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Original article

Plant-pathogenic fungi in seeds of different pea cultivars in Poland

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Legume crops are exposed to infection by fungal pathogens, which often results in contamination with mycotoxins. The aim of this study was to evaluate the level of field resistance/susceptibility of edible and fodder pea cultivars to the colonization of seeds by fungal pathogens in two subsequent seasons, as well as to identify the pathogens present in the seeds of the tested cultivars. *Alternaria* spp. were the most common fungi isolated from pea seeds in both seasons, followed by *Fusarium* spp., *Stemphylium* spp., *Ulocladium* spp., *Botrytis cinerea* Pers., *Epicoccum nigrum* Link., and *Phoma pinodella* L. K. Jones. The highest percentage of infected seeds (55 %) was recorded for cultivar Ezop. The presence of a large number of fungi was found in 2012 for cultivars Santana, Tarchalska, Medal, Cysterski, Mentor, Lasso, and Ezop. Fodder cultivars displayed a lower infection level than edible cultivars. We can conclude that *Alternaria* spp. were the most frequent fungi present in pea seeds in Poland and *Fusarium* spp. were likely the most dangerous, having in mind their established mycotoxigenic abilities.

KEY WORDS: Alternaria*;* Fusarium*; molecular identification; mycotoxins; seed-borne pathogens; seed-transmitted diseases*

As many other plant species, legume crops are exposed to infection by fungal pathogens, which often cause the accumulation of mycotoxins in plant tissues. The consumption of contaminated plant material poses a serious risk to human and animal health. Fungal species, including *Fusarium* spp., *Phoma pinodella* L. K. Jones, *Ascochyta pisi* Lib., and *Alternaria* spp., are among the most important agents involved in field pea (*Pisum sativum* L.) diseases (1).

Ascochyta pea diseases (blights), caused by *Ascochyta* spp. and *Phoma* spp., are responsible for limiting seed yield worldwide. The first disease symptoms relate to the formation of spores on aerial organs. The infection progresses quickly and results in necrotic lesions (2). Pea-infecting *Ascochyta* and *Phoma* fungi are able to produce several phytotoxins that have a key role in the damage of plant cells (3, 4).

Fusarium species are widely spread and infect plants independently of developmental stage. The growth of the *Fusarium* fungi and the accumulation of their mycotoxins can affect grain quality (5). Occurrence of pea root rot is associated with two pathogens: *F. solani* f. sp. *pisi* F. R. Jones (6) and *F. avenaceum* (Fr.) Sacc. (7). A vascular pathogen, *Fusarium oxysporum* f.sp. *pisi* (*Fop*) Schltdl., which infects the xylem tissue and is a known factor in Fusarium wilt (8). The progress of symptoms depends on the *Fop* race (9).

The *Alternaria* genus includes saprobiotic, endophytic, and pathogenic species that may cause various plant diseases. According to Thomma (10), melanin production by fungal isolates has significant

impact on the virulence of the isolate. Tentoxin plays a role in pathogenesis (11) by blocking the ATPase responsible for the hydrolysis of ATP in plant chloroplasts. This leads to the chlorosis of sensitive hosts.

Infections of plant tissues by pathogenic fungi can cause yield losses as high as 50-75 % (12). It is therefore important to know the specific susceptibility level of different pea cultivars to fungal pathogens. Many fungal species belong to seed-borne pathogens, as they spread along the plant during growth. One possible reaction of a plant resistant to fungal infection is hypersensitivity and seed abortion. Genotypes that do not respond to the infection process may produce fungi-contaminated seeds.

The aim of the present study was to evaluate the level of resistance/susceptibility of edible and fodder cultivars of field pea to infection by fungal pathogens and identify the pathogens present in the seeds of the tested cultivars. This knowledge could help single out and grow only resistant cultivars, which would in turn prevent the contamination of food and agricultural products with harmful mycotoxins. This study is the first to provide such an extensive and detailed analysis of seed contamination in pea cultivars in Poland. In addition, we also compared the specific weather conditions at the two studied locations in order to analyse the factors affecting fungal contamination and emphasise the differences in local pathogen populations.

