 1 and 2 improved the visual interpretation of the data, representing *A. brassicae* as Group 1 and *A. brassicicola* and *A. japonica* as Group 2, while *A. alternata* isolates were segregated into

TABLE 3 Description of morphological characters, colony growth and sporulation pattern of *Alternaria* spp. from rapeseed

Morphological characters	*Species* (number of isolates)			
	***A. brassicae* (48)**	***A. brassicicola* (33)**	***A. japonica* (6)**	***A. alternata* (26)**
Mycelial texture and shape	Cottony, circular	Cottony, circular	Cottony, circular	Aerial or cottony, circular
Colony colour	White and brownish	Brown-black	Grey greenish	Grey, brown, and green
Colony margin	Cream and pale	Yellowish or cream	Various	Various
Colony growth (mm/day)	3.3 ± 0.7 (3, 4)	5.9 ± 0.5 (5, 7)	4.0 ± 0.5 (3, 5)	10.0 ± 1.1 (8, 12)
Conidiophores (µm)	125.3 ± 19.4a (90, 185)	112.7 ± 45.6ab (19, 197)	105.1 ± 44.4b (21, 189)	72.9 ± 23.2 (19, 120)
Conidial shape	Obclavate mostly	Ellipsoid, ovoid, or obclavate	Ellipsoid, ovoid, or obclavate	Obclavate to long-ellipsoid
Conidial length (µm)	152.2 ± 32.7 (53, 214)	36.5 ± 8.7a (20, 52)	66.9 ± 9.9 (47, 88)	36.9 ± 5.8a (24, 55)
Conidial width (µm)	13.1 ± 1.7 (10, 16)	9.0 ± 2.1ab (8, 14)	10.4 ± 4.0a (4, 20)	9.1 ± 3.2b (4, 16)
Beak length (µm)	62.3 ± 12.4 (38, 88)	2.4 ± 0.5a (2, 7)	3.0 ± 1.3a (2, 7)	4.6 ± 1.6 (2, 7)
Beak width (µm)	9.3 ± 2.9 (4, 15)	4.5 ± 1.6a (2, 7)	5.2 ± 0.9a (2, 7)	2.0 ± 0.9 (1, 5)
Number of transversal septa (min.–max.)	3–15	1–8	1–8	1–8
Number of longitudinal septa	2–5 (10%)	0–5 (7%)	2–7 (76%)	0–6 (44%)

Note: Values within the same row followed by the same letter are not significantly different based on Tukey's test at $p < .05$; conidiophores and conidial morphology are valued in average of 50.

Values are presented as mean ± *SD* (minimum, maximum) for each measured character.

Groups 3 and 4. Isolates of *A. alternata* in Group 3 coincided with the *A. alternata* isolates that were pathogenic to leaves of rapeseed and cabbage, while isolates of *A. alternata* Group 4 from PCA plot proved to be nonpathogenic (Figure 5).

Further statistical analyses were conducted with representative isolates of *A. brassicae*, *A. brassicicola*, and *A. japonica*; however, *A. alternata* representative isolates consisted of two groups distinguished in PCA analyses. There was a statistically significant interaction of the species and temperature on growth and sporulation rates ($F_{16.436,156.134} = 29.31$, $p = .000$; $F_{10.4,98.802} = 19.721$, $p = .000$, respectively), as two-way mixed ANOVA indicated. Fast growth was recorded for *A. alternata* Groups 3 and 4, while *A. brassicae*, *A. brassicicola*, and *A. japonica* showed slow to moderate growth (Figure 6). At temperatures of 0 and 40 °C, none of the isolates grew or produced spores. At temperatures of 5, 10, 15, and 20 °C, the growth of *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* Group 4 did not show significant differences, while *A. alternata* Group 3 grew considerably faster. Temperatures of 20 and 25 °C were optimal for growth of *A. brassicae* (5.6 mm/day), *A. brassicicola* (5.8 mm/day), and *A. japonica* (4.4 mm/day), whilst maximum growth for *A. alternata* Group 4 was measured at 25 °C (optimum was up to 30 °C) (6.5 mm/day), similar to *A. brassicae* and *A. brassicicola* but significantly different from growth rate of *A. japonica* and *A. alternata* Group 3. For *A. alternata* Group 3, the optimal range was widest, extending from 20 to 30 °C, with an average growth of 8 mm/day.

A notable decrease in growth in the range of 25 to 30 °C was recorded for *A. brassicae* and *A. japonica*, while *A. brassicicola* showed a slower decline. None of *A. brassicae* isolates grew at 35 °C, while *A. brassicicola*, *A. japonica*, and *A. alternata* grew very little. *A. brassicicola* showed higher sporulation than *A. japonica* and *A. alternata*, while *A. brassicae* had the lowest sporulation ability. Optimum temperatures for sporulation of *A. brassicae* were from 15 to 20 °C, which were lower than optimal temperatures for growth. There were no significant differences among sporulation rates of *A. alternata* Groups 3 and 4, and optimal temperatures were in the same range as for the growth. Optimal temperatures for sporulation for *A. brassicicola* and *A. japonica* ranged from 20 to 25 °C, with no difference between 20 and 30 °C. Although the growth of *A. japonica* showed a rapid decrease at 30 °C, high temperatures did not affect sporulation. With increased temperatures, the colour of colonies changed to darker shades, texture was more cottony, and margins were more uniform and exhibited white to yellowish colours.

