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Production and Migration of Mycotoxins in Sweet Pepper Analyzed by Multimycotoxin LC-MS/MS

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In this work the presence and migration behavior of mycotoxins formed in sweet pepper, inoculated by *Fusarium* species involved in internal fruit rot, were investigated. Two different commercial sweet pepper cultivars were inoculated with two different *Fusarium proliferatum* isolates that were sampled from diseased peppers. After 10 days of incubation at 20 °C in a closed container, the lesion caused by the fungal infection was dissected. Around the lesion, up to three concentric rings of pepper fruit tissue with a width of 5 mm were cut out and analyzed using a multimycotoxin LC-MS/MS method. The analyses resulted in the detection of beauvericin and fumonisins B_1, B_2, and B_3. Beauvericin was detected only in the lesions (95%), and the levels varied between 67 and 73800 μg/kg. Fumonisins B_1, B_2, and B_3 were detected in the lesions and in the surrounding tissue, indicating migration of these toxins into healthy parts of the sweet pepper. In the lesion the fumonisin B_1 level varied between 690 and 104000 μg/kg. Even in the outer ring fumonisin B_1 was still present. Mostly it was present at a lower level than in the lesion, with a maximum level of 556 μg/kg. A similar migration behavior was obtained for fumonisins B_2 and B_3, but lower levels were detected in the lesions, up to 10900 and 1287 μg/kg, respectively. The analysis of 20 pepper samples resulted in the detection of beauvericin or alternariol. Seven samples were contaminated, and the level of beauvericin was 124 μg/kg (N = 1), whereas the level of alternariol varied from below the LOQ (6.6 μg/kg) to 101 μg/kg (N = 6).

KEYWORDS: LC-MS/MS; multimycotoxin; sweet pepper; internal fruit rot; migration of mycotoxins

INTRODUCTION

Mycotoxins are toxic fungal secondary metabolites mainly produced by *Fusarium*, *Penicillium*, *Aspergillus*, and *Alternaria* species. The occurrence of mycotoxins in foodstuffs such as maize, grains, nuts, and different kinds of seeds have been extensively investigated (*1*). Data describing the occurrence of mycotoxins in vegetables are rare. However, the high water activity and a nearly neutral pH increase the susceptibility for fungal spoilage in these products (*2*). Fumonisin B_1 and moniliformin were detected in asparagus spears sampled in Poland (*3*). Alternariol, alternariol methyl ether, and altenuene were detected in tomatoes, peppers, and olives (*4*). The presence of HT-2 toxin, diacetoxyscirpenol, and zearalenone in potato products and zearalenone in pepper and beans has been reported (*1*). In dried vegetable products such as okra, hot chili, tomato, onion, and baobab leaves sampled from Benin, Mali, and Togo mycotoxigenic *Aspergillus* species were identified. The analysis of aflatoxins in these products resulted in the detection of aflatoxins B_1 and B_2 in okra and hot chili (*5*).

Internal fruit rot of sweet peppers is a fungal disease that has caused considerable yield losses in commercial greenhouses in British Columbia and Alberta (Canada) and several European countries since 2003 (*6*). In the early stages of infection, there are no symptoms visible on the outside of the fruit. External soft rot symptoms may appear following further mycelial colonization of the internal parts, but frequently these symptoms become visible only after the fruits have been harvested or even commercialized. The etiology and epidemiology of this disease are still under investigation. In a recent study (*6*) 56 *Fusarium* isolates were collected from infected pepper fruits and stems: 32 were identified as *F. lactis*, 18 as *F. solani*, 3 as *F. proliferatum*, and 3 as *F. oxysporum* species. Two of the identified *Fusarium* species (*F. lactis* and *F. proliferatum*) belong to the *Gibberella fujikuroi* species complex, and they are mycotoxin-producing fungi. Species belonging to this complex are able to produce fumonisins, beauvericin, fusaric acid, moniliformin, zearalenone, and type A and B trichothecenes (*7*). In a multimycotoxin study (*8*) four sweet peppers were inoculated with two different *Fusarium* isolates obtained from peppers affected with internal fruit rot, namely, a *F. proliferatum* isolate and an isolate with high EF-1α gene sequence homology to *F. lactis*. Beauvericin and fumonisins B_1, B_2, and B_3 were detected in the samples inoculated with *F. proliferatum*, whereas only beauvericin was detected in the samples inoculated with the *Fusarium* species related to *F. lactis*.

