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Original Research

Occurrence of *Fusarium* Fungi and Mycotoxins in Marketable *Asparagus* Spears

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Abstract

Fusarium oxysporum and/or *F. proliferatum* were isolated from all asparagus spears with brown spots (which indicate an infection) and from almost all spears without spots. The presence of *Fusarium* spp. and their toxins in the basal parts of asparagus spears was analyzed. Fumonisin B_1 (FB_1) and moniliformin (MON) were found in spears with brown spots and those without disease symptoms. FB_1 was determined in the concentration range 0.16-152.68 ng g^{-1} (mean 7.52), while moniliformin was detected in the range 15.30-585.00 ng g^{-1} (mean 121.00). Only in 10% analyzed spears were metabolites not detected.

Keywords: asparagus spears, fumonisin B_1, *Fusarium oxysporum*, *Fusarium proliferatum*, HPLC, moniliformin, PCR

Introduction

Asparagus (*Asparagus officinalis* L.), due to its exceptional taste, high nutritional value, and high content of biologically active compounds, is becoming a highly appreciated vegetable worldwide. Spears appear in markets in spring, when people are dramatically missing these valuable substances [1]. In the cultivation of perennial crops, such as asparagus, protection against *Fusarium* stem and crown rot with mycotoxin accumulation in plant tissues is important [2]. The disease develops in plants, slowly reducing the quality of spears and causing a risk to for human health and significant loss in production. Moreover, the quality and shelf-life of *Fusarium*-infected commercial asparagus spears to be significantly reduced. Nigh et al. [3] reported that storage of infected spears for periods of 5 or 10 days reduces quality by 31% and 67%, respectively, while uninfected spears, harvested under identical conditions, retain their quality. Worldwide asparagus acreage is greatest in Europe (52,500 ha), followed by North America (45,500 ha) [4]. Although infection of asparagus spears by *Fusarium* spp. has previously been reported [3, 5-11], there is a lack of detailed evaluation on the quality and safety of asparagus spears in the Polish market.

Apart from known sources, several new sources of mycotoxins (e.g. cornflakes, maize bread, beer, black and herbal tea, medicinal plants) have been reported recently [12-16]. It is difficult to forecast the human health hazard in connection with the disease, since *Fusarium* species have been isolated both from tissues with no spots and those with disease symptoms [17]. It has been shown that the consumption of fresh and processed asparagus spears infected by *Fusarium* spp. may constitute a significant risk for human health, connected with exposure to mycotoxins, e.g. fumonisin B_1 (FB_1) or moniliformin (MON) – toxins usually accompanying some *Fusarium* species [18, 19].

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Both toxins are stable at high temperature, so most operations in food processing do not result in a significant reduction of their content. FB_1 exhibits nephrotoxic action, activates liver and oesophageal cancers, and causes cellular disorders, as well as numerous changes in the immune system [20]. On the basis of existing knowledge, the International Cancer Research Agency in 2002 classified FB_1 as a substance of probably carcinogenic action, class 2B for humans [21]. In turn, moniliformin exhibits cytotoxic and cardiotoxic activity, causes developmental disorders and may also induce the development of Keshan disease [22]. Acceptable concentration levels of some mycotoxins in foods, feeds and their components are regulated in several countries [23].

Seeds, crowns and soil are known sources of fungi, where they can live for many years on crop residue and debris. Since asparagus fusariosis influences human health, causes considerable economic losses and is difficult to control, the importance of the undertaken studies is obvious.

The aim of the study was to assess the presence of *Fusarium* spp. and their toxins in asparagus spears with and without brown spots. Also, contamination levels with fumonisin B_1 and moniliformin in basal part of spears were analyzed.

Materials and Methods

Experimental material was collected in 2007. Twenty white asparagus spears with brown spots (recognized as necrotic changes indicating fusariosis of asparagus spears), and 20 with no disease symptoms were collected at a farmers market. All spears were divided into three parts - basal, central and apical, for fungi isolation and identification while mycotoxins were determined in the basal part only.

Isolation and Morphological Identification of *Fusarium* spp.