3.5 | Molecular characterization

A BLAST search with each individual sequence of ITS, *Alt a1*, *GAPDH*, and *ATP* suggested that the tested isolates from rapeseed corresponded to *A. brassicae*, *A. brassicicola*, *A. japonica*, or *A. alternata* (Table 1). Comparing obtained isolates with reference GenBank sequences, 10 different haplotypes were identified, distributed in

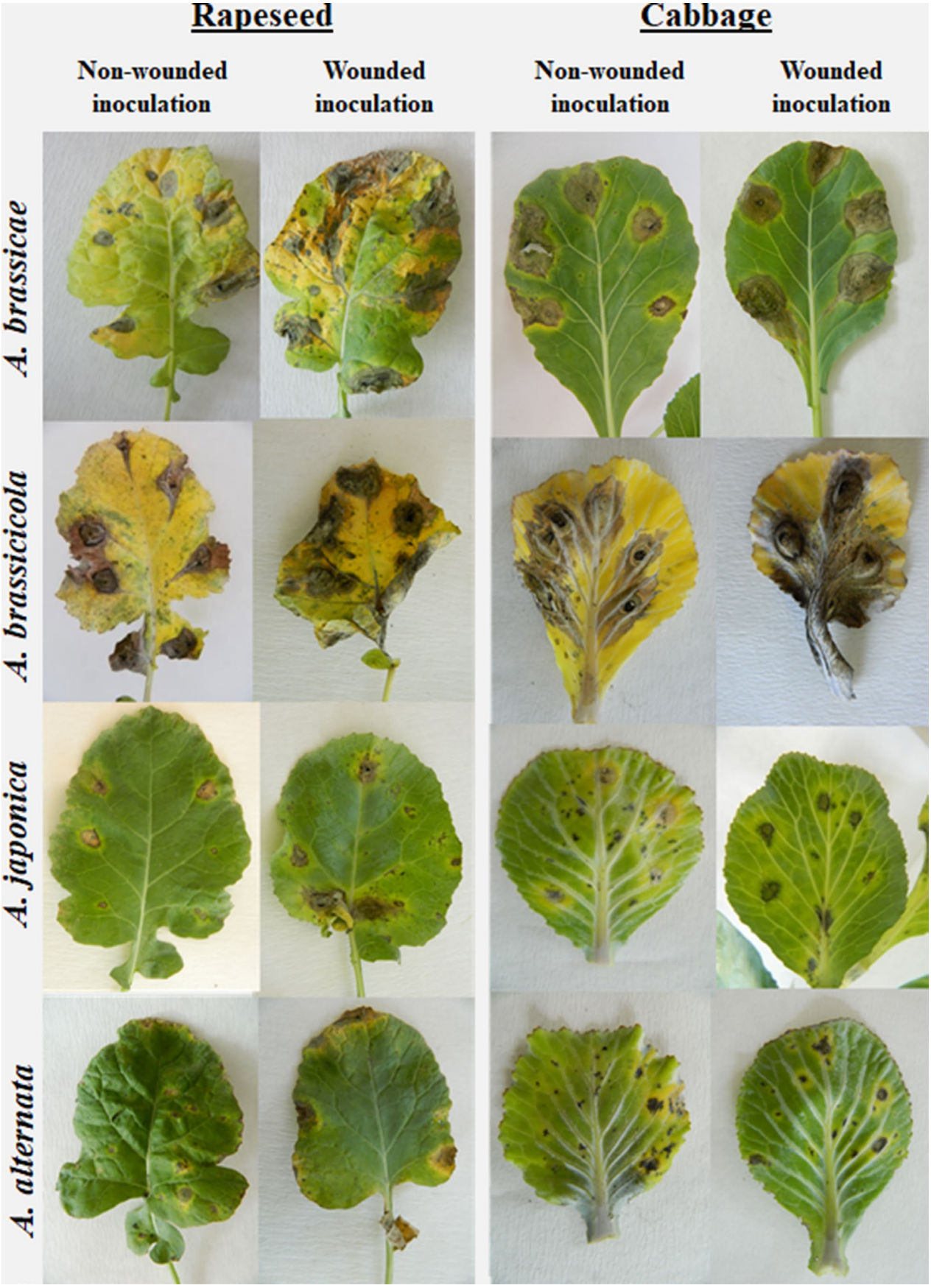

FIGURE 3 Symptoms of leaf spot disease developed 7 days after inoculation with drop of a conidial suspension of horseradish and cabbage leaves with *Alternaria brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* [Colour figure can be viewed at wileyonlinelibrary.com]

