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The contamination of Fabaceae plants with fungi and mycotoxins

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This study aimed to assess the contamination of Fabaceae plants with fungi and mycotoxins, which have a negative effect on the health of consumers of plant products. Quantitative PCR was used to analyse the DNA of *Alternaria*, *Cladosporium* and *Fusarium* fungi in 69 plant samples harvested from four Russian regions in 2015. Additionally, mycotoxins were analysed using ELISA. *Cladosporium* fungi DNA was found in all the analysed samples; the occurrence of *Alternaria* and *Fusarium* DNA was 84% and 51%, respectively. Mycotoxin alternariol was detected in 100% and emodin in 90% of the samples. The occurrence of deoxynivalenol, diacetoxyscirpenol and T-2/HT-2 mycotoxins was 32%, 42% and 41%, respectively. Plant species had no significant effect on the *Alternaria*, *Cladosporium* and *Fusarium* DNA content of the samples; however, the alternariol, emodin, deoxynivalenol and diacetoxyscirpenol contents differed significantly between plant species. The geographical origin had a statistically significant effect on the *Alternaria* and *Fusarium* DNA contents, likely due to differences in weather conditions.

Key words: legumes, *Alternaria*, *Cladosporium*, *Fusarium*, quantitative PCR, ELISA

Introduction

Forage legumes are characterised by high nutritive and protein-balanced values and are the main component of animal feed. They also ensure the preservation and maintenance of soil fertility. The cultivation of legumes is associated with a variety of factors that affect the quality of the final yield. One of these is the effect of fungal diseases, which are often characterised by high harmfulness (Tivoli et al. 1996, Allen and Lenné 1998).

According to many researchers, *Alternaria* Nees, *Cladosporium* Link and *Fusarium* Link fungi are the dominant group of mycobiota in forage legumes (Leach 1955, Lager and Johnsson 2002, Al-Askar et al. 2012, Kononenko et al. 2015). Most identified *Alternaria* and *Cladosporium* species are characterised as saprotrophic organisms, whereas many *Fusarium* species although being highly aggressive pathogens for legumes can also lead a saprotrophic life (Leach 1955, Kellock et al. 1978, Zaccardelli et al. 2006, Chittem et al. 2015, Gossen et al. 2016).

Analysis of the occurrence of isolated fungi revealed that *Alternaria* and *Cladosporium* fungi were the most abundant in the mycobiota of alfalfa seeds, with 100% prevalence; the average infection rate of these fungi was 10% and 22%, respectively (Al-Askar et al. 2012).

Mycological analysis of the seed samples of four *Trifolium* species grown in the northwest of the USA found that *Cladosporium cladosporioides* (Fresen) G.A. de Vries significantly exceeded the occurrence of all other fungal species. This species was present in 11.5% of surface-sterilised seeds and almost 60% of untreated seeds. *Alternaria* fungi were the next most abundant (Leach 1955). *Alternaria* fungi prevailed in the microbiota of field peas in western Canada (Esmaeili Taheri et al. 2017).

Fusarium spp. appear to be quite common in Australia in *Medicago* plants, including, *F. acuminatum* Ellis et Everh., *F. avenaceum* (Fr.) Sacc., *F. equiseti* (Corda) Sacc., *F. chlamydosporum* Wollenw. & Reinking, and *F. graminearum* Schwabe (Barbetti et al. 2006). The infection of legume seeds can also be associated with *F. incarnatum* (Desm.) Sacc., *F. verticillioides* (Sacc.) Nirenberg and *F. solani* (Mart.) Sacc. (Lamprecht et al. 1988, Zaccardelli et al. 2006, Al-Askar et al. 2012). Some of these species impede the germination of seedlings and the vigour of germinated seedlings. *Fusarium* fungi were the most frequently isolated pathogens from the roots of grain legumes in the USA and Canada (Chittem et al. 2015, Gossen et al. 2016).