The boundary between brownish and healthy tissues or 1 cm^2 pieces of asymptomatic tissues from the particular parts of each spear were used for fungi isolation. After disinfection with 1% sodium hypochlorite, five sections of tissue (2 mm in diameter) were cut out from each of three parts of the spear and transferred onto separate Petri dishes containing potato dextrose agar (PDA) (Merck, Darmstadt, Germany) amended with streptomycin at 100 µg mL^{-1} for *Fusarium* spp. isolation. Cultures of fungi grown from the sections and then from a single spore were transferred onto standard media and identified according to the manuals of Booth [24], Gerlach and Nirenberg [25] and Kwasna et al. [26].

Molecular Identification of *Fusarium* spp.

Mycelia from 9-day old single-spore cultures of *F. proliferatum* and *F. oxysporum*, grown on liquid medium (5 g L^{-1} of glucose, 1 g L^{-1} of yeast extract), were collected by vacuum filtration using a Büchner funnel. DNA was extracted and purified using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's recommendations. Two pairs of primers were used in individual PCRs: the forward primer 5'-TGCATCAGACCACTCAAATCCT-3' and reverse primer 5'-TGTCAGTAACTCGACGTTGTTGTT-3' for detection of *F. oxysporum,* and the forward primer 5'-TGCATCAGACCACTCAAATCCT-3' and reverse primer 5'-TGTCAGTAACTCGACGTTGTTGTT-3' for detection of *F. proliferatum* (Sigma-Genosys, Pampisford, UK) [27]. *F. proliferatum* strain (DSM No. 62261). *F. oxysporum* strain (DSM No. 62287), obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), were used as reference isolates. The amplification reactions were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Valencia, USA). The reaction mixture was described earlier by Irzykowska [28]. PCR was carried out in a Biometra T*personal* 48 thermocycler (Whatman Biometra, Goettingen, Germany) using the following program: initial denaturation for 3 min at 94ºC, followed by 35 cycles of denaturation at 94°C for 40 seconds, primer annealing at 60°C for 40 seconds and extension at 72°C for 1 min. The amplification was ended with an additional extension at 72°C for 3 minutes. The PCR products were separated by electrophoresis in 1.5% agarose gel with 1x TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0) and visualized under UV light following ethidium bromide staining. A Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas GMBH, St. Leon-Rot, Germany) was used as a molecular size standard.

Extraction and Purification of Fumonisin B_1 and Moniliformin

Samples (10 g) from the basal parts of asparagus spears were homogenized for 3 min with 20 mL of methanol-water (3:1, v/v) and filtered through Whatman No. 4 filter paper according to the method described by Sydenham et al. [29]. The supernatant was then divided into two equal subsamples for FB_1 and MON analyses.

The fraction used for FB_1 analysis was adjusted to the pH value of 5.8-6.5 by 0.1 M KOH water solution. A SAX cartridge was attached to the SPE manifold unit (Supelco, Bellefonte, PA, USA) and conditioned at a flow rate of 2 mL min^{-1} successively with 5 mL of methanol followed by 5 mL of methanol-water (3:1, v/v). An aliquot of 5 g filtered subsample extract (10 mL) was applied to the top of the conditioned cartridge at a flow rate of 2 mL min^{-1}, and washed with 8 mL of methanol-water (3:1, v/v), immediately followed by 3 mL of methanol. FB_1 was eluted from the column to a glass collection vial with 10 mL of 1% acetic acid in methanol, at a flow rate of 1 mL min^{-1}. The eluate was evaporated to dryness at 40°C under a stream of nitrogen. Dry residue was stored at 4°C until high performance liquid chromatography (HPLC) analyses.

The fraction used for MON analysis was defatted with *n*-hexane (3×50 mL), concentrated and later purified on

glass columns containing 1.5 g of Florisil gel (60-100 mesh, No. 12994, Merck, Darmstadt, Germany) according to the method described by Kostecki et al. [30]. Gel was activated for 1.5 h at 110°C prior to column preparation and the columns were conditioned with 5 mL of acetonitrile and washed with 5 mL of chloroform. The extract was applied to the top of the column and washed with 5 mL of chloroform followed by 5 mL of water. After solvent evaporation, the toxin residue was dissolved in 5 mL of methanol to be quantified.