MATERIALS AND METHODS

Isolation of fungal strains

Seeds of seven pea cultivars (Santana, Medal, Lasso, Sokolik, Turnia, Ezop, Tarchalska) were tested for fungal occurrence. Each cultivar was sown at two distinct localities in central Poland (Radzików and Wiatrowo) during the 2011 and 2012 seasons. Additionally, five other cultivars, not sown purely for the purposes of this study, were examined in 2011 (Wiato, Hubal, Wenus, Eureka, Gwarek) and three in 2012 (Milwa, Cysterski, Mentor). Table 1 contains the full list of cultivars tested along with their description (edible or fodder cultivar). Among the cultivars tested, 8 were edible and 7 grown for fodder. Each cultivar was grown in four replicates and two localities; therefore, the overall number of seeds tested was 400 per cultivar. Plant material was harvested in July of each year of cultivation and samples of 50 seeds from each cultivar in four replicates were grown at two localities were used for evaluating presence of fungi. Seeds were surface disinfected with 0.5 % sodium hypochlorite (Javel, Warsaw, Poland) for 30 s. After surface treatment, the plant material was rinsed three times with sterile water. The seeds were placed on a single layer of water-soaked, sterile filter paper (Whatman no. 1) in Petri dishes, which were incubated for 7 days at 20-25 °C and checked by observation of fungal presence every day. Mycelia from seeds with visible fungal growth were transferred to new plates with potato dextrose agar (PDA) medium (Oxoid, Wesel, Germany).

Table 1 *Pea cultivars used for the evaluation of fungal species presence on the seeds harvested in 2011 and 2012 seasons.(+) cultivar tested for fungal occurrence in season, (-) cultivar not tested for fungal occurrence in season*

Cultivar	2011	2012
Edible		
SANTANA	+	+
TARCHALSKA	+	+
EZOP	+	+
LASSO	+	+
MENTOR	-	+
MEDAL	+	+
WENUS	+	-
CYSTERSKI	-	+
Fodder		
WIATO	+	-
SOKOLIK	+	+
EUREKA	+	-
GWAREK	+	-
HUBAL	+	-
MILWA	-	+
TURNIA	+	+

Morphological identification

The purified isolates were cultured for 7 days on the synthetic nutrient agar (SNA) medium (glucose, sucrose, potassium dihydrogen phosphate, potassium nitrate, magnesium sulfate anhydrous, potassium chloride from Sigma-Aldrich, Steinheim, Germany; agar from Oxoid, Wesel, Germany). Subsequently, the strains were identified according to their morphological characteristics, structure of hyphaae,

phialides and conidia (13). An optical microscope (Olympus, Tokyo, Japan), set at 200x of total magnification was used for genus identification of fungal isolates.

Molecular analyses

Mycelia for DNA extraction were scraped from 7-day-old PDA cultures of individual isolates. Isolation of total DNA was done using a Cetyltrimethyl Ammonium Bromide (CTAB method) (14). Fungal material was incubated at 65 °C for 20 min in 800 µL CTAB buffer (Sigma-Aldrich, Steinheim, Germany), 0.4 % β-merkaptoethanol (Sigma-Aldrich, Steinheim, Germany) and 150 µL chloroform/isoamyl alcohol (24:1/volumes mix, Sigma-Aldrich, Steinheim, Germany). Then the tubes were incubated at room temperature for 10 min. After incubation and addition of 150 µL of chloroform/isoamyl mixture, the samples were mixed and centrifuged at 12,000 rpm (equivalent to 15.294 x *g*) for 15 min. In the next step, the aqueous phase was transferred to new tubes and 60 µL of 3 mol L^{-1} sodium acetate were added. For the DNA precipitation, two volumes of 96 % cold solution of ethanol were used. The tubes were then left at -20 °C for 20 min. The samples were centrifuged at 12,000 rpm for 20 min and the pellet was dissolved in 150 µL TE buffer (10 mmol L^{-1} Tris-HCl, 1 mmol L^{-1} EDTA from Sigma-Aldrich, Steinheim, Germany). Molecular identification of isolates was performed based on partial sequences of the translation elongation factor *tef-1α* and ITS1-ITS2 region of the rDNA gene cluster. Primer pairs Ef728M (CATCGAGAA GTTCGAGAAGG)/Tef1R (GCCATCCTTGGA GATACCAGC) and ITS4 (TCCTCCGCTTA TTGATATGC)/ITS5 (GGAAGTAAAAG TCGTAAC AAGG), validated in previous studies (15, 16), were used for the *tef-1α* gene fragment and the ITS region amplification.