Although consumers will reject a visibly rotten food product, processed products may still form a source of mycotoxins.

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Consumers can also cut out the rotten part of the food product and consume the remainder not knowing or realizing the possible risk for mycotoxin contamination. Only a few studies have been published about the occurrence of mycotoxins in noncolonized parts of the food product. Some studies report the migration of patulin in pears, apples, tomatoes, and wheat bread and the occurrence of ochratoxin A in moldy vegetables after removal of rotten tissues (*9*−*12*). These results demonstrate the possible risk of mycotoxin contamination in noninfected parts of food products. On the one hand, this study was designed to investigate the migration behavior of mycotoxins formed in sweet pepper, inoculated by *Fusarium* species known to cause internal fruit rot. Twenty commercial sweet pepper samples were also analyzed using a multimycotoxin LC-MS/MS method (*8*).

MATERIALS AND METHODS

Reagents and Chemicals. Methanol and acetonitrile were of high-performance liquid chromatography grade (VWR International, Zaventem, Belgium). Ethyl acetate and *N*,*N*-dimethylformamide were purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). Sodium hydroxide, acetic acid, and formic acid from Merck (Darmstadt, Germany) were used. Ammonium acetate was supplied by Grauwmeer (Leuven, Belgium). Mycotoxin standards nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, fusarenon-X, aflatoxins B_1, B_2, G_1, and G_2, HT-2 toxin, alternariol, alternariol methyl ether, altenuene, ochratoxin A, zearalenone, fumonisins B_1 and B_2, beauvericin, sterigmatocystin, and zearalanone were purchased from Sigma Chemical Co. (Bornem, Belgium). Diacetoxyscirpenol and T-2 toxin were purchased from Biopure (Tulln, Austria). Fumonisin B_3 was obtained from Promec unit (Tygerberg, South Africa). Nivalenol (100 μg/mL), neosolaniol (100 μg/mL), and diacetoxyscirpenol (101.8 μg/mL) stock solutions were certified solutions in acetonitrile. The fumonisin B_3 standard (1 mg) was dissolved in 1 mL of acetonitrile/water (50:50, v/v). Stock solutions of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X, aflatoxins B_1, B_2, G_1, and G_2, HT-2 toxin, T-2 toxin, altenuene, ochratoxin A, zearalenone, beauvericin, fumonisins B_1 and B_2, sterigmatocystin, and zearalanone (1 mg/mL) were prepared in methanol. Alternariol and alternariol methyl ether stock solutions (1 mg/mL) were prepared in methanol/dimethylformamide (60:40, v/v). All stock solutions were stored for 1 year at −18 °C except fumonisin B_3, which was stored at 4 °C. Working standard solutions were made by diluting the stock standard solutions in methanol and were stored at −18 °C for 6 months. From the individual stock standard solutions and working solutions was prepared a standard mixture with the following concentrations in methanol: nivalenol (7.5 ng/μL); alternariol methyl ether (5 ng/μL); fusarenon-X, fumonisins B_1, B_2, and B_3 (2.5 ng/μL); deoxynivalenol, 3-acetyldeoxynivalenol, alternariol, HT-2 toxin, and zearalenone (1 ng/μL); 15-acetyldeoxynivalenol and beauvericin (0.5 ng/μL); neosolanil (0.3 ng/μL); aflatoxins G_2, G_1, B_2, and B_1, altenuene, and sterigmatocystin (0.2 ng/μL); and diacetoxyscirpenol, T-2 toxin, and ochratoxin A (0.1 ng/μL). The mixture was stored at −18 °C for 3 months.

Extraction and Cleanup Materials. Bakerbond aminopropyl (NH_2) solid phase extraction (SPE) columns were purchased from Achrom (Zulte, Belgium). The NH_2-SPE column was conditioned by passing through 3 mL of ethyl acetate/formic acid (99:1, v/v). Octadecyl (C_{18}) SPE columns were purchased from Grace Discovery Sciences (Lokeren, Belgium). The C_{18}-SPE column was conditioned by passing through 5 mL of acetonitrile/water (84:16, v/v). Bond Elut strong anion exchange (SAX) SPE columns were obtained from Varian (Sint-Katelijne Waver, Belgium). The SAX column was conditioned by passing through 4 mL of methanol followed by 4 mL of methanol/water (75:25, v/v). Ultrafree-MC centrifugal filter devices (0.22 μm) of Millipore (Bedford, MA) and Nanosep MF centrifugal devices (0.22 μm) of Pall (Zaventem, Belgium) were used.