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Forage plants contaminated with fungi often cause various allergies in farmers and animals (Lugauskas et al. 2004, Ajoudanifar et al. 2011, Żukiewicz-Sobczak et al. 2013). Spores of *Cladosporium* and *Alternaria* fungi have been shown to be present in significant amounts in the air and can cause severe allergic reactions leading to serious health problems (Hjelmroos 1993, Damialis et al. 2017). Quantitative PCR (qPCR), which reveals the biomass of fungi through the content of their DNA in plant tissues, was used in the current study, has been established as the most convenient method for the quick and objective assessment of infection levels in crops (Nicholson et al. 1998, Pavón et al. 2012, Kulik et al. 2015). This method was successfully applied for the detection and quantification of the DNA of seven *Fusarium* species associated with legumes (Zitnick-Anderson et al. 2018, Żelechowski et al. 2019). Some fungal species existent in plant mycobiota have the ability to produce mycotoxins, which have a negative effect on human and animal health (Barbetti and Allen 2005). Used as feed, forage legumes can also be a source of mycotoxins that affect the quality of agricultural products. The species-specific character of mycotoxin production is well known, but it is also affected by environmental factors (Schmidt-Heydt et al. 2009, 2011, Magan and Medina 2016). Previous studies have demonstrated the contamination of forage grasses with *Alternaria* and *Fusarium* mycotoxins (Tan et al. 2011, Nichea et al. 2015). An analysis of mycotoxins in legumes in Russia also revealed a high occurrence and accumulation of mycotoxin alternariol (AOH) produced by *Alternaria* fungi, emodin (EMO) associated with *Cladosporium* fungi, and *Fusarium* mycotoxins (Gavrilova et al. 2017, Burkin and Kononenko 2018, Kononenko and Burkin 2018, 2019).

The aim of the current study was to assess the presence of *Alternaria*, *Cladosporium* and *Fusarium* fungi, and the mycotoxins they produce in the tissues of Fabaceae plants collected from various regions of European Russia in the context of plant traits.

Materials and methods

In total, 69 samples belonging to 12 plant species (*Galega orientalis*, *Lathyrus pratensis*, *Medicago falcata*, *Medicago sativa*, *Melilotus albus*, *Melilotus officinalis*, *Trifolium hybridum*, *T. pratense*, *T. repens*, *Vicia cracca*, *V. sativa* and *V. sepium*) of six genera from the Fabaceae family were collected from different regions of European Russia during the summer of 2015 (Table 1). Manuals were used to determine the plant species, life cycle, growth habit and melliferous capacity (Burmistrov and Nikitina 1990, Gubanov et al. 2003, Skvorcov 2004).

Table 1. Characteristics of the plant species used in this study

Genus	Species		Number of samples	Stem growth	Life cycle	Melliferous capacity (kg ha^{-1})
Galega Tourn. ex L.	*G. orientalis* Lam.	fodder galega	4	erect	perennial	+ (60)
Lathyrus L.	*L. pratensis* L.	meadow vetchling	6	climbing	perennial	− (30)
Medicago L.	*M. sativa* L.	alfalfa	4	erect	perennial	+ (170)
	M. falcata L.	sickle medick	2	erect	perennial	+ (170)
Melilotus Mill.	*M. albus* Medik.	white sweet clover	6	erect	annual or biennial	+ (200)
	M. officinalis (L.) Pall.	yellow sweet clover	3	erect	biennial	+ (200)
Trifolium L.	*T. hybridium* L.	alsike clover	9	creeping	perennial	+ (145)
	T. pratense L.	red clover	13	creeping	perennial	+ (90)
	T. repens L.	shamrock	7	creeping	perennial	+ (120)
Vicia L.	*V. sepium* L.	bush vetch	3	climbing	perennial	− (9)
	V. cracca L.	tufted vetch	10	creeping / climbing	perennial	+ (69)
	V. sativa L.	common vetch	2	erect	annual or biennial	− (9)

Geographically, the analysed samples originated from Leningrad Oblast (n=44), Novgorod Oblast (n=5), Pskov Oblast (n=11) and Smolensk Oblast (n=9). These geographical areas are located throughout the north-western (Leningrad Oblast) and central European regions (Smolensk Oblast).

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The plant samples were collected in May and June (n=26), in July (n=20) and in August (n=23). The weather conditions are presented in the Table 2.

Table 2. Weather conditions during 2015 in the sampled regions

Region (number of samples)	Month	Month temperature, °C			Average humidity, %	Month rainfall, mm
		mean	min	max		
Leningrad Oblast (n=44)	May-June	13.8	5.3	25.1	64	68
	July	16.9	7.9	25.1	73	86
	August	18.2	9.1	28.2	68	47
Novgorod Oblast (n=5)	May–June	14.1	3.1	25.4	68	66
	July	16.9	7.8	29.7	73	45
	August	17.3	5	28.2	71	38
Pskov Oblast (n=11)	May–June	16.7	4.4	27.5	93	90
	July	18.2	4.2	29.2	72	94
	August	19.0	3.8	30.4	71	24
Smolensk Oblast (n=9)	May–June	15.9	6.0	27.8	63	62
	July	18.8	8.5	31.4	69	61
	August	20.1	7.0	32.8	60	7

Vegetative plants were cut at a height of 5 cm above the soil surface. The plants were dried at a temperature not higher than 50 °C. The dried plant samples were then separately homogenised in the sterilised grinding chambers of a batch mill (Tube Mill Control, IKA, Königswinter, Germany).