The polymerase chain reaction was done in 20 µL reaction mixture containing 1 unit of Phire II HotStart Taq DNA polymerase (Thermo Scientific, Espoo, Finland), 4 µL of 5× PCR buffer, 12.5 pmol L^{-1} of forward/reverse primers, 2.5 mmol L^{-1} of each dNTP and 1 µL 50 ng of fungal DNA. The amplification conditions were as follows: initial denaturation 30 s at 98 °C, 35 cycles of 10 s at 98 °C, 5 s at 58 °C (for the ITS region) or 63 °C (for the tef-1α fragment), 15 s at 72 °C with a final elongation of 1 min at 72 °C.

Amplification products were electrophoresed in 1.5 % agarose gels (Invitrogen, Carlsbad, CA, USA) in 1×TBE buffer (0.178 mol L^{-1} Tris-borate, 0.178 mol L^{-1} boric acid, 0.004 mol L^{-1} EDTA from Sigma-Aldrich, Steinheim, Germany) containing ethidium bromide.

For sequence reading, the PCR products were purified and sequenced according to the previously described protocol (17). Sequences were edited using Chromas v. 1.43 (Technelysium, Tewantin, Australia) and analysed using BLASTn algorhitm (18).

Weather conditions

Temperature and total rainfall were obtained at WeatherOnline Poland (19). The weather conditions were monitored for two localities in Poland (Radzików and Wiatrowo). The average monthly temperature and rainfall were recorded from April to July in 2011 and 2012.

Statistical analyses

An infection degree (frequency of infected seeds) was estimated for each sample. This average number of infected seeds for each cultivar was calculated taking into account four replicates of each pea cultivar, which enabled us to determine the exact degree of fungal infection in the pea seeds.

Chi-square test (20) was used to exclude the differences between the infection levels of the same cultivar at two distinct localities. Due to the differences in weather conditions within the two years as well as in the susceptibility of the cultivars, the analysis was conducted separately for each cultivar in 2011 and 2012.

The test resulted in the equation:

$$\chi^2 = \sum_{j=1}^{k} \frac{(O_j - E_j)^2}{E_j}$$

O_j – number of infected seeds,

E_j – expected number of infected seeds.

The critical value was read from the Chi-Square Distribution Table for degrees of freedom df=2, and α=0.01.

RESULTS

The percentage of seed material contaminated with fungal pathogens was different for individual cultivars (Figure 1). Edible cultivars (e.g., Ezop, Santana, Cysterski) displayed a higher contamination level than fodder cultivars (e.g., Sokolik, Turnia, Wiato). Fungal

infection of cultivars Hubal, Gwarek, Sokolik, and Wiato was very low in 2011 (Figure 1A). The seeds of cv. Wiato from Radzików were free from detectable fungi. Three of the edible pea cultivars (Santana, Tarchalska, and Ezop) displayed a considerably high infection degree, nevertheless, the highest percentage (55 %) of cv. Ezop infection was noted in Wiatrowo. The infection degree of cv. Santana was higher in 2012 compared to 2011(Figure 1B). Seed contamination of cv. Tarchalska was similar to that recorded in the previous year (reaching 30 % of seeds contaminated with fungi). A high degree of contamination was also found for the seeds of cultivars Medal, Cysterski, Mentor, Lasso (about 40 % at both localities), and Ezop (Wiatrowo). Seeds of cv. Sokolik were not infected in any of the two distinct regions and the seed material of cv. Turnia in Radzików was also not contaminated (Figure 1B).

