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Effects of genotype and environmental factors on rape seed contamination with mycotoxins and mycotoxin-producing fungi

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Studies on pathogenic infection level and the content of pathogen-produced toxins on the seed of different winter rape cultivars as affected by different factors were conducted at the Lithuanian Institute of Agriculture. Microbiological seed contamination of different winter rape cultivars ranged within 1.4–2.6 cfu $g^{-1} \times 10^4$. In the seed samples of all winter rape cultivars, the dominant fungi were those belonging to the genera *Cladosporium*, *Alternaria*, *Penicillium* and *Fusarium*. For the first time we investigated toxins produced by rape seed fungi and found the mycotoxin deoxynivalenol in separate cultivars to amount to 164–183 μg kg^{-1} seed. Aflatoxin content ranged from 1.0 μg kg^{-1} in the seed of cv. 'Alaska' to 3.1 μg kg^{-1} in the seed of cv. 'Triangle'. Even greater variations between the cultivars were identified for ochratoxin A content in seed. Having determined the level of aflatoxin and ochratoxin A on the seed of various winter rape cultivars after an eight-month storage, a reduction trend was observed for both toxins tested. Experimental evidence suggests that the effect of anthropogenic factors on the surface and internal fungal contamination of seed of winter rape cultivars was not very pronounced, however, during the eight-month storage the seed surface fungal infection declined, and an especially marked reduction occurred in the level of infection with the fungi of the genera *Alternaria* and *Botrytis*.

Key words: winter oilseed rape, seed contamination, mycotoxins

INTRODUCTION

Grains, seeds and many spices are a major source of food and feed and the raw material for many industrial products. From the early stage of their formation on growing plants until their use and consumption, they are subjected to damage by several biological agents, mainly fungi (Christensen, 1991). Fungi that invade grains, seeds and spices in storage may be responsible for reduction in germinability, discolouration and total spoilage including heating and losses of dry matter (Lacey, Magan, 1991). Both in the field and in storage, seed invading fungi can produce mycotoxins that may cause illness to man and animals after their ingestion (Frisvad, Samson, 1991; Miller, 1995; Rizk, Botros, 2006). Mycotoxins are relatively small molecules characterized by a diversity of chemical structure and biological activity. They are often genotypically specific of a group of species, but the same compound can also be formed by fungi belonging to different genera (Abarca et al., 2000). Mycotoxin contamination of food and feed presents a serious food safety issue on a global scale, causing tremendous yield and economic losses (Brodhagen, Keller, 2006).

Conventional plant breeding for resistance to pathogens, although successful, is in many cases still too slow to keep pace with pathogen adaptation, and suffers from the lack of genetic variability in cultivated varieties. Phytotoxins, because of their role in disease development, have been proposed as convenient markers for early screening of resistant genotypes and as selective agents for in vitro selection (Buiatti, Ingram, 1991).

Singly or collectively the fungi attack and blacken the fruit-bearing branches and cause seed shedding. Seventy fungi species attributed to 36 genera have been identified and found to spread with rape seed in Canada (Babadoost, Gabrielson, 1979; Clear, Patrick, 1995). Russian research evidence suggests that before thrashing siliques were heavily infected by *Alternaria brassicae* and *Alternaria brassisicola* (Schwein.) micromycetes as well as by saprotrophic fungi (*Cladosporium* spp., *Alternaria alternata)* (Портенко, 1997). In Poland, seed analyses showed that *Alternaria brassicae*

was more frequent on rape seed compared with *Alternaria brassisicola* (Sadowski, Klepin, 1991; Lewartowska et al., 1993). A specific composition of fungi in Lithuania was detected on seeds of rape, maize, soy and on their products. The most frequent fungi were as follows: *Aspergillus niger*, *A clavatus*, *A. vesicolor*, *Fusarium oxysporum*, *F. avenaceum*, *Penicillium expansum*, *P. palitans*, *P. roquefortii*, *P. viridicatum*, *Alternaria alternata*, *Rhizomucor pusillus*. Fungi most intensively producing toxins were identified and the intensity of their synthesis was ascertained. More active strains belong to the species *Alternaria brassicae*, *A. pluriseptata*, *Chrysosporium merdarium*, *Fusarium solani*, *Fusarium* sp., *Penicillium expansum*, *Penicillium* sp. (Kačergius et al., 2005).

