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IDENTIFICATION OF FUNGI INHABITING UNDERGROUND PLANT PARTS OF SOYBEAN [*Glycine max* (L.) Merrill] IN TWO DEVELOPMENTAL STAGES

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ABSTRACT

Soybean [*Glycine max* (L.) Merrill] plants are potential hosts for different pathogens. Soil-borne diseases, caused by pathogenic fungi, are considered to be the main reason for the loss of soybean yields worldwide. The aim of the research was to isolate fungi inhabiting underground parts of soybean in order to identify potential pathogens present in south-east Poland. Research material comprised of seven soybean cultivars grown in field in 2017–2019. Samples collected in three subsequent vegetative seasons constituted soybean plants with disease symptoms on cotyledons, hypocotyls, roots and ungerminated seeds. Fragments of the infected plant tissues were subjected to mycological analysis. A total of 1692 pure fungal isolates were obtained from the sampled plants and almost 80% of these isolates were assigned to *Fusarium* genus. Among *Fusarium* spp. isolates, the most frequently detected species was *F. oxysporum* (71.3%). Other detected *Fusarium* species included mainly: *F. graminearum*, *F. poae*, *F. culmorum*, *F. solani* (syn. *Neocosmospora solani*), *F. fujikuroi*. *F. avenaceum.* Other fungi accounted for approx. 19% of the obtained isolates and their main representatives were: *Alternaria alternata*, *Trichoderma* sp., *Rhizoctonia solani*, *Mucor* sp., *Cladosporium* sp. and *Rhizopus* sp. Comparison of these results with published data from other regions of Poland shows differences in prevalence of different species of soil-borne fungi.

Key words: soybean, soil-borne pathogens, fungal diseases, species identification, PCR

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] belongs to *Fabaceae* family. Soybean seeds combine a number of important compounds essential for animal nutrition. Therefore, around three-quarters of soy produced worldwide is used for animal feed, especially for pigs and poultry [WWF 2014]. The most important advantages of soybean include high concentration of protein and fat in seeds of approximately 40% and 20%, respectively. Apart from a high protein content, soybean is rich in fatty acid omega-3, isoflavones and dietary fiber; therefore, it is important component of food [Hartman et al. 2015]. There are many uses of soybean in the world, especially in Asia and the USA. Soybean is used to produce soybean milk, tofu, tempeh, soybean paste as well as cosmetics and biofuel. From among many different soybean products available on the market, the most popular is soybean oil, which is the most frequently manufactured and consumed oil in the world [Guzeler and Yildirim 2016].

In addition to its nutritional value, soybean has other advantages. As a typical legume it is associated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, which bind atmospheric nitrogen. The symbiotic nitrogen fixing of legumes, including soybean,

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plays an important role in agriculture as it leads to improving soil fertility and availability of nitrogen and phosphorus as well as beneficial changes in soil microbiological properties. In addition, it reduces the use of chemical plant protection agents and fertilizers. It also increases yield of other plants grown in the field after soybean. For this reason, soybean is an excellent forecrop for succeeding plants [Jensen et al. 2010, Kopke and Nemecek 2010, Stagnari et al. 2017].

Soybean was cultivated since at least the beginning of the ninth century in China and then in Japan and Korea, where the first evidence for its large-scale cultivation was discovered [Hymowitz 1990]. Currently, the largest soybean producers in the world include USA, Brazil and Argentina. These three countries account for 82% of global soybean production, therefore they have been called the 'Big-3' producers in the world [Agarwal 2013]. The global growth of soybean production is spectacular; it has increased by about 350% since 1987. In 2018, the world soybean production was 361 million tons [SoyStats 2019]. In Poland soybean production is also dynamically increasing. According to FAOSTAT [2012, 2016, 2017], in 2012, the cultivation area of this crop was approximately 855 ha, but four years later it was already approximately 9 times higher and in 2017, it reached 9333 ha.

