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FUSARIUM SPOROTRICHIOIDES SHERB. TOXINS EVALUATED IN CEREAL GRAIN WITH *TRICHODERMA HARZIANUM*

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Abstract: *Fusarium* head blight is one of the most severe diseases of small grain cereals and is caused by several toxigenic *Fusarium* species. Yield losses and mycotoxin accumulation in grain are caused by the disease. *F. sporotrichioides* and *F. poae* produce type A trichothecenes. Saprophyte fungal antagonists, especially *Trichoderma harzianum*, are effective biocontrol agents against several fungal soil-borne plant pathogens. These fungal antagonists can reduce the production of *Fusarium* spp. mycotoxins in some crop plants. The aim of this study was to examine the influence of *T. harzianum* AN4 on the production of type A trichothecenes by *F. sporotrichioides* in cereals. The accumulation of six trichothecene mycotoxins (scirpentriol, *i.e.* STO, T-2 tetraol, T-2 triol, HT-2 toxin *i.e.*, HT-2, T-2 toxin *i.e.*, T-2, diacetoxyscirpenol, *i.e.*, DAS) was reduced on average, by over 89% in bioassays of *F. sporotrichioides* and *Trichoderma* isolate AN4 on a liquid medium and on solid substrates (seeds of naked and husked oats and wheat). The reduction in our experiments depended on fungal isolate and substrate. From the three isolates of *F. sporotrichioides* used in the experiments, the highest accumulation of all the metabolites after 21 days by *F. sporotrichioides* in nearly all substrates, was recorded for strain ZFR 159. On the liquid medium inoculated with *F. sporotrichioides* ZFR 159, the amount of type A trichothecenes was the lowest (STO, T-2 tetraol, and T-2 triol not detected, HT-2 toxin 0.02 ppm, DAS 0.10 ppm, T-2 toxin 0.99 ppm). The highest total concentration of these toxins was produced by this isolate in husked oat cv. German (180.16 ppm), but in naked oat cv. Akt the toxin concentration was low (27.62 ppm). Trichothecene accumulation by *T. harzianum* AN4 was reduced the most in oat cv. Akt (98.48%) in the liquid medium (98.22%), while the lowest reduction was in oat cv. German (48.77%). The non-toxigenic *T. harzianum* AN4 isolate proved to be a useful biocontrol agent against the toxigenic *F. sporotrichioides* in cereals, significantly reducing the production of six type A trichothecenes. This is the first report on effective biocontrol of *F. sporotrichioides* in cereals by *T. harzianum*.

Key words: biocontrol, *Fusarium sporotrichioides*, *Trichoderma harzianum*, trichothecenes

INTRODUCTION

More than 20 *Fusarium* species are associated with *Fusarium* head blight (FHB) of cereals. Those *Fusarium* species that predominate vary depending on the crop species involved, the region, and season (Edwards 2004; Logrieco and Visconti 2004). *Fusarium* species are necrotrophic pathogens important in cereal-growing regions. These pathogens cause various diseases, such as: root rot, foot rot (culm rot), stem base rot, crown rot, *Fusarium* seedling blight, and *Fusarium* head (ear) blight or scab. *Fusarium* species produce toxic metabolites, which may contaminate animal and human food (Snijders 2004; Schollenberger *et al*. 2005; Wiśniewska 2005).

The species *F. sporotrichioides,* of the section *Sporotrichiella,* is widespread on plants and in the soil throughout the cold and cool regions of the world (Kulik *et al*. 2004; Visconti *et al*. 1985). This species was often isolated from oat panicles and kernels. In Poland, especially in its southeastern part, FHB in oats was frequently observed, caused by *F. avenaceum, F. culmorum, F. poae, F. crokwelense*, and *F. sporotrichioides* (Mielniczuk *et al*. 2004).

F. sporotrichioides, like *F. acuminatum, F. sambucinum* and *F. poae*, produce type A trichothecenes: T-2 toxin, HT-2 toxin, T-2 tetraol, T-2 triol, scirpentriol (STO), and diacetoxyscirpenol (DAS). Those mycotoxins are accumulated in infected grain (Brown *et al*. 2001; Mateo *et al*. 2002; Moss 2002; Moss and Thrane 2004; Niesen *et al*. 2004; Perkowski and Basiński 2002; Perkowski *et al*. 2003; Perkowski *et al*. 2007; Thrane *et al*. 2004).