Alternaria spp. were the most commonly isolated fungal species among all of the isolates obtained in 2011 (90 % of fungal isolates, Table 3). Turnia and Ezop cultivars were infected by *Fusarium* species at both localities. Ezop from Wiatrowo was the most contaminated cultivar (110 fungi were found, including 19 % belonging to the *Fusarium* genus). *Fusarium* species were also detected on seeds of other genotypes. In Wiatrowo, these included cvs. Wiato, Sokolik, and Tarchalska, and in Radzików cv. Eureka. Using molecular techniques, three *Fusarium* species were identified: *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsush.) Nirenberg, and *F. poae* (Peck) Wollenw. *Stemphylium sp., Ulocladium sp*., and *Botrytis cinerea* Pers. were isolated from the seeds only in a smaller number of occasions (2 %, Table 3).

Similarly, in the 2012 season *Alternaria sp.* were the most common fungi isolated from pea seeds. Their frequency was about 94 %, regardless of the cultivar or locality. The average frequency of samples containing *Fusarium spp*. was 3.2 % and the identified species included *F. avenaceum*, *F. proliferatum*, *F. equiseti* (Corda) Sacc., and *F. poae*. *Fusarium*-infected cultivars were genotypes Santana, Medal, Turnia, Tarchalska, Milwa, and Cysterski from Wiatrowo and Mentor from Radzików. Other fungal strains were represented by *Stemphylium spp*., *Ulocladium spp*., *Epicoccum nigrum* Link., and *Phoma pinodella* (Table 2).

The average air temperatures for April-July of 2011 and 2012 at the two localities did not differ significantly (Figure 2). Moreover, the amounts of rainfall were also similar. High rainfalls were reported only in July 2011.

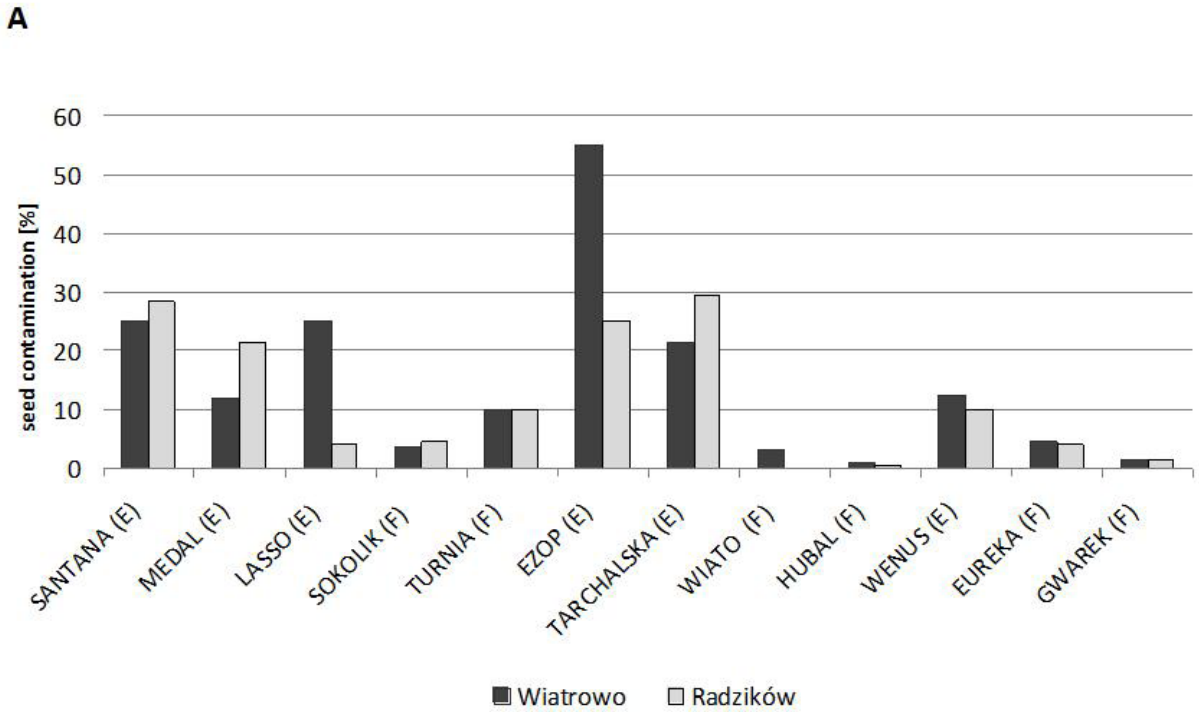

Figure 1A *Level of fungal contamination [in % of contaminated seeds] of the seed material of pea cultivars grown in Radzików and Wiatrowo in 2011. (E) edible cultivars used in human diet, (F) fodder cultivars for feed production. First seven cultivars have been tested in 2011 and 2012 seasons*

