Occurrence of *Alternaria* Toxins in Fibre Flax, Linseed, and Peas Grown in Organic and Conventional Farms: Monitoring Pilot Study

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Abstract

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Fungi representing *Alternaria* spp. are ubiquitous pathogens that may under certain conditions cause spoilage of various food crops. Several *Alternaria* species are known producers of toxic secondary metabolites in some fruits and vegetables, nevertheless, only limited knowledge is available on the occurrence of these mycotoxins in legumes and/or oilseeds used for human nutrition. In the first part of the presented study, the analytical method employing reversed phase high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD) was implemented to enable the examination of these food commodities for the presence of altenuene (AE), alternariol (AOH), and alternariol monomethyl ether (AME); the limits of detection were 1, 3 and 2 µg/kg for AE, AOH, and AME, respectively. Altogether 122 flax and 84 pea seed samples grown under organic and/or conventional farming conditions were analysed in the years 2002–2003. AME was detected in 20 flax seed samples; AE and AOH were present in only 2 and 4 samples, respectively. More frequent incidence of *Alternaria* toxins was recognised in fibre flax seeds as compared to linseed samples. Compared to the crops from the conventional farming, the concentrations of these mycotoxins found in positive organic samples were higher. No *Alternaria* mycotoxins were detected in the pea samples, probably due to the presence of antifungal compounds in the respective crop.

Keywords: *Alternaria*; fungi; altenuene; alternariol; alternariol monomethyl ether; mycotoxins; fibre flax seeds; linseed seeds; pea seeds

Growing information on the importance of the plant fibre, polyunsaturated fatty acids, antioxidants, and other health-promoting components has resulted in changing dietary habits of the European population. An increased demand for some oilseeds such as linseed that until now have not represented common dietary items has recently occurred on this account. However, the growing

consumption of foods of plant origin unavoidably poses the risk of increased consumers' exposure to various mycotoxins.

While extensive attention has been paid in Europe to the producers of *Fusarium* toxins and Ochratoxin A, which are frequently found in cereals and oilseed plants, only very few studies have been concerned with other pathogenic fungi infecting various edible plants.

Species of the *Alternaria* genus are examples of ubiquitous plant pathogens that may contaminate a wide variety of crops in the field and cause post-harvest decay of various fruits, grains, and vegetables (Chelkowski & Visconti 1992; Weidenborner 2001). Due to their growth even at low temperatures, they may be also responsible for spoilage of these commodities during their refrigerated transport and storage, which not only results in economic losses to growers and commercial marketers, but may also threaten consumers' health (Torres *et al.* 1998; Tournas & Stack 2001).

It has been documented in several studies (Chelkowski & Visconti 1992; Visconti & Sibilia 1994; Torres *et al.* 1998; Feng-Gin & Yoshizawa 2000), that *Alternaria alternata* (the most known representative of *Alternaria* genus) produces several toxic chemicals belonging to different structural groups, among them the benzopyrone derivatives such as altenuene (2α,3β,4aβ-tetrahydro-2,3,7-trihydroxy-9-methoxy-4a-methyl-6H-dibenzo[b,d]pyran-6-one; AE), alternariol (3,7,9-trihydroxy-1-methyl-6H-dibenzo[b,d]pyran-6-one; AOH), and alternariol monomethyl ether (3,7-dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one; AME) (Figure 1) being typically the most abundant secondary metabolites of concern. As shown in those few available studies focused on *Alternaria* toxins production in fruits and vegetables, the most frequently detected co-metabolite is AOH, which is occasionally ac-

companied by AME. The maximum levels of these mycotoxins reported in marketed products were in the range 10^0–10^3 µg/kg, higher levels were found in samples visibly infected by *Alternaria* rot, i.e. in products obviously not suitable for consumption (Stinson *et al.* 1981; Scott *et al.* 1997; Lau *et al.* 2003).

These compounds are lethally toxic to young birds, and when administered to mammals, they produce various adverse effects such as salivation, emesis (vomiting), erythema, gastrointestinal haemorrhage, convulsions, an increase in the packed cell volume (an immune parameter), and other toxicity symptoms. The relationship between the contamination of food crops by *Alternaria alternata* and the incidence of oesophageal cancer in some regions was discussed by Liu *et al.* (1991). Recently, estrogenic potential of AOH was demonstrated by Lehmann *et al.* (2005). Until now, due to the lack of comprehensive toxicological data, no hygienic limits have been established for the representatives of this mycotoxins group.

Several, mainly chromatographic techniques have been used for the determination of *Alternaria* toxins in food crops. In the earliest studies, the procedure most commonly used for the examination of food extracts was thin layer chromatography (Chelkowski & Visconti 1992). At present, gas chromatography (GC) coupled to either flame ionisation (FID) and/or mass spectrometric detector (MSD) is used for the determination of AE, AOH, AME, and other *Alternaria* toxins (Scott *et al.* 1997). It should be noted that derivatisation of the target analytes has to be carried out prior to the determinative step when using GC. Alternatively, high-performance liquid chromatography (HPLC) employing ultraviolet (UV), fluorescence (FLD), electrochemical (ECD), and mass spectrometric (MSD) detectors is employed for the direct analysis of these toxins in food matrices (Delgado *et al.* 1996; Delgado & Gómez-Cordovés 1998; Fente
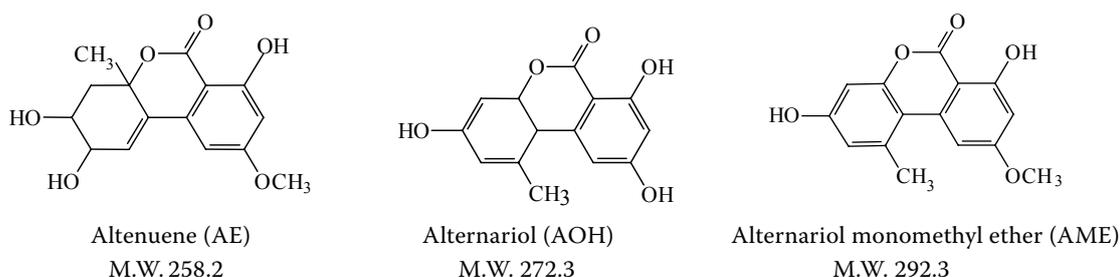

Figure 1. Chemical structures of target *Alternaria* toxins

Altenuene (AE)
M.W. 258.2

Alternariol (AOH)
M.W. 272.3

Alternariol monomethyl ether (AME)
M.W. 292.3

et al. 1998; GIACOMELLI et al. 1998; SCOTT 2001; MOLINA et al. 2002; LAU et al. 2003).

The aim of this two-year study was to evaluate the relationship between various agricultural practices (conventional and organic), used for the production of flax and pea seeds, and the extent of *Alternaria* toxins occurrence in these crops. A simple HPLC/FLD method was implemented for the target mycotoxins control.

MATERIALS AND METHODS

Plant materials. The following samples of the investigated food crops were used for the evaluation of the influence of agricultural practices on *Alternaria* mycotoxins occurrence: (*i*) 79 fibre flax samples (cultivars Jitka and Venica); (*ii*) 43 linseed samples (cultivars Atalante, Lola and Jupiter); and (*iii*) 84 pea samples (cultivars Grana, Gotik, Zekon, Jacpot, Lantra and Komet), all grown in the Czech Republic. The flax and pea samples were supplied by the project partner AGRITEC, Research, Breeding and Service Ltd., Šumperk, Czech Republic. The experimental growing and harvesting conditions are described below.

Flax seeds (*Linum usitatissimum* L.). Both flax seed products of the fibre flax and the linseed samples were obtained from small-plot field tests conducted in the years 2002 and 2003. The size of the individual plots was 10 m^2.

The linseed cultivars in all localities were harvested by the direct harvest method – with the cutting small-plot corn harvester. The fibre flax varieties grown in the conventional fields in Šumperk were harvested by diphasic method – the stems of the fibre flax were pulled out with the capsules, placed in lines on the field, and after four days they were deseeded. The stems of fibre flax samples from the organic fields in the localities Komňátky, Ocmanice, and Jedlí were plucked and dried out of fields and were also deseeded after four days. After they were dried and purified, the examination of *Alternaria* mycotoxins (AE, AOH and AME) contents was carried out. Several factors (field fertilisation and the way of farming) potentially influencing fungal growth (and subsequent toxins production) were tested. The conditions of cultivation are characterised in the following paragraphs.

Field fertilisation. The relationship between the total nutrients input and the types of fertilisers applied in the production of the fibre flax

Table 1. Application of fertilisers in fibre flax seeds (cultivar Venica) production

kg N/ha	NPK 10-10-10	AN	AS
Control	0	0	0
AN 40	0	40	0
AN 60	0	60	0
AN 80	0	80	0
AS 40	0	0	40
AS 60	0	0	60
AS 80	0	0	80
NPK 40	40	0	0
NPK 60	60	0	0
NPK 80	80	0	0

NPK 10-10-10 – compound fertiliser containing nitrogen, phosphorus and potassium; AN – ammonium nitrate; AS – ammonium sulphate

cultivar Venica and the content of *Alternaria* mycotoxins in this crop was investigated. The experimental crop was grown in conventional fields in the locality Šumperk (average monthly precipitations are shown in Figure 2). In these experiments, the following fertilisers were used: NPK 10-10-10 (compound fertiliser containing nitrogen, phosphorus, and potassium in a form which is easily acceptable for plants) and/or ammonium nitrate (AN) and/or ammonium sulphate (AS); their dosages were: 40, 60, and 80 kg N/ha (Table 1). The pesticide preparations applied in the year 2002 were as follows (active ingredients in parentheses): Karate (lambda-cyhalothrin), Glean (chlorsulfuron), Basagran Super (bentazone-sodium) and Pantera (quizalofop-P-tefuryl), in the year 2003 Nurelle (chlorpyrifos and cypermethrin), Glean, Basagran Super, Lontrel (clopyralid), and Pantera. The treatments were carried out during the vegetation period according to the registered use (http://ec.europa.eu/food/plant/protection/pesticides/legislation_en.h) and the methodology approved for the fibre flax breeding strategy in the intensive way of farming (Tables 2 and 3).

Way of farming. Small-plot trials were established both in organic farms (locality Komňátky, Ocmanice and Jedlí) and conventional fields (locality Šumperk). While no treatment with fungicides was used for the protection of organic flax seeds, in the later farming system the seeds used for
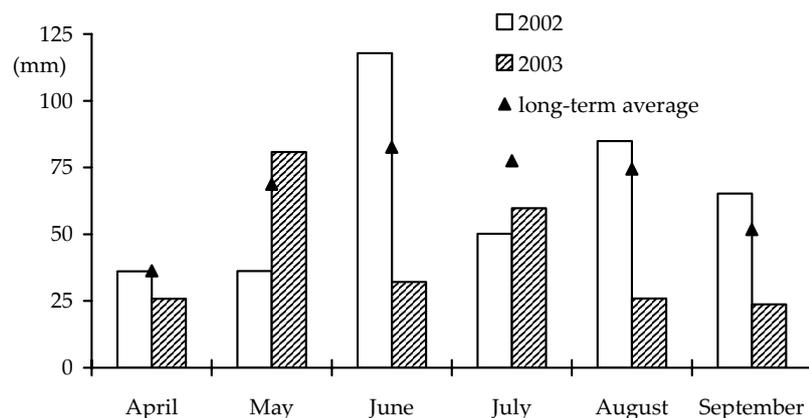
Figure 2. Average monthly amount of precipitation in the years 2002 and 2003 in locality Šumperk

sowing were treated with Vitavax 200FF (active ingredients carboxin and thiram). The treatment of flax plants during vegetation was realised according to the registered use (http://ec.europa.eu/food/plant/protection/pesticides/legislation_en.htm) and the methodologies characterised as "intensive", "extensive" and "organic"; for details see Tables 2 and 3. It should be noted that, in spite of the "organic" methodology (no fertiliser, no pesticides used), the crops from the locality Šumperk cannot be classified as organic, due to the conventional agricultural practices performed in the previous crop season.

Pea seeds (*Pisum setivum* L.). The relationship between nitrogen nutrition in the conventional intensive agriculture as well as the way of farming (intensive, extensive and organic) and the content of *Alternaria* mycotoxins was assessed. All tests were established in the locality Šumperk. The

pesticide preparations (Duett – active ingredients carbendazim and epoxiconazole, Stomp 300 E – active ingredient pendimethalin, and Karate 2.5 WG – active ingredient lambda-cyhalothrin) were used for the post-emergence treatment in conventional "extensive" and "intensive" farming. For desiccation applied 10 days before plants harvest, Roundup (active ingredient glyphosate) and Harvade (active ingredient dimethipin) were used. No pesticides and fertilisers were used in the organic way of agriculture.

Determination of *Alternaria* **toxins**. An analytical method employing reversed phase high-performance liquid chromatography (HPLC) coupled with fluorescence detection (FLD) was used for the determination of *Alternaria* toxins (AE, AOH and AME) in flax and pea seed samples.

Analytical standards and chemicals. The standards of altenuene (AE, 98% purity), alternariol

Table 2. Application of fertiliser and pesticides in experimental fields – year 2002

Type of agriculture	Fertiliser NPK 10-10-10	Pesticide preparation			
		Karate	Glean	Basagran Super	Pantera
Intensive	40 kg N/ha	0.3 l/ha	12 g/ha	1.2 l/ha	2.5 l/ha
Extensive	25 kg N/ha	0.3 l/ha	7 g/ha	1.2 l/ha	2.5 l/ha
Organic	none	none			

Table 3. Application of fertiliser and pesticides in experimental fields – year 2003

Type of agriculture	Fertiliser NPK 10-10-10	Pesticide preparation				
		Nurell	Glean	Basagran Super	Lontrel	Pantera
Intensive	40 kg N/ha	0.6 l/ha	12 g/ha	1.2 l/ha	0.3 kg/ha	2.5 l/ha
Extensive	25 kg N/ha	0.6 l/ha	7 g/ha	1.2 l/ha	0.3 kg/ha	2.5 l/ha
Organic	none	none				

(AOH, 96% purity), and alternariol monomethyl ether (AME, 99% purity) were purchased from Sigma-Aldrich (Steinheim, Germany). Each of them was dissolved separately in methanol to make 0.2 mg/ml stock solutions, from which mixed standard solutions in methanol were prepared and kept at 4°C in refrigerator.

The solvents used were methanol and cyclohexane from Merck (Germany), and ethyl acetate from Scharlau (Spain). Redistilled water was prepared using Milli-Q system (Millipore, France), sodium bicarbonate was obtained from Merck (Germany). Sodium sulphate, anhydrous (Penta, Czech Republic), used for filtration, was heated up to 500°C for 6 h and stored in a tightly closed glass vessel before use.

Apparatus. Gel permeation chromatography (GPC) – The clean up of the extracts was carried out using an automated gel permeation chromatography (GPC) system Gilson (France), consisting of Gilson 305 MASTER pump, fraction collector ASPEC^{TM} XL, Gilson, dilutor Gilson 401C, microcomputer (software 731 PC via RS232C), and a stainless steel column (600 mm × 7.5 mm) packed with PL gel (size of particles 10 µm, dimension of pores 50A, Polymer Laboratories, USA).

High-performance liquid chromatography (HPLC) – For quantification high-performance liquid chromatography system was used composed of Hewlett-Packard 1050 series quaternary pump system, HP 1050 series autosampler, and a HP 1046 A fluorescence detector (FLD) (HP, USA), and a LiChroCART 250-4 column (250 × 4 mm I.D.) with the sorbent LiChroCART 100 RP-C18 (5 µm, Merck, Darmstandt, Germany) protected by a guard column (4 × 4 mm I.D.) with the same stationary phase.

Analytical procedure. Extraction – A homogenous representative sample of flax or pea seeds was extracted with 80 ml of methanol in a 250-ml Erlenmeyer flask for 60 min employing a rotary shaker. The extract was filtered through filter paper No. 390 (Filtrak, Niederschlag, Germany) to 100-ml volumetric flask, made up with methanol and stored in a refrigerator at 4°C.

Clean-up – Before removing matrix co-extracts, 20 ml aliquot of the filtrate was evaporated and redissolved in 20 ml of 5% NaHCO$_3$. This solution was transferred into a 100 ml separating funnel and 20 ml of ethyl acetate was added. The mixture was vigorously shaken for about 7 min and then the organic phase was filtered through anhydrous sodium sulphate layer and evaporated to dryness. The residue was dissolved in 3 ml of cyclohexane: ethyl acetate mixture (1:1, v/v) and 1.5 ml of this solution was loaded onto the GPC column (PL gel – rigid gel, mobile phase cyclohexane: ethyl acetate, 1:1 v/v, flow rate 1.0 ml/min). The first portion of eluate was discarded and the second one (15–20 ml) was collected. This 'mycotoxin' fraction was evaporated in a rotary vacuum evaporator at 40°C and the residual solvent was removed with a gentle stream of nitrogen; the residue was then dissolved in 0.5 ml of methanol for HPLC analysis.

HPLC – FLD determination. For the separation of the target analytes from the interfering matters, gradient elution employing a mixture of water and methanol was used. The initial composition of the mobile phase (40% of methanol) was held for 2 min, followed by raising the concentration of methanol linearly to 100% within 10 min. After maintaining these conditions for 8 min, the concentration of methanol was adjusted back to the initial composition and the column was equilibrated for 2 min. 20 µl of the extract was injected onto the HPLC column. The mobile phase flow rate was 1.0 ml/min and the column temperature was kept at 35°C.

The detection of the target analytes was carried out with fluorescence detector (FLD) under the following excitation/emission wavelength settings: 243/460 nm for AE and 252/408 nm for AOH and AME. The peaks were identified by the retention times. For quantification, external calibration (calibration curve) was employed. Spiked samples were used for validation (repeated analyses at 3 spiking levels), the generated data are summarised in Results and Discussion.

RESULTS AND DISCUSSION

Method performance characteristics

In the first phase of this study, the analytical method was searched applicable for the examination of flax and pea seed samples for the presence of *Alternaria* mycotoxins. The possibility of using GC/MS for this purpose was considered. Due to a relatively polar nature of the target analytes, their conversion into volatile derivatives has to be carried out prior to the GC determinative step. Trifluoroacetanhydride, pentafluoropropionanhydride (acylation agents), and diazomethane (methylation

Table 4. Performance characteristics obtained by repeated analyses (*n* = 5) of spiked flax samples

Spiking level (µg/kg)	AE (%)		AOH (%)		AME (%)	
	recovery	RSD	recovery	RSD	recovery	RSD
AE [50], AOH [100], AME [100]	74.0	3.4	81.0	5.8	74.4	3.4
AE [24], AOH [48], AME [48]	74.8	1.8	78.9	9.5	81.4	2.3
AE [8], AOH [16], AME [16]	80.2	1.5	94.2	2.5	85.0	2.5
LOD	1 µg/kg		3 µg/kg		2 µg/kg	
LOQ	3 µg/kg		8 µg/kg		6 µg/kg	

agent) were used for the derivatisation of *Alternaria* toxins involved in our study. Unfortunately, this approach (not described in Experimental) proved to be unsuitable, because of either rather poor stability of the derivatives obtained (products of acylation) or the formation of identical methylated products from AOH and AME. With regard to these problems, a reversed phase high-performance liquid chromatography employing fluorescence detection was implemented for the analysis of AE, AOH, and AME in all three plant matrices involved in this study. Two-step clean-up procedure consisting of liquid-liquid partition and gel permeation chromatography enabled to remove most of the matrix components contained in the methanolic crude extract. The performance characteristics of the validated procedure are summarised in Table 4. In Figures 3 and 4, chroma-

tograms of blank, spiked, and real contaminated samples are shown for illustration.

Examination of various linseed and fibre flax seeds contamination

Field fertilisation. In the years 2002 and 2003, altogether 49 fibre flax seed samples grown under conventional farming conditions differing in the form/amount of the nitrogen fertiliser input were examined for *Alternaria* toxins contents. The only secondary metabolite detected, representing these mycotoxins, was AME. Its incidence was fairly higher in the first experimental year (46% of samples), as compared to the second crop year (only 5% of samples). This difference might be due to more favourable conditions for the crop infection in the first growing season (significantly
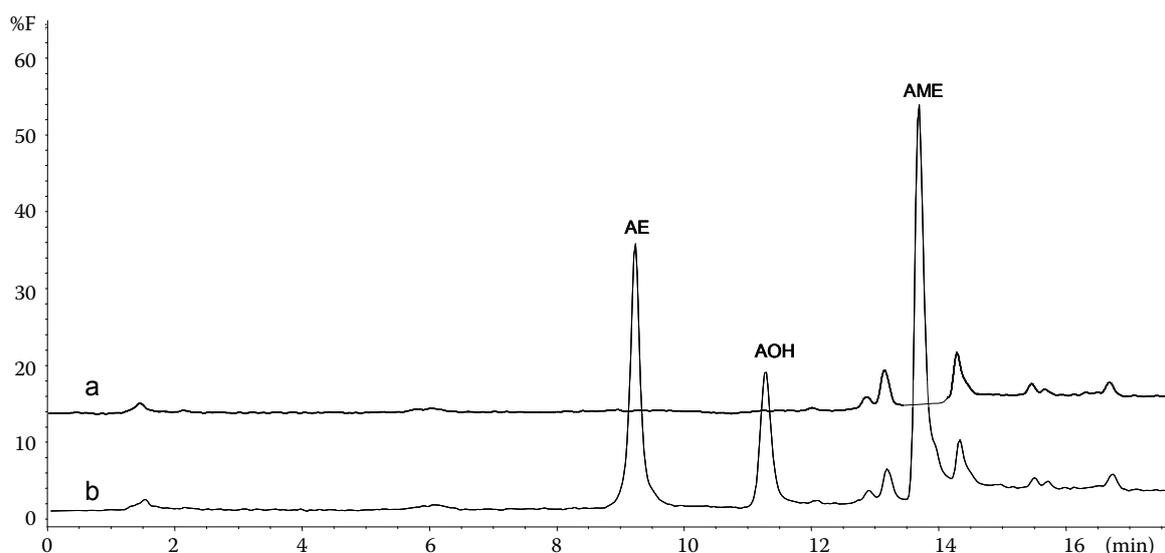

Figure 3. HPLC/FLD chromatograms of (a) blank and (b) spiked linseed sample (spiking level 100 µg/kg AE and 200 µg/kg AOH and AME, each)
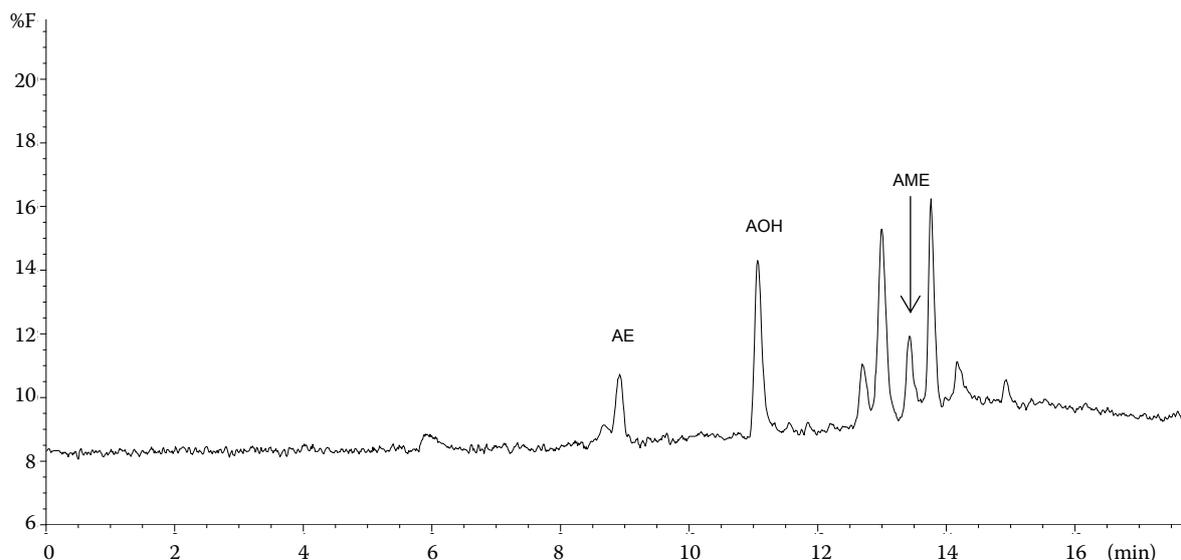
Figure 4. HPLC/FLD chromatogram of a naturally contaminated linseed sample (4 μg/kg AE, 69 μg/kg AOH and 16 μg/kg AME)

higher precipitations as shown in Figure 2). The overview of the results is shown in Table 5, no distinct relationship was found between the form and/or amount of nitrogen fertiliser input into the soil and the frequency of *Alternaria* toxins occurrence in fibre flax seeds.

Way of farming. In the year 2002, altogether 36 flax seed samples from the locality Šumperk (12 per each of the three farming systems) were analysed. The occurrence of toxic *Alternaria* secondary co-metabolites was proved only in three samples of fibre flax seeds (all of them were of the cultivar Jitka – one from the intensive and two from the extensive systems). Similarly to the above experiments concerned with the assessment of the fertilisation influence, AME was the only *Alternaria* mycotoxin exceeding the detection limit in the samples examined, its values, however, were low – in the range of 11–17 μg/kg.

A small extent of flax seeds contamination was documented also in the year 2003. Contrary to the previous year, positive samples were obtained from the organic farm. All three target mycotoxins were determined in the linseed cultivar Jupiter: 4 μg/kg AE, 69 μg/kg AOH, and 16 μg/kg AME, respectively. Rather surprisingly, in the second positive sample of fibre flax seeds (cultivar Jitka), no AME exceeding the method limit of detection was found, only AE (9 μg/kg) and AOH (19 μg/kg) were present. Probably a different *Alternaria* strain infested the fibre flax seeds in this case.

In the next set of experiments, one linseed sample and one fibre flax seed sample obtained from each of the organic farms in the localities Jedlí, Ocmanice and Komňátky in the year 2002 were analysed. *Alternaria* toxins were found in two linseed samples. One of them (from the locality Jedlí) contained relatively high levels of AOH (104 μg/kg) and AME (30 μg/kg), in the second one (from the locality Komňátky) only a low level of AOH (14 μg/kg) was detected. Similarly to the results of the experiments conducted in the local-

Table 5. *Alternaria* toxins in fibre flax seeds in experiments differing in field fertiliser input (experiments described in paragraph "Field fertilisation")

Crop year	Number of experimental samples	Mean content of AME (μg/kg)*									
		control	AN 40	AN 60	AN 80	AS 40	AS 60	AS 80	NPK 40	NPK 60	NPK 80
2002	30	21	19	23	–	14	–	9	–	7	6
2003	19	–	–	–	–	–	–	–	–	–	10

*$n = 3$

ity Šumperk, no *Alternaria* toxins were present in flax seeds harvested in the organic farms in the year 2003.

Pea samples

54 and 30 pea samples harvested in the years 2002 and 2003, respectively, were examined for the presence of *Alternaria* toxins. None of them contained detectable levels of the target analytes although the presence of *Alternaria* toxins was proved in the flax seeds grown in the same locality (Šumperk). This phenomenon might be due to the presence of an antifungal protein (designated pisumin), the presence of which in legumes has recently been reported by several authors (Cabral *et al.* 2003; Ye & Ng 2003).

CONCLUSIONS

Based on the results of two-year study concerned with the incidence of *Alternaria* toxins in flax seeds and pea seeds grown in various agricultural systems, the following conclusions can be drawn.

(*i*) AE, AOH, and AME, exceeding levels 1, 3, and 2 µg/kg(LODs), respectively, can be reliably determined in flax seeds and pea seeds by HPLC/FLD method implemented in this study.

(*ii*) No distinct relationship was documented between the N-fertilisers input and *Alternaria* toxin levels in fibre flax seeds.

(*iii*) No distinct relationship was documented between the intensity of agrochemicals use (fertilisation, pesticides use) in conventional fields and *Alternaria* toxin levels in flax seeds.

(*iv*) Higher levels of *Alternaria* toxins in flax seeds were found in the samples grown in the year with higher precipitations during the growth season (i.e. higher humidity of the environment). Climatic conditions seem to play a more important role as compared to the influence of agricultural practices.

(*v*) Due to a limited number of samples from the certified organic farms, it was rather difficult to draw general conclusions on the influence of the field history (past use of agrochemicals) on the target mycotoxins incidence.

(*vi*) A higher incidence of *Alternaria* toxins was found in fibre flax seeds as compared to linseed samples harvested in a particular locality in the same year. This fact is probably associated with the harvest practices. Contrary to the direct har-

vesting of linseed samples, diphasic harvest was employed in the case of fibre flax seeds. The latter conditions were obviously more favourable for the fungal development.

(*vii*) The pea samples examined seem to be resistant against *Alternaria* infection since no occurrence of *Alternaria* toxins was proved in this commodity. Antifungal proteins contained in this crop are probably responsible for the protective effect.

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