T-2 toxin is a highly toxic type A trichothecene produced by various species of *Fusarium*. The most important producer of this mycotoxin is *F. sporotrichioides,* a saprophyte (*i.e.* not pathogenic to plants) with optimum growth at 2 to 35°C, only at high humidity (over 0.88). As a consequence, T-2 and HT-2 toxins are not normally found in grain at harvest but result from water damage to grain, *e.g.* when it remains for extended periods in the field at or after harvest, especially in cold weather, or in grain that becomes wet during storage. Surveys have revealed the presence of T-2 and HT-2 toxins in seeds, *e.g.* of wheat, maize, barley, oats, rice, beans and soy beans, as well as in some cereal-based products. For instance, the toxins

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have been found in oats growing in northern temperate climates and in Poland, especially in the southeastern regions (Kiecana and Perkowski 1998). T-2 toxin is a potential inhibitor of protein synthesis both *in vivo* and *in vitro*. T-2 toxin caused some cases of poisoning in domestic animals, causing changes in leukocyte counts, weight loss, vomiting, depression, and antibody formation and diarrhoea (Pascale *et al*. 2003). However, natural degradation of T-2 and HT-2 toxins has been observed in cereal grain both in the field and during storage. It is difficult to understand how concentrations of mycotoxin are decreased in a natural system, although several explanations have been put forward. The concentration of free mycotoxins and biomassis (Eged 2002; Buśko and Wiśniewska 2005; Perkowski *et al*. 2008) in a natural ecosystem may be the result of concurrent synthesis, transport, conjugation, release from bound forms, and degradation by the plant or by other microbes (Karlovsky 1999).

Physical, chemical and biological methods have been used to decontaminate grain-containing trichothecenes, but they have usually proved to be ineffective.

The trichothecene T-2 toxin is deacylated to HT-2 toxin by rumen microbes (Kiessling *et al*. 1984; Beeton and Bull 1989; Swanson *et al*. 1987). The main degradation pathway in most isolates involved side-chain cleavage of the acetyl residue and transformed HT-2 toxin and T-2 triol. In all cases the complete bacterial communities were more active against T-2 toxin in terms of rates of degradation than any single bacterial component.

Trichoderma spp. are fungi that are common in the soil and in the rhizosphere and phyllosphere. A significant number of studies from over many years have proved the potential of *Trichoderma* spp. as biological agents antagonistic to several soil-borne pathogens (Elad *et al*. 1982; Ganassi *et al*. 2001; Kucuk 2003; Kucuk and Kivanc 2004). This species can be used as an alternative to synthetic pesticides and thus, greater public acceptance and reduced environmental impact.

A number of *Trichoderma* strains, such as chitinases and glucanases, are able to secrete lytic enzymes. Secretion is possible when grown in liquid media supplemented with either polymers, such as laminarin or chitin, or with fungal cell walls (Elad *et al*. 1982; Kucuk and Kivac 2004).

T. harzianum (Persoon: Fries) is an effective biocontrol agent against several fungal soil-borne plant pathogens (Green *et al*. 1999). It has been used successfully against a range of pathogenic fungi belonging to various genera: *Phytophthora, Sklerotinia, Gaeumannomyces*, *Rhizoctonia, Drechslera* and *Fusarium* (Chaverri *et al.* 2003; Kucuk, 2003). This species has a potential for the enhancement of plant growth and resistance to plant pathogens (Bailey and Lumsdem 1998).

A biological control agent comprising the antifungal agent *T. harzianum* (ATCC No. 20691) is characterized by antifungal activity against fungi of the genus *Fusarium*. That strain is useful for protecting most crops affected by the fungus *Fusarium* spp. and it is more active than other previously disclosed strains. Biocontrol compositions containing that strain of *T. harzianum,* provide antifungal protection to a broad spectrum of plants, including wheat, cotton, melons, banana (Nel *et al*. 2006), and tomatoes (www.freepatentsonline.com/4748021.html). The non-toxigenic *T. harzianum* isolate AN4 may be a very useful biocontrol agent against toxigenic *Fusarium* species in cereals, reducing the inoculum of *Fusarium* spp. (Buśko *et al*. 2008). However, there has been no previous research done about whether it really can prevent the production of the more toxic type A trichothecenes, by *Fusarium* in kernels. That is why the aim of this study was to examine the influence of *T. harzianum* AN4 on the production of type A trichothecenes by *F. sporotrichioides* in cereals.

