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Diaporthe pseudolongicolla – the new pathogen on soybean seed in Serbia

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Summary: Seed decay is one of the most important diseases of soybean (*Glycine max* (L.) Merr.) that has a negative impact on the market grade of soybeans. The disease is mainly caused by *Diaporthe longicolla*, along with other *Diaporthe* species. Screening of soybean seeds health status in Vojvodina Province, Serbia, showed cultural and morphological variability among isolates identified as *D. longicolla*. With the use of DNA sequences of internal transcribed spacer (ITS1-5.8S-ITS2) region and partial translation elongation factor 1-alpha (EF1-α), the new species was determined. BLAST analysis showed 100% identity with *D. pseudolongicolla* (syn. *D. novem*) that was described in this study and its taxonomic revision is discussed. Pathogenicity trial showed that both species, *D. longicolla* and *D. pseudolongicolla*, are highly pathogenic on soybean stem and seed, causing 100% of stem wilting and more than 82% of seed decay.
Key words: *D. longicolla*, *D. pseudolongicolla*, *Diaporthe*, seed decay, soybean

Introduction

Soybean (*Glycine max* (L.) Merr.) is listed as the second most significant oil crop, just after oil palm (FAO, 2014). It occupies prominent position with the highest protein level among legumes. Soybean is used for livestock feeding, as edible and industrial oil, as well as high protein food crop for human consumption. More than 200 phytopathogenic microorganisms have been described on soybean (Hartman et al. 2015). Most of them attack soybean seed and thus negatively affect germination and seed quality. One of the major causes of poor seed quality in most soybean-growing regions worldwide is seed decay. It is caused primary by *Diaporthe longicolla*, along with *D. sojae*, *D. caulivora*, *D. aspalathi*, *D. eres* species complex, *D. foeniculina*, and *D. rudis* (Li, 2011; Petrović et al., 2015; Petrović et al. 2016). Soybean seeds infected with *Diaporthe* species are usually small, shrunken, flattened and elongated, with cracked seed coats and chalky-white mycelium (Fig 1). This has negative influence on the market grade of soybeans. On the other hand, latent infected seeds have a normal appearance, without symptoms of disease, but with reduced germination, vitality and quality (Kmetz, Schmitthenner & Ellett, 1978).

In the 1980s and 1990s, causal agents of seed decay were distinguished based on the symptomatological, morphological, cultural and pathogenic characteristics (Hobbs, Schmitthenner & Kuter, 1985; Morgan-Jones, 1989; Sinclair, 1993). In many *Diaporthe* species, both anamorph (asexual) and teleomorph (sexual) stages have been described as well as their ability to form pycnidia and perithecia. However, *D. longicolla* has long been described as a fungus without the teleomorph stage whose pycnidia have very long necks that release only alpha conidia. Recent studies revealed new details on this fungus such as atypical symptoms on stems in the field, pycnidia with beta conidia and the ability to form teleomorph (Santos, Vrandečić, Ćosić, Duvnjak & Phillips, 2011; Vidić et al. 2013; Olson et al. 2015).

During monitoring of soybean seeds health status in Vojvodina province, Serbia, slight differences among *D. longicolla* isolates were noticed. Detailed molecular study showed the presence of new species. For this reason, study of a new parasite and its possible pathogenicity on the soybean has been described in this paper as well as its taxonomical relation to *D. longicolla*.