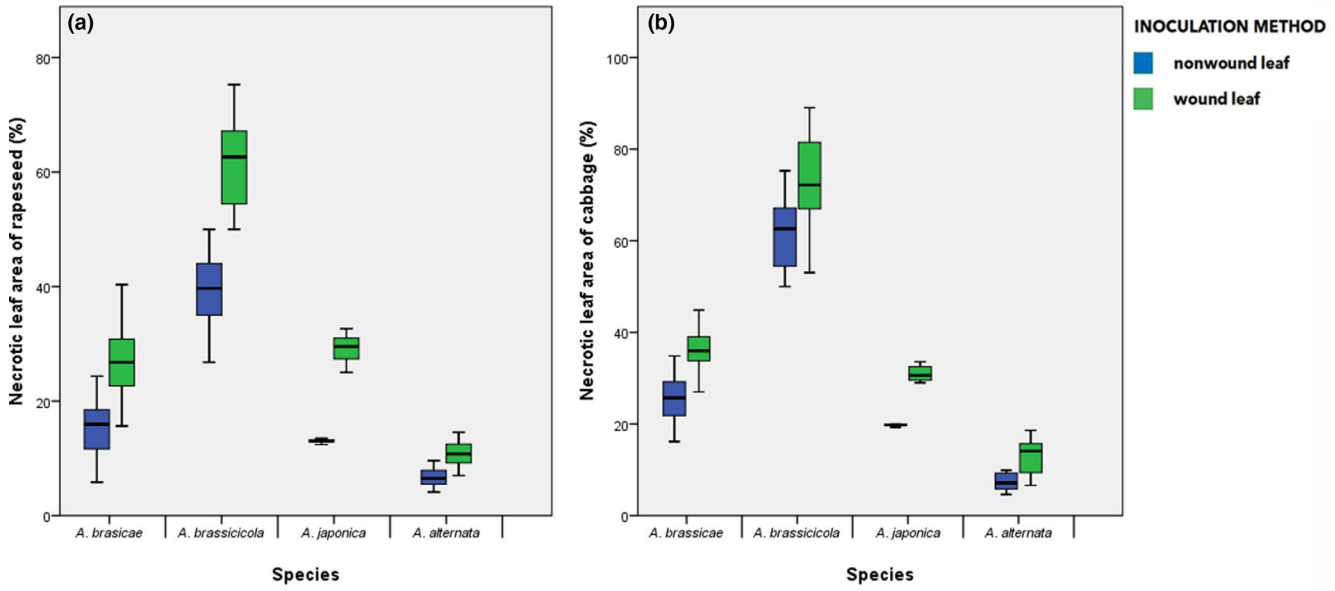

FIGURE 4 Virulence of *Alternaria brassicae*, *A. brasicicola*, *A. japonica*, and *A. alternata* in inoculation assays with (a) rapeseed and (b) cabbage plants [Colour figure can be viewed at wileyonlinelibrary.com]

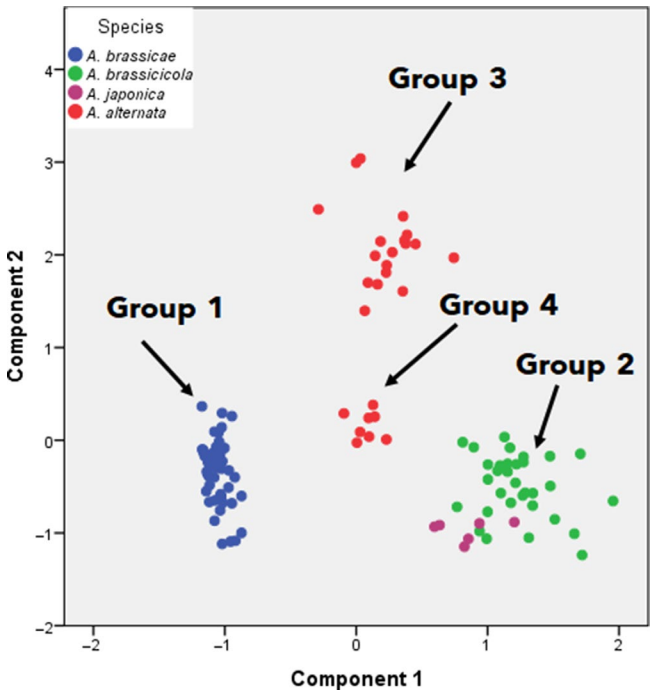

FIGURE 5 A principal component analysis (PCA) of *Alternaria brassicae* (Group 1), *A. brassicicola* (Group 2), *A. japonica* (Group 2) and *A. alternata* (Groups 3 and 4) from rapeseed based on growth rates and sporulation intensity at different temperatures. The main contributing variables for component 1 were sporulation rate at 25, 15, 20, 10, 30, and 5 °C, respectively, and for component 2, the strongest loadings were variable measuring growth rates at 20, 10, 5, 25, and 15 °C, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

four groups, where isolates in Group 1 were confirmed as *A. brassicae*, the isolates of Group 2 shared homology to *A. brassicicola*, Group 3 were identified as *A. japonica*, and Group 4 corresponded to the sequences of *A. alternata*, which was in agreement with the results of morphological analyses. The amplicon lengths of the ITS, *Alt a1*, *GAPDH*, and *ATP* sequences varied between species (Table 4). Sequences of each of the 10 haplotypes were deposited in GenBank with accession numbers (Table 1).

In the partition homogeneity test, ITS and *GAPDH*; *Alt a1* and *ATP*; ITS, *Alt a1*, *GAPDH* and *ATP* data set combinations showed no significant inconcordance between data (p = .014, p = .02, and p = .005, respectively), enabling further Bayesian and maximum-likelihood analysis on the combined data set. Concatenated sequences of the ITS and *GAPDH* sequences were used for separate phylogenetic analyses of *A. brassicae*, *A. brassicicola*, and *A. japonica*, while *A. alternata* was analysed using concatenated sequences of *Alt a1* and *ATP* genes. In the first phylogenetic analyses of *A. brassicae*, *A. brassicicola*, and *A. japonica*, the Bayesian information criterion (BIC) employed in jModelTest recognized a general time reversible model with gamma distribution (GTR + G) as the most appropriate substitution model. The length of the ITS and *GAPDH* concatenated sequences used in this analysis was 1,038 bp. Concatenated sequences of four analysed gene regions, ITS, *Alt a1*, *GAPDH*, and *ATP*, were established as a single multilocus data set for all isolated species. The reference isolate of *A. japonica* was used only in analyses of ITS and *GAPDH* based on sequence gene availability in the GenBank database. For the phylogenetic analysis of 1,563 bp *Alt a1* and *ATP* concatenated sequences of *A. alternata*, the GTR + I + G model was chosen as the best-fit model. For joint phylogenetic analysis of ITS, *Alt a1*, *GAPDH*, and *ATP* concatenated sequences of all 10 haplotypes, the best-fit model was chosen to be GTR + G. The length of concatenated sequences was 2,444 bp. Tree topologies from maximum-likelihood and Bayesian analyses were congruent; therefore, one overlapped phylogenetic tree for every phylogenetic analysis was shown with both bootstrap values (Figure 7).