More than 200 pathogens are known to affect soybean. Fungal pathogens constitute a large group among them [Hartman et al. 2015]. In Argentina, the most important disease infecting soybean roots was sudden death syndrome, which is caused by species of *Fusarium*. Due to this disease, from year 2015, yield losses are up to 15% and 90% in different parts of the country. Similarly in Canada, sudden death syndrome has become permanently established and losses occur every year. Yield reductions due to this disease have been as high as 20% in some fields. In China not all fungal diseases cause economic losses. Seedling diseases and soybean rust are the most significant in this country. In the United States among the fungal diseases of soybean, the most important are the seedling diseases caused by *Fusarium*, *Phytophthora* and *Phytium*. These pathogens have great economic significance not only regionally but also country-wide [Hartman et al. 2015]. In Poland, the most dangerous diseases in soybean fields are caused by soil-borne pathogenic fungi. Soybean roots are exposed to these pathogens during the whole vegetation period [Patkowska 2001, Horoszkiewicz-Janka et al. 2013]. However, it is known that young plants are most often infected, when they are under low temperature stress. Such conditions may occur when seed are sown into a cold soil or when there is a sudden change in the weather during germination.

Some of soybean pathogens colonize only the phyllosphere while others occur only on underground plant parts. However, many pathogenic fungi colonizing soybean plant infect multiple organs and can be found in all plant parts [Hartman et al. 2015]. The most pathogenic fungi in the germination stage of soybean and young seedlings include: *Fusarium culmorum* (Wm. G. Sm.) Sacc., *F. oxysporum* Schltdl., *Neocosmospora solani* (Mart.) L. Lombard & Crous [(syn. *Fusarium solani* (Mart.) Sacc.)], *Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm., (anamorph *Phomopsis sojae* Lehman), *Colletotrichum* spp., *Ascochyta* spp., *Phytophthora* spp., and *Globisporangium* spp. [Killebrew 1993, Hartman et al. 2015]. These pathogens cause root and stem diseases. Additionally, some of them (*F. oxysporum, Colletotrichum* spp., *Septoria glycines*) inhibit the growth of plants, because they destroy vascular tissue in the stem, lateral shoots and petioles [Hartman et al. 2015]. It is particularly harmful for soybean to be infected by several species of fungi at the same time. According to Hartman et al. [2015], diseases caused by several types of soil-borne fungi are a limiting factor in the production of soybean seeds which has a negative effect on plant quality and yields.

Fungal diseases are not the primary concern for soybean production in Poland at the moment. Although, according to Horoszkiewicz-Janka et al. [2013], losses in legume production, including soybean, caused by fungal diseases are on average 15%, but they can also be significantly higher, up to 70%. The dynamic growth of soybean cultivation area in Poland and frequently repeating weather anomalies, that are particularly dangerous for soybean seedlings, may increase the development of fungal diseases in the field. For this reason, we should monitor soybean fields in Poland for the presence of pathogenic fungi. Here, we aim at characterizing fungal communities inhabiting soybean plants grown in the south-east of Poland, in Podkarpackie voivodship. Neither this study area nor seven soybean cultivars included in this study, were subjected to similar investigations before.

MATERIAL AND METHODS

Collection and preparation of samples. This study was conducted on experimental field located in Makowisko (50°2'43"N, 22°47'10"E) – Podkarpackie voivodeship, south east of Poland – in three subsequent vegetation seasons between 2017 and 2019. Each year, experimental field was set up in the area where the forecrop was corn, and where the soybean was not grown before. The experiment was carried out on sandy loam soil, which was a good wheat soil complex. Chemical soil properties were described in detail for the same experimental field by Jarecki et al. [2020]. Phosphorus and potassium fertilization was 17.44 kg/ha and 49.8 kg/ha respectively. Nitrogen fertilizer was not used. Weather conditions were recorded at the Experimental Stations for Variety Testing in Skołoszów, which was located 12 km away from this experimental field (Tab. 1)

The following seven soybean cultivars were sown in the field: 'Annushka', 'Mavka', 'Violetta', 'Atlanta', 'Madlen', 'Lajma' and 'Smuglyanka' (all cultivars were obtained from Age Soya – European soybean breeding company). Seeds of the selected cultivars were sown in experimental plots, each with an area of appox. 19 m^2. Before sowing seeds were inoculated with a commercial *Bradyrhizobium japonicum* inoculant.

In each year of the survey, samples were collected twice: in June, when the best growing soybean plants reached BBCH phase 14–17, and in July, when plants were at the anthesis (BBCH phase 65–69). At both collection times, seven plants retarded in growth or/and with disease symptoms on roots, hypocotyls or cotyledons were obtained from each cultivar. In the laboratory, samples were washed in running water for half hour and then they were surface disinfected with 1.4% sodium hypochlorite solution for one minute. Then rinsed three times in sterile water and dried on sterile tissue paper. Surface-disinfected plant material was cut into 1cm long fragments. Then up to six explants from each plant were placed in Petri dish on mineral SNA medium [Leslie and Summerell 2006] with tetracycline hydrochloride (2.5 mg $\cdot$ l^{-1}). In total, 1662 explants were placed on 339 Petri dishes. Most of the explants (1210 of them) were sampled from hypocotyls, while 412 explants originated from roots and 25 – from cotyledons. Additionally, 15 ungerminated seeds with clear disease symptoms were also included in this mycological analysis. All explants were incubated at room temperature for 14 days in order to observe fungal growth.