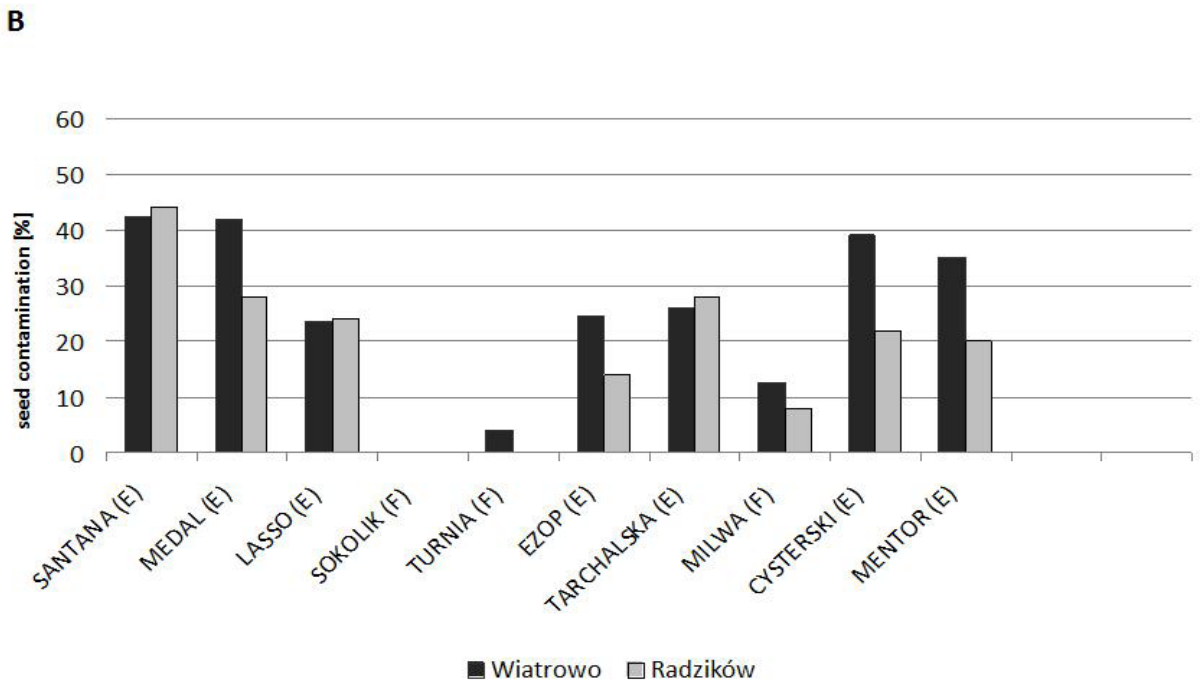

Figure 1B *Level of fungal contamination [in % of contaminated seeds] of the seed material of pea cultivars grown in Radzików and Wiatrowo in 2012. (E) edible cultivars used in human diet, (F) fodder cultivars for feed production. First seven cultivars have been tested in 2011 and 2012 seasons*

Table 2A *Results of chi-square test for seeds harvested in 2011.Critical value is equal to 9.21034 for the degrees of freedom df=2, and α=0.01 (data from Chi-Square Distribution Table). Low value χ^2 excludes the differences between the seeds infection level dependent on the localities*

Cultivar	Number of infected seeds in Radzików	Expected number of infected seeds in Radzików	Number of infected seeds in Wiatrowo	Expected number of infected seeds in Wiatrowo	χ^2
WIATO	0	3	6	3	6.100
HUBAL	1	1.5	2	1.5	0.342
WENUS	22	23.5	25	23.5	0.194
EUREKA	8	8.5	9	8.5	0.062
GWAREK	3	3.5	4	3.5	0.144
SANTANA	62	58.5	55	58.5	0.592
MEDAL	43	33	23	33	0.592
LASSO	7	28.5	50	28.5	37.828 *
SOKOLIK	9	8	7	8	0.260
TURNIA	19	19.5	20	19.5	0.028
EZOP	50	79	108	79	70.363 *
TARCHALSKA	45	44.5	44	44.5	0.016