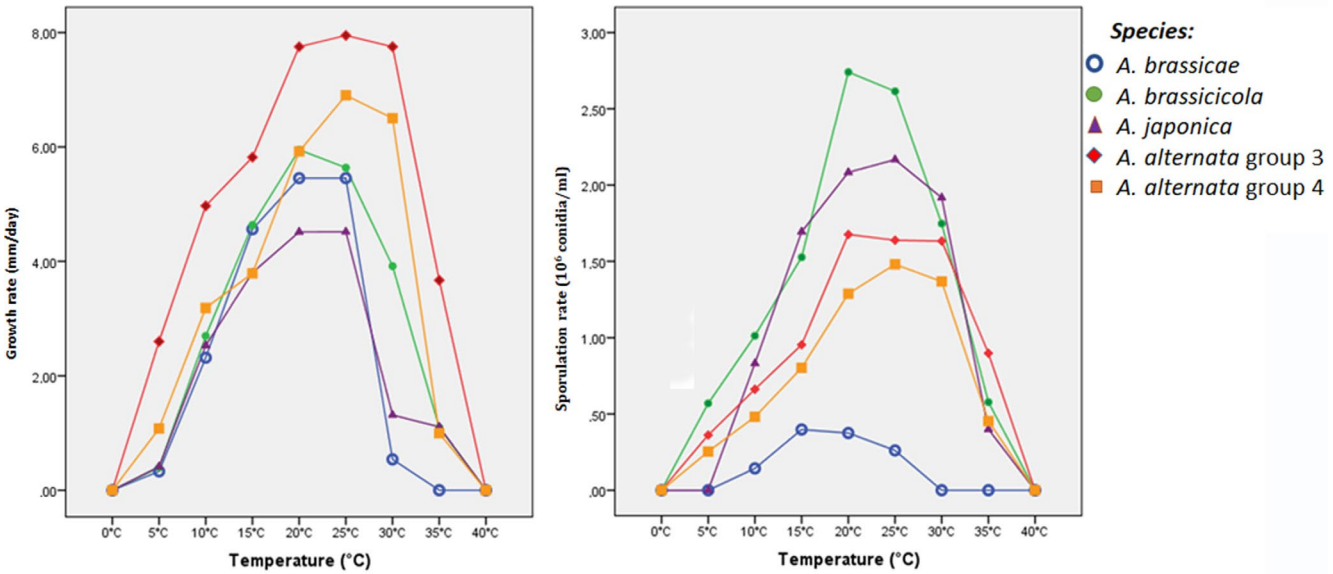

FIGURE 6 Mean rates of colony growth and sporulation of *Alternaria* isolates in the temperature range 0–40 °C on potato dextrose agar [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 PCR amplicon lengths (bp) with gene region, reference of original PCR protocol and changed conditions for PCR amplification of Serbian *Alternaria* isolates from rapeseed

Locus	Reference	Changed PCR conditions	Species (no. of isolates)			
			A. brassicae (48)	*A. brassicicola* (33)	*A. japonica* (2)	*A. alternata* (26)
ITS	Woudenberg *et al.* (2013)	Annealing 58 °C for 30 s in 37 cycles	509	511	549	504
Alt a1	Hong *et al.* (2005)	Annealing 55 °C for 30 s in 35 cycles	457	452	463	448
GAPDH	Lawrence *et al.* (2016)	Annealing 58 °C for 30 s in 35 cycles	571	554	580	588
ATP	Lawrence *et al.* (2016)	Annealing 62 °C for 30 s in 37 cycles	1,109	1,161	1,224	1,174

The PCR products of the ITS and *GAPDH* regions separated three clades (Figure 7a). The first branch, separated as a monophyletic lineage with a bootstrap value of 100%, segregated all 48 *A. brassicae* isolates into one clade along with the reference strain EGS 38-032. The next clade, comprising 33 *A. brassicicola* isolates, was divided into three subclades, among which was the reference strain EEB 2232. In the third branch, six isolates of *A. japonica* joined with the reference strain EGS 50-099. The second phylogenetic analyses of *A. alternata* (Figure 7b) using *Alt a1* and *ATP* sequences showed segregation of 26 isolates into five clades. Eight *A. alternata* isolates that were non-pathogenic (Group 4 in Figure 5) were classified in separate group, haplotype 9; while pathogenic isolates were classified as haplotypes 6, 7, 8, and 10. The phylogenetic tree revealed that *A. alternata* isolates from rapeseed were separated from *A. tenuissima* and *A. arborescens* reference isolates. Combined analyses of ITS, *Alt a1*, *GAPDH*, and *ATP* sequences of all representative haplotype sequences from Serbia, reference isolates and outgroup sequences resulted in a phylogenetic tree with similar topology to the previous ITS–*GAPDH* and *ATP–Alt a1* separate phylogenies (Figure 7c). *Alternaria* isolates were separated into two subgroups (100% bootstrap value) or four clades. The first subgroup was composed of two clades, *A. alternata* and *A. brassicae* haplotypes with separate *A. japonica* clade, while the second subgroup was composed of *A. brassicicola* haplotypes.