Macro- and microscopic identification of fungi. The fungi, that grew on SNA medium were isolated by cutting a small piece from the edge of the mycelium of each individual colony using a preparation needle or a scalpel. The mycelial pieces were transferred into separate Petri-dishes with Potato Dextrose Agar medium (PDA, Difco) and then incubated at 20°C for 14 days. Colonies of fungi that could not be identified under the microscope were additionally transferred to the selective media SNA or CLA and incubated at 20°C for 14 days in order to induce sporulation. This way, mainly *Fusarium* genus was identified. Some cultures, which were grown on SNA and CLA medium, were maintained in dark conditions to promote sporulation. Pure cultures of all isolates were characterized

Table 1. Weather conditions recorded during this survey in 2017–2019 compared to long term averages recorded for years 1978–2015

Months	Sum of precipitation (mm)				Mean temperature (°C)			
	2017	2018	2019	1978–2015	2017	2018	2019	1978–2015
April	42.7	24.3	46.7	46.0	6.4	10.9	7.3	9.0
May	68.4	47.0	158.6	71.6	12.6	14.9	11.8	14.1
June	48.7	104.7	25.4	79.2	17.3	17.0	19.5	16.6
July	43.0	98.1	60.2	94.3	18.0	18.5	17.9	18.5
August	21.2	84.3	101.9	63.0	18.4	18.4	17.7	18.1

morphologically and identified to the level of genus or species using microscope NIKON Eclipse 80i and taxonomic descriptions [Barnett and Hunter 1998, Leslie and Summerell 2006]. In addition, colonies were classified into unique mycelial phenotypes based on texture, pigmentation and relative growth. For comparative purposes, fungal isolates obtained from the Bank of Pathogens (from the Institute of Plant Protection-National Research Institute, Poznań, Poland) were used as reference. The same isolates were also used as positive controls in polymerase chain reaction (PCR) assays.

Molecular identification of fungal isolates. All fungal isolates obtained in this study were subjected to DNA extraction and PCR identification. For that purpose, small fragments of mycelium were scraped and placed in a 2-ml tubes, with a sterile steel beads. Then they were homogenized at a frequency 30Hz for three minutes by means of Tissue Lyser II (Qiagen). Fungal genomic DNA was extracted according to a modified CTAB method [Doyle and Doyle 1987]. The extraction buffer contained: 3% w/v CTAB, 100 mM Tris-base, 20 mM EDTA, 1.4 M NaCl, pH = 8. After extraction, DNA concentration was measured using spectrophotometer NanoDrop2000 (Thermo Scientific).

Then DNA was amplified in PCR using genus- or species-specific primers designed for: *Alternaria alternata*, *Fusarium* sp., *F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. oxysporum, F. poae, F. proliferatum, F. solani* (syn. *Neocosmospora solani*), *F. sporotrichioides, F. subglutinans* (currently named as *Fusarium fujikuroi*) and F. verticillioides (Tab. 2). PCR amplification was conducted in a volume of 20 µl containing: 1xPCR buffer [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20], 2.5 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.5 U-Taq (Thermo Fisher Scientific) and 20 ng genomic DNA. Primers designed to amplify selected species or genus (Tab. 2) were used at concentration of 0.5 mM. Amplification was performed on C1000 and S1000 thermal cyclers (Bio-Rad) using temperature profiles described in Table 2. It was tested if PCRs gave genus- or species-specific results by testing amplification using DNA from reference isolates from Bank of Pathogens which served as PCR controls. Negative controls were DNA free.

After amplification, PCR products were separated on a 2% agarose gel by electrophoresis in 1x TBE buffer (Tris base, boric acid and 2 mM EDTA, pH 8.0), with ethidium bromide (0.02 µl · ml^{-1}). The DNA bands were visualized with UV light. Amplicon lengths were estimated by comparison with a ladder (GeneRuler 100-bp DNA Ladder, Thermo Scientific).

