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Original Research

Fungi Presence and their Mycotoxin Distribution in Asparagus Spears

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Abstract

Fusarium oxysporum, *F. proliferatum*, *F. culmorum*, *F. heterosporum*, and *F. solani* were detected in asparagus spears. Identification based on morphology of *F. proliferatum* and *F. oxysporum* was confirmed by PCR with species-specific primers. The incidence of *Fusarium* colonization was the highest in the basal epidermal tissue of spears. The studies revealed high levels of MON and low levels of FB_1 in spears infected by *F. oxysporum*. There was no association between *F. proliferatum* occurrence and FB_1 or MON presence. Mycotoxins were found in spears with and without the disease symptoms, originating from Poznań and Świdwowiec, although fungi were common only in spears from the second location. However, in healthy spears the mycotoxin concentrations were low or undetectable. The presence of mycotoxins in uninfected spears may suggest their transport through vascular tissue from possible sources such as crowns and roots to spears.

Keywords: *Asparagus officinalis*, fumonisin B_1, moniliformin, HPLC, species-specific PCR

Introduction

Asparagus (*Asparagus officinalis* L.) is a vegetable grown worldwide, with well documented knowledge on its nutritional value. Its spears are known as a source of protein, vitamins, saponins, and microelements [1-4].

Today, throughout the world, over 210,000 ha are under asparagus production [5]. Europe has the largest share of the world's asparagus acreage (52,500 ha), followed by North America (45,500 ha). The EU's production made up 9% of the world's production. Asparagus is grown in Europe mostly in Spain (30% of EU total), Germany (18%), France (15%), Italy (15%), and Greece (14%) [6].

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It is important for consumers to have asparagus spears free of pathogenic fungi and their toxins. Fungi of *Fusarium* genus are one of the most serious pathogens of asparagus [7]. The severity of particular *Fusarium* species depends on asparagus cultivar susceptibility, environmental conditions, agricultural practices, and other factors [8-10]. Some of the pathogens are able to form mycotoxins – secondary metabolites with possible health hazards and significant influence on food safety.

Fusarium proliferatum is one of the most common asparagus pathogens, producing fumonisins [11, 12]. Among known metabolites of fungi, fumonisin B_1 (FB_1) presence is one of the greatest toxicological concerns. FB_1 is toxic to the liver in all species and to the kidney in a range of laboratory and farm animal species, causing apoptosis

followed by mitosis in the affected tissues. Moreover, FB_1 inhibits ceramide synthase and disrupts sphingolipid metabolism [13]. Contamination of food with FB_1 has been associated with oesophageal cancer of humans in Africa and China [14], and is a proposed risk factor for birth defects in United States and South Africa [15]. The evidence that FB_1 causes liver and kidney cancer in rats [16, 17] led to the classification of fumonisins as carcinogenic to animals and as possible human carcinogens [18]. Fumonisins exhibit various immunological and reproductive effects under experimental conditions, but their impact on food safety is still poorly understood [19]. Moniliformin (MON) and other mycotoxins are also produced by *F. proliferatum*. Diets containing material naturally contaminated with MON can cause reduced performance, hematological disorders, and mortality in rodents, chicks, and pigs. In poultry, both acute and chronic effects, with progressive muscular weakness, respiratory distress, cyanosis, coma, and death being the most prominent symptoms were described [20]. At present, MON is regarded as cytotoxic and is a suspected cause of Keshan disease, a human heart problem that occurs in rural regions of China and South Africa [21-23]. The natural occurrence of mycotoxins in asparagus tissues indicates a possible risk for human health. The influence of food processing on secondary metabolite toxicity remains unclear - there is the evidence, for example, that some fumonisin decomposition products are even more toxic than fumonisin itself [24]. However, hydrolized FB_1 is less toxic than FB_1 [25, 26] as toxin-sugar reaction products [27].

The aim of this study was identification of *Fusarium* spp. and determination of fumonisin B_1 and moniliformin concentration in green and white asparagus spears. The impact of location and host origin on distribution of different *Fusarium* species and the association between mycotoxin levels and the presence of particular *Fusarium* species were evaluated.

Experimental Procedures

Asparagus Spear Samples

A field experiment was performed to investigate a subset of *Fusarium* species naturally infecting asparagus spears. Spears of two asparagus cultivars, Eposs and Gijnlim, were sampled in two locations in the Poznań region – Marcelin and Świdwowiec. Green spears of both cultivars in Marcelin and the Gijnlim in Świdwowiec were cut just above the soil surface. White spears of Eposs in Świdwowiec were cut about 5 cm above the crown. In Świdwowiec, Gijnlim has been planted since 1993 on sandy soil, and Eposs since 1997 on the same soil type. Therefore, spear samples were collected from this location in the 12th and the 8th years of harvest, respectively. In Marcelin, both cultivars were planted on sandy loam soil in 2000 and spears were sampled in the 5th year of harvest. The total monthly precipitation before harvest was 76 mm in Świdwowiec and 65 mm in Poznań, and temperature was 13.6 and 13.7ºC, respectively.