Researchers of various countries indicate that the genus *Alternaria* produces mycotoxins alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins I, II, and III (ATX-I, -II, and -III), and L-tenuazonic acid (TeA) (Montemuro, Visconti, 1992; Silvana da Mottia, Soares, 2000). The post-harvest fungus *Penicillium verrucosum* var. *cyclopium* is the predominant *Penicillium* species on stored cereals and oilseeds in western Canada, and one of the main causes of spoilage (Mills, 1980; Sinha et al., 1969; Mills, Abramson, 1982). The fungus is known to produce penicillic acid (Northolt et al., 1979b; Scott et al., 1972) and ochratoxin A, a potent nephrotoxin which also demonstrates teratogenic activity (Northolt et al., 1979a). The predominant fungal species present in Spain were *Alternaria alternata* (Fries) Keissler, *Penicillium* spp. and *Aspergillus flavus*. Only aflatoxin B_1 was detected in 1 of the 20 samples analyzed, with a concentration of 0.25 μg kg^{-1}. Of the 40 *Aspergillus flavus* strains isolated from oilseed rape samples, only three revealed aflatoxigenic capacity. None of the *Penicillium* spp. isolated from oilseed rape samples showed mycotoxigenic capacity (citreoviridin, griseofulvin, citrinin, patulin and penicillic acid) (Vińas et al., 1994). A large number of metabolites have been reported, so that the situation is similar to the *Fusarium* mycotoxins in that, in the present state of knowledge, only a few occur naturally in food commodities or are of major toxicological significance.

It is the first study of toxins produced by rape seed fungi in Lithuania. This paper describes the effects of genotype and various factors on seed contamination with mycotoxins and fungi producing mycotoxins.

MATERIALS AND METHODS

Field trials. Different winter oilseed rape cultivars (Alaska, Courage, Digger, Milena and Triangle) were used in field experiments. Winter oilseed rape was grown following the conventional technology. The disease control agents (a.i. tebuconazole 250 g ha^{-1}, a.i. metconazole 90 g ha^{-1} and a.i. azoxistrobine 250 g ha^{-1}) were applied at the end of winter oilseed rape flowering (BBCH 69) (Lancashire et al., 1991). The seed of winter oilseed rape was harvested by a Sampo 500 combine harvester, separately for each plot. One thousand g seed samples were taken for laboratory analyses. In the laboratory, samples of seed of each cultivar were analysed. Analysis of seed surface contamination with fungi and internal infection were made at harvesting and after eight months of storage. Seed samples for analysis were stored at a 48–50% relative air humidity and at a temperature of 16–18 ˚C.

Fungi occurring on oilseed rape seed. Internal infection of seeds was determined on agarized potato dextrose medium (PDA) (Fluka, Switzerland), in Petri dishes. Seed surface was sterilised for 30 s by soaking seeds in 70% ethyl alcohol. Then the seeds were rinsed in sterile water, dried on sterile filter paper and placed on PDA, 20 seed per plate, in a sterile chamber. The plates were incubated for 7 days at 20 ˚C in a thermostat (Binder, Germany) for 12 h in the light and 12 h in the dark. The spores of fungi were identified by microscope (Leica DM LS) examination. For the identification of fungal genera according to conidia, descriptors and other literature were used (Lugauskas et al., 1997; Malone, Muskett, 1997).

Mycological analysis. For the evaluation of seed surface contamination with fungi, the dilution plating technique was applied. Ten grams of seed was placed in 100 ml of sterile water, shaken for 10 min, dilutions (1:100, 1:1000, 1:10000) were made and 1 ml of suspension was drawn into a Petri dish and poured over with malt agar medium containing chloramphenicol (50 mg l^{-1}) (Samson et al., 1992). The analysis of each sample was performed in three replications. Fungi were cultivated for 7–10 days at a temperature of 28 ˚C. Colony forming units (cfu) of fungi per 1 g of seed and the distribution frequency (as a relation of the number of samples where the species were found to the total number of examined samples, expressed in %) of the dominating species were calculated. Pure fungal strains were isolated on Czapek–Dox agar (Merck), malt extract agar (MEA, Merck), potato dextrose agar (PDA, Merck) media and identified according to manuals. Fungal species were determined according to Satton et al., 2002; Mathur, Kongsdal, 2003; Malone, Muskett, 1997; Lugauskas et al., 2002; Malloch, 1997.