Analysis of Mycotoxins by HPLC Method

Fumonisin B_1 in asparagus tissue was quantified according to the method described by Shepard et al. [31] and Sydenham et al. [29]. The FB_1 standard (1 ng μL^{-1} in methanol-water 1:1, v/v) was prepared and stored at 4°C. The OPA reagent (20 mg per 0.5 mL of methanol) was prepared and diluted with 2.5 mL of 0.1 M $Na_2B_4O_7$, then combined with 25 µL of 2-mercaptoethanol. The FB_1 standard or spear extracts were derivatized with OPA reagent. After 3 min, the reaction mixture (10 µL) was injected onto the HPLC column. Methanol-water solution of 0.1 M NaH_2PO_4 (77:23, v/v) was adjusted to pH=3.35 with *o*-phosphoric acid after filtration through a 0.45 µm Waters HV membrane and used as the mobile phase with a flow rate of 0.5 mL min^{-1}. A Waters 2695 apparatus, with C_{18} Nova Pack column (3.9×150 mm) and Waters 2475 fluorescence detector (λ_{EX}=335 nm; λ_{EM}=440 nm), were used for quantitative analysis of the metabolite. The FB_1 retention time was 7.35 min.

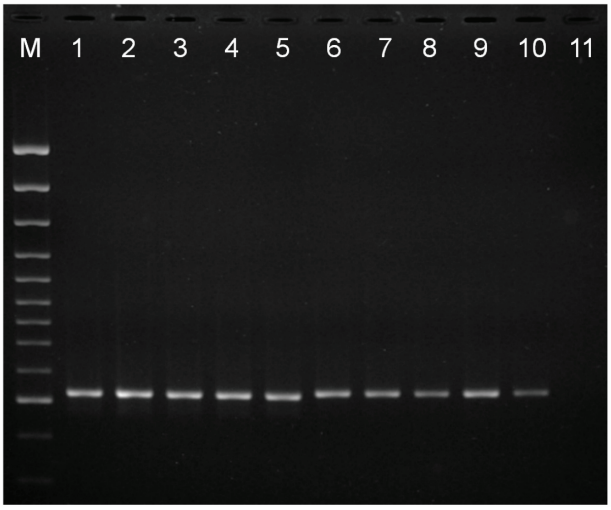

Fig. 1. Species-specific PCR for *F. proliferatum* and *F. oxysporum* identification.
Lane M - Gene Ruler™ 100 bp DNA Ladder Plus;
lanes 1-4 - *F. proliferatum* isolates;
lane 5 - a reference *F. proliferatum* isolate used as a positive control,
lanes 6 - 9 *F. oxysporum* isolates;
lane 10 - a reference *F. oxysporum* isolate used as a positive control;
lane 11 - a negative control.

Moniliformin amount was determined by the HPLC method using a Waters 501 apparatus with C_{18} Nova Pack column (3.9×300 mm) and Waters 486 UV detector (λ_{max}=229 nm). Acetonitrile-water solvent (15:85, v/v) buffered with 10 mL of 0.1 M K_2HPO_4 in 40% *t*-butyl-ammonium hydroxide in 1 L of solvent [32] was used as the mobile phase at a flow rate of 0.6 mL min^{-1}. The retention time of MON was 11.5 min.

Reagents

Standards: fumonisin B_1 and moniliformin provided from Sigma-Aldrich (Steinheim, Germany).

Solvents: acetonitrile, chloroform, *n*-hexane, methanol provided from Sigma-Aldrich (Steinheim, Germany).

Reagents: glacial acetic acid, 2-mercaptoethanol, *o*-phosphoric acid, *t*-butyl-ammonium hydroxide, sodium tetraborate, sodium dihydrogen phosphate, potassium hydrogen phosphate, potassium hydroxide, and paper filter (Whatman 4) were provided by Sigma-Aldrich (Steinheim, Germany). Water was obtained from a Milli Q system (Millipore, Billerica, Ma., USA).