**value χ^2 > critical value (9.21034), statistically significant result*

Table 2B *Results of chi-square test for seeds harvested in 2012.Critical value is equal to 9.21034 for the degrees of freedom df=2, and α=0.01 (data from Chi-Square Distribution Table). Low value χ^2 excludes the differences between the seeds infection level dependent on the localities*

Cultivar	Number of infected seeds in Radzików	Expected number of infected seeds in Radzików	Number of infected seeds in Wiatrowo	Expected number of infected seeds in Wiatrowo	χ^2
SANTANA	22	22.6	81	82.4	0.203
MEDAL	14	16.8	70	67.2	0.879
LASSO	12	12.2	49	48.8	0.005
SOKOLIK	0	0	0	0	0.000
TURNIA	9	3.4	8	13.6	12.371 *
EZOP	7	10.4	45	41.6	1.755
TARCHALSKA	14	13.4	53	53.6	0.046
MILWA	4	6	26	24	0.948
CYSTERSKI	11	16.4	71	65.6	3.308
MENTOR	10	15.4	67	61.6	3.487

**value χ^2 > critical value (9.21034), statistically significant result*

DISCUSSION

Legume crops, including field pea, are frequently colonized by pathogenic or endophytic fungi (21). The overall frequency of fungal isolate detection in pea seeds of different cultivars was calculated in order to evaluate the degree of infection. A decreased level of pea infestation was observed for fodder cultivars. However, increased infestations were observed in the case of edible cultivars (Figures 1A, 1B), which corresponds to Marcinkowska (22), who concluded that edible cultivars, due to the influence of weather, are more susceptible to infection by pathogens. In a study by Embaby et al. (23), an increase of the infestation degree was observed with high moisture content in the seed samples. In the present study, seed samples were collected after the very rainy season of 2011 (Figure 2) and their high fungal infection was likely the result of high moisture content during the formation of pods.

However, it seems that water availability after that stage did not have such a strong impact. High rainfalls in July 2011 did not result in higher seed contamination, while in 2012 higher precipitation in May and June resulted in an overall increase of fungal contamination (Figures 1 and 2). Weather conditions play the most significant role and dramatically affect fungal growth and mycotoxin biosynthesis (24). Water availability and temperature at specific plant growth stage are particularly crucial for disease development and severity. Woudenberg et al. (25) indicated that fungi can infect different parts of a plant at various growth stages. Nevertheless, the major factors causing plant contamination with pathogens are local and temporary weather conditions that affect the development of fungal infection (26).

The influence of precipitation on fungal incidence is particularly well visible for Radzików in 2011, where it was considerably rainier than in Wiatrowo. The overall contamination of cultivars was higher, as well (Figures 1 and 2).

F. proliferatum and *F. verticillioides* colonise a wide range of hosts, but they are primarily known as maize pathogens (27, 28). However, Ivić et al. (29) managed to isolate *F. proliferatum* and *F. verticillioides* from pea.

In the present study, *F. proliferatum* was predominantly isolated from seeds of cultivar Turnia, whereas *F. verticillioides* was found in seeds of cultivar Ezop - both harvested in 2011 (Table 3). The observed presence of *Fusarium* spp. in the analysed pea seeds suggests the possibility of contamination with mycotoxins, as both *F. proliferatum* and *F. verticillioides* have the ability to produce fumonisins. Fumonisin biosynthesis is determined by the activity of the *FUM* gene cluster comprising of 17 *FUM* genes (30, 31) and numerous studies have shown that the sequence analysis of *FUM1* gene may be successfully used to identify *Fusarium* strains (32-35). An analysis of the genetic divergences of this particular gene among the pea-derived strains and the strains originating from different host species has shown a higher level of similarity between the strains of *F. proliferatum*