The total DNA from 200 mg of milled plant material was isolated using the Genomic DNA Purification Kit (Thermo Fisher Scientific, Vilnus, Lithuania) following the manufacturer's protocol. Using the same kit, DNA was also isolated from the mycelium of strains of *F. graminearum* MFG 58775, *A. tenuissima* MFP 556081 and *Cladosporium* sp. MFP 235011 cultivated on potato-sucrose agar. All the fungal strains were maintained in the collection of the Laboratory of Mycology and Phytopathology of the All-Russian Institute of Plant Protection, St. Petersburg, Russia. DNA concentrations from the samples and fungal strains were determined using a Qubit 2.0 fluorometer with a Quant-iT dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and aligned to 2–50 pg ng^{-1}.

The DNA content of *Alternaria*, *Cladosporium* and the group of *Fusarium* fungi producing trichothecene mycotoxins (Tri-*Fusarium*) were evaluated using qPCR with TaqMan probes (Halstensen et al. 2006, Zeng et al. 2006, Yli-Mattila et al. 2008, Pavon et al. 2012). The reaction was carried out in a 20 µl volume containing 10 µl of a 2 × TaqAB master mix (AlkorBio, St. Petersburg, Russia), 300 nM of each primer, 100 nM of a fluorescent sample (Evrogen, Moscow, Russia) and 2 µl of the DNA sample. All the qPCR assays were run using a thermocycler (CFX 96 Real-Time System; BioRad, Hercules, CA, USA), and the DNA content was presented as the ratio of fungal DNA to total DNA in each sample (pg ng^{-1}). A low quantification limit of 5×10^{-4} pg fungal DNA on one ng of total DNA was established as the DNA threshold value for the samples; this could be quantitatively determined with high precision. All the samples were analysed at least twice.

For the mycotoxin detection, 1 g of milled plant material was placed in a tube containing 10 ml of a mix of acetonitrile and water (84:16 v/v, respectively). The tube was shaken vigorously and left to incubate for 12–14 h. After repeated shaking and 10-fold dilution with a buffer solution, the extract was used for an indirect competitive used enzyme-linked immunosorbent assay (ELISA). The determination of T-2/HT-2 toxins, deoxynivalenol (DON), diacetoxyscirpenol (DAS), AOH and EMO was performed using certified kits of immunoreagents and calibrants intended for the control of objects of plant origin (VNIIVSGE, Moscow, Russia). The lower limit of the quantitative measurements corresponded to an 85% level of antibody binding.

The data were analysed using Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) and Statistica 10.0 (StatSoft, Tulsa, OK, USA). A standard t-test was used to determine statistically significant differences between the means. Relationships between the quantitative traits were evaluated using the linear Pearson correlation coefficient (r). The results were considered significant when $p < 0.05$.

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Results

The DNA of *Alternaria* fungi was detected in 84% of the samples. The maximum DNA content of these fungi was found in a *Melilotus officinalis* sample from Pskov Oblast: 3620×10^{-4} pg ng^{-1}. The average *Alternaria* DNA content in the analysed samples was 42×10^{-4} pg ng^{-1}. The DNA of *Cladosporium* fungi was found in 100% of the samples in the range of 7×10^{-4} to 5490×10^{-4} pg ng^{-1}, with an average content of 586×10^{-4} pg ng^{-1}. The maximum *Cladosporium* DNA content was found in a sample of red clover from the Leningrad Oblast. The DNA of Tri-*Fusarium* fungi was detected in 51% of the samples in amounts of up to 492×10^{-4} pg ng^{-1} (i.e. meadow vetchling from Smolensk Oblast).

Mycotoxin AOH was detected in 100% of the samples in amounts ranging from 20–1549 ppb, with an average content of 196 ppb. EMO was detected in 90% of the samples in amounts ranging from 17–10000 ppb, with an average content of 652 ppb. DON mycotoxin was detected in 32% of the samples ranging from 100–631 ppb, DAS in 42% of the samples ranging from 141–1892 ppb and T-2/HT-2 toxins in 41% of the samples ranging from 4–27 ppb. The average contents of DON, DAS and T-2/HT-2 toxins were 221, 437 and 10 ppb, respectively.