3.6 | Haplotype network

To determine evolutionary relationships among haplotypes, haplotype networks were constructed for the *GAPDH* gene for *A. brassicicola* (Figure 8a) and *ATP* sequences for *A. alternata* by the most parsimonious pathways (Figure 8b). The haplotype network showed a relationship pattern similar to the taxonomic indices given by the phylogenetic tree by revealing three *A. brassicicola* haplotype groups, and five groups of *A. alternata* isolates (haplotypes 6 and

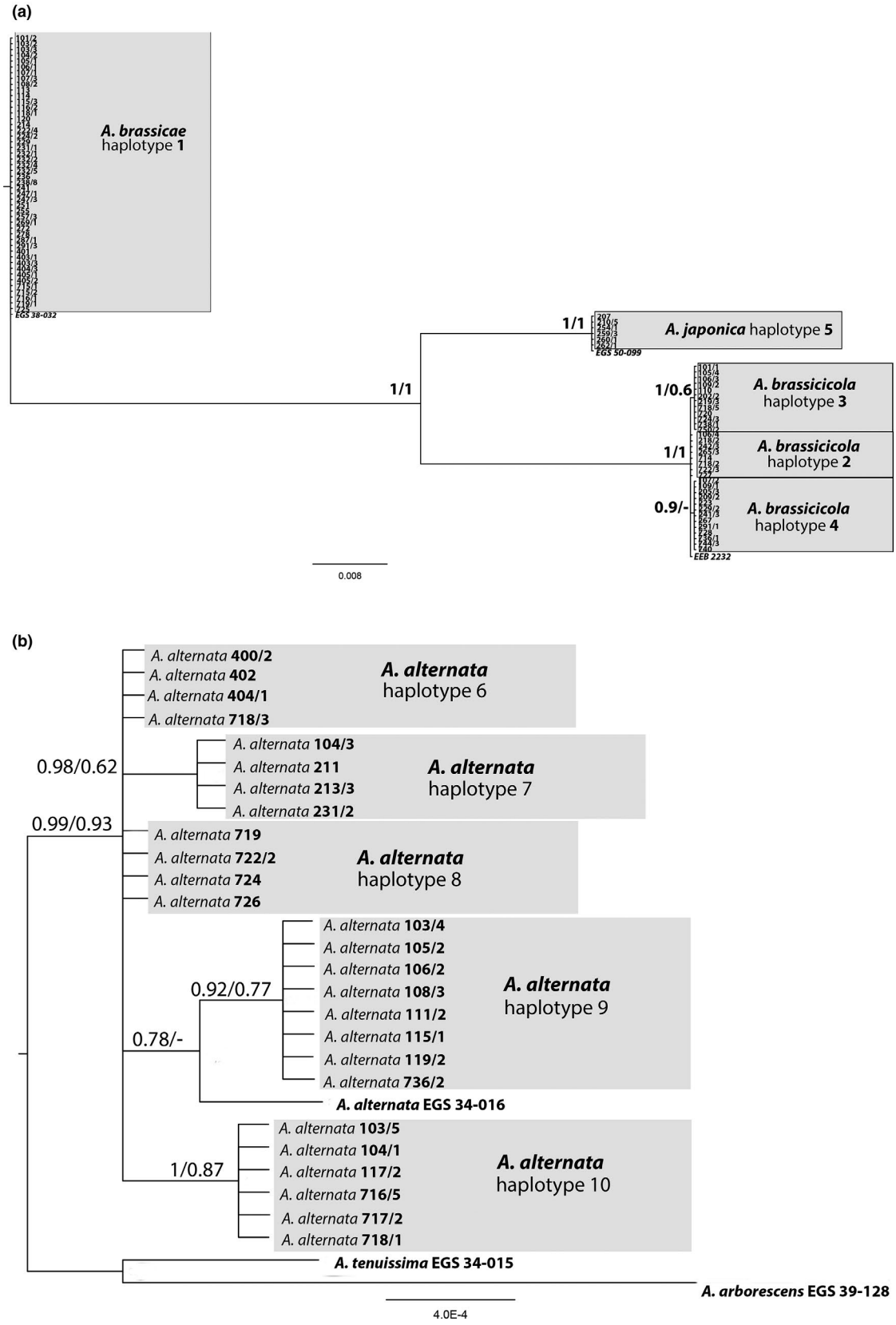

FIGURE 7 The consensus phylogenetic tree based on: (a) Bayesian and maximum-likelihood analysis of combined ITS and *GAPDH* genes sequences of *Alternaria brassicae*, *A. brassicicola*, and *A. japonica* from rapeseed and reference isolates from the GenBank; (b) *Alt a1* and *ATP* gene for the *A. alternata* isolates and reference isolates from the GenBank; (c) ITS, *GAPDH*, *Alt a1* and *ATP* genes for the one representative isolate of the haplotype for *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* isolates and reference isolates from GenBank. Bootstrap support values (expressed as percentages of 1,000 replications) are given at the nodes (Bayesian/maximum-likelihood bootstrap values of only >60 are shown)