MATERIALS AND METHODS

Fungal isolates

Two isolates of *F. sporotrichioides* (ZFR 159, ZFR 41) were kindly supplied by dr. Góral from the Plant Breeding and Acclimatization Institute, Radzików, Poland, while isolate KF 2006 was provided by the Institute of Plant Genetics, Poznań, and *T. harzianum* AN4 by Dr. Logrieco from the Institute of Food Production Sciences, CNR 70125, Bari, Italy.

Bioassays

Solid and liquid substrates were tested: naked oat cultivar Akt, husked oat cv. German, oat line CHD 1296, winter wheat cv. Begra, spring wheat cv. Torka, and a liquid medium (30 g sucrose, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 3 g $NaNO_3$, 0.01 g $FeSO_4$, in 1 l of H_2O).

Each sample of cereal grain (50 g) was placed in a 300-ml Erlenmeyer flask with 15 ml of distilled water for 24 hours. Next, the flasks were autoclaved for 30 min at 121°C, cooled to room temperature, and inoculated with 5-mm discs of 7-day fungal culture on potato dextrose agar (3 discs per flask), and 16 ml of a suspension of *T. harzianum* AN4 (48,000 colony-forming units/ml) was added per 50 g of substrate. Control substrates were incubated only with isolates of *F. sporotrichioides*. The cultures were incubated at 24°C and were shaken every day from the 3rd day of incubation. After 3 weeks, the substrates were dried at room temperature and then used for mycotoxin analysis.

Analysis of type A trichothecenes

Subsamples of 10 g each were used for analysis of each toxin. All the subsamples were prepared in the same way. They were ground in a WŻ-1 laboratory mill (Research Institute of Baking Industry Ltd., Bydgoszcz, Poland), designed especially for grinding cereal samples.

Samples were extracted overnight with 100 ml of acetonitrile-water solvent (82:18, v/v), and filtered (Whatman no. 5 filter paper). Then the extracts were purified on a 5 ml column of mixed alumina (neutral activated, 70–230 mesh) Merck, Darmstadt, Germany, Darco G 60 - charcoal (100 mesh; Aldrich, Steinheim, Germany), and Celite 545 (Serva, Heidelberg, Germany) 4:3:4 [w/w/w]. The extracts were evaporated to dryness by using a rotary evaporator. The residue was dissolved by using two aliquots of 2 ml ethyl acetate and 2 ml of chloroform-acetonitrile (4:1, v/v).

Type A trichothecenes (H-2 toxin, T-2 toxin, T-2 tetraol, T-2 triol, STO and DAS) were analysed as trifluoroacetic anhydride (TFAA) derivatives. To the dried sample, 100 µl of TFAA was added. After 20 min, the reacting substance was evaporated to dryness under nitrogen. The residue was dissolved in 500 µl of isooctane, and 1–2 µl was injected onto a gas chromatograph-mass spectrometer (Hewlett Packard GC 6890, MS 5972 A, Waldbronn, Germany).

The HP-5MS capillary column (0.25 mm x 30 m) was used. The injection port temperature was 280°C, the transfer line temperature was 280°C, and the analysis was performed with programmed temperature (from 80°C [1 min] to 280°C at 25°C [min]), the final temperature being kept for 10 min. The helium flow rate was constant at 0.7 ml min. Each sample was run twice: in full scan mode (m/z 100–600) for identification, and in selected ion-monitoring (SIM) mode for quantification in comparison with trichothecenes group A standards supplied by Sigma (St Louis, MO, USA). The following ions were used for trichothecene detection: HT-2 toxin, m/z 455, 327; T-2 toxin, m/z 327, 401; T-2 tetraol, m/z 455, 568; T-2 triol, m/z 455, 374, 402, 569; DAS, m/z 402, 374, 455, 569; STO, m/z 456, 555. The first ion in each set was used for quantification. The detection limit was 0.01 ppm. Average recoveries (n = 9) of the toxins by the above method were: 86±3.8% for T-2, 88±4.0% for T-2 tetraol, 82±4.2% for T-2 triol, 91±3.2% for HT-2, 84±4.6% for DAS, and 82.3±3.8% for STO.