The effect of plant species

The occurrence of *Alternaria* fungi varied widely among the sampled plant species and genera: from 33% in the bush vetch samples to 100% in the samples of common vetch, red clover, shamrock and sickle medick. On average, *Melilotus* plants accumulated the largest amount of *Alternaria* DNA compared to plants of other genera (Fig. 1). Plants from the *Lathyrus* and *Trifolium* genera were characterised by high levels of *Cladosporium* DNA compared to other legumes, whereas *G. orientalis* plants were the least contaminated by these fungi. The occurrence of Tri-*Fusarium* varied from 25% in the *Melilotus officinalis* samples to 89% in the *Trifolium hybridum* samples, but the largest amounts of Tri-*Fusarium* DNA were detected in *Lathyrus* plants.
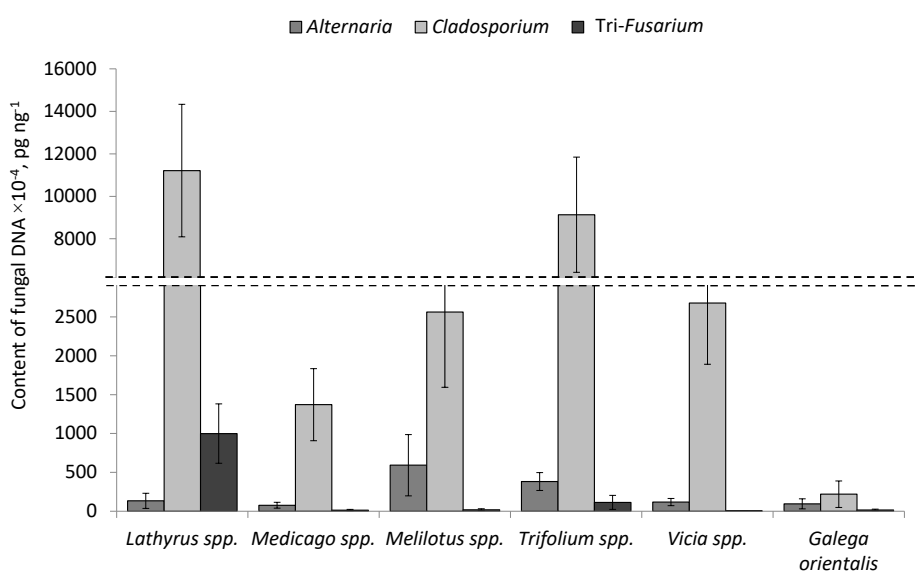

Fig. 1. *Alternaria*, *Cladosporium* and Tri-*Fusarium* DNA contents in samples of legume plants. The bars indicate confidence intervals with a 95% significance level.

On average, the *Lathyrus* plant samples were the most contaminated by AOH (694±176 ppb), while *Vicia* and *Melilotus* plants were the least contaminated (53±9 and 91±16 ppb, respectively). On average, samples of *Trifolium* plants (1284±402 ppb) were the most contaminated by EMO compared to the other legumes (from 19±11 ppb in *Melilotus* plants to 257±86 ppb in *Lathyrus* plants). *Lathyrus* plant samples were most contaminated by *Fusarium* mycotoxins (DON = 295±99 ppb, DAS = 879 ± 279 ppb and T-2/HT-2 toxins = 11 ± 4 ppb), while none of the *Fusarium* mycotoxins were detected in the *Vicia sepium* samples.

An absence of a significant effect for plant species (12 species) on the amount of *Alternaria*, *Cladosporium* and Tri-*Fusarium* DNA in the samples was established. The effect of the genus (six genera) was substantial only for the amount of Tri-*Fusarium* DNA. The content of AOH, EMO, DON and DAS mycotoxins in the samples depended on

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the plant species. The effect of plant genus on the accumulation of mycotoxins also proved to be significant. However, the accumulation of T-2/HT-2 toxins was not dependent on either the plant species or genera.

The effect of the geographical origin of the plant samples

The average amounts of fungal DNA and mycotoxins differed in the legume samples depending on the region of origin (Table 3).

The occurrence of *Alternaria* fungi varied from 56% (Smolensk Oblast) to 93% (Leningrad Oblast), and *Cladosporium* fungi was detected in 100% of the examined samples. On average, the samples from Pskov Oblast were most infected by both these fungi. The occurrence of Tri-*Fusarium* fungi in the regions ranged from 45% (Pskov Oblast) to 60% (Novgorod Oblast), but on average, the samples from Smolensk Oblast were 30–60 times more infected by these fungi. The geographical origins of the plant samples had a significant effect on the amount of *Alternaria* and Tri-*Fusarium* DNA, but an effect by this factor on *Cladosporium* infection was not seen.

AOH was found everywhere, whereas the occurrence of EMO in the samples varied from 60% (Novgorod Oblast) to 93% (Leningrad Oblast). DON was not detected in the samples from Novgorod Oblast; the highest frequency of this mycotoxin was found in the samples from Leningrad Oblast and Pskov Oblast (36% each). The occurrence of DAS in the samples ranged from 27% to 48%, and the occurrence of T-2/HT-2 toxins ranged from 18% to 56%.