RESULTS

Most of the collected plants had symptoms on roots and the lower parts of hypocotyls, which had contact with the soil. Therefore, most of the isolated fungi come from these organs (Tab. 3). A total number of 1692 pure fungi isolates were obtained within this study (250, 613 and 829 isolates obtained in 2017, 2018 and 2019, respectively). These isolates were assigned to 12 species and 8 genera (Tab. 4). However, majority of them belonged to *Fusarium* genus, what was confirmed by results of microscopic observation and amplification with primer set ITS-Fu1f/ITS-Fu1r designed for this genus. PCR product of expected size (approximately 466 bp, Fig. 1-A) was obtained for 1352 (79.9%) tested isolates using these primers. Subsequent PCR assays with 11 primer sets specific for different *Fusarium* species have been successful in identifying and differentiating *Fusarium* species for 1293 isolates. Species specificity of the used primer sets was confirmed using control DNA samples from isolates obtained from the Bank of Pathogens (Fig. 1)

The most common *Fusarium* species detected in this study was *F. oxysporum*. A pair of primers Fof1/Fof2, used to identify this species, gave amplification DNA product of 340 bp (Fig.1-B) for 964 isolates which comprise 57.0% of all isolates and 71.3% of *Fusarium* isolates. The percentage of *F. oxysporum* isolates was higher in June compared to July, with exception of 2019, when the opposite was true.

The second most common *Fusarium* species identified among the tested isolates was *F. graminearum*; it was found in 112 isolates (8.3% of *Fusarium* isolates). This species was detected using Fg16NF/Fg16NR primers which amplified a fragment of approx. 280 bp (Fig.1-D). Next most common Fusarim species identified among the obtained fungal isolates was *F. poae.* Primers Fp82F and Fp82R amplified a product of expected size for 58 isolates (220 bp; Fig 1F).

Table 2. Primers used for the identification of fungal isolates

Fungal species	Primer name	Sequence 5'–3'	Product size (bp)	Source of primers	PCR thermal profiles used for amplification
Alternaria alternata	AAF2 AAR3	TGCAATCAGCGTCAGTAACAAAT ATGGATGCTAGACCTTTGCTGAT	340	Konstantinova et al. 2001	95°C 2 min; (95°C 45 s, 58°C 45 s, 72°C 2 min) ×40; 72°C 10 min
Fusarium sp.	ITS-Fu1 ITS-Fu1	ACAACTCATAACCCTGTGAACAT CAGAAGTTGGGTGTTTTACGG	466	Arif et al. 2012	95°C 5 min; (94°C 50 s, 56°C 50 s, 72°C 1 min) ×35; 72°C 7 min
Fusarium avenaceum	JiAF JiAR	GCTAATTCTTAACTTACTAGGGGCC CTGTAATAGGTTATTTACATGGGCG	220	Turner et al. 1998	95°C 3 min; (94°C 30 s, 58°C 30 s, 72°C 2 min) ×40; 72°C 5 min
Fusarium culmorum	Fc01F Fc01R	ATGGTGAACTCGTCGTGGC CCCTTCTTACGCCAATCTCG	570	Nicholson et al. 1998	95°C 5 min; (94°C 20 s, 66°C 1 min, 72°C 45 s) ×5; (94°C 20 s, 64°C 1 min, 72°C 45 s) ×5; (94°C 20 s, 62°C 1 min, 72°C 45 s) ×25; 72°C 5 min
Fusarium equiseti	Feq-F Feq-R	GGCCTGCCGATGCGTC CGATACTGAAACCGACCTC	900	Jurado et al. 2005	95°C 1 min 25s; (95°C 35 s, 66°C 30 s, 72°C 30 s) ×30, (95°C 35 s, 54°C 30 s, 72°C 30 s) ×20; 72°C 5 min
Fusarium graminearum	Fg16NF Fg16NR	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	280	Nicholson et al. 1998	95°C 5 min; (94°C 50 s, 56°C 50 s, 72°C 1 min) ×35; 72°C 7 min
Fusarium oxysporum	Fof1 Fof2	ACATACCACTTGTTGCCTCG CGCCAATCAATTTGAGGAACG	340	Mishra et al. 2003	95°C 5 min; (94°C 50 s, 58°C 50 s, 72°C 1 min) ×35; 72°C 7 min
Fusarium poae	Fp82F Fp82R	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGTT	220	Parry and Nicholson 1996	94°C 2 min ; (94°C 1 min, 55°C 1 min, 72°C 2 min) ×40; 72°C 5 min
Fusarium proliferatum	Pro1 Pro2	CTTTCCGCCAAGTTTCTTC TGTCAGTAACTCGACGTTGTTG	585	Mule et al. 2004	95°C 5 min; (94°C 50 s, 56°C 50 s, 72°C 1 min) ×35; 72°C 7 min
Fusarium solani	TEF-Fs4f TEF-Fs4r	ATCGGCCACGTCGACTCT GGCGTCTGTTGATTGTTAGC	658	Arif et al. 2012	95°C 2 min; (95°C 45 s, 58°C 45 s, 72°C 2 min) ×40; 72°C 10 min
Fusarium sporotrichioides	FspIT2K P28SL	CTTGGTGTTGGGATCTGTGTGCAA ACAAATTACAACTCGGGCCCGAGA	288	Kulik et al. 2004	95°C 3 min; (95°C 30 s, 68°C 30 s, 72°C 40 s) ×40; 72°C 7 min
Fusarium subglutinans (*Fusarium fujikuroi*)	Sub 1-F Sub 2-R	CTGTCGCTAACCTCTTTATCCA CAGTATGGACGTTGGTATTATATCTAA	631	Mule et al. 2004	95°C 5 min; (94°C 50 s, 56°C 50 s, 72°C 1 min) ×35; 72°C 7 min
Fusarium verticillioides	Ver1 Ver2	CTTCCTGCGATGTTTCTCC AATTGGCCATTGGTATTATATATCTA	578	Mule et al. 2004	95°C 5 min; (94°C 50 s, 56°C 50 s, 72°C 1 min) ×35; 72°C 7 min