RESULTS AND DISCUSSION

In the analyzed experiment all the *F. sporotrichioides* isolates (ZRF 159, ZRF 41, KF 2006) used on different substrates produced group A trichothecenes (T-2 toxin, HT-2 toxin, DAS, T-2 triol, T-2 tetretraol, STO) at different concentrations, which is consistent with the literature data presented in the Introduction. T-2 toxin and HT-2 were the main metabolites produced by the isolates of *F. sporotrichioides* (Table 1); however, toxigenicity of individual isolates varied significantly. The total concentration of type A toxins produced by isolate ZFR 159 was 525.85 ppm, compared to 314.17 by isolate ZFR 41 and 85.68 ppm by isolate KF 2006, respectively (Table 1).

The yield of type A trichothecenes (T-2 toxin, HT-2 toxin, DAS, T-2 triol, T-2 tetretraol, STO) in our experiments was the highest for wheat in the Begra cultivar. In the case of naked oat or husked oat, toxin concentration depended on the used *F. sporotrichioides* isolate. Isolate ZFR 159 generally produced the highest amounts of toxins in kernels of husked oat cv. German: 110.87 ppm T-2 toxin, 65.53 ppm HT-2, 1.72 ppm T-2 triol, 1.47 ppm T-2 tetraol, 0.41 ppm DAS and 0.18 ppm STO (Table 1). The total concentration of trichothecenes was significantly higher in husked oat cv. German (180.18 ppm) than in a naked oat cv. Akt (27.62 ppm). This situation seems consistent with other data available in literature. Kiecana and Perkowski (1998) reported a high susceptibility of cv. German to *F. sporotrichioides* in natural infection in southeastern Poland. A high concentration of HT-2 toxin (0.40 ppm) was detected in kernels from natural infection, and several other type A trichothecenes (T-2 toxin, T-2 tetraol, and DAS) were also detected in this material. Additionally, in a study by (Perkowski and Basiński 2008) it was found that naked-grained oat cultivars had a significantly lower percentage of contamination with trichothecenes, in relation to husked cultivars. The concentration of trichothecenes in the grain of naked-grained oat cultivars also turned out to be significantly lower. This is probably caused by the fact that the highest amount of type A trichothecenes is accumulated in chaff. Perkowski *et al*. (2003) reported that trichothecene levels in oats were the highest in chaff, lower in the apical internode of the stalk, and the lowest in grain. In case of isolate ZRF 41, the opposite situation was observed, *i.e.* a higher toxin concentration was recorded in naked oat and it was on average, approx. 4 times higher in relation to the husked cultivar, which hardly seems to be a plausible scenario. It may only be explained by laboratory conditions, under which biosynthesis is run, obviously being inadequate in comparison to field cultivation.

Trichothecene content in grain may be reduced by growing resistant cultivars, and by the application of fungicides or biological antagonists. Parry *et al*. (1995) suggested that biological control measures could be a useful alternative to fungicide treatment, since the period during which cereals are sensitive to the disease is short. There are only a few reports on biological control of *Fusarium* (Mao *et al*. 1998; Hoefanagles and Linderman 1999).

Buśko *et al*. (2008) found that a *T. artroviride* isolate significantly reduced the biosynthesis of five type B trichothecenes (DON, 3-AcDON, 15-AcDON, FUS and NIV) produced by *F. culmorum* and *F. graminearum* in rice. Cooney et al. (2001) also showed that DON production by *F. graminearum* in culture media was inhibited (up to 80%), using a *Trichoderma* metabolite (6PAP) produced by a *Trichoderma* isolate. In response to the presence of pathogenic fungi *Trichoderma* gave enhanced production of the antibiotic 6-pentyl-α-pyrone (6PAP) (Cooney *et al*. 2001). In turn, to date no potential reduction was shown in the amounts of highly toxic type A trichothecenes produced in grain by the fungus F. *sporotrichioides*.