The maximum amounts of AOH, DON, DAS and T-2/HT-2 toxins were detected in the samples from Smolensk Oblast. The samples from Leningrad Oblast were more contaminated by EMO. The geographical origin of the plant samples had a substantial effect on the accumulation of DON and DAS, while the influence of this factor on the accumulation of the other mycotoxins was not statistically significant.

Table 3. Fungal DNA and mycotoxin contents in legume plants collected from different regions of Russia in 2015

Geographical origin of plant samples (number of samples)	The average amount of fungal DNA ±CI ×10^{-4}, pg ng^{-1}			The average amount of mycotoxins ±CI, ppb				
	Alternaria	*Cladosporium*	Tri-*Fusarium*	AOH	EMO	DON	DAS	T-2/HT-2 toxins
Leningrad Oblast (n=44)	204±43	4306±1356	14±3	182±26	836±279	63±14	152±26	5±1
Novgorod Oblast (n=5)	176±65	2707±445	33±22	58±12	55±28	0	60±36	2±1
Pskov Oblast (n=11)	857±387	10572±4741	17±13	192±79	99±27	55±25	94±47	1.4±0.7
Smolensk Oblast (n=9)	62±33	9466±3191	965±567	343±162	252±73	192±95	564±267	9±3

CI = confidence interval with significance level of 95%

The effect of the month of sample collection

The distribution of samples contamination by fungi and mycotoxins was determined to depend on the month the plants were collected (Table 4).

The occurrence of *Alternaria* fungi increased from 85% in samples collected in May and June to 90% in samples collected in July, but it decreased to 78% in the August samples. Similarly, the occurrence of Tri-*Fusarium* fungi was 50%, 60% and 52%, respectively. The occurrence of T-2/HT-2 toxins, DON and DAS mycotoxins increased at the end of the growing season. The proportion of samples in which at least one trichothecene mycotoxin was detected was 46%, 55% and 70% in May to June, July and August, respectively.

The total fungal DNA and all analysed mycotoxin contents in the forage legume samples increased during the growing season. An exception was the content of *Alternaria* DNA and EMO, which reached maximums in July. The effect of toxins by sampling month on the accumulation of metabolites in the samples was only significant for *Alternaria* and *Cladosporium* DNA and T-2/HT-2 toxins.

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Table 4. *Alternaria*, *Cladosporium* and Tri-*Fusarium* DNA and mycotoxins in samples of legume plants collected at different times during the 2015 growing period

The month (number of samples)	The average amount of fungal DNA ±CI ×10^{-4}, pg ng^{-1}			The average amount of mycotoxins ±CI, ppb				
	Alternaria	*Cladosporium*	Tri-*Fusarium*	AOH	EMO	DON	DAS	T-2/HT-2 toxins
May-June (n=26)	164±51	285±54	18±6	156±28	580±254	53±15	111±30	1.5±0.5
July (n=20)	585±224	8133±2680	17±7	199±52	118±24	40±15	136±41	3±1
August (n=23)	169±46	10192±2714	385±235	239±71	999±448	126±42	325±111	8±2

CI = confidence interval with significance level of 95%

The effect of plant habit

Sampled plants with erect stems (*V. sativa*, *Melilotus* spp., *G. orientalis* and *Medicago* spp.) had a lower occurrence of *Alternaria* (71%) and Tri-*Fusarium* (42%) fungi than the samples of plants with creeping stems (*Lathyrus* spp., *Trifolium* spp., *V. sepium* and *V. cracca*): 91% and 60%, respectively.

On average, the *Alternaria* and Tri-*Fusarium* DNA contents did not differ significantly for plant samples with erect or creeping stems (Fig. 2). However, a significant effect of stem growth on infection with *Cladosporium* fungi was revealed. On average, plants with creeping stems accumulated the DNA of these fungi 4.5 times more than plants with erect stems.