Morphological Identification of *Fusarium* spp.

Subsets of ten white spears of Eposs asparagus and ten green spears of Gijnlim asparagus from Świdwowiec, as well as ten green spears of both asparagus cultivars from Marcelin, were collected May 23, 2005. All spears were divided into six parts (epidermal: basal, central, and apical, as well as pericambium and vascular tissue: basal, central, and apical). For fungi isolation, from each of six parts, after surface disinfection with 1% sodium hypochlorite, five sections of tissue (2 mm in diameter) were cut out and transferred onto separate Petri dishes containing potato dextrose agar medium (PDA) (Merck, Darmstadt, Germany) with streptomycin (100 μg/mL). Single spore cultures of fungi grown from the mentioned sections were transferred onto standard media and identified according to mycological keys by Booth [28], Gerlach and Nirenberg [29], and Kwasna et al. [30].

Molecular Identification of *F. oxysporum* and *F. proliferatum*

Mycelia from 9-day old single-spore cultures of *F. oxysporum* and *F. proliferatum* grown on liquid medium (5 g/L of glucose, 1 g/L 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) [31, 32]. *F. proliferatum* strain (DSM No. 62261) and *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 species-specific primers were designed based on a partial sequence of the calmodulin gene.

The amplification reactions were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Valencia, USA) in a small total volume (5 μL). The reaction mixture has been described before [33]. Amplification was carried out in a Biometra T*personal* 48 thermocycler (Whatman Biometra, Goettingen, Germany) using the following programme: initial denaturation for 3 minutes at 94ºC, followed by 35 cycles of denaturation at 94ºC for 40 seconds, primer annealing at 60ºC for 40 seconds and extension at 72ºC for 1 minute. The amplification was ended with an additional extension at 72ºC for 3 minutes. The PCR products were separated by electrophoresis in 1.5% agarose gels with 1× 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.

Chemicals and Reagents

Standards of pure fumonisin B_1 and moniliformin were purchased from Sigma (St. Louis, MO, USA).

Acetonitrile, methanol (HPLC grade), disodium tetraborate, 2-mercaptoethanol and *t*-butyl-ammonium hydroxide were purchased from Sigma-Aldrich. Potassium hydroxide, sodium dihydrogen phosphate, acetic acid, *n*-hexane, chloroform, *o*-phosphoric acid were purchased from POCh (Poland). Water for the HPLC mobile phase was purified using a Milli-Q system (Milipore, Bedford, MA, USA).

Mycotoxin Analyses

Samples (10 g) of asparagus tissue from the basal parts of spears were homogenized for 3 min in 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. [34]. 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 a pH of 5.8-6.5 with 0.1 M KOH water solution. A SAX cartridge was attached to the SPE manifold unit (Supelco, Bellefonte, PA, USA) which was conditioned successively with 5 mL of methanol followed by 5 mL of methanol-water (3:1, v/v). An aliquot of 5 g of filtered subsample extract (10 mL) was applied on the top of the conditioned cartridge and washed with 8 mL of methanol-water (3:1, v/v), immediately followed by 3 mL of methanol. FB_1 was eluted with 10 mL of 1% acetic acid in methanol at a flow rate of 1 mL/min from the column to a glass collection vial. The eluate was evaporated to dryness under a nitrogen stream. Fumonisin B_1 in purified extract of asparagus tissue was quantified by the HPLC method described by Shepard et al. [35] and Sydenham et al. [34]. The FB_1 standard or spear extracts were derivatized with OPA reagent solution. After 3 minutes, 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 the flow rate of 0.5 mL/min. Waters 2695 apparatus, with a C_{18} Nova Pack column (3.9×150 mm) and a Waters 2475 fluorescence detector (λ_{EX}=335 nm and λ_{EM}=440 nm) were used to quantify the metabolite (on the basis of peak area). The FB_1 retention time was 7.35 min. The detection limit was 1.0 ng/g for FB_1. Positive results (on the basis of retention time) were confirmed by HPLC analysis and compared with the relevant calibration curve (correlation coefficient for FB_1 was 0.9967). Recovery for FB_1 was 93%, which was measured in triplicates by extracting the mycotoxin from blank samples spiked with 1.0-10.0 ng/g of the compound. The relative standard deviations were less than 8%.

