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Microfungi on the kernels of transgenic and non-transgenic maize damaged by the European corn borer

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From 2002–2004 isolations were carried out to determine the kinds and abundance of microfungi from non-transgenic maize kernels damaged by the European corn borer (ECB) and from transgenic Bt-maize (enriched with delta-endotoxin from the soil bacterium *Bacillus thuringiensis*). Bt-maize and non-transgenic maize (*Zea mays*) were grown at Praha-Ruzyně and Ivanovice na Hané, Czech Republic. Thirty-one taxa of filamentous microfungi were isolated, including eight zygomycetes and twenty-three ascomycetes (anamorphic stage). Presence of ECB, corn treatment, year, locality and isolation method significantly accounted for differences in fungus communities. Bt-maize was significantly different from the treatments with non-transgenic hybrids and was often associated with the potentially toxinogenic fungi *Alternaria alternata* and *Epicoccum nigrum*. Conversely, Bt-maize had lower incidences of *Fusarium* spp. and *Acremonium strictum*.

Key words: Bt-maize, microfungi, plant protection, European corn borer, *Zea mays*

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V letech 2002–2004 byly izolovány a identifikovány druhy a množství mikroskopických hub z obilek netransgenních hybridů kukuřice poškozených zavíječem kukuřičným a z transgenní Bt-kukuřice (obohacená o delta-endotoxin z půdní bakterie *Bacillus thuringiensis*). Bt-kukuřice a netransgenní kukuřice (*Zea mays*) byla pěstována v Praze-Ruzyni a v Ivanovicích na Hané v České republice. Bylo izolováno 31 taxonů vláknitých mikromycetů, 8 zygomycetů a 23 askomycetů (v anamorfním stadium). Přítomnost zavíječe, varianta kukuřice, rok, lokalita a metoda izolace byly významně důležité faktory pro sledované rozdíly houbových společenstev. Varianta Bt-kukuřice byla odlišná od netransgenních hybridů, byly zde častěji izolovány některé potenciálně škodlivé houby jako *Alternaria alternata* a *Epicoccum nigrum*. Na druhou stranu Bt-kukuřice měla nižší výskyt druhů rodu *Fusarium* a druhu *Acremonium strictum*.

INTRODUCTION

Maize kernels are colonised by over 100 species of fungi (Jesenská 1993), including beneficial endophytes (Wicklow et al. 2005) and pathogens, which seriously reduce the pre-harvest yields. Some kernel-inhabiting fungi also produce mycotoxins (Malíř and Ostrý 2003). The kinds and frequency of fungi on pre-harvest maize kernels is an indicator of the degree of fungus contamination. Among the most prevalent fungi found on kernels are common saprobes and parasites, such as species of *Fusarium*, *Alternaria*, *Cladosporium* and *Epicoccum*, some of which produce mycotoxins (Fisher et al. 1992, Gonzalez et al. 2002, Ono et al. 2002, Ono et al. 1999, Pitt et al. 1993, Pitt et al. 1998).

Some of the factors affecting the kinds and abundance of fungi on maize kernels is the frequency at which maize is grown in rotation with other cereals and variations in climate (Ono et al. 1999). Another factor is the presence of parasitic invertebrates, which enhance physically damage to the kernels and passively transmit fungal spores. However, it is unknown how much insect damage affects the degree of colonisation by fungi.

One of the most important insect pests of maize in Central Europe and the Czech Republic is the European corn borer (ECB, *Ostrinia nubilalis* Hubner; Lepidoptera, *Pyralidae*) (Christensen and Schneider 1950, Kazda et al. 2001, Magg et al. 2001, Mason et al. 1996, Miller 1956). The most effective method of protecting maize against ECB is by growing transgenic maize (Bt-maize), which contains the Cry genes from the bacterium *Bacillus thuringiensis* (Magg et al. 2001, Munkvold et al. 1999).

The work reported here had two purposes. The first one was to establish which fungi prefer damaged kernel tissue and also how exposure to field condition followed by contact with associated insects, air or water splash affect such fungi living on healthy and injured kernels, and to determine the relationships between the levels of microfungi contamination and damage of maize ears caused by ECB. ECB damage to plant should provide entrance to the infection by various microfungi. Our presumption was that maize plants which are protected against ECB will have a lower incidences of microfungi. As such, the use of Bt-maize should result in higher quality kernels. The qualitative and quantitative spectrum of the fungi was studied on ECB damaged kernels and kernels damaged in other ways, e.g. by other insects and mechanical injury. In addition, fungi were isolated from intact kernels from the same corn ear. The second purpose was to determine the affect of maize protection by Bt-toxin on the associated mycoflora, especially toxinogenic fungi.