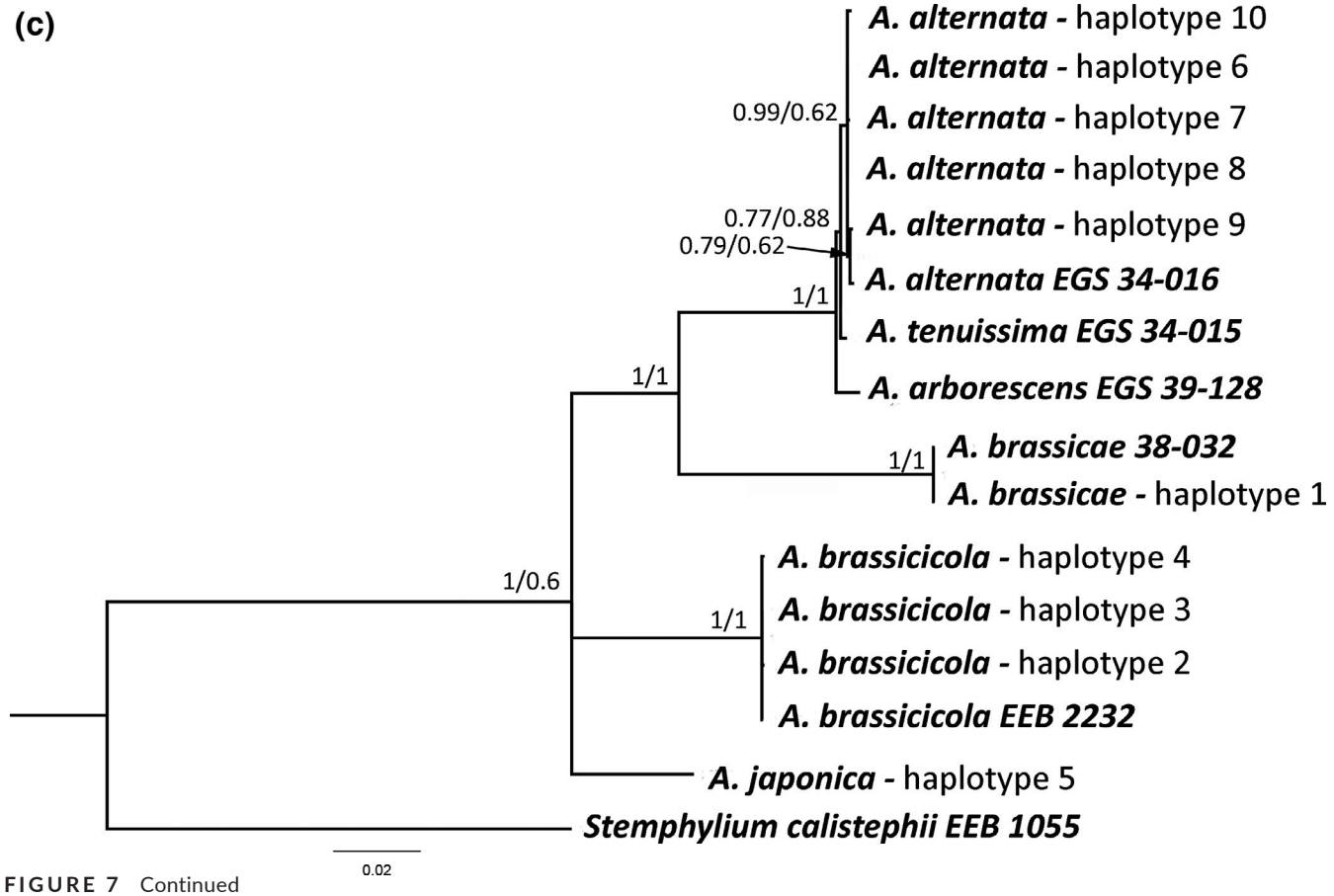

FIGURE 7 Continued

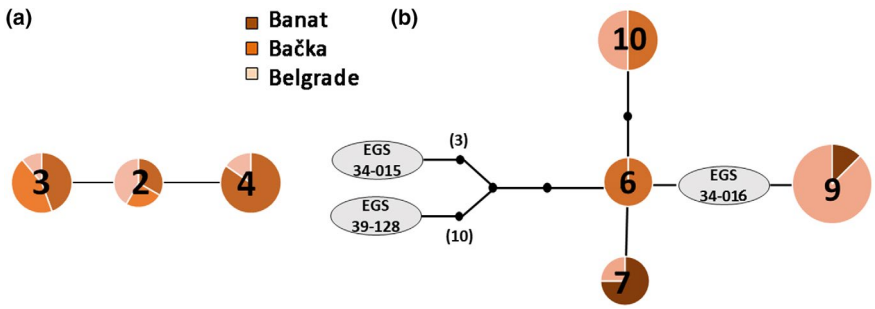

FIGURE 8 Haplotype genealogical networks obtained for *Alternaria brassicicola* (a) and *A. alternata* isolates (b) originated from rapeseed, supplemented with the reference strains from the NCBI gene base marked in grey (EGS 34-016 – *A. alternata*; EGS 34-015 - *A. tenuissima*; EGS 39-128 – *A. arborescens*). Haplotypes are represented as a circle with number, proportional in size to the number of isolates belonging to a specific haplotype; interconnecting dots represent missing or unsampled intermediate haplotypes differing by a single mutation or more, which is given in parentheses [Colour figure can be viewed at wileyonlinelibrary.com]

8 of *A. alternata* isolates from Serbia were presented as the same) (Figure 8). The haplotype network enabled a clearer representation of the *A. brassicicola* distribution, where haplotype 2 was characterized as central or ancestral and was found in all regions together with haplotype 3, while haplotype 4 was isolated in Banat and Belgrade but not in the Bačka district. The haplotype network of *A. alternata* revealed haplotype 6, found in Bačka and haplotype 8, found in Banat and Bačka, as ancestral, which differed from haplotypes 7 and 9, found in Banat and Belgrade, and haplotype 10 found in Bačka and Belgrade.

4 | DISCUSSION

The current study describes the occurrence and distribution of *Alternaria* spp. obtained from rapeseed in Serbia in 2015 and 2016. Based on morphological and molecular characterizations, 48 large-spored and 65 small-spored *Alternaria* isolates were grouped into four well-supported phylogenetic clades by using concatenated sequences of the ITS, *Alt a1*, *GAPDH*, and *ATP* genes. The phylogenetic tree represents *A. brassicae* in clade 1, *A. brassicicola* in clade 2, *A. japonica* in clade 3, and *A. alternata* in clade 4. Molecular analyses were

required for revealing complex species composition and inter- and intraspecies genetic relations. Although morphological and cultural characteristics are often unreliable identification factors because of their variability (Lawrence *et al*., 2016), genetic analyses supported the results of morphological identification showing discrimination among the groups. These results are first to report the involvement of multiple *Alternaria* species as causal agents of rapeseed leaf spot disease in Serbia and present the first occurrence of *A. japonica* and *A. alternata* on rapeseed (*B. napus*) in Europe. As the study confirmed, these four *Alternaria* species cause similar symptoms on rapeseed and cannot be reliably identified just by their effects on field plants.