Sample Preparation. The stem and seeds of the sweet pepper were removed, and the edible tissue was ground and homogenized with a Homex 6 (Bioreba, Reinach, Switzerland). Three grams was extracted with 15 mL of ethyl acetate/formic acid (99:1, v/v) and shaken for 15 min on a horizontal shaker and centrifuged for 5 min at 3300*g*. The upper layer was filtered through a folded Ederol, 12.5 cm, quality 15, filter paper (VWR) and collected. This process was repeated with 10 mL of ethyl acetate/formic acid (99:1, v/v). The extraction volume was adjusted to 30 mL. Ten milliliters was kept apart for the SAX cleanup, whereas the remaining part of the extract was evaporated to 5 mL and cleaned up by passing through the NH_2-SPE column. After evaporation of the acidified ethyl acetate, the dry extract was dissolved in 3 mL of acetonitrile/water (84:16, v/v) and cleaned up by passing through the C_{18}-SPE column. The eluate was directly collected in a test tube.

The 10 mL sample extract for SAX cleanup was evaporated to dryness. The residue was dissolved in 5 mL of methanol/water (75:25, v/v). Sodium hydroxide (0.25 M) was added to obtain pH 5.8−6. Afterward, the extract was cleaned up with the SAX column, which was washed by passing through 4 mL of methanol/water (75:25, v/v) followed by 4 mL of methanol. Four milliliters of methanol/acetic acid (99:1, v/v) was used to elute and to collect the mycotoxins in the above-mentioned test tube, by which both eluates were combined in one test tube Afterward, the combined solvents were evaporated and the residue was dissolved in 100 μL of mobile phase containing methanol/water/acetic acid (57.2:41.8:1, v/v) and 5 mM ammonium acetate. Before LC-MS/MS analysis the mycotoxins dissolved in mobile phase were centrifuged in a Ultrafree-MC centrifugal device for 15 min at 14000*g*.

Apparatus. A Waters Acquity UPLC system coupled to a Micromass Quatro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA) was used to analyze the samples, equipped with the Masslynx software for data processing. The column used was a 150 mm × 2.1 mm i.d., 5 μm, Symmetry C_{18}, with a 10 mm × 2.1 mm i.d., 3.5 μm, guard column of the same material (Waters, Zellik, Belgium).

HPLC Conditions. The column was kept at room temperature. The injection volume was 20 μL. The mobile phase consisted of variable mixtures of mobile phase A (water/methanol/acetic acid, 94:5:1 (v/v), and 5 mM ammonium acetate) and mobile phase B (methanol/water/acetic acid, 97:2:1 (v/v), and 5 mM ammonium acetate) at a flow rate of 0.3 mL min^{-1} with a gradient elution program. The gradient elution started at 95% mobile phase A with a linear decrease to 35% in 7 min. Over the next 4 min mobile phase A decreased to 25%. An isocratic gradient of 100% mobile phase B started at 11 min for 2 min. Initial column conditions were reached at 25 min.

MS/MS Detection. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. Capillary voltage was 3.2 kV, and nitrogen was used as spray gas. Source and desolvation temperatures were set at 150 and 350 °C, respectively. Mycotoxins were analyzed using selected reaction monitoring channels. The detailed conditions have been described (*8*). The described multimycotoxin LC-MS/MS method was validated and fulfilled the method performance criteria required by Commission Regulation (EC) 401/2006 (*13*).