Microscopic species determination confirmed by PCR tests allowed for detection *F. culmorum* and *F. solani* in 45 and 41 isolates, respectively. Primers Fc01F/Fc01R, used for *F. culmorum* detection, produced for the above-mentioned number of isolates a fragment of expected size (570 bp; Fig.1-C). In turn for *F. solani,* primer set TEF-Fs4/TEF-Fs4r amplified a PCR product of approx. 658 bp (Fig.1-E). All *F. solani* isolates were obtained from samples collected in June.

PCR assay was also used to confirm microscopic identification of *Alternaria alternata* among the obtained isolates. Primers AAF2/AAF3 amplified a PCR product of approx. 340 bp (Fig.1-H) for 95 isolates. The genus *Alternaria* was the second largest genus after *Fusarium* spp. detected in this study. Majority of the obtained *A. alternata* isolates (91.5% of all obtained isolates of this species) originated from samples collected in July.

Based on taxonomic identification using microscopic methods, the remaining isolates were identified mainly as: *Trichoderma* sp., *Rhizoctonia solani*, *Mucor* sp., *Cladosporium* sp., *Rhizopus* sp. (Tab. 4).

Table 3. Number of fungi isolated from individual plant organs

Fungus species	Roots	Hypocotyls	Cotyledons	Ungerminated seeds
Acremonium sp.	4	2	0	0
Alternaria alternate (Fr.) Keissler	39	56	0	0
Aspergillus sp.	2	2	0	0
Botrycis cinerea Pers.	3	3	0	0
Cladosporium sp.	10	11	0	0
Fusarium avenaceum (Fr.) Sacc.	13	12	0	0
Fusarium culmorum (Wm. G. Sm.) Sacc.	21	21	3	0
Fusarium equiseti (Corda) Sacc.	0	4	0	0
Fusarium graminearum Schwabe	53	59	0	0
Fusarium oxysporum Schltdl.	360	541	43	20
Fusarium poae (Peck) Wollenw.	22	36	0	0
Neocosmospora solani (Mart.) L. Lombard & Crous (syn. *Fusarium solani* (Mart.) Sacc.)	12	28	0	1
Fusarium sporotrichioides Sherb.	1	2	0	0
Fusarium fujikuroi Nirenberg (earlier *Fusarium subglutinans*)	15	26	0	0
Other *Fusarium* sp.	25	28	1	5
Mucor sp.	10	22	0	2
Penicillium sp.	3	8	0	0
Rhizopus sp.	11	9	0	1
Rhizoctonia solani J.G. Kühn	21	34	3	0
Trichoderma sp.	28	53	1	2
Total	656	954	51	31

Table 4. Fungi isolated from the infected plants of soybean collected in three vegetative seasons