The occurrence of trichothecene mycotoxins in the erect-stem plant samples was 2.5–5 times lower than in the creeping-stem plant samples. The proportions of plant samples with erect and creeping stems, in which at least one trichothecene mycotoxin was found, were 28% and 70%, respectively. Plants with erect stems were significantly less contaminated by all the analysed mycotoxins. The most substantial difference was for EMO; its content in creeping plants was 17 times higher than in erect plants. The AOH and trichothecene mycotoxin contamination of the creeping plants was 2.5–6 times more than that of the erect plants. Plant habit had a significant effect on the accumulation of all the analysed mycotoxins.
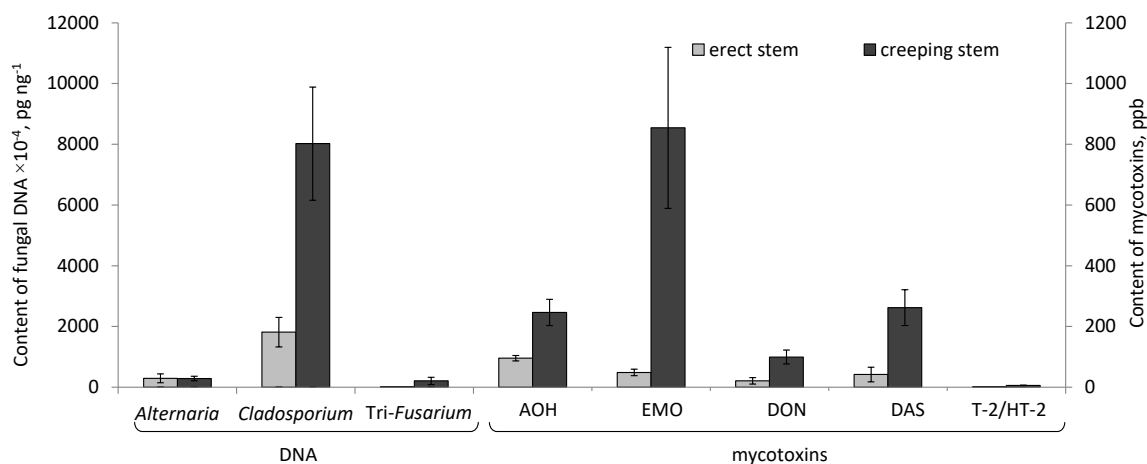

Fig. 2. Fungal DNA and mycotoxins contents in legume plant samples with different stem growths. The bars indicate confidence intervals with a 95% significance level.

The effects of life cycle and melliferous capacity

The occurrence of *Alternaria* fungi in the annual/biennial legume (*Melilotus* spp. and *V. sativa*) and perennial legume plant samples (*G. orientalis*, *Lathyrus* spp., *Medicago* spp., *Trifolium* spp., *V. sepium* and *V. cracca*) was 73% and 88%, respectively. At the same time, the perennial legume samples were 2 times less contaminated with *Alternaria* fungi compared to the annual/biennial plants. *Cladosporium* fungi were found in 100% of the samples, but no significant effect of life cycle on the accumulation of *Cladosporium* DNA was found. The occurrence of Tri-*Fusarium* fungi did not depend on life cycle either; they were detected in half of the samples in each group: 55% and 53%, respectively. However, perennial legume plants contained an average of 10 times more Tri-*Fusarium* DNA than the annual/biennial plants (Fig. 3A).

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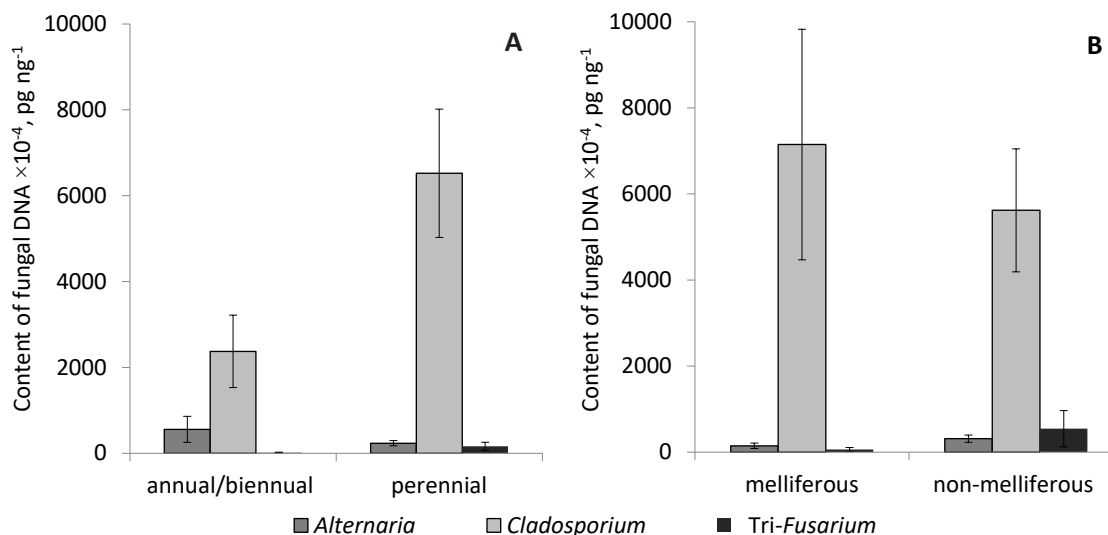
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Fig. 3. Fungal DNA content in legume plant samples with different (A) life cycles and (B) melliferous capacity. The bars indicate confidence intervals with a 95% significance level.

The occurrence of all analysed mycotoxins in the annual/biennial plant samples was 2–6 times less than in the perennial grasses. The perennial legumes also contained more of all the analysed mycotoxins compared to the annual/biennial plants, but this difference was not statistically significant for any of the mycotoxins.