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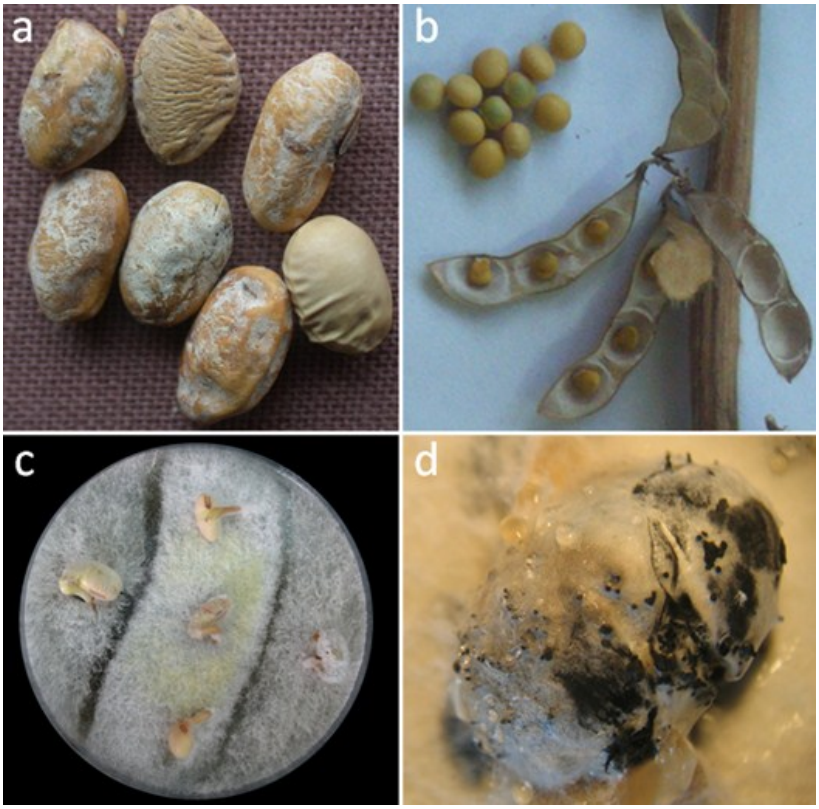

Figure 1. Seed decay. a-b: rotten seeds, c: latently infected seeds, d: stroma with pycnidia on soybean seed

Materials and Methods

Isolation and morphology

Symptomatic plants and seeds were collected throughout the soybean-producing area in Vojvodina Province, Serbia in period 2002-2012. Using standard phytopathological procedures 64 *D. longicolla* single-conidial isolates were obtained, 33 from seeds and 31 from diseased stem tissues. A total of 64 isolates were confirmed on potato-dextrose agar (PDA), while the sporulation of representative isolates was monitored during two months on PDA with 5-cm-long pieces of autoclaved soybean stems. Morphological features (pycnidial conidiomata, alpha and beta conidia) were described and measured (100 conidia and pycnidial conidiomata).

Sequence Analysis

DNA was extracted from all isolates using modified protocol of Cenis (1992). PCR (Polymerase Chain Reaction) amplification, sequencing of the internal transcribed spacer (ITS1-5.8S-ITS2) region of rDNA and partial translation elongation factor 1-alpha (EF1-α) were performed as described by Vidić et al. (2013). BLAST searches were carried out to select closely related sequences from GenBank. Using ITS identification, representative isolates were selected for sequencing of EF1-α and further morphological and pathogenic characterization (Table 1).

Pathogenicity testing

Pathogenicity of selected isolates was tested on soybean cultivar Sava, plants and seeds. Soybean plants were cultivated in the greenhouse: five plants per pot in four replications. Plants were inoculated at V2 growth stage according to the plug method by Vidić et al. (2013), while conidial suspensions (10^6 conidia/ml) were used in seed test inoculation following the method by Vidić et al. (2013). Seeds, inoculated with each isolate, were placed on wet filter paper in 90 mm Petri dishes, 25 seeds per dish. The experiment was set up in four replications and incubation was at 24°C. After seven days, number of decayed germinated and ungerminated seeds was counted. The significance of differences between obtained data was tested by ANOVA, using the Dunnett's test.

Results and Discussion

Morphological characteristics

Using morphological profile given by Hobbs et al. (1985), cultural and morphological differences among isolates that were previously identified as *D. longicolla* were noticed. Both, *D. longicolla* (Fig 2) and *D. pseudolongicolla* (Fig 3) had similar appearance in Petri dishes. White, compact mycelia with yellowish-green ring around the centre of the colony were a typical feature for both species. Inside of their black stromatic structure pycnidial conidiomata with alpha conidia and

Table 1. List of representative isolates of *D. longicolla* and *D. pseudolongicolla* used in this study