MATERIALS AND METHODS

The following maize varieties and hybrids were used in these studies: Bt-maize (hybrid MEB307 Bt-Monumental in 2002 and 2003, DKC 3421YG in 2004, both Monsanto), Monumental hybrid (isogenic hybrid Monumental in 2002 and 2003 and DKC 3420 in 2004) and Raissa (a Czech hybrid). One experimental plot with Bt-maize and two plots of both non-transgenic hybrids were established during the years 2002–2004 in the growing seasons at Praha-Ruzyně (Central Bohemia) and Ivanovice na Hané (south Moravia), Czech Republic. The experimental design included five treatments per year and location. The ECB was preset in all treatments except treatment 1 (Fig. 1).

At each locality, a 1 ha site was divided into three, adjacent 0.3 ha surrounding plots. The area was planted with an 8 m wide strip of the Raissa hybrid. All the plots were treated with a pre-emergent herbicide (Guardian, 2.5 l/ha) and a post-emergent (Grid 20 g/ha) herbicide. On the plots with Bt-maize, no other herbicide was applied. During the years 2002–2004, three collections of ears (different maturity stages) were made from August to October (before harvest). In total 180 ears (36 ears from each treatment) were collected. Two methods were used to isolate the microfungi. First, visible mycelium was aseptically removed from the surface of three ECB-damaged kernels from the non-transgenic maize hybrids and by other insects on the transgenic hybrids. They were plated onto each of three isolation media. ECB damage was determined based on the presence of ECB excrements. Damage caused to Bt-maize by other insects (aphids) was identified by visible mycelium (mostly at the terminal part). Secondly, three intact kernels from each ear were washed separately in sterile water using an ultrasonic shaker (3 x 1 min, 44 kHz) and plated onto each of three isolation media. Thus, nine random kernels from each ear were processed using both methods.

The media used for isolating the fungi (Atlas 1997) were malt extract agar (MEA), cornmeal agar (CMA) and soil extract agar with glucose and rose bengal (SEGA). The Petri dishes were incubated in the dark at 25 °C. The isolated fungi were grown on: malt agar (MA), MEA, oatmeal agar (OA), and potato carrot agar (PCA) to induce fungus sporulation for identification. *Aspergillus*, *Fusarium* and *Penicillium* were not identified to species.

The occurrence of each fungus on each kernel was recorded, and the effect of environmental factors and the treatment methods were analysed using multivariate methods with Canoco version 4.5 (ter Braak and Šmilauer 2002). Only fungus species isolated more than once were included in the analysis. Data on the annual abundance of each species were first subjected to multivariate analysis and detrended correspondence analysis (DCA). The short gradient (2.0) suggested a linear response between abundance and the different factors; therefore redundancy analysis (RDA) was used. The factors used in the analyses were: (1)