On average, the occurrence of *Alternaria* and Tri-*Fusarium* fungi in melliferous legume plants was higher (90% and 57%, respectively) than in non-melliferous legumes (64% and 36%, respectively), while *Cladosporium* fungi were found in 100% of the samples.

The contamination of melliferous and non-melliferous legumes with *Alternaria* and *Cladosporium* fungi was not significantly different (Fig. 3B). Non-melliferous plants were 12 times more contaminated with Tri-*Fusarium* fungi than melliferous plants, and this difference was significant.

The occurrence of AOH and EMO mycotoxins in the samples of melliferous and non-melliferous legumes was approximately the same, ranging from 88% to 100%. The occurrence of trichothecene mycotoxins in the non-melliferous legumes was in the range of 28%–40%, which was less than the similar values for melliferous plants, in which the occurrence of these mycotoxins was 55%–64%. The contamination of non-melliferous legumes by AOH, DON and DAS trichothecene mycotoxins was 1.5–5 times higher than for melliferous plants and the difference was significant. The opposite tendency was observed for EMO: this toxin accumulated in non-melliferous legumes 4.5 times less than in melliferous ones, but it was not statistically significant.

Discussion

Differences in the habits and life cycles of legumes complicate the correct analysis of their microbiological contamination. The facility of molecular methods, whereby DNA is extracted in the first stage from an average homogeneous sample obtained by grinding whole plants, represents great advantages compared with traditional methods. The active application of molecular methods to assess the microbiological status of plants has been observed in recent years (Kulik et al. 2015, Gagkaeva et al. 2017, Zitnick-Anderson et al. 2018).

In the mycobiota of forage legumes, *Alternaria*, *Cladosporium* and *Fusarium* fungi have been found to be the dominant group (Leach 1955, Lager and Johnsson 2002, Al-Askar et al. 2012, Kononenko et al. 2015). In our study, *Cladosporium* fungi were also the most common among the three analysed groups of fungi, and they were found in all the samples. The most favourable substrates for these fungi were *Lathyrus* and *Trifolium* plants, in which a high content of *Cladosporium* DNA was found. The DNA content of these fungi in the group of plants with creeping stems was significantly higher than in plants with erect stems. According to Tivoli et al. (1996), as a result of higher humidity at the base of the pea canopy, greater development of leaf blight is observed in the lower part of pea plants. In support of this, accessions of *Trifolium pratense* with erect growth habit were less susceptible to rot compared with creeping forms of red clover (Vleugels et al. 2013).

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In the spring samples, the content of *Cladosporium* DNA was significantly lower compared with the plants collected later in the year. The colonisation of pea (*Pisum sativum*) leaves by fungi has been previously described; an increase of predominant *Cladosporium* spp. by the end of the growing season has been observed (Dix and Webster 1995). Probably, the dramatic prevalence of *Cladosporium* conidia in the air compared to the number of other fungal conidia (Grinn-Gofroń et al. 2016) is associated with the significant contamination of plants by these fungi.

On average, the highest amount of *Alternaria* DNA was found in the *Melilotus* plants. The geographical origin and the month sampled had a statistically significant effect on the content of *Alternaria* DNA, while the effects of plant species, genus and traits were accidental. Therefore, environmental conditions had the greatest effect on the distribution of these fungi. The contamination of legume plants by *Alternaria* fungi reached a maximum in July compared with samples collected earlier and later. According to available weather conditions in the analysed regions, July 2015 was characterised by the highest rainfall in most areas. The correlation between rainfall and *Alternaria* DNA content was significant (r=+0.28), while the average temperature and humidity were not substantial. Differences in the *Alternaria* DNA content between the regions were congruent: samples from the Pskov Oblast with the highest rainfall were the most infected, while samples from the Smolensk Oblast with the minimum rainfall were the least infected. It is known that the penetration and growth of fungi in plant tissues are affected by environmental conditions, but according to some researchers, the effect of rainfall during the growing season has a more significant effect on the development of *Alternaria* leaf blight than temperature (Saharan and Saharan 2004, Meno et al. 2019).

Tri-*Fusarium* fungi producing trichothecene mycotoxins were found in half of the analysed legume samples. On average, a significantly higher content of their DNA was found in the *Lathyrus* plants. The geographical origin of the samples had a statistically significant effect on the content of Tri-*Fusarium* DNA, while the effects of sampling month, plant species, genus and traits were accidental. In the plant samples from the Smolensk Oblast, the average content of Tri-*Fusarium* fungi was higher than in samples from other regions. The weather conditions in this region were characterised by the highest average temperature for all the summer months during 2015 compared with the other analysed regions. The correlation between the average monthly temperature and the Tri-*Fusarium* DNA content was significant (r=+0.29).