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 earlier by Kostecki et al. [36]. The gel was activated for 1.5 h at 110ºC 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 on 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. Moniliformin concentration level was determined by the HPLC method and Waters 501 apparatus with C_{18} Nova Pack column (3.9×300 mm) and a Waters 486 UV detector (λ_{max}=229 nm) was used. 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 was used as the mobile phase at a flow rate of 0.6 mL/min. The MON retention time was 11.5 min. Positive results (on the basis of retention time) were confirmed by HPLC analysis (peak area) and comparison with the relevant calibration curve (correlation coefficient for MON was 0.9990). Recovery for MON was 90%, which was measured in triplicates by extracting the mycotoxins from blank samples spiked with 10-100 ng/g of the compounds. The relative standard deviation (R.S.D.) value was below 7%.

Statistical Analysis

Uniformity of distribution of *F. oxysporum* and *F. proliferatum* occurrence in vertical (V) parts, (basal, central and apical) and horizontal (H) parts, (epidermal part as well as pericambium and vascular tissue) was tested by a chi-square test for small sample sizes independent for the Eposs and Gijnlim cultivars. Two-sample *t*-test was used for statistical verification of the hypothesis of lack of differences in *F. oxysporum* and *F. proliferatum* occurrence in white and green spears [37]. Also, two-way analysis of variance was carried out to determine the effects of location, L (Poznań and Świdwowiec), cultivars, C (Eposs and Gijnlim), and the location × cultivars (L×C) interaction on the variability of FB_1 and MON amount accumulated. The least significant differences (LSD) for traits were calculated. The relationship between FB_1 and MON was calculated using the correlation coefficient [38]. The association between *F. oxysporum* and *F. proliferatum* (different parts of spears) occurrence and amount of FB_1 and MON synthesized was estimated using analysis of regression [39]. The occurrence of *F. oxysporum* and *F. proliferatum* in each analyzed part of spears was treated and considered as an independent variable in individual models.

Results

Fusarium oxysporum, *F. proliferatum*, *F. culmorum*, *F. heterosporum*, and *F. solani* were detected in analyzed asparagus spears both with or without brown spots. Molecular verification of mycological identification of *F.*

Table 1. Distribution of *Fusarium* spp. in asparagus spears in Świdwowiec (n=50).

Part of spear		Eposs cv.		Gijnlim cv.	
		Sections infected by [%]			
		F. oxysporum	*F. proliferatum*	*F. oxysporum*	*F. proliferatum*
Epidermal	basal	36	20	16	4
	central	16	12	0	0
	apical	8	8	0	0
Pericambium and vascular tissue	basal	6	8	4	0
	central	4	2	0	0
	apical	0	0	0	0
Chi-square		1.87	3.57	3.36	2.40
p value		0.399	0.169	0.186	0.301

Table 2. Means and standard deviations for fumonisin B_1 and moniliformin concentration detected in asparagus spears infected* by *Fusarium* spp (n=50).

Cultivar	Fumonisin B_1 [ng/g]				Moniliformin [ng/g]			
	Marcelin		Świdwowiec		Marcelin		Świdwowiec	
	Mean	Standard deviations	Mean	Standard deviations	Mean	Standard deviations	Mean	Standard deviations
Eposs	195.8b	80.59	273.0a	103.63	37.65b	67.09	48.30b	46.22
Gijnlim	312.0a	68.42	288.7a	65.16	1.20b	3.79	146.20a	147.08
Mean for cultivars	253.9A	93.99	280.9A	84.64	19.43D	49.92	97.25C	117.38
Grand mean	256.6				89.7			
$LSD_{0.05}$ Location	52.2				53.9			
$LSD_{0.05}$ Cultivar	52.2				53.9			
$LSD_{0.05}$ L×C	73.8				76.3			

In columns followed by the same small letters means not significantly different.
In row followed by the same big letters means not significantly different.
* The concentration of mycotoxins in healthy spears was low or undetectable.

proliferatum and *F. oxysporum* was done. Species-specific PCR products were 526 bp and 534 bp in length from *F. proliferatum* and *F. oxysporum*, respectively [40].