Species	Isolate	Source of isolation	Origin	Collector	GenBank accession numbers	
					ITS	EF1-α
Diaporthe longicolla	PDS157A	stem	Rimski Šančevi	Vidić M.	JQ697845	JQ697858
	PL/KR19	seed	Rimski Šančevi	Petrović K.	JF430483	JF461469
Diaporthe pseudolongicolla (syn. *D. novem*)	PL42	seed	Loznica	Vidić M.	JQ697843	JQ697856
	PL44	seed	Žabalj	Vidić M.	KU672724	KU672725
	PL68	seed	Sombor	Petrović K.	JQ697842	JQ697855
	PL75	seed	Subotica	Petrović K.	JQ697841	JQ697854
	PL/KR6	seed	Ruma	Petrović K.	JF704181	JF704182

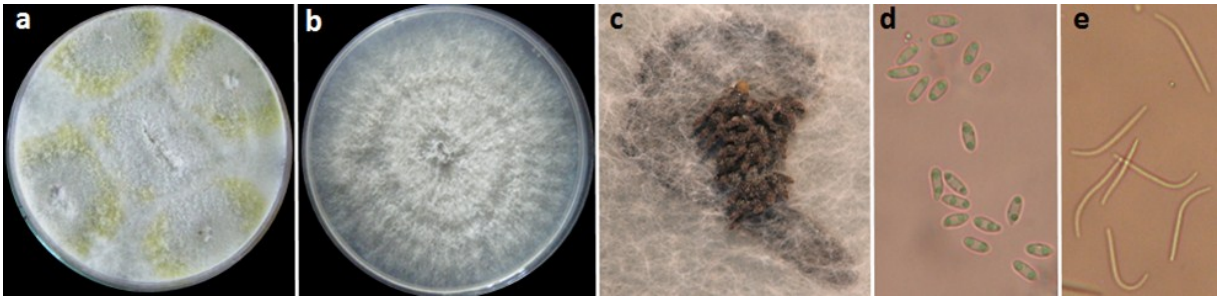

Figure 2. *Diaporthe longicolla.* a: young colony of pathogen isolated from soybean stem, b: six days old colony, c: pycnidial conidiomata on stroma, d: alpha conidia, e: beta conidia

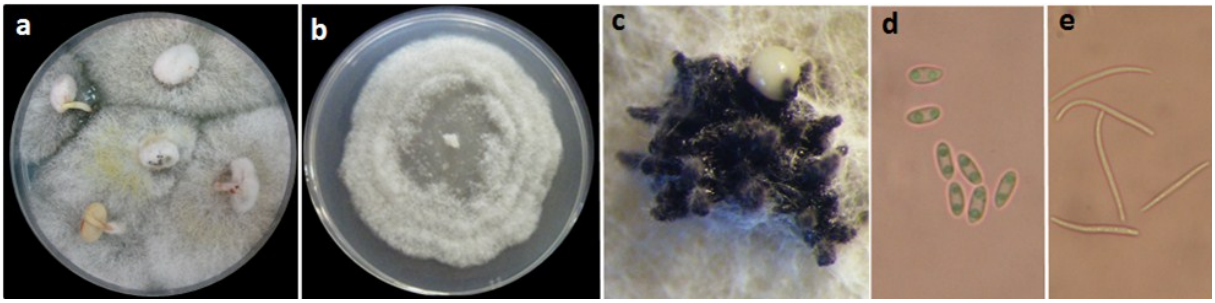

Figure 3. *Diaporthe pseudolongicolla*. a: young colony of pathogen isolated from soybean seeds, b: six days old sterile colony, c: pycnidial conidiomata on stroma, d: alpha conidia, e: beta conidia

rarely beta conidia were observed. Both species lacked of perithecia. The only observed difference among them was that *D. pseudolongicolla* isolates rapidly became sterile and lost their ability to form reproductive organs.