Reduced production of type A trichothecenes was found in liquid and solid substrates, co-inoculated with *F. sporotrichioides* ZFR 159 and *T. harzianum* AN4. In the liquid medium, the reduction ranged from 85% for HT-2 toxin to 100% for DAS, on average amounting to 98.22% (Table 2).

The reduction was the highest in kernels of naked oat cv. Akt (mean 98.48%), and the lowest in husked oat cv. German (mean 48.77%). In winter wheat cv. Begra, reduction varied for different toxins from 67.65% (T-2 tetraol) to 91.58% for T-2 triol (Table 2).

When comparing media used in the experiment, it was found that among all substrates, the average concentration of type A trichothecenes was the lowest in the liquid medium (1.11 ppm) and oat cv. Akt (27.62 ppm), moderate in winter wheat cv. Begra (100.73 ppm) and the highest in the cv. German (180.16 ppm) (Table 2).

Considering all substrates jointly, the production of type A trichothecenes was markedly reduced by *Trichoderma* isolate AN4: from 57.42% for HT-2 to 85.22% for T-2 tetraol (Table 2).

Table 1. Mean concentrations of type A trichothecenes produced in several substrates by isolates of *F. sporotrichioides* (ZFR159, ZFR 41, KF 2006), after 21 days at 24°C

Isolate	Substrate	Type A trichothecenes [ppm]						
		STO	T-2 tetraol	T-2 triol	DAS	HT-2	T-2	total
ZFR 159	liquid medium	ND	ND	ND	0.10	0.02	0.99	1.11
	seeds of naked oat cv. Akt	0.02	0.28	0.12	0.12	3.65	23.43	27.62
	seeds of husked oat cv. German	0.18	1.47	1.72	0.41	65.53	110.87	180.18
	seeds of wheat cv. Begra	0.67	37.03	1.70	0.20	20.66	40.47	100.73
	seeds of wheat cv. Torka	0.29	8.56	2.25	0.40	29.10	48.33	88.93
	seeds of oat line CHD129	ND	0.73	0.56	0.11	31.38	94.51	127.29
ZFR 159	∑							525.85
ZFR 41	liquid medium	ND	ND	ND	ND	ND	0.01	0.01
	seeds of naked oat cv. Akt	0.13	1.89	0.81	0.31	24.60	140.96	168.69
	seeds of husked oat cv. German	ND	0.39	0.18	ND	9.04	31.71	41.32
	seeds of wheat cv. Begra	0.22	7.82	0.87	0.04	7.73	17.47	34.15
	seeds of wheat cv. Torka	0.03	0.86	0.08	0.04	3.87	7.39	12.27
	seeds of oat line CHD129	ND	ND	ND	ND	11.18	46.55	57.73
ZFR 41	∑							314.17
KF 2006	liquid medium	ND	ND	ND	0.01	0.01	0.05	0.07
	seeds of naked oat cv. Akt	0.03	1.16	0.54	0.28	3.29	18.85	24.15
	seeds of husked oat cv. German	ND	0.15	0.12	0.07	5.57	20.67	26.58
	seeds of wheat cv. Begra	ND	1.08	0.21	ND	3.05	2.28	6.62
	seeds of wheat cv. Torka	ND	ND	ND	ND	4.21	5.46	9.67
	seeds of oat line CHD 129	ND	ND	ND	ND	3.48	15.11	18.59
KF 2006	∑							85.68

ND – not detected, STO – scirpentriol, DAS – diacetoxyscirpenol

Table 2. Mean concentrations of type A trichothecenes produced by *F. sporotrichioides* ZFR 159, and reduction [%] of mycotoxin accumulation by competitive *T. harzianum* isolate AN4 in the liquid medium and solid substrates, after 21 days at 24°C