Inoculation of Peppers. Pure cultures of *Fusarium* spp. were isolated from greenhouse-grown sweet peppers that were affected by internal fruit rot. Two single-spore isolates were selected for artificial inoculations. On the basis of EF-1α gene sequence determination and BLASTn alignment with the sequences in GenBank, these were identified as *F. proliferatum* (*14*). The isolates are hereafter referred to as pro1 and pro2. A culture of each isolate was deposited in the MUCL fungal collection and received accession numbers MUCL53013 (pro1) and MUCL53014 (pro2). For inoculation studies, 2-week-old cultures were produced on potato dextrose agar (Formedium, U.K.). Each isolate was separately inoculated onto two red sweet peppers. The peppers were pin-wounded with a needle, after which a mycelium plug (diameter = 5 mm) from the margin of the cultures was placed onto the wound and fixed to the pepper with a drop of 0.2% water agar. Control treatments included peppers that were inoculated with sterile agar plugs.

Migration Experiment. For the migration study two different commercially available sweet pepper cultivars were inoculated with pro1 and pro2 in at least four replicates, resulting in the formation of four experimental setups, namely, pepper cultivar 1 inoculated with pro1, pepper cultivar 1 inoculated with pro2, pepper cultivar 2 inoculated with pro1, and pepper cultivar 2 inoculated with pro2. After 10 days of incubation at 20 °C in a closed container, the lesion that was caused by the fungal infection was dissected from the pepper. Around the lesion up to three concentric rings of pepper tissue with a width of 5 mm were indicated (**Figure 1**). Each ring was cut out of the peppers using a flame-sterilized

Table 1. Results of the Detected Mycotoxins in the Different Sweet Pepper Samples

	cultivar 1						cultivar 2					
	pro1			pro2			pro1			pro2		
	N/TR[a]	mean (μg/kg)	SD (μg/kg)	*N*/TR	mean (μg/kg)	SD (μg/kg)	*N*/TR	mean (μg/kg)	SD (μg/kg)	*N*/TR	mean (μg/kg)	SD (μg/kg)
					Fumonisin B_1 (LOD = 13 μg/kg)							
lesion	5/5	33857	45295	4/4	3115	2250	5/5	13196	12160	5/5	6969	8268
ring 1	5/5	1802	1786	4/4	330	209	5/5	674	498	4/5	608	839
ring 2	5/5	236	48	4/4	165	70	3/5	232	156	5/5	224	197
ring 3	1/3	236		4/4	99	14	3/3	131	103	2/2	147	128
					Fumonisin B_2 (LOD = 6.5 μg/kg)							
lesion	3/5	2492	2396	4/4	568	298	4/5	783	402	5/5	2736	4577
ring 1	2/5	124	151	2/4	143	154	0/5			1/5	204	
ring 2	0/5			2/4	73	67	0/5			2/5	106	8
ring 3	0/3			1/4	26		0/3			1/2	108	
					Fumonisin B_3 (LOD = 8.4 μg/kg)							
lesion	2/5	1093	59	4/4	177	58	3/5	624	577	4/5	274	209
ring 1	2/5	61	45	0/4			0/5			0/5		
ring 2	0/5			1/4	28		0/5			0/5		
ring 3	0/3			0/4			0/3			1/2	26	
					Beauvericin (LOD = 4.3 μg/kg)							
lesion	4/5	1169	1592	4/4	1487	1044	5/5	637	1053	5/5	15356	32696
ring 1	0/5			0/4			0/5			0/5		
ring 2	0/5			0/4			0/5			0/5		
ring 3	0/3			0/4			0/3			0/2		

[a] *N*, number of samples contaminated; TR, total samples analyzed.

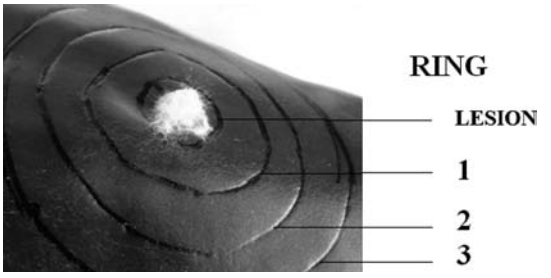


Figure 1. Inoculated sweet pepper with the indication of three concentric rings around the lesion.

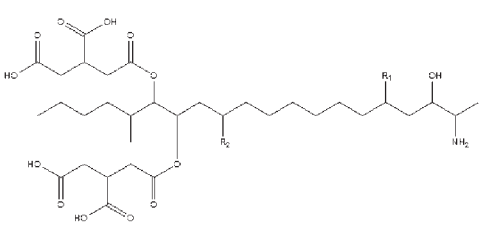
scalpel for the dissection. All samples were frozen (−18 °C) until processing for mycotoxin analysis.