Identification

At the time of the morphological identification concept, it was believed that only *Diaporthe* species present on soybean seeds were *D. longicolla*, *D. sojae*, *D. caulivora* and *D. aspalathi* (Morgan-Jones, 1989; Sinclair, 1993). However, in the most studies at that time, the variability of *Diaporthe* species was reported in terms of morphological characteristics, which led many authors to conclusion that there exist more *Diaporthe* species among well-known pathogens (Fernández & Hanlin, 1996; Zhang et al., 1997; Zhang et al. 1998; Zhang, Hartman, Curio-Penny, Pedersen & Becker, 1999; Li, 2011). Introduction of molecular tools helped to resolve variability among *Diaporthe* isolates from soybean and was beneficial in detection of new species (Santos et al., 2011; Petrović et al. 2015; 2016; Mathew, Gulya, Jordahl & Markell, 2018). Using ITS sequences, from a total of 64 isolates, 59 strains were identified as *D. longicolla*, while five strains were separated as a new species named *D. pseudolongicolla* (Table 2). These five strains were previously determined as *D. longicolla* by morphological identification concept. All 59 *D. longicolla* isolates had uniform ITS and EF1-α sequences, and PDS157A and PL/KR19 were designated as reference isolates of *D. longicolla*. Isolates of *D. longicolla* were equally present on soybean seeds and stems, while *D. pseudolongicolla* was isolated only from seeds. Symptoms of seed decay produced by both species were identical and causal agents could not be visually identified. Newly

Table 2. Identification of *D. longicolla* and *D. pseudolongicolla* isolates by the BLAST analysis

Locus	Isolate	*Diaporthe longicolla*		*Diaporthe pseudolongicolla*	
		Type sequence[a]	% of identity	Type sequence[a]	% of identity
ITS1-5.8S-ITS2	PDS157A	CBS127267 (HM347700)	100	CBS117165 (DQ286285)	96
	PL/KR19		100		96
	PL42		96		100
	PL44		96		100
	PL68		96		100
	PL75		96		100
	PL/KR6		96		100
EF1-α	PDS157A	CBS127267 (HM347685)	100	CBS117165 (DQ286259)	71
	PL/KR19		100		71
	PL42		70		100
	PL44		70		100
	PL68		70		100
	PL75		71		100
	PL/KR6		70		100

[a] CBS, The Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands

detected species was probably present on soybean seeds for a long time masked by well-known seedborne pathogens.

Taxonomy

Diaporthe longicolla (Hobbs) J.M. Santos, Vrandečić & A.J.L. Phillips, Persoonia 27: 13. 2011. (Fig 2)

Basionym. *Phomopsis longicolla* Hobbs, Mycologia 77: 542. 1985.

Cultural characteristics: On PDA had white, compact aerial mycelium with typical yellowish-green ring around the centre of the colony. After 5-7 days, colony formed massive, black stromatic structures, irregular in shape, which completely covered the bottom of Petri dishes.

Pycnidial conidiomata on autoclaved soybean stem with PDA in culture were globose, black, aggregated and rarely solitary. They had long necks, ranging 250-700 µm in length. Pycnidia formed within black stroma had white to yellowish mucous droplets observed on ostiole. *Alpha conidia* were ellipsoidal, 2-guttules, dimensions 4.9-7.5 × 2.2-3.0 µm. *Beta conidia* were found in two-months -old cultures. They were unicellular, comma-shaped, size 22.3-29.2 × 1.0-1.3 µm.

Perithecia were not formed.

Notes - Pycnidia formed into black zone line symptoms (Vidić et al. 2013) on the lower part of the soybean stem, were always releasing alpha and beta conidia. Santos et al. (2011) reported that *D. longicolla* possesses both mating-types indicating that this species is self-fertile. Moreover, Vidić et al. (2013) have found several perithecia on overwintered stems inoculated with isolate PDS157A. The perithecial body varied in size from 117 to 406 µm in diameter, with the average 234 µm (Vidić, *unpublished data*). Asci and ascospores were typical for the genus *Diaporthe*, but perithecia were never found in culture. However, authors were not successful in repeating the trial and proving presence of the perithecia of *D. longicolla*. On the other hand, Fernández and Hanlin (1996) noted that one isolate (PI 526), morphologically identified as *D. lonigicolla* produced few fertile perithecia only when grown on autoclaved elm bark, but they were also not able to repeat that finding.