Fungus species	Number of isolates							
	2017		2018		2019		Total	
	VI	VII	VI	VII	VI	VII		%
Acremonium sp.*	1				3	2	6	0.4
Alternaria alternata (Fr.) Keissler		6	1	33	7	48	95	5.6
Aspergillus sp.*						4	4	0.2
Botrytis cinerea Pers.*	4					2	6	0.4
Cladosporium sp.*	2		5	3	8	3	21	1.2
Fusarium avenaceum (Fr.) Sacc.		3	2	11		9	25	1.5
Fusarium culmorum (Wm. G. Sm.) Sacc.	16	5	5	14		5	45	2.7
Fusarium equiseti (Corda) Sacc.					4		4	0.2
Fusarium graminearum Schwabe	5	5	5	13	34	50	112	6.6
Fusarium oxysporum Schltdl.	122	30	219	208	185	200	964	57.0
Fusarium poae (Peck) Wollenw.		3			13	42	58	3.4
Neocosmospora solani (Mart.) L. Lombard & Crous (syn. *Fusarium solani* (Mart.) Sacc.)	7		15		19		41	2.4
Fusarium sporotrichioides Sherb.				1	2		3	0.2
Fusarium fujikuroi Nirenberg (earlier *Fusarium subglutinans*)		1			16	24	41	2.4
Other *Fusarium* sp.	18	1		27		13	59	3.5
Mucor sp.*	2		3	5	9	15	34	2.0
Penicillium sp.*	4				2	5	11	0.7
Rhizopus sp.*	5				9	7	21	1.2
Rhizoctonia solani J.G. Kühn		1	24		27	6	58	3.4
Trichoderma sp.*	9		10	9	31	25	84	5.0
Total	195	55	289	324	369	460	1692	100

*for indicated fungi all PCR tests with primers used in this study gave negative results and taxonomic determination was done using microscopic methods

DISCUSSION

This research was performed mainly to identify fungi inhabiting soybean plants under field conditions with a particular focus on pathogenic fungi. Studies of this subject in many environments are an active area for research, because fungal diseases are often the cause of yield losses and economic damage to soybean producers.

This work shows that most fungi isolated from soybean plants under field conditions in Podkarpackie region in Poland belong to the *Fusarium* genus, with *F. oxysporum* and *F. graminearum* being the most frequently isolated members of this genus. In another region of Poland, Patkowska [2001] and Pastucha [1998] carried similar studies and they found that, among *Fusarium* isolates, *F. solani* and *F. oxysporum* were the most common. On another continent, Diaz Arias et al. [2013b] isolated *Fusarium* species from soybean grown in the fields in Iowa State (USA). In this three-year survey, they consistently found that *F. oxysporum*, *F. acuminatum* and *F. solani* were the