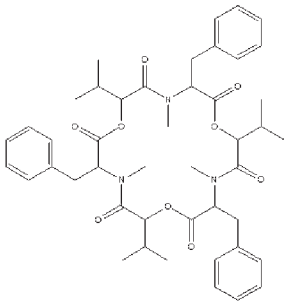
Analysis of Symptomatic Commercial Sweet Peppers. Twenty peppers with moldy or brown spots originating from a grocery (Oostkamp, Belgium) were selected in the period June−October 2008 by the grocer and transferred to the first author. Before each sample was ground, homogenized, and analyzed as described above, a description of the status (spots, external and/or internal mold growth) of the pepper was recorded and a photograph was taken.

Statistical Analysis. In the migration study four experimental setups were obtained and for each pepper the lesion and up to three concentric rings were analyzed. In a first step the data of the experimental setups were tested for normality using the Shapiro−Wilk test, which is the recommended test for a limited number of replicates ($N \leq 50$). For fumonisin B_1 an ANOVA analysis, an independent samples test to compare more than two test groups, was performed to investigate a statistically significant difference between the four experimental setups. After combination of the data of the four research groups, the conditions to perform the parametric repeated-measures ANOVA test were not fulfilled. Therefore, the Friedman test, which is the nonparametric alternative, was performed to investigate statistically significant differences between the concentric rings. If significant effects were revealed, a paired *t* test or a Wilcoxon test taking account of the Bonferroni correction was used to locate the pairwise differences between the rings. All calculations were executed in Excel 2007 or SPSS 17.

RESULTS AND DISCUSSION

In a previous study (*8*) peppers inoculated with *F. proliferatum* produced beauvericin and fumonisins B_1, B_2, and B_3. Frequently, another *Fusarium* species is recovered from pepper with internal fruit rot, but isolates of this species produce only beauvericin. For the migration study of different mycotoxins through the tissues of the pepper, two different *F. proliferatum* isolates were used to inoculate two different commercial pepper cultivars. The multimycotoxin analysis resulted in the detection of beauvericin and fumonisins B_1, B_2, and B_3. The results of the migration study are summarized in **Table 1**. Beauvericin was found in 95% of all replicate lesions, but never in the tissue surrounding the lesion. The levels varied between 67 and 73800 μg/kg. The large variation in beauvericin levels in the individual samples resulted in no statistically significant difference between the four experimental setups. Unlike beauvericin, fumonisins B_1, B_2, and B_3 were detected in all of the concentric rings, indicating migration of fumonisins in pepper tissue. In contrast to beauvericin, fumonisins are hydrophilic compounds (**Figure 2**) and are very soluble in the aqueous environment (*15*). Vegetables, and sweet peppers in particular, contain >90% water, so probably this is why fumonisins were detected in all of the concentric rings and beauvericin only in the lesion. In the lesion, fumonisin B_1 was detected in all of the replicates. In the first ring it was still detected in 95% of the replicates. In rings 2 and 3, the percentage of fumonisin B_1-contaminated replicates decreased to 89 and 83%, respectively. The detected levels in the lesion varied from 690 to 104000 μg/kg. The maximum levels in rings 1, 2, and 3 were, respectively, 4405, 556, and 248 μg/kg. The large variation in detected fumonisin





BEAUVERICIN

FORMULAE

ANALYTE	R_1	R_2
Fumonisin B_1	OH	OH
Fumonisin B_2	OH	H
Fumonisin B_3	H	OH

Figure 2. Structural formulas of fumonisins and beauvericin.