Substrate	Isolate	Mean concentration [ppm] of type A trichothecenes in positive samples, and reduction [%] of mycotoxin accumulation						
		STO	T-2 tetraol	T-2 triol	DAS	HT-2	T-2	total
Liquid medium	ZFR 159	ND	ND	ND	0.10	0.02	1.00	1.12
	ZFR 159 + AN4	ND	ND	ND	ND	0.003	0.02	0.02
	Reduction [%]				100	85.00	98.00	98.22
Oat cv. Akt	ZFR 159	0.02	0.28	0.12	0.12	3.65	23.43	27.62
	ZFR 159 + AN4	ND	ND	ND	ND	ND	0.42	0.42
	Reduction [%]	100	100	100	100	100	98.20	98.48
Oat cv. German	ZFR 159	0.17	1.47	1.71	0.41	65.53	110.87	180.16
	ZFR 159 + AN4	0.05	1.15	1.01	0.35	38.08	51.65	92.29
	Reduction [%]	70.59	21.77	40.94	14.64	48.89	53.41	48.77
Wheat cv. Begra	ZFR 159	0.67	37.03	1.70	0.20	20.66	40.47	100.73
	ZFR 159 + AN4	0.10	3.12	0.55	0.02	4.61	4.56	12.96
	Reduction [%]	85.08	91.58	67.65	90.00	77.69	88.73	87.13
Mean	ZFR 159	0.23	9.61	1.26	0.22	25.06	53.11	89.49
	ZFR 159 + AN4	0.05	1.42	0.52	0.09	10.67	14.16	26.91
	Reduction [%]	78.26	85.22	58.73	59.09	57.42	73.34	69.93

As was shown for the first time in this study, *T. harzianum* AN 4 seems to be a very useful fungus in the biological control against aggressive and toxigenic *F. sporotrichioides*, especially to reduce the production of type A trichothecenes in cereal grain.

In conclusion, literature data and this study show that *Trichoderm*a spp. and other antagonists significantly reduce the growth of *Fusarium* spp. colonies in culture media. *Trichoderma* spp. proved to be very effective, as an antagonist inhibiting toxin accumulation in cereal grain.

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POLISH SUMMARY

TWORZENIE TOKSYN PRZEZ *FUSARIUM SPOROTRICHIOIDES* SHERB W ZIARNIE ZBÓŻ ZAPRAWIANYM *TRICHODERMA HARZIANUM*

Fuzarioza kłosów jest jedną z najważniejszych chorób zbóż powodowaną przez patogeniczne gatunki z rodzaju *Fusarium*. Saprotroficzne grzyby, szczególnie *Trichoderma harzianum*, mogą być efektywnym antagonistą glebowych grzybów i mogą redukować tworzenie mikotoksyn fuzaryjnych w ziarnie. Celem pracy było zbadanie wpływu *T. harzianum* AN4 na tworzenie sześciu toksyn trichotecenowych typu A (scirpentriol, tj. STO, T-2 tetraol, T-2 triol, HT-2, T-2, diacetoxyscirpenol, DAS) tworzonych przez *F. sporotrichioides* w podłożu płynnym i stałym (ziarno jęczmienia oplewionego i nagiego oraz pszenicy). Stwierdzono, że redukcja poziomu toksyn zależała od izolatu grzyba i podłoża. Z użytych w badaniach trzech izolatów najwyższy poziom akumulacji wszystkich metabolitów po 20 dniach inkubacji, zaobserwowano dla szczepu ZFR 159. Najmniejszy poziom toksyn przy użyciu tego izolatu stwierdzono na podłożu płynnym (0,00–0,99 ppm, a najwyższy w oplewionym ziarnie jęczmienia odmiany German (180,16 ppm).

W ziarnie nieoplewionego jęczmienia odmiany Akt, sumaryczny poziom toksyn wynosił 27,62 ppm. Najwyższą redukcję kumulowanych toksyn typu A pod wpływem *T. harzianum* (AN4), stwierdzono w ziarnie owsa nagiego odmiany Akt (98,48%) i w płynnym podłożu (98,22%), najmniejszy u podlewanego jęczmienia odmiany German (48,77%).

T. harzianum (AN4) – nietworząca toksyn może być antagonistą toksynotwórczego szczepu *F. sporotrichioides* w zbożach, znacząco redukując tworzenie sześciu toksyn trichotecenowych typu A, po inokulacji toksynotwórczym gatunkiem *F. sporotrichioides.*