Statistical Analysis

One-way analysis of variance was carried out for statistical verification of significant differences between sections of spears in regard to *Fusarium oxysporum* occurrence in different parts of asparagus (basal, central, apical), *F. proliferatum* (basal, central, apical), and fumonisin B_1 and moniliformin concentration levels. The least significant difference ($LSD_{0.05}$) for each trait was calculated. The relationship between *F. oxysporum* and *F. proliferatum* occurrence in different parts of plants, as well as between FB_1 and MON concentration levels, was estimated using correlation coefficients. The influence of *F. oxysporum* and *F. proliferatum* on FB_1 and MON concentration levels was estimated using regression analysis. The presence of *F. oxysporum* and the presence of *F. proliferatum* in different parts of asparagus spears were treated as independent variables and considered in individual models.

Results

In asparagus samples from both sets (with and without brown spots indicating fusariosis), the presence of *F. proliferatum* and *F. oxysporum* as well as the formation of mycotoxins (fumonisin B_1 and moniliformin) were observed. Morphological identification of *F. proliferatum* and *F. oxysporum* was confirmed by molecular method. Species-specific PCR products were 526 bp and 534 bp in length from *F. proliferatum* and *F. oxysporum*, respectively (Fig. 1). *F. oxysporum* was isolated from 75% of asymptomatic spears and from all spears with brown spots. On the other hand, *F. proliferatum* was identified in 35% of spears without and in 15% of samples with brown spots.

Table 1. Mean squares from the analysis of variance for number of asparagus spear sections infected by *Fusarium* fungi and mycotoxin concentrations (FB_1 and MON).

Source of variation	Degrees of freedom	*F. oxysporum*			*F. proliferatum*			FB_1	MON
		basal	central	apical	basal	central	apical		
Spears	1	0.841**	0.784**	0.169*	0.025	0.025*	0.001	339.8	3,300
Residual	38	0.089	0.069	0.035	0.0096	0.006	0.003	642.2	17,505

* significant at P<0.05
** significant at P<0.01

Table 2. Occurrence of *Fusarium* spp. in asparagus spears (mean values and least significant differences – LSD).

Values for spears infected by *Fusarium* spp.						
Section of spear	*F. oxysporum*			*F. proliferatum*		
	without spots	with spots	$LSD_{0.05}$	without spots	with spots	$LSD_{0.05}$
Basal	16	45	19	6	1	6
Central	18	46	17	5	0	5
Apical	6	19	12	1	2	3

Only 20% of spears were free from both species and disease symptoms. On the basis of analysis of variance it was proved that the occurrence of *F. oxysporum* in all analyzed spear parts (basal, central, apical) and *F. proliferatum* in the central part was significantly different between spears with brown spots and those without disease symptoms (Table 1). *F. oxysporum* was observed more often in all parts of spears with brown spots, while *F. proliferatum* was observed more often in central parts without disease symptoms (Table 2).

The HPLC analysis revealed that both mycotoxins (FB_1 and MON) were found at different contamination levels. FB_1 was determined in the concentration range 0.16-152.68 ng g^{-1} (average 7.52), while moniliformin was detected in the range 15.30-585.00 ng g^{-1} (mean 121.00) (Figs. 2 and 3). Only 10% of analyzed spears were free from both metabolites.

In both groups (with and without brown spots), the influence of *F. proliferatum* presence (percentage of positive samples) on concentrations of both metabolites was not found. However, it is worth noting the inversely proportional influence (P<0.05) of *F. oxysporum* occurrence in the central part of spears with brown spots on moniliformin contamination levels. *F. oxysporum* occurrence in the apical part of spears influenced FB_1 concentration (P<0.001).

We have observed a significant correlation between *F. oxysporum* occurrence in different parts of spears:
(i) basal and central (r=0.319, P<0.05),
(ii) basal and apical (r=0.363, P<0.05),
(iii) central and apical (r=0.407, P<0.01).