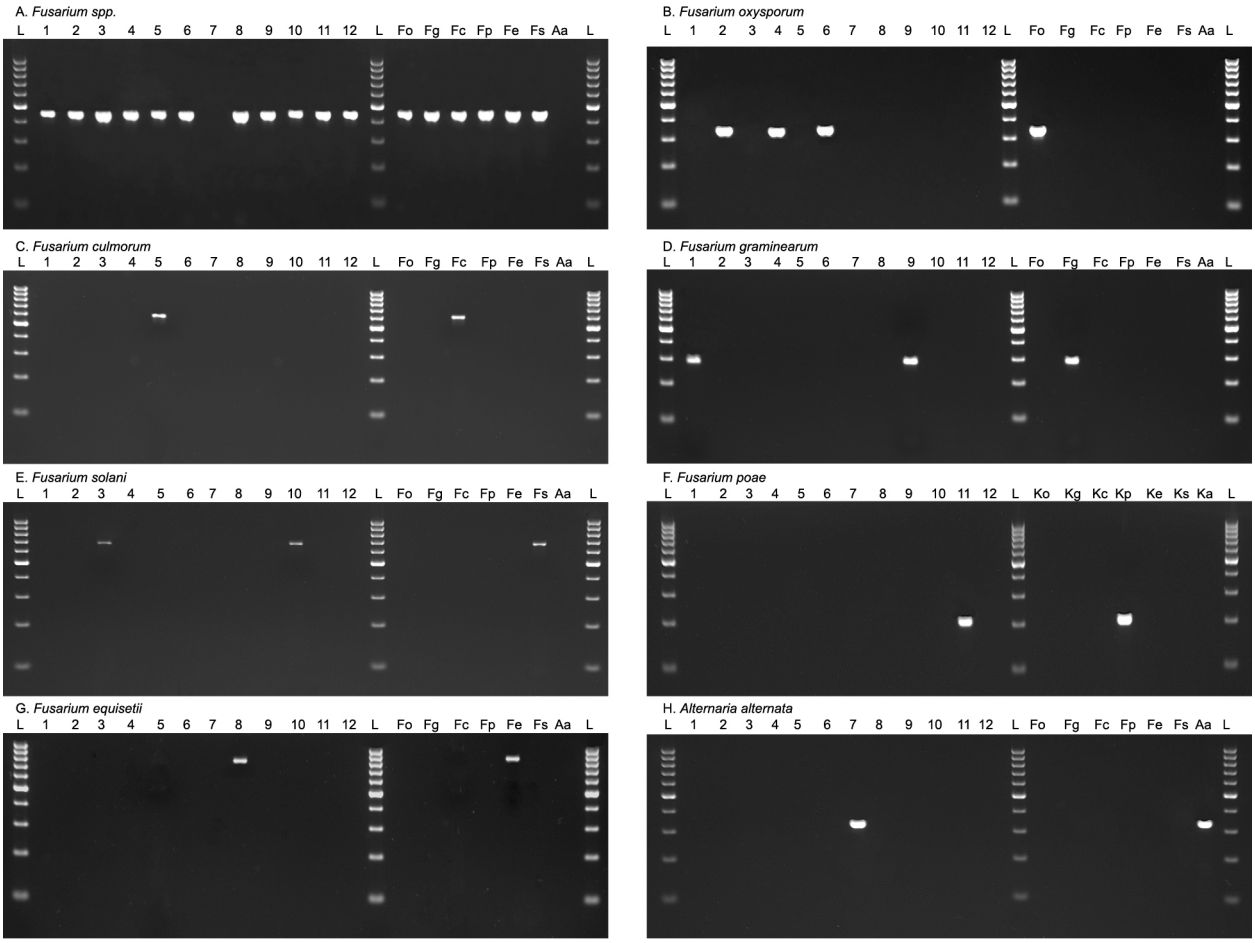

Fig. 1. Example results of PCR species identification of 12 fungi isolates. Figures A-H visualize product of PCRs performed separately using genus- and species-specific primers. Amplified samples loaded on gel include: tested isolates (1–12), and controls from fungal isolates obtained from the Bank of Pathogens belonging to the following species: *Fusarium oxysporum* (Fo), *F. graminearum* (Fg), *F. culmorum* (Fc), *F. poae* (Fp), *F. equiseti* (Fe), *F. solani* (Fs; currently named *Neocosmospora solani*) and *Alternaria alternata* (Aa). Every gel contained also size standard GeneRuler 100-bp Ladder (L)

most commonly isolated species. Consistent detection of the same species in subsequent years of research on the same location suggests significant influence of environment on colonization of plants by fungi. Surveys performed on a larger geographic scale give opportunity to record regional differences in relative frequencies of *Fusarium* species. Leslie et al. [1990] isolated fungi belonging to this genus from three crops, including soybean, grown in 34 fields located in the central and southeastern United States. Generally, *F. oxysporum*, *F. solani* and *F. equiseti* were most commonly recovered from soybean plants. However, *Fusarium* isolates obtained from soybean grown in different fields showed a considerable variation in the number and identity of detected species. From some fields only *F. oxysporum* or only *F. solani* was obtained, while in others five different *Fusarium* species were found. This shows that, although *Fusarium* spp. are considered to be cosmopolitan, a local occurrence of a particular species is difficult to predict and multiple studies on this topic are valuable.

Fusarium spp. are well recognized as soybean pathogens affecting plants in different developmental stages [Hartman et al. 2015]. For example, *F. oxysporum,*

which dominated isolates obtained in this study, is a species associated with Fusarium blight or wilt on soybean. Symptoms of this disease appear usually in July when temperature exceeds 28°C, particularly on plants growing in sandy soils. Such conditions are conducive to the development of conidial spores and mycelium growth [Hartman et al. 2015]. Patkowska [2001] in the three-year study noted a high proportion of *F. oxysporum* isolated from soybean plants at anthesis. The same results were obtained earlier by Armstrong et al. [1950], as well as Sinclair and Backman [1989]. Whereas Dias Arias et al. [2013b] more frequently isolated *F. oxysporum* from soybean plants in the vegetative stage (equivalent of BBCH phase 13–15), before flowering. Leslie et al. [1990] and Sinclair and Backman [1989] pointed out that Fusarium wilt is an important disease in many countries and causes a reduction of soybean yields up to 60%. *F. oxysporum* is also reported to cause root rot and seedling disease; therefore, it can be found on young plants under cool conditions (below 14°C). Jansić et al. [2005] described rotting and damping off of soybean seedlings associated with low emergence in fields. The same authors obtained isolates, belonging to four different species, from tissues with disease symptoms and showed that isolates of *F. oxysporum* showed the highest level of pathogenicity