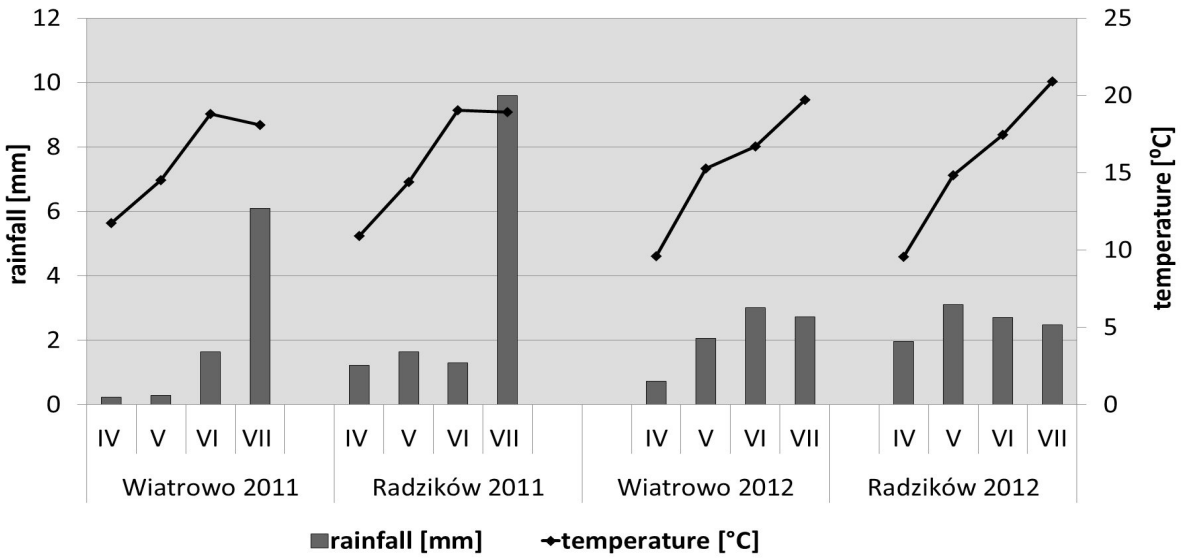

Figure 2 *Mean monthly temperatures and rainfall for two localities studied in April-July period in 2011 and 2012 seasons*

isolated from the same plant host (33, 35). Moreover, analysis of *FUM1* sequence can be used to evaluate the contamination with fumonisins; however, significant intraspecific differences in mycotoxigenic abilities have been reported (33). Though mycotoxin analyses were not planned for this study, they definitely would contribute to knowledge in the field (some analyses have already been performed and more extensive research is planned on this material). In fact, significant amounts of fumonisins have been found in the seeds of cultivars Eureka and Turnia (35). Taking our results into account, it can be concluded that some pea-originating *Fusarium* strains can be regarded as specific for the host, although results have also shown that susceptibility of individual pea cultivars to pathogenic fungi vary.

Table 3 *Number of isolates of different fungal species isolated from total number of 200 seeds of each cultivar grown in two distinct localities in Poland (W-Wiatrowo, R-Radzików) in 2011 and 2012 seasons*

Fungal species	2011		2012	
	W	R	W	R
Alternaria spp.	304	270	440	91
Botrytis cinerea	2	-	-	-
Epicoccum nigrum	-	-	2	-
Fusarium spp.*	38	12	15	3
Phoma medicaginis var. *pinodella*	-	-	3	-
Stemphylium spp.	2	-	3	-
Ulocladium spp.	7	1	7	-
Total number of isolates	353	283	470	94

**In the* Fusarium *genus* F. avenaceum, F. equiseti, F. poae, F. verticillioides *and* F. proliferatum *were identified*