In contrast, we observed a correlation between *F. proliferatum* occurrence in basal and central parts of spears only. No correlation between the occurrence of both species (*F. oxysporum*, *F. proliferatum*) and concentration of both metabolites (FB_1, MON) was found in analyzed spears (P>0.05).

Discussion of Results

The presented results indicate high contamination of asparagus spears by mycotoxins – metabolic products of *Fusarium* spp. colonizing plants. Both *F. proliferatum* and *F. oxysporum* are known as mycotoxin producers, although with different profiles, so correct identification of them is of great importance, because each species possesses different toxicological risks for humans. Quick and precise molecular identification of pathogenic species is useful for the prevention of pathogenic and toxigenic risks connected with *Fusarium* infection [27]. Various PCR-based techniques have been developed to detect and distinguish *Fusarium* spp.

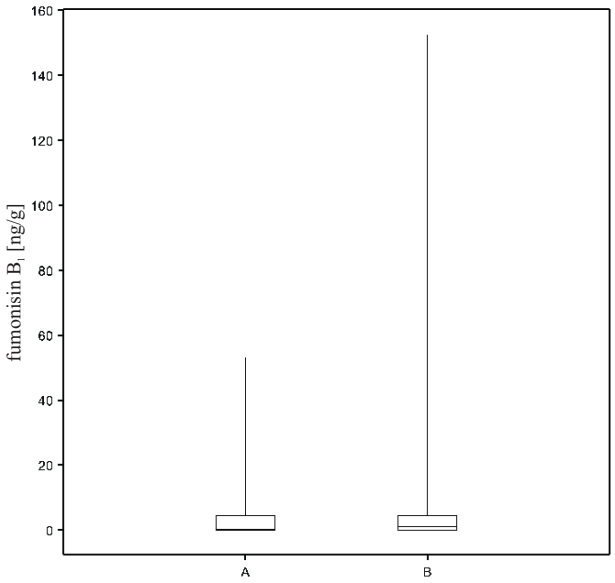

Fig. 2. Boxplot of the fumonisin B_1 concentration in asparagus spears: (A) with brown spots, (B) without symptoms.

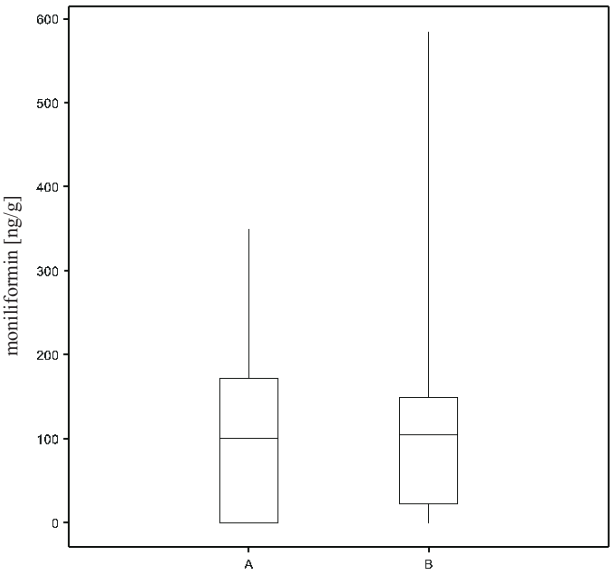

Fig. 3. Boxplot of the moniliformin concentration in asparagus spears: (A) with brown spots, (B) without symptoms.

These fungi are usually differentiated by either mycotoxigenic genes, other genes, ribosomal DNA or SCAR (sequence-characterized amplified region) markers [27, 33].

Asparagus spears from Poland and Germany have been previously reported to be infected with *Fusarium* spp. [25]. The fungi grow from infected crowns into emerging spears. In California, the predominant species recovered from marketable spears is *F. oxysporum*, while in the warmer, desert regions of northern Mexico, *F. proliferatum* was isolated from up to 98% of spears taken directly from packed commercial 5-kilo cartons [3]. Asparagus spears available to consumers in Connecticut were colonized by *Fusarium* spp. with variable frequency depending on the part of the spear, origin of the crop, and time of spear collection, but the majority of isolates were identified as *F. proliferatum* and *F. oxysporum* [6].