A. brassicae is considered the main pathogen of the *B. napus* and *B. campestris* complex (Saharan *et al*., 2016), together with *A. brassicicola*, most commonly found on the *B. oleracea* complex (Reis and Boiteux, 2010). Our study revealed *A. brassicae* as the predominant species in the rapeseed production areas in Serbia, which supports previous studies in India, China, Canada, Brazil, Australia, and other notable rapeseed-producing countries (Reis and Boiteux, 2010; Saharan *et al*., 2016; Al-Lami *et al*., 2019). The third species mentioned is *A. japonica*, which was recently reported to be widespread mostly on cabbage, turnip, and rocket (Ren *et al*., 2012; Bassimba *et al*., 2013, Siciliano *et al*., 2017). *A. alternata* is a widespread pathogen that infects tomato, potato, apples, pears, mandarins, and pistachio (Pryor and Michailides, 2002; Serdani *et al*., 2002; Peever *et al*., 2004; Landschoot *et al*., 2017) and has also been reported as a causal agent with prevalence in the *B. olearacea* complex (Saharan *et al*., 2016). Recently, studies of whole-genome alignments suggested that several species pathogenic on brassica plants (*A. alternata*, *A. brassicinae*, *A. tenuissima*, *A. tenuis*) should be classified under one taxonomical category, species *A. alternata* (Woudenberg *et al*., 2013; 2015). In the present study, molecular analyses of ITS and *GAPDH* sequences, supported by a 100% bootstrap value, indicated that *A. brassicae* was genetically uniform and that morphological differences should be attributed to phenotype plasticity, confirming previous reports from different hosts and different geographic origins of isolates (Cooke *et al*., 1998). Genetic variability in nucleotide sequence of ITS and *GAPDH* region or any protein-coding region for *A. brassicae* has not been described in a published study so far (in sequences uploaded to GenBank database some differences in these regions can be noticed), which was confirmed in studies on canola in Canada and India (Saharan *et al*., 2016). Although phylogenetic monophyly was not observed for many other *Alternaria* species, studies of *A. brassicae* suggested that these populations are monoclonal, of recent origin, or with a lack of selection pressure in their environment. Only one ITS haplotype of *A. brassicicola* has been recorded in Serbia on rapeseed, but also on broccoli in China, *Isatis indigotica* in China, canola in Canada, and cruciferous crops in Thailand and India (Pattanamahakul and Strange, 1999; Gao *et al*., 2014; Saharan *et al*., 2016; Akram *et al*., 2019). Phylogenetic analyses of *GAPDH* sequences revealed three haplotypes among *A. brassicicola* isolates in Serbia. Sequences of haplotype 2 showed to be the same as reference sequences of *A. brassicicola* EEB 2233; haplotypes 2 and 3 were also previously isolated in Serbia from horseradish, while haplotype 4 was recorded on rapeseed for the first time. The results for *A. brassicicola* presented highly uniform morphological and cultural characteristics that were in keeping with previous results (Thrall and Burdon, 2005). For *A. japonica* isolates, molecular analyses of ITS and *GAPDH* gene regions showed the presence of one haplotype and homology with the reference isolate *A. japonica* EGS 50-099, which was in accordance with studies of Gilardi *et al*. (2012) and Bassimba *et al*. (2013). Morphological variability of *A. alternata* has been observed in isolates from rapeseed, but it has also previously been reported on pear, hazelnut, blueberry, and potato (Pryor and Michailides, 2002; Armitage *et al*., 2015). In the present study, molecular analyses based on the *ATP* and *Alt a1* genes revealed five haplotype groups of *A. alternata* (five variable positions in the *ATP* gene region). Nucleotide sequences of the *ATP* gene of *A. alternata* isolates from rapeseed in Serbia revealed high genotypic diversity, which was in agreement with studies on blueberry in China and pistachio in Turkey (Ozkilinc and Sevinc, 2018). Only one haplotype of the *ATP* region of *A. alternata* was described on rapeseed in Australia, but only a small number of isolates was tested (Al-Lami *et al*. 2019). Although separation between *A. alternata* isolates and the *A. tenuissima* reference isolate was indicated in this study, genetic differences were small as has been previously shown (Andrew *et al*., 2009; Rotondo *et al*., 2012; Lawrence *et al*., 2013; Armitage *et al*., 2015; Ozkilinc *et al*., 2018).

The highest virulence was expressed by *A. brassicicola*, whilst *A. brassicae* and *A. japonica* showed no significant differences in pathogenicity, and *A. alternata* had the lowest potential for leaf necrosis. Our results were partially in agreement with a study in Australia that suggested that *A. japonica* was the most virulent among all isolated species in rapeseed while *A. alternata* and *A. brassicae* expressed moderate to high levels of virulence (Al-Lami *et al*., 2019). The same authors also demonstrated lack of variability in virulence among isolates of *A. brassicae* and *A. brassicicola*, while Siciliano *et al*. (2017) and Nowakowska *et al*. (2019) reported differences in virulence for *A. alternata* and *A. japonica* from rocket in Italy and *A. alternata*, *A. brassicicola*, and *A. japonica* in Poland. A group of eight *A. alternata* isolates were identified as nonpathogenic (one isolate from Banat and seven from Belgrade district) for rapeseed and cabbage. Molecular and PCA analyses confirmed that this group is the separate haplotype group with significant differences in temperature response for growth and sporulation rates compared with the rest of the isolates in the *A. alternata* group. This finding is in keeping with those of studies reporting that *A. alternata* has a saprobic and pathogenic nature, indicating its pathogenic capability as a unstable or variable (Thomma *et al*. 2003). It was suggested that the pathogenicity of *A. alternata* could be changed due to certain "triggers" needed for starting pathogenicity processes, such as climate conditions, developmental stage of plants, and the presence of other pathogens under natural epidemiological conditions (Pryor and Michailides, 2002; Rotondo *et al*., 2012). A study of brown spot disease of lemon in Florida reported groups of *A. alternata* isolates from two related hosts, pathogenic only on the original host (species specific), pathogenic for the original and