F. oxysporum is an important yield-limiting factor not only in soybean, but also in other legumes worldwide. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is considered to be the major soil-borne fungus affecting chickpea globally. This pathogen can devastate crops and cause up to 100% production losses [Jendoubi et al. 2017]. It is now found in the majority chickpea growing areas in Asia, Africa and southern Europe. For pea Fusarium wilt is also a global problem. Several races of this pathogen are economically important in most areas of the world [Kraft et al. 1988]. Similarly in lentils, *Fusarium oxysporum* f. sp. *lentis* is responsible for causing significant yield losses in dry and warm conditions. Under favorable conditions this disease can cause the complete loss of the crop [Pouralibaba et al. 2015].

Another important *Fusarium* species that causes diseases of soybean plants is *F. graminearum*. This pathogen infects seeds and seedlings especially in cold field conditions and it can be responsible for pre-emergence damping-off. *F. graminearum* can also cause a root rot and pod infection [Hartman et al. 2015]. Broders et al. [2007] obtained *Fusarium* spp. isolates from symptomatic seedlings of soybean collected in Ohio (USA). In subsequent pathogenicity tests, they showed that *F. graminearum* was significantly more pathogenic than other isolated species. Dias Arias et al. [2013a] confirmed that *F. graminearum* is an important pathogen for soybean also by performing pathogenicity tests. They compared the severity of root rot symptoms caused by isolates of nine *Fusarium* species and recorded the highest aggressiveness for *F. graminearum* isolate, which caused darkening of 96% of root area and other significant detrimental effects on root morphology.

Apart from *F. oxysporum* and *F. graminearum,* other soil-borne fungi can colonize soybean plants. Some of them cause diseases of seedlings and plants before flowering. For example *F. solani* (syn. *Neocosmospora solani*) and *Rhizoctonia solani* were less frequently isolated in our study, but all or almost all isolates of these fungi originate from samples collected in June. Older plants may be more resistant to these pathogens. The fact that *F. solani* and *R. solani* are more frequently isolated from young plants compared to plants at anthesis was confirmed by earlier reports from Poland [Pastucha 1998, Patkowska 2001]. Both species are associated with diseases of soybean plants at vegetative stage. *Fusarium solani* causes a rapid death of soybean (sudden death syndrom) whereas *Rhizoctonia solani* is responsible for damping-off and root rot [Hartman et al. 2015].

It is well known that soybean plants are hosts for *Alternaria* genus. The most frequently detected species are usually *A. alternata* and *A. tenuissima*, which are widely recognized as leaf and pod parasites Sinclair and Backman [1989]. In this study, *A. alternata* was next most common species isolated from soybean, after *Fusarium* genus. According to Sinclair and Backman [1989], these fungi occur mainly on seeds, although they also cause diseases affecting leaves and stems. Symptoms of Alternaria leaf spot usually appear on mature plants at anthesis and during the podding stage [Hartman et al. 2015]. In this context, it is not surprising that in our study majority of *Alternaria* isolates were obtained from July collections.

The current work shows that potentially pathogenic fungi can be isolated from soybean plants during

the whole vegetation. Both, young plants at vegetative stage and mature soybean plants can be hosts for different species of fungi. The increase of cultivation area of soybean and growing it in monoculture can cause accumulation of pathogenic fungi in the soil leading to increased losses of the crop. Therefore, various strategies to control fungal diseases in soybean fields should be developed and implemented.

CONCLUSIONS

1. Soybean seedlings as well as mature soybean plants are infected by different species of pathogenic fungi, among which the most common are members of *Fusarium* genus.

2. Among all *Fusarium* species *F. oxysporum* was most frequently isolated.

3. *Rhizoctonia solani* and *Fusarium solani* are more frequently isolated from young plants, while *Alternaria alternata* from plants in flowering stage.

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