Among all fungi, *F. oxysporum* and *F. proliferatum* occurred the most often. Both species were also found more often in white (>70%) than in green (<30%) spears cultivated in Świdwowiec. *F. oxysporum* was present in 71.55%, and *F. proliferatum* in 79.63% of white spears ($P<0.05$). The incidence of *Fusarium* colonization was the highest in the basal epidermal tissue of spears (Table 1). In green spears grown in Marcelin, only a few *F. heterosporum* isolates were found. Chemical analysis revealed different levels of both mycotoxins – FB_1 and MON (Table 2). FB_1 was determined in the concentration range (ng/g) 31.2-436.5 (mean 256.6), while moniliformin was detected in the range (ng/g) 48.0-870.0 (mean 89.7). Fumonisin B_1 was found in all analyzed spears, while MON was detected in 40.5% of samples.

Field location, beside of other factors, had a significant influence on the amount of MON with no impact on FB_1 presence in spears. Higher levels of MON and FB_1 were rather found in spears of asparagus grown in Świdwowiec than in Marcelin (Table 2, Figs. 1, 2). FB_1 concentration did not depend on agronomic factors (location, cultivar) because the significance of the differences resulted from the difference between Eposs and Gijnlim in Marcelin. However, MON concentration might be affected by factors other than location and cultivar, although it is not possible to estimate these factors based on the data presented in this paper. The amount of FB_1 detected in Eposs spears was

inversely proportional to the occurrence of *F. oxysporum* (y=238.2-83x). On the other hand, the amount of MON was directly proportional to the occurrence of *F. oxysporum* detected in the basal part of the pericambium and vascular tissue (y=38.0+249x). The correlation coefficient between FB_1 and MON was not significant (-0.036, P>0.05). *F. oxysporum* occurrence in basal pericambium tissue (explanatory variable, x) influenced FB_1 concentration (response variable, y). Linear regression analysis of the data gave the following equation: y=302.4-432x. Percentage of variation accounting for the explanatory variable was 27.7 (standard error of observation = 72).

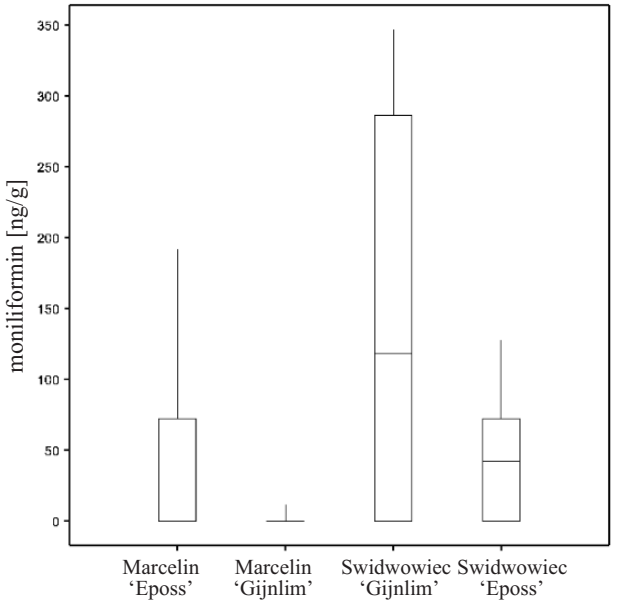

Fig. 1. Box-and-whisker diagram of moniliformin concentration, classified by locations and cultivars. The box spans the interquartile range of the values in the variate, so that the middle 50% of the data lie within the box, with a line indicating the median; whiskers extend beyond the ends of the box as far as the minimum and maximum values. $LSD_{0.05}$=76.3

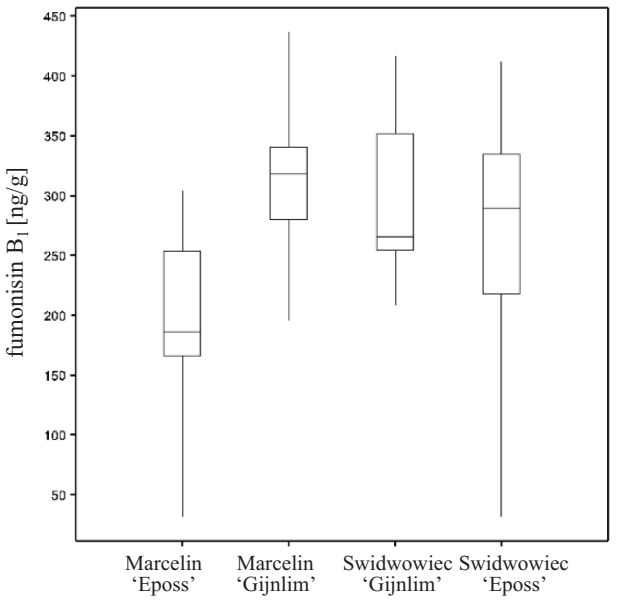

Fig. 2. Box-and-whisker diagram of fumonisin B_1 concentration, classified by locations and cultivars. $LSD_{0.05}$=73.8