Analysis of the relationships between the analysed fungi revealed a significant positive correlation between the *Alternaria* and *Cladosporium* fungi DNA contents in the plant samples (r=+0.37). In the samples collected in July and August, this connection was more substantial (r=+0.46 and +0.56, respectively). When analysing the fungi contamination of samples grouped according to traits, this connection was even more significant: in plants with creeping stems, the correlation coefficient between the *Alternaria* and *Cladosporium* fungi DNA contents was r=+0.47, and in the annual/biennial plant samples, its value reached r=+0.50. This demonstrates the similarity of the conditions required for the growth of *Alternaria* and *Cladosporium* fungi, especially in conditions favourable for saprotrophic fungi (i.e. proximity of the soil and aging plant tissue). Previous molecular analyses have revealed a similarly significant relationship between *Alternaria* and *Cladosporium* fungi coexisting in small grain cereals (Kulik et al. 2015, Gagkaeva et al. 2017) and legumes (Orina et al. 2018).

Our study did not reveal any significant effect of sampling month on the accumulation of mycotoxins in legumes tissues. A tendency for mycotoxin accumulation over the growing season was not found in *T. pratense*, *Medicago* spp. or *Melilotus* spp. plants collected from other regions of Russia (Burkin and Kononenko 2018, Kononenko and Burkin 2018). In addition, there were no differences in the content of *Fusarium* mycotoxins in naturally growing herbs from the Poaceae family collected in spring and summer in one region of Argentina (Nichea et al. 2015). However, the EMO, AOH, DON and DAS contents in samples of *Lathyrus pratensis* collected in August were significantly higher than in plant samples collected in May and June (Kononenko and Burkin 2019).

No statistically significant effect of life cycle on the amount of fungal DNA or mycotoxin content was detected, although, according to our results, the perennial legumes contained more of all the analysed mycotoxins compared with the annual/biennial plants. This is consistent with Engels and Krämer (1996), who found high T-2 toxin, ZEA and, especially, DAS content in perennial ryegrass compared with annual ryegrass.

According to our results, the content of *Alternaria* and *Cladosporium* DNA in melliferous and non-melliferous legumes was not significantly different. The opposite situation was found in the case of the Tri-*Fusarium* fungi: non-melliferous legumes were contaminated with significantly higher amounts of Tri-*Fusarium* DNA than melliferous ones, as well as with DON and DAS mycotoxins. According to some researchers, flowering plant pollen can stimulate conidia germination and the growth of fungi on leaf surfaces by reducing nutritional competition.

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The abundance of pollen in the atmosphere coincides with an increase in the numbers of *Alternaria* and *Cladosporium* conidia during the summer months, and pollen and spores are deposited together on plant surfaces in great numbers (Dix and Webster 1995). Bees can also contribute to plant infection by transferring fungal conidia. Analyses of bee pollen have revealed the ubiquitous presence and predominance of *Alternaria*, *Mucor*, *Aspergillus* and *Cladosporium* fungi, and *Aureobasidium* and *Penicillium* have also often been found, whereas *Fusarium* fungi have not been detected (Osintseva and Chekryga 2008, Kačániová et al. 2011, Deveza et al. 2015).

A correlation between the content of *Alternaria* DNA and mycotoxin AOH in all the analysed legume samples was not detectable. This was probably because the current study analysed the presence of these fungi at the genus level, while different *Alternaria* species are characterised by a contrasting ability to produce this mycotoxin (Andersen et al. 2002). Additionally, the correlation between the *Cladosporium* DNA and mycotoxin EMO contents was significant (r=+0.38). Various fungi can produce EMO (Kusari et al. 2008), and this is also a known metabolite of some plants (Izhaki 2002). *Cladosporium* fungi are probably not the exclusive producers of this mycotoxin in legume tissues; however, the contribution of these fungi is substantial. A correlation was established between the Tri-*Fusarium* DNA and DON and DAS mycotoxins contents (r=+0.39 for each) observed in the group of creeping-stem plants (r=+0.31 and +0.33, respectively) and perennial plant samples (r=+0.34 and +0.36, respectively). The correlation between the trichothecene mycotoxin contents was also significant (T-2/HT-2 toxins and DON=+0.48, T-2/HT-2 toxins and DAS=+0.52, and DON and DAS=+0.85).

The current study's findings on the distribution of fungi and their relationships with host plants and each other are of high scientific and practical interest. Knowledge about the contamination of legumes with fungi and mycotoxins is important for human and animal health. It is obligatory to use fast, highly sensitive methods for the detection of fungi and their metabolites in plants to reliably predict possible dangers to consumers.

Acknowledgements

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