Known hosts – *Abutilon theophrasti* (Li, Bradley, Hartman & Pedersen, 2001; Vrandečić et al. 2004), *Acer truncatum* (Sun, Guo & Hyde, 2011), *Amaranthus spinosus* and *Leonorus sibiricus* (Cerkauskas, Dhingra, Sinclair & Asmus, 1983), *Ambrosia trifida, Euphorbia maculata, Rumex crispus, Xanthium strumarium* and *Vigna unguiculata* (Roy, Ratnayake & McLean, 1997), *Arachis hypogaea* (Sanogo & Etarock, 2009), *Aster exilis, Caperonia palustris, Desmanthus illinoensis, Eclipta prostrata, Euphorbia nutans, Ipomoea lacunose, Polygonum aviculare* and *Sida spinosa* (Mengistu & Reddy, 2005), *Chamaesyce nutans* (Mengistu, Castlebury, Smith, Rossman & Reddy, 2007), *Cucumis melo, Glycine max* (Hobbs et al. 1985; Vidić, Jasnić & Stojšin, 1996; Zhang et al. 1997; Santos et al. 2011; Gomes et al. 2013), *Helianthus annuus* (Mathew et al. 2012), *Pyrus pyrifolia* (Bai et al. 2015), *Solanum melongena* (Shu et al. 2014), and *Trichilia elegans* (Flores, Pamphile, Sarragiotto & Clemente, 2013).

Distribution – Argentina (Hernandez, Pioli, Peruzzo, Formento & Pratta, 2015), Australia (Ash et al. 2010), China (Cui, Duan, Wang, Li & Zhu, 2009; Shu, Chen, Huang, He & Zhou, 2014), Croatia (Vrandečić et al. 2004), Greece (Holevas et al. 2000), New Mexico (Sanogo & Etarock, 2009), Serbia (Vidić & Jasnić, 1994) and USA (Hobbs et al. 1985; Zhang et al. 1997).

Diaporthe pseudolongicolla, K. Petrović, L. Riccioni & M. Vidić, nom. nov. — MycoBank MB564245 (Fig 3);

Etymology: The prefix *pseudo* is used to mark very similar fungus with *D. longicolla* in terms of morphological and pathogenic features.

= *D. novem*, J.M. Santos, Vrandečić & A.J.L. Phillips, Persoonia 27: 14. 2011.

Basionym. *Phomopsis* sp. 9 (van Rensburg et al. 2006)
Cultural characteristics: Fresh strains were identical with *D. longicolla* in terms of white, compact aerial mycelia on PDA. Main difference between these two species was that isolates of *D. pseudolongicolla* quickly become sterile and lost ability to produce reproductive organs.
Pycnidial conidiomata on autoclaved soybean stems with PDA in culture were sphaerical, up to 550 µm in diameter. They were black, aggregated within stroma, without necks or with long necks, up to 450 µm long. Yellowish drop with alpha and beta conidia exuded from the ostiole. *Alpha conidia* were unicellular, bi- to multiguttulate, ellipsoidal with dimension 6.2-9.7 × 2.3-3.1 µm. *Beta conidia* were unicellular, filiform, curved at one end, size 19.5-29.1 × 0.9-1.2 µm and rarely formed.
Perithecia were not formed.
Notes – The reference isolate *Phomopsis* sp. 9 could not be determined, because it was sterile when deposited in the CBS collection, and marked by numerological classification system (van Rensburg, Lamprech, Groenewald, Castlebury & Crous, 2006). Later, Santos et al. (2011) proposed the formal name *D. novem*, but the problem of numerological classification remained (*novem* = nine, *Latin*). Numerology classification system cannot be considered as valid, since it provides only temporary solution during identification of an organism. For that reason, the name of *D. novem* appears to be incorrect, because it is not in consistence with the Code of classification (http://www.iapt-taxon.org/nomen/main.php?page=art23). Moreover, Santos et al. (2011) have established that *D. novem* is a heterothallic fungus, which means that perithecia will form only when the isolates with opposite mating type were crossed and this is the reason why perithecia are rarely observed. Since strong similarity in terms of morphological characteristics as well as pathogenicity was observed among *D. longicolla* and *Phomopsis* sp. 9 (syn. *D. novem*) isolates, it is considered that *Diaporthe pseudolongicolla* (MB564245) is appropriate name for the unidentified group *Phomopsis* sp. 9 (Petrović, 2012).