Discussion

F. proliferatum and *F. oxysporum* are known as the main pathogens of asparagus plants worldwide. Although *F. oxysporum* is cosmopolitan, *F. proliferatum* tends to be more prevalent in soil of warmer regions than in cooler ones [41]. In these studies several *Fusarium* species were detected in asparagus spears, including *F. oxysporum* and *F. proliferatum* as the dominant ones. Variation among countries in the occurrence of these species has been reported. For example *F. oxysporum* was the most common species in the Netherlands and was isolated from 80% of examined plants, while *F. proliferatum* was not observed in analyzed samples [42]. *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* were the main pathogens of asparagus in Spain and were found at rates of about 40% each [5]. In contrast, in Italy and Connecticut (USA) diseased asparagus plants were colonized mainly by *F. proliferatum* [41, 21]. Besides the species mentioned above, in our studies *F. culmorum*, *F. heterosporum*, and *F. solani* were found rarely, while in the studies of Gossmann et al. [9]. *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. redolens*, *F. sambucinum*, *F. solani*, and *F. subglutinans* were identified occasionally.

Because *Fusarium* species infecting asparagus differ in pathogenicity and in their capacity for mycotoxin, biosynthesis, correct identification is of paramount importance for risk assessment of spears for human consumption. Fusarial populations are traditionally identified by their cultural and morphological characteristics. However, this approach may be inappropriate due to the limited number of taxonomic characters and small differences of available features [43]. As a consequence, proper identification is carried out in different ways, including molecular detection. Species-specific primers for *Fusarium* detection have been generated from sequences of diverse origin: anonymous DNA fragments [34], rDNA [45, 46] or on structural genes [31, 32, 47]. In our study, specific PCR products for *F. proliferatum* and *F. oxysporum* were obtained, confirming the utility of the primer used. This result demonstrates that analyzed isolates did not exhibit genomic alterations in the primers annealing sites and the length of the genomic region tagged by specific primers was not changed by insertion or deletion events.

Presented results revealed that the incidence of *Fusarium* colonization was the highest in the basal epidermal tissue of spears, which is in accordance with other studies [41, 10]. Guerrero et al. [48] considered the crown as a probable source of these species what may explain why basal parts of spears were colonized more often than other segments. Moreover, Elmer [41] claimed that rapid growth of the spear may allow the apical segments to escape the advancing hyphae.

There was considerable variation in the number of *F. proliferatum* strains producing fumonisin B_1 and in the

amounts of FB_1 produced by strains from different sources [49]. *F. proliferatum* can produce both mycotoxins (FB_1 and MON) at high concentrations, whereas only some isolates of *F. oxysporum* can produce FBs at much lower concentration levels [40]. Because *F. oxysporum* taxonomic status is unclear, the concept of a genetic variation (e.g. mycotoxin profile) is still discussed [50]. These studies revealed a high level of MON and low level of FB_1 associated with the occurrence of *F. oxysporum* in infected spears. The higher the level of MON, the higher the percentage of infection caused by this pathogen. Moreover, there was no significant association between *F. proliferatum* occurrence and FB_1 and MON presence. Logrieco et al. [11] reported that FB_1 was present in the *F. proliferatum* – infected crown tissue and stems at concentrations of 7.4 μg/g and 0.83 μg/g, respectively. In Liu et al. studies [51] the mean FB_1 concentration in analyzed samples from China was 123 ng/g and the toxin was found in 80% of spears. Nigh et al. [52] reported lower incidence of *F. proliferatum* infection of spears harvested from the northern fields of California than from Mexican fields. No fumonisin was found in Mexican and Californian spears that were colonized by *F. proliferatum*.

In contrast, presented results demonstrate that mycotoxins were found in spears with and without disease symptoms, even in spears where fungi were not detected. However, in healthy spears the mycotoxin concentration was low or undetectable. The presence of mycotoxins in uninfected asparagus spears may suggest their transport through vascular tissue from crowns and roots to spears. It is interesting that FB_1 or FB_2 were not translocated in maize seedlings growing in the autoclaved soil when administered in the watering solution [53]. This statement suggests that the fungus-plant interaction is necessary for FB_1 translocation in maize seedlings. It seems that such transport of FB_1 might appear in asparagus plants naturally infected by fungi. However, it should be noted that in this experiment fungi and mycotoxins were only isolated from spears and more extensive studies are necessary to clarify the method of mycotoxin transport. Asparagus alone is not a significant source of mycotoxins, but under some circumstances could contribute to exposure when consumed with other food products containing FB_1 and MON.

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