related host, or cases having only some baseline pathogenicity that enabled them to colonize but not infect healthy tissue of the related host (Peever *et al*., 1999; Andrew *et al*., 2009). Given that fungal pathogens evolve together with the host (Abdullah *et al*., 2017), the observed differences suggest that isolates belonging to haplotype 9 could be originally derived from another host plant and under intensive crop conditions manage to survive, adapting to rapeseed where they show no pathogenicity. In Serbia, especially Vojvodina, rapeseed has a long crop-rotation history with wheat plants, where it was previously shown that *A. alternata* occurs in most wheat-growing regions. Iram *et al*. (2005) also reported isolates of *A. alternata* of wheat–rice crop system that showed differences in aggressiveness on rice compared to wheat; some isolates showed to be nonaggressive for wheat, others were nonaggressive for rice, but there were no nonaggressive isolates on both hosts. Molecular clustering based on random amplification of polymorphic DNA (RAPD) also showed grouping of nonpathogenic isolates from rice together (Iram *et al*. 2005). Wounding of the plant stem increased necrotic leaf area compared to nonwounding treatment, which emphasized the importance of effective insect management to reduce tissue necrosis that was frequently observed during the field survey. The presented data support the idea that different brassica species can be efficient sources of inoculum of *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* and therefore present a risk for further transmission and new epidemics on host plants in proximity. Thus, more research on this disease is needed, including the effects of crop rotation, fungicides, development of genetic resistance, and other control measures.

Because the main producing regions of rapeseed, namely, Europe, Canada, China, Australia, and the USA, have variable climate conditions with different rapeseed cultivars, growing trends and practice, it is expected that species composition, disease incidence, severity, and epidemiology of certain pathogens vary between regions and years (Al-Lami *et al*., 2019). Temperature, beside humidity, is one of the most important factors for fungal growth and reproduction, which can impact species distribution and disease outbreaks in *A. porri*, *A. helianthi*, *A. cirsinoxia* and also *A. brassicae* and *A. brassicicola* (Green and Bailey, 2000). In the climate conditions of southern Australia, during 2017 and 2018, Al-Lami *et al*. (2019) reported 10 *Alternaria* species, some of which were reported first time on rapeseed, while Van de Wouw *et al*. (2016) registered a decrease of almost 40% of *A. brassicae* incidence on canola in Australia in 2015 compared to 2013. In India, the relation of climate conditions and disease development and progress has been reported for *Alternaria* spp. pathogens on rapeseed crops (Saharan *et al*., 2016). Optimal temperatures indicated for *A. brassicae* in this study were the same as reported for isolates from the Netherlands and Canada, while for *A. brassicae* from France, India, and the UK, optimal temperatures were slightly lower (Saharan *et al*., 2016). Optimal temperatures for the mycelial growth of *A. brassicicola* and *A. japonica* were higher than for *A. brassicae*, as also reported in India (Saharan *et al*. 2016). *A. alternata* expressed a wider range of optimal temperatures, extending from 20 to 30 °C, which was in accordance with results for *A. alternata* isolates from citrus (Timmer *et al*., 2000). The most abundant sporulation was measured for *A. brassicicola*, while *A. brassicae* had a lower sporulation intensity and lower sporulation temperature optimum (15–20 °C) than the other three species (20–25 °C). In rapeseed surveys, during 2015 and 2016, seasonal prevalence of species has not been noticed. Results in this study indicated that *A. brassicae* isolates from rapeseed favour lower temperatures for sporulation compared to other isolated species of *Alternaria*; however, in field conditions, temperature appeared to have a lesser effect for isolates from rapeseed. This case could be due to the short vegetation period of rapeseed plants, where there are relatively few days during spring and early summer with average temperatures above 20 °C. Results of this study support field observations in Serbia where development of the disease on rapeseed was most severe during flowering of plants in May and June, when temperature and precipitation were high, while symptoms remained localized on leaves during winter. Studies of different epidemiological aspects, such as variation in the pathogen species among regions, crops, and seasons, are important to establish the most suitable fungicide treatment and the most efficient application.

Alternaria spp. has been described as some of the most important pathogens of the Brassicaceae family. The present work identified *A. brassicae*, *A. brassicicola*, *A. japonica*, and *A. alternata* occurring simultaneously in the field, plant, and leaf. Severe difficulties in the management of these pathogens arise from the fact that the disease is seedborne and shows pathogenicity for a variety of hosts and plant parts, and it spreads infective propagules able to survive in infected plant debris. Our study highlights the importance of the polyphasic approach to predict the ecological and evolutionary dynamics of *Alternaria* pathogens and find effective management strategies, especially in the current global circumstances when climate change has already caused a decrease in suitable areas for rapeseed cultivation in Europe (Jaime *et al*., 2018). Further analyses on a wider regional scale should be conducted in Europe, given that high genetic diversity can influence better adaptation of pathogens to novel selection pressures and the evolutionary potential of spreading to alternative hosts, such as other crops or native flora of the Brassicaceae family, which could disrupt effective disease management practices. The cultivation of susceptible cultivars will contribute to an increase in the level of seed infection, while a shift in the presence and distribution of species could be a new reason for reducing the yield and inefficiency of fungicides. Screening of oilseed pathogens from importing countries is of great importance for the EU oilseed market; therefore, detailed studies such as this one could provide practical benefit in improving monitoring and resistance management in this wide trade area. Increasing global demand for rapeseed products, both for consumption and for biofuels, makes it one of the fastest growing markets in recent years, further necessitating increased production area and productivity, both of which depend on effective strategies for controlling pathogens.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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