B_1 level in the individual samples resulted in no statistically significant difference among the four experimental setups. In 16% (3 of 19 peppers) there was an increase of the fumonisin B_1 levels in adjacent rings (ring 2 > ring 1 or ring 3 > ring 2), and only in 21% ($N = 4$) was no fumonisin B_1 detected in the outer ring (ring 2 or 3). The data of the concentric rings were compared and, as could be expected, the detected fumonisin B_1 levels decreased from the lesion to ring 3. The Friedman test was performed and resulted in a statistically significant difference in the detected fumonisin B_1 levels between the concentric rings ($p \leq 0.001$). A statistically significant difference was obtained between the lesion and ring 1 ($p \leq 0.001$) and between rings 1 and 2 ($p = 0.001$). The p value of 0.028 between rings 2 and 3 exceeds the Bonferroni-corrected significance level of 0.0083, resulting in no statistically significant difference between these two rings. In the lesion fumonisin B_2 was detected in 84% of the analyzed samples. This frequency decreased in rings 1, 2, and 3 to 26, 21, and 17%, respectively. In 68% of the analyzed pepper samples, fumonisin B_3 was detected in the lesion and decreases were observed to 11, 11, and 9% in rings 1, 2, and 3, respectively. The maximum fumonisin B_2 levels detected were 10900, 252, 120, and 108 μg/kg in the lesion and rings 1, 2, and 3, respectively. For fumonisin B_3 these levels were 1287, 93, 28, and 26 μg/kg. There was a statistically significant difference only between the lesion and rings 1, 2, and 3 ($p < 0.0083$). There was no statistically significant difference in the detected fumonisin B_2 or fumonisin B_3 level present in the rings.

Analysis of Symptomatic Commercial Sweet Peppers. Analysis of the 20 selected pepper samples showed a mycotoxin contamination in 7 peppers (35%). In one sample beauvericin was detected at a level of 124 μg/kg. The affected pepper had a dark spot on the outside, but there was no mold visible externally. A small moldy spot was present on the inside. In the six other contaminated samples alternariol was detected. In four samples the detected level was below the LOQ, which is 6.6 μg/kg, whereas 13 and 101 μg/kg levels were detected in two other samples. The alternariol-contaminated peppers showed mold growth on the pepper stem as shown in **Figure 3**. These symptoms are different from the symptoms that occur with internal fruit rot. In one of these samples no mold growth was visible inside the fruit, and in five samples there was moderate to strong internal mold growth. The pepper with 13 μg/kg had no internal mold growth, whereas the pepper with 101 μg/kg showed black decayed spots on top of the mold growth. The obtained results suggest the occurrence of alternariol in unaffected pepper tissue by migration because the stem was removed for analysis. The results of these analyses do not demonstrate a high consumer risk, as these peppers were sorted out by the grocer and were not for sale. As mentioned in the Introduction, it is not always possible to detect an infection on the inside of the fruit, so this can result in the purchase of a moldy pepper by the consumer. If the consumer cuts out the affected tissue of the pepper, there is no risk for beauvericin consumption because this toxin is present only in the moldy tissue and does not migrate to unaffected tissue as mentioned in the migration study. Until now, no data concerning the migration of alternariol in fruit or vegetable tissue are available.

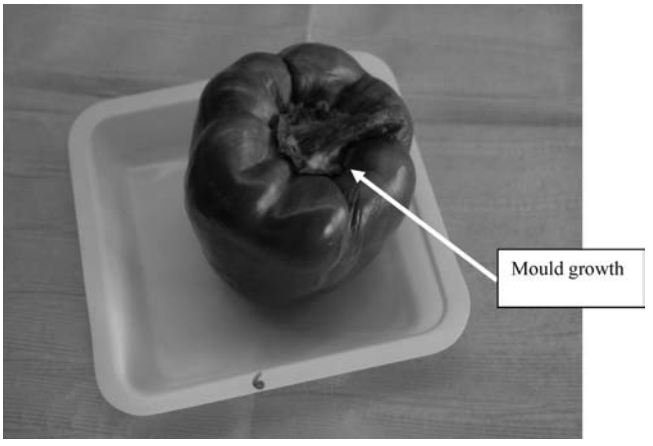


Figure 3. Pepper selected from a grocery with stem mold growth and alternariol detected in the tissue.

In general, the multimycotoxin analysis of the inoculated peppers in the migration study resulted in the detection of beauvericin and fumonisins B_1, B_2, and B_3. Beavericin was detected only in the lesion, whereas fumonisins B_1, B_2, and B_3 were also present in the surrounding tissue, indicating migration of these toxins into healthy parts of the sweet pepper. The analysis of suspected commercial sweet peppers showed a contamination of seven peppers with beauvericin or alternariol. The beauvericin-contaminated pepper showed internal fruit rot symptoms, whereas the alternariol-contaminated peppers showed proceeding stem rot symptoms. Despite removal of the stem for analysis, alternariol was detected in unaffected pepper tissue.

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