Mycotoxin analysis. Rape seed samples (200 g) were stored at –18 ˚C prior to examination for mycotoxins. The mycotoxins deoxynivalenol (DON), ochratoxin A, aflatoxin (total) concentration in 2005 was determined on sub-samples of 50 g of seed per each plot. Seed samples were analysed by the ELISA (enzyme-linked immunosorbent assay) method (Bennet et al., 1994; Wilkinson et al., 1991). The Veratox® aflatoxin, Veratox® DON 5/5, Veratox® ochratoxin A test kits (Neogen, USA) were used for the analysis. Mycotoxin extraction and tests were performed according to the manufacturer's instructions. Multiskan MS was used for the reading of immunoenzymic microstrips.

Statistical analysis. The experimental data were processed by the analysis of variance and correlation–regression analysis method. Fisher's Protected Least Significant Difference method was used to determine significant differences between the means.

RESULTS AND DISCUSSION

The seed samples of different winter oilseed rape cultivars showed a variable incidence of fungal contamination. The highest fungal contamination, 2.6 cfu $g^{-1}\times10^4$, was recorded for the seeds of cv. 'Courage' and the lowest contamination for the seeds of cv. 'Milena', which was twice as low as that of cv. 'Courage' (Table 1). The predominant fungi present in the seed samples were *Cladosporium* spp., *Alternaria* spp., *Penicillium* spp. and *Fusarium* spp. The results showed that different species of fungi were able to produce mycotoxins: deoxynivalenol (DON), aflatoxins (AF) and ochratoxins A (OTA). The highest concentrations were identified of the mycotoxin DON, in the seeds of separate cultivars its content ranging from 164 to 183 µg kg^{-1}, and the variations between the cultivars were not very marked (Table 2). The content of AF toxins ranged from 1.0 µg kg^{-1} on cv. 'Alaska' to 3.1 µg kg^{-1} on cv. 'Triangle' seed. Even higher variations between the cultivars were recorded according to the contents of OTA toxins in seeds. The highest contents of all three mycotoxins were identified in the seeds of cv. 'Triangle', however, their microbiological contamination was not the highest.

Table 1. **Microbiological contamination (cfu $g^{-1}\times10^4$) of winter rape seed of different cultivars and incidence of fungi**

Cultivar	Fungi, genus	cfu g^{-1} x10^4
'Alaska'	*Cladosporium, Alternaria, Penicillium, Botrytis*, *Aspergillus*	2.2
'Courage'	*Cladosporium, Alternaria, Fusarium, Penicillium*	2.6
'Digger'	*Cladosporium, Alternaria, Botrytis*, *Penicillium, Fusarium*	1.9
'Milena'	*Cladosporium, Alternaria, Botrytis, Fusarium, Penicillium*	1.4
'Triangle'	*Cladosporium, Alternaria, Fusarium, Penicillium, Aspergillus, Botrytis*	1.8

Surface and internal fungal infection of seeds of various winter rape cultivars was determined twice – shortly after harvesting (before drying) and after 8 months of storage (Tables 3, 4). The composition of fungi shortly after harvesting and after 8 months was very similar, in both cases fungi of the genera *Alternaria* and *Cladosporium* being predominant. The content of these fungi on seed surface varied inappreciably during storage, and a reduction trend was identified in seeds samples of all cultivars. *Botrytis* spp. on seed surface shortly after harvesting was rather frequent, the seed with surface infection of this fungus accounting for 22.5–40.0%. During storage, the incidence of this fungus on seeds considerably declined, and only 4.0 – 9.0% of seeds were found to have surface infection of this fungus. Fungi of the genus *Penicillium* were identified on 3.8–15.0% of rape seeds shortly after harvesting, a similar level being identified after 8 months. *Fusarium* fungi were identified on up to 33.8% of seeds shortly after harvesting and on up to 12% after 8 months of storage. There is a report that during an investigation of the mycoflora on oilseed rape, the predominant fungal species present in 20 samples collected from Catalonia (Spain) were *Alternaria alternata* (Fr.) Keissl., *Penicillium* spp. and *Aspergillus flavus* (Vińas et. al., 1994).

Analysis of seed internal infection shortly after harvesting revealed that among the fungi most prevalent were *Botrytis* spp. (incidence 40.0–52.5%.), *Alternaria* spp. (22.5–46.3%) and *Cladosporium* spp. (21.3–40.0% of seed with internal infection). Seeds with *Penicillium* spp. internal infection accounted for 8.8%, and *Fusarium* fungi were identified only in one cultivar ('Triangle'). After 8 months of storage winter rape seed internal infection with *Alternaria*, *Cladosporium* and *Botrytis* fungi declined very markedly, however, internal infection with *Fusarium* spp. fungi increased.

The contents of AF and OTA toxins measured in winter rape seeds after 8 months of storage revealed a trend of reduction in the contents of both toxins during storage (Table 2). These toxins could be produced by *Penicillium* spp. ir *Fusarium* spp. fungi, and as other

Table 2. **The content of mycotoxins on winter rape seed of different cultivars at harvesting and during storage**

Cultivar	Mycotoxins µg kg^{-1}				
	At harvesting			After 8 months	
	DON	Aflatoxin	Ochratoxin A	Aflatoxin	Ochratoxin A
'Alaska'	164	1.0	1.9	2.1	1.9
'Courage'	181	1.8	2.0	3.3	1.3
'Digger'	169	1.8	5.1	2.7	1.7
'Milena'	183	1.5	4.7	0.0	1.6
'Triangle'	183	3.1	7.0	0.0	1.8
Average	**176**	**1.8**	**4.1**	**1.6**	**1.7**

Table 3. **Fungi composition on harvested seed of winter oilseed rape in 2005**

Cultivar	Surface infection, %					
	Fungi, genus					
	Alternaria	*Cladosporium*	*Botrytis*	*Penicillium*	*Fusarium*	Other
At harvesting						
'Alaska'	57.5	55.0	37.5	15.0	8.8	5.0
'Courage'	72.5	67.5	22.5	6.3	16.3	3.8
'Digger'	73.8	73.8	31.3	5.0	6.3	2.5
'Milena'	71.3	68.8	40.0	6.3	33.8	3.8
'Triangle'	77.5	73.8	25.0	3.8	7.5	2.5
LSD_{05}	**13.76**	**11.66**	**11.21**	**7.34**	**7.12**	**4.04**
After 8 months						
'Alaska'	58.0	55.0	7.0	13.0	12.0	6.0
'Courage'	71.0	62.0	5.0	7.0	10.0	7.0
'Digger'	63.0	54.0	9.0	6.0	7.0	3.0
'Milena'	65.0	52.0	5.0	8.0	6.0	4.0
'Triangle'	65.0	53.0	4.0	13.0	12.0	6.0
LSD_{05}	**8.51**	**7.31**	**3.67**	**5.26**	**5.24**	**4.02**

Table 4. **Internal infection of harvested seed of winter oilseed rape in 2005**

Cultivar	Infected seed (internal infection), %					
	Fungi, genus					
	Alternaria	*Cladosporium*	*Botrytis*	*Penicillium*	*Fusarium*	Other
At harvesting						
'Alaska'	46.3	21.3	41.3	8.8	0.0	8.8
'Courage'	26.3	27.5	45.0	2.5	0.0.	8.8
'Digger'	38.8	23.8	40.0	5.0	0.0	1.3
'Milena'	22.5	30.0	46.3	3.8	0.0	5.0
'Triangle'	22.5	40.0	52.5	5.0	1.3	6.3
LSD_{05}	**14.90**	**17.72**	**18.40**	**6.33**	**1.72**	**5.71**
After 8 months						
'Alaska'	8.0	5.0	1.0	3.0	3.0	4.0
'Courage'	13.0	7.0	3.0	3.0	2.0	2.0
'Digger'	13.0	6.0	2.0	1.0	2.0	1.0
'Milena'	9.0	3.0	2.0	3.0	1.0	2.0
'Triangle'	15.0	3.0	2.0	2.0	2.0	4.0
LSD_{05}	**6.22**	**5.96**	**3.67**	**4.55**	**4.50**	**3.56**

researchers indicate, most of the mycotoxins have been recognized as metabolic products of *Aspergillus*, *Penicillium* and *Fusarium* species. The production of aflatoxins and ochratoxins have been usually associated with a small number of species, but some recent studies have reported the production of these mycotoxins by some other species (Abarca et al., 2000). Other authors suggest that none of the *Penicillium* spp. isolated from oilseed rape samples revealed mycotoxigenic capacity (citreoviridin, griseofulvin, citrinin, patulin and penicillic acid) (Vińas et al., 1994). Only aflatoxin B_1 was detected in one of the 20 samples analyzed, with a concentration of 0.25 μg kg^{-1}. Of the 40 *Aspergillus flavus* strains isolated from oilseed rape samples, only three revealed aflatoxigenic capacity (Vińas et al., 1994). Mills and Abramson (1982) indicate that ochratoxin A was reported as present if levels exceeding 2.5 μg kg^{-1} and the confirmation was positive. Only seven of 34 *Penicillium* strains produced detectable ochratoxin A. The frequency of occurrence of *Penicillium* spp. or other fungi on seeds in a lot did not appear to be related to ochratoxin A production. The findings of our research also confirmed that the abundance of fungi on seeds did not have any direct effect on mycotoxin content in seeds. Our experimental

Table 5. **Fungi composition on harvested seed of winter oilseed rape as affected by anthropogenic factors in 2005**

Cultivar	Surface infection, %					
	Fungi, genus					
	Alternaria	*Cladosporium*	*Botrytis*	*Penicillium*	*Fusarium*	Other
At harvesting						
			not sprayed			
'Alaska'	57.5	55.0	37.5	15.0	8.8	5.0
'Courage'	72.5	67.5	22.5	6.3	16.3	3.8
'Digger'	73.8	73.8	31.3	5.0	6.3	2.5
'Milena'	71.3	68.8	40.0	6.3	33.8	3.8
'Triangle'	77.5	73.8	25.0	3.8	7.5	2.5
Min-max	**57.5–77.5**	**55.0–73.8**	**22.5–40.0**	**3.8–15.0**	**6.3–33.8**	**2.5–5.0**
			sprayed			
'Alaska'	60.4	56.7	28.4	14.2	3.8	4.6
'Courage'	58.4	55.4	30.0	11.7	5.4	7.5
'Digger'	61.7	59.6	30.4	11.3	1.3	7.9
'Milena'	53.0	53.8	25.0	18.3	3.0	8.4
'Triangle'	65.0	65.9	29.2	11.7	2.9	6.3
Min-max	**53.0–65.0**	**53.8–65.9**	**25.0–30.4**	**11.7–18.3**	**1.3–5.4**	**4.6–8.4**
After 8 months						
			not sprayed			
'Alaska'	58.0	55.0	7.0	13.0	12.0	6.0
'Courage'	71.0	62.0	5.0	7.0	10.0	7.0
'Digger'	63.0	54.0	9.0	6.0	7.0	3.0
'Milena'	65.0	52.0	5.0	8.0	6.0	4.0
'Triangle'	65.0	53.0	4.0	13.0	12.0	6.0
Min-max	**58.0–71.0**	**52.0–62.0**	**4.0–9.0**	**6.0–13.0**	**6.0–12.0**	**3.0–7.0**
			sprayed			
'Alaska'	48.0	42.3	11.0	14.3	8.3	5.0
'Courage'	47.3	43.7	7.3	11.3	11.3	5.3
'Digger'	48.3	43.0	5.7	8.0	5.7	3.0
'Milena'	42.0	43.3	9.7	11.3	17.3	4.3
'Triangle'	49.7	48.3	5.7	9.0	12.0	3.3
Min-max	**42.0–49.7**	**42.3–48.3**	**5.7–11.0**	**8.0–14.3**	**5.7–17.3**	**3.0–5.3**

Averaged data of 3 fungicides.

evidence suggests that *Aspergillus* spp. was not identified, although other researchers have reported that oilseed crops are recognized to be potentially suitable substrates for the production of toxic secondary metabolites by molds, notably the production of aflatoxins by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* (Hesseltine et al., 1966).

Fungicides are used for disease control in oilseed rape crops. In our experiments, we identified surface infection of seeds of five winter rape cultivars as affected by this anthropogenic factor. Seed analyses were done shortly after harvesting and after 8 months of storage. Our experimental evidence shows that there were no marked differences between seed samples collected from unsprayed plots and from those sprayed with fungicides at the end of flowering (Table 5). However, during the eight-month storage seed surface infection with various fungi declined and especially markedly with *Alternaria* spp. (sprayed plots) and *Botrytis* spp. (unsprayed and sprayed plots). Internal seed infection was lower than surface infection. When internal seed infection was determined shortly after harvesting, the same fungi were found to be prevalent as on seed surface (*Botrytis* spp., *Alternaria* spp. and *Cladosporium* spp.). During storage, *Alternaria* spp. internal seed infection of different cultivars was reduced about 5.8 times (cv. 'Alaska', untreated), *Botrytis* spp. internal infection 41.3 times and did not exceed 3 per cent. Conversely, after storage the number of seeds with internal *Fusarium* spp. infection increased both in the samples from untreated and treated plots.

Infected rape seeds are considered to be one of the chief *Alternaria* blight infection sources (Humpherson-Jones et al., 1982; Howlider et al., 1985; Meah et al., 1988). *Alternaria brassicicola* surface infection on seeds remains viable for only about two years, whereas internal infection of this fungus persists in seeds for a much

longer period. Some authors indicate that in rape seeds *Alternaria* spp. remains viable for up to 12 years (Maude and Humpherson-Jones, 1980). In order to reduce the risk of internal infection with *Alternaria*, it is recommended to separate small, light seeds during the seed cleaning process or to store seeds for a longer period. Storage of *Alternaria* spp.-infected seed at 25 °C for 6–8 months can reduce the possible infection level by 50%, and seed storage for 2–3 months at 35 and 45 °C cleans rape seeds from *Alternaria* infection (Chahal and Sekhon, 1981). At 5 °C the fungus remains viable for a relatively long period. Due to this, *Alternaria* seed infection is not as important in southern countries with the prevalent warm weather as in cooler northern regions (Smith et al., 1988). In our experiments we determined seed internal infection reduction trends during storage, but they were not very distinct, because the seed storage temperature was lower than indicated by other researchers.

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GENOTIPO IR APLINKOS VEIKSNIŲ ĮTAKA RAPSŲ SĖKLŲ UŽTERŠTUMUI MIKOTOKSINAIS BEI JUOS GAMINANČIAIS GRYBAIS

S a n t r a u k a

Žieminių rapsų įvairių veislių sėklų užsikrėtimas patogenais bei jų gaminamų toksinų kiekio tyrimai dėl įvairių veiksnių įtakos atlikti Lietuvos žemdirbystės institute. Skirtingų žieminių rapsų veislių sėklų mikrobiologinė tarša buvo nuo 1,4–2,6 kvs g^{-1} x10^4. Visų žieminių rapsų veislių sėklų ėminiuose vyravo *Cladosporium*, *Alternaria*, *Penicillium* ir *Fusarium* genčių grybai. Pirmą kartą buvo atlikti rapsų sėklų grybų gaminamų toksinų tyrimai. Nustatyta, kad mikotoksino deoksinivalenolio koncentracija kai kurių veislių sėklose buvo 164–183 µg kg^{-1}. Aflatoksino kiekis nustatytas nuo 1,0 µg kg^{-1} 'Alaska' veislės sėklose iki 3,1 µg kg^{-1} 'Triangle' veislės sėklose. Dar didesnė kaita tarp veislių buvo nustatyta pagal ochratoksino A kiekį sėklose. Nustačius aflatoksino ir ochratoksino A kiekį įvairių veislių žieminių rapsų sėklose po 8 mėnesių sandėliavimo, pastebėta abiejų toksinų kiekio mažėjimo tendencijos. Tyrimų duomenimis, antropogeninių veiksnių įtaka žieminių rapsų veislių sėklų paviršiniam užterštumui ir vidiniam užsikrėtimui grybais nebuvo labai ryški, tačiau sandėliuojant sėklas 8 mėnesius sėklų paviršinis užsikrėtimas įvairiais grybais sumažėjo, ypač ryškiai – užsikrėtimas *Alternaria* ir *Botrytis* genties grybais.

Raktažodžiai: žieminiai rapsai, sėklų užterštumas, mikotoksinai