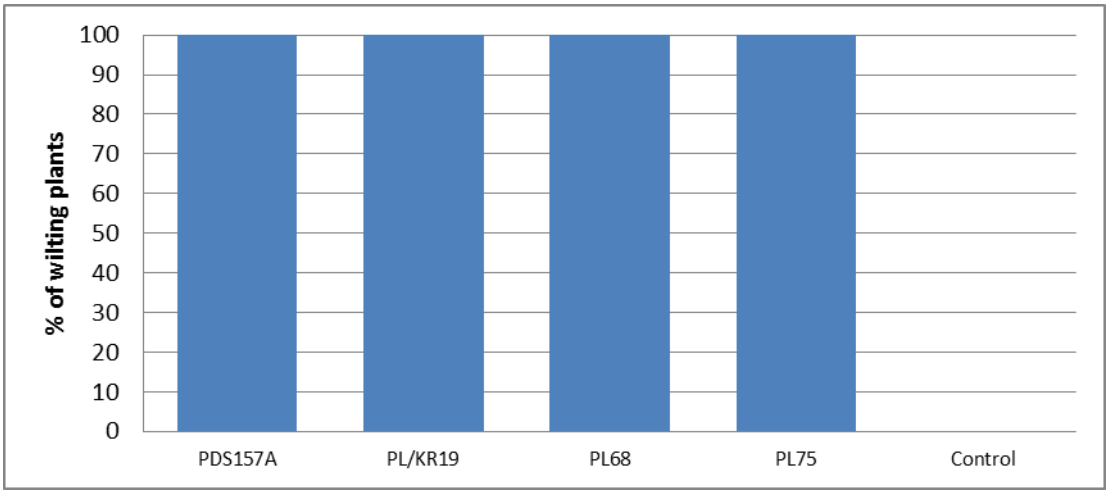

Figure 4. Pathogenicity of *D. longicolla* and *D. pseudolongicolla* isolates on soybean plants

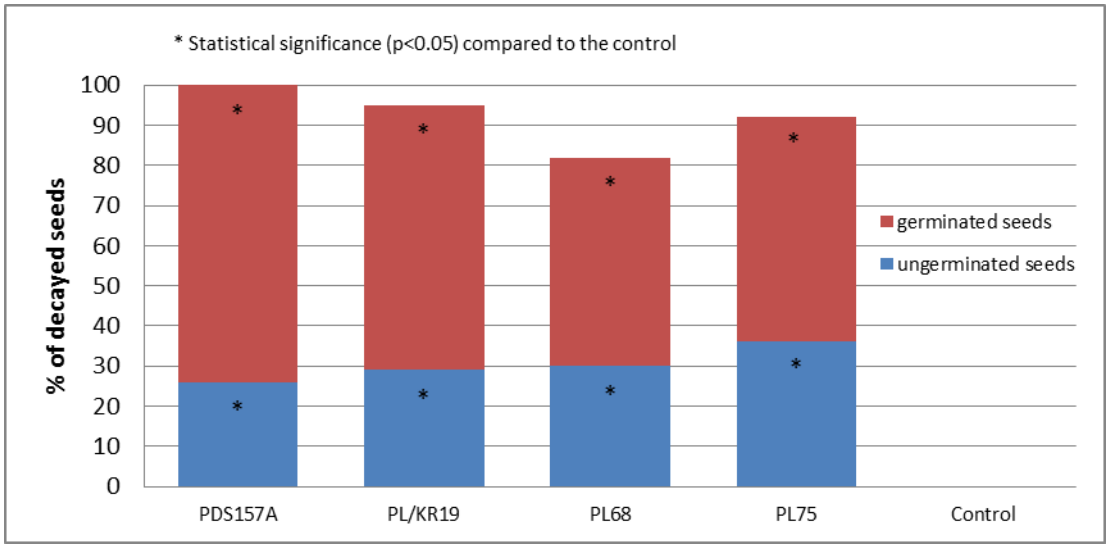

Figure 5. Pathogenicity of *D. longicolla* and *D. pseudolongicolla* isolates on soybean seeds

Known hosts – *Asclepias syriaca*, *Aspalathus linearis* (van Rensburg et al. 2006), *Actinidia deliciosa* (Díaz et al. 2014), *Glycine max*, *Helianthus annuus* (Rekab, Del Sorbo, Reggio, Zoina & Forrao, 2004), *Hydrangea macrophylla* (Santos, Correia & Phillips, 2010), and *Vitis vinifera* (van Niekerk et al. 2005).
Distribution – Chile (Díaz et al. 2014), Croatia (Santos et al. 2011), Italy (Rekab et al. 2004), Portugal (Santos et al. 2010), South Africa (van Niekerk et al. 2005; van Rensburg et al. 2006).

Pathogenicity testing

Both species had high degree of virulence on soybean plants causing 100% wilting (Fig 4). The plug method of inoculation managed to reproduce the typical symptoms of pod and stem blight on soybean stem. In the beginning, pycnidial lesions were visible near the injection site. Later on, pycnidia spread up and down along the stem. Inoculation of soybean seeds by immersing them into conidia suspension showed that *D. longicolla* and *D. pseudolongicolla* significantly reduced the germination rate of the seeds compared to the control. Results showed that *D. longicolla* isolates PDS157A and PL/KR19 caused 100% of seed decay, while *D. pseudolongicolla* isolates PL68 and PL75 caused 82% and 92% of seed decay, respectively (Fig 5). Control plants and seeds were symptomless. Pathogenicity of *D. longicolla* isolates on soybean stems and seeds were observed by Vidić et al. (2013), who noted variability in aggressiveness, but pathogenicity data of *D. pseudolongicolla* was missing and this study represents the first results of pathogenicity of this species on soybean.

Conclusion

Diaporthe species are one of the major causes of soybean seed decay and the most aggressive is *D. longicolla* along with *D. pseudolongicolla*. The difference between these two species is that isolates of *D. pseudolongicolla* rapidly became sterile when grown in culture and lost their ability to form reproductive organs. Using ITS and EF1-α sequences, from a total of 64 isolates, 59 strains were identified as *D. longicolla*, while five strains separated as a new species named *D. pseudolongicolla*. Isolates of *D. longicolla* were equally present on soybean seeds and stems, while *D. pseudolongicolla* was isolated only from seeds. Isolates of *D. longicolla* PDS157A and PL/KR19 caused 100% of seed decay, while *D. pseudolongicolla* isolates PL68 and PL75 caused 82% and 92% of seed decay, respectively. This study represents the first result of pathogenicity of *D. pseudolongicolla* on soybean.

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Diaporthe pseudolongicolla – novi patogen na semenu soje u Srbiji

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Sažetak: Trulež semena je jedna od najvažnijih bolesti soje (*Glycine max* (L.) Merr.), koja negativno utiče na vrednost soje na tržištu. Najčešće je uzrokuje *Diaporthe longicolla*, zajedno sa drugim *Diaporthe* vrstama. Pregledom zdravstvenog stanja semena soje u Vojvodini (Srbija) uočena je morfološka varijabilnost između izolata identifikovanih kao *D. longicolla*. Upotrebom sekvenci DNK iz ITS regiona (ITS1-5.8S-ITS2) i elongacionog faktora 1-alfa (EF1-α) potvrđeno je prisustvo nove vrste na semenu soje. BLAST analizom je dokazana identičnost od 100% sa *D. pseudolongicolla* (sin. *D. novem*) koja je opisana u ovom radu i čija taksonomska revizija se razmatra. Ispitivanja patogenosti su pokazala da su obe vrste, *D. longicolla* i *D. pseudolongicolla,* visoko patogene na stablu i semenu soje uzrokujući 100% uvenuće biljaka kao i trulež više od 82% semena.

Ključne reči: *D. pseudolongicolla*, *D. longicolla*, Diaporthe, soja, trulež semena

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