Fusarium avenaceum is the major agent causing rot in pea roots (36). The prevalence of this species in pea seeds was high in 2012 (Table 3). Moreover, the domination of this pathogen in field pea has already been reported in earlier studies (37). Compared to 2011, it can be suggested that the presence of *F. avenaceum* was related to weather conditions (especially to the air temperature of about 25-30 °C). The emergence of *F. avenaceum* on pea seeds could have also been related to the rotation of crop species, due to a wide range of hosts potentially infected by this fungus, which would indicate a lack of host-specificity among the strains of this species (38, 39). Sørensen et al. (40) described naturally occurring *F. avenaceum* isolates collected from apples that were able to produce enniatins. These cytotoxic compounds may pose a risk to consumer health in cases other than just those involving apples. The fact that *F. avenaceum* had the highest frequency in cultivars from Wiatrowo indicated a possibility of the presence of enniatins in pea tissues. In our study, four out of five *F. avenaceum*-infected cultivars were edible peas: Tarchalska, Santana, Medal and Cysterski, while only one strain was isolated from the fodder cultivar Turnia. The results obtained are in good accordance with earlier observations stating that edible cultivars are more susceptible to fungal infections (22).

The ability to synthesise mycotoxins is perhaps the most important threat arising from plant-pathogenic fungi. Certain reports have stated the specific profile of toxic secondary metabolites produced by individual fungal strains (16, 23). In the present study, it would be extremely difficult to perform mycotoxin analyses for all of the isolates. The identification and quantification of this mycotoxin group in collected pea plant material has been planned for future research. However, for some of the pathogens identified, the ability to produce specific mycotoxins (e.g., moniliformin and enniatins for *F. avenaceum* and fumonisins for *F. proliferatum* and *F. verticillioides*) is well-studied and proven for virtually all strains isolated from plant species (17, 33, 34). As for seed material, some of the samples were tested for the presence of mycotoxins. The measured levels, however, were extremely low; therefore, the results were not given.

A few isolates of *Phoma medicaginis var. pinodella* were found on the tested pea seeds. Davidson et al. (41) described the symptoms in pea seedlings caused by *Phoma sp*. and indicated that this fungus might be a seed-borne pathogen. However, in our experiments the pathogen was not commonly present in seed material. The most likely explanation of the low level of seed infection was probably related to the accumulation of reactive oxygen species (ROS) in plant tissues. The increase of ROS in aging pea seeds was observed in a study by Yao et al. (42) and higher ROS levels may have resulted in the inhibition of fungal growth (43).

In conclusion, the results obtained in the present study clearly show different levels of susceptibility to fungal infestations presented by pea cultivars grown in Poland. They also serve as a source of information on mycotoxins possibly present in seed material. Our future research will focus mainly on investigating the content of mycotoxins specific for individual fungal species in pea cultivar seed material. Such an approach could contribute to general knowledge in the area of common plant pathogens in agricultural environments of central Europe.

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Conflict of interest

Authors declare no conflict of interest.

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Sažetak

Patogene plijesni u sjemenkama različitih sorti graška u Poljskoj

Mahunasti usjevi izloženi su infekcijama patogenih plijesni, što često rezultira zarazom mikotoksinima. Cilj ovoga istraživanja bio je procijeniti stupanj otpornosti/podložnosti jestivih sorti graška i onih koji se koriste za krmivo na kolonizaciju sjemenki patogenim plijesnima tijekom dviju sezona te identificirati patogene u sjemenkama istraživanih sorti. Najčešća plijesan izdvojena iz sjemenki tijekom obiju sezona bila je *Alternaria* spp., a nju su brojnošću pratile *Fusarium* spp., *Stemphylium* spp., *Ulocladium* spp., *Botrytis cinerea* Pers., *Epicoccum nigrum* Link. i *Phoma pinodella* L. K. Jones. Najviši postotak zaraženih sjemenki (55 %) zabilježen je za sortu Ezop. Prisutnost većeg broja plijesni pronađen je 2012. u sortama Santana, Tarchalska, Medal, Cysterski, Mentor, Lasso i Ezop. Sorte korištene za krmivo pokazale su općenito nižu razinu zaraženosti od jestivih. Možemo zaključiti kako je *Alternaria* spp. bila najčešća plijesan u sjemenkama graška u Poljskoj, a *Fusarium* spp. vjerojatno najopasnija, uzimajući u obzir njene ustanovljene mikotoksigenične sposobnosti.

KLJUČNE RIJEČI: Alternaria; *bolesti prenosive sjemenkama;* Fusarium*; molekularna identifikacija; mikotoksini; patogeni sjemenki*

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