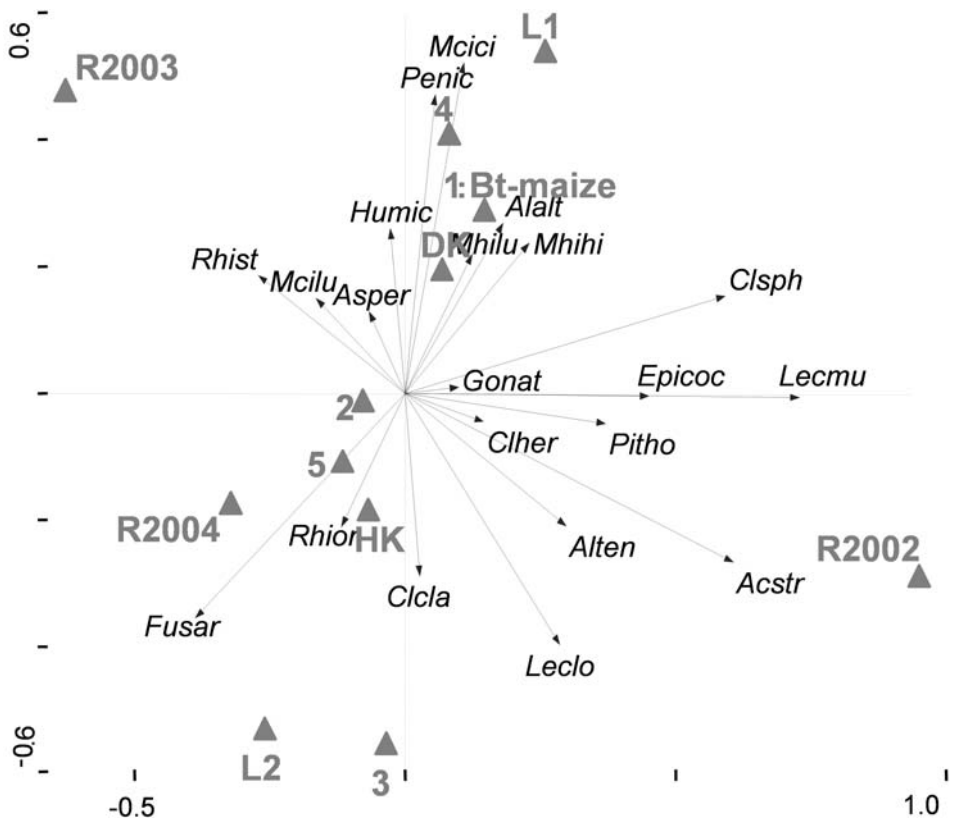

Fig. 1. Biplot of the redundancy analysis, showing the combined effect of year, treatment (1–5), method, locality and presence of European corn borer on the kinds of fungi isolated over 3 years (2002–2004) from healthy and ECB damaged kernels of Bt and non-transgenic maize (the first axes explain 58.1 % of the variance and the sum of all canonical eigenvalues is 0.291).

Abbreviations: treatment 1 – Bt-maize, ECB absent, 2 – Monumental plot 1, 3 – Raissa plot 1, 4 – Monumental plot 2, 5 – Raissa plot 2, L1 – locality Praha-Ruzyně, L2 – locality Ivanovice na Hané, HK – healthy kernels, DK – damaged kernels, Acrst – *Acremonium strictum*, Alalt – *Alternaria alternata*, Alten – *Alternaria tenuissima*, Asper – *Aspergillus* sp. div., Clcla – *Cladosporium cladosporioides*, Clher – *Cladosporium herbarum*, Clsph – *Cladosporium sphaerospermum*, Epicoc – *Epicoccum nigrum*, Fusar – *Fusarium* sp. div., Gonat – *Gonatobotrys simplex*, Leclo – *Lecanicillium* cf. *longisporum*, Lecmu – *Lecanicillium muscarium*, Mcici – *Mucor circinelloides* f. *circinelloides*, Mcilu – *Mucor circinelloides* f. *lusitanicus*, Mhihi – *Mucor hiemalis* f. *hiemalis*, Mhilu – *Mucor hiemalis* f. *luteus*, Penic – *Penicillium* sp. div., Pitho – *Pithomyces chartarum*, Rhior – *Rhizopus oryzae*, Rhist – *Rhizopus stolonifer*.

Bt-maize, ECB absent, (2) treatment of non-transgenic hybrids, ECB present (Raissa plots 1, 2; Monumental plots 1, 2), (3) year (2002, 2003, 2004), (4) locality (Praha-Ruzyně, Ivanovice na Hané) and (5) isolation method (damaged and healthy kernels). They were assessed using the Monte Carlo permutation test (MCPT) with 499 simulations.

RESULTS

Tab. 1 shows the frequencies of all the fungi isolated from healthy and damaged maize kernels. Thirty-one filamentous microfungi were isolated, eight zygomycetes and twenty-three ascomycetes (anamorphic stage). *Aspergillus*, *Fusarium* and *Penicillium* were not identified to species. Regardless of the treatment or year, the most common fungi isolated were *Fusarium* spp., *Cladosporium cladosporioides*, *Mucor circinelloides* f. *circinelloides*, *Acremonium strictum* and *Alternaria alternata* (Tab. 1, grey cells). All of the treatments and methods resulted in the isolation of similar fungi, but the fungi differed in isolation frequency.

The principal component analysis (PCA) showed no apparent clusters of samples correlated to variables (data not shown). Results of the RDA revealed that all the test variables were significantly ($p = 0.001$) responsible for the observed variability in the data set. The most easily detectable variable was the absence of ECB (first two axes explained 40.3 % of the variation). Isolation method and year were the next most important factors (both together on the first two axes explained 37.1 % of the variation). The effect of the non-transgenic hybrid treatment explained the smallest portion of the variance (first two axes 18.2 %).

The RDA analysis showed a positive correlation between *Alternaria alternata*, *Epicoccum nigrum* and Bt-maize and, consequently, as a result the absence of ECB (Fig. 1, Tab. 1). These fungi were also more abundant on damaged than on healthy kernels. *Fusarium* spp. and *Acremonium strictum* were strongly negatively correlated with Bt-maize. *Fusarium* species were isolated from 27.8–37.5 % of the transgenic maize kernels and from 62.5–86.1 % of the non-transgenic hybrid kernels. The occurrence of the fungus *Acremonium strictum* was 16.7 % on transgenic hybrids and 19.5–41.7 % on non-transgenic hybrids (Tab. 1). These same fungi were also more common on healthy than on damaged kernels.

Tab. 1. Microfungi isolated from healthy and damaged maize kernels at Praha-Ruzyně and Ivanovice na Hané, Czech Republic from pre-harvested maize kernels between 2002 and 2004 (fungi in the grey cells were isolated most often).

	Frequency of fungi from treatments 1–5									
	Healthy kernels					Damaged kernels				
Microfungi	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Acremonium strictum W. Gams