Available literature on the presence (in different plant materials) of fumonisin B_1 and moniliformin (as metabolic products of mainly *Fusarium proliferatum*) gives highly diverse information. In the USA and Slovakia concentrations of FB_1 and MON were analyzed in poultry feeds. Contamination with these toxins was much higher in the USA (FB_1 61-546 μg g^{-1}, MON 66-367 μg g^{-1}) [18]. In Slovakia moniliformin concentration was at a similar level as in this study but for fumonisin B_1 was much higher [34]. Other American studies concerning maize and its products showed a level of contamination with fumonisin B_1 and moniliformin in the ranges of 43-1642 and 26-774 ng g^{-1}, respectively. Simultaneous occurrence of FB_1 and MON was recorded in 34% of individual samples [35]. The first report on the occurrence of FB_1 in asparagus spears infected by *F. proliferatum*, originating from Italy, indicated the presence of mycotoxins in crowns and assimilation shoots [3, 8] and reported that the incidence of *F. proliferatum* colonization can be high in spears with no detectable level of fumonisins. In contrast, in this study a high level of mycotoxins was detected in asymptomatic and uninfected spears.

Our present studies confirm earlier results [10]. No influence of *F. proliferatum* occurrence on accumulation of either mycotoxin in spears is the most important observation. It is worth mentioning that only *F. proliferatum* is known as an FB_1 producer, while *F. oxysporum* is recognized as a species not able to form this mycotoxin [10, 36]. Both mycotoxins were more often found in spears without symptoms of fusariosis than in diseased ones. A possible explanation of the above phenomenon is as follows: stem and crown rot of asparagus is associated with a higher abundance of toxigenic fungi (able to form FB_1 and MON). In consequence, the mycotoxins accumulated in crowns can be transported with nutrients to developing spears. To some extent this concept is similar to the results indicating penetration of deoxynivalenol into kernels of cereals with scab symptoms and pathogen development [37]. It is necessary to point out the differences in the structure of kernels and spears that result in only limited penetration of mycotoxins in the former (hard tissue). Since the metabolites' migration into the kernel is also limited, mycotoxins accumulate mostly on the surface, and in consequence such processes as cleaning, washing, hulling, grinding and milling of cereal kernels can significantly reduce the concentration levels of the mycotoxins in final products [38, 39]. In spear tissue, because of intensive transpiration, the mycotoxins can be more uniformly distributed in all kinds of tissues, including those not infected by toxigenic fungi and devoid of disease symptoms.

In successive studies from Germany, fumonisin B_1 was found in 90% of analyzed asparagus samples [9, 40]. In a report from China the mean concentration of FB_1 in analyzed samples was 123 ng g^{-1} [19]. The differentiation of toxin accumulation levels in analyzed asparagus spears, found in this study, can be explained by different susceptibility of cultivars to pathogen infection and disease development. It might also depend on soil conditions and plant stress caused by harvesting [41, 42]. Higher concentrations of both toxins (FB_1 and MON) in the basal part of spears may frequently result from the higher incidence of colonization by toxigenic fungi in this part. In cereals, four types of plant resistance mechanisms to fusariosis have been suggested: resistance of the host plant to infection with pathogenic fungi (mechanism I); resistance of the host to pathogen spread in the tissue (II); ability of the plant to degrade mycotoxins (III); and tolerance of the host tissue to a high concentration of toxic metabolites (IV) [43]. In asparagus, the above-suggested transport of mycotoxins with nutrients, and in consequence probable uniform (lower) concentration in the tissue, could be a new mechanism of plant resistance to the pathogen.

Considering the harmfulness of analyzed mycotoxins formed by fungal pathogens, it is necessary to improve the quality of produced asparagus spears and protect the health of consumers. Genetic selection of asparagus cultivars resistant to fusariosis on the one hand and screening of

spears for the presence of fungi and accumulation of toxic secondary metabolites on the other hand are very important for the production of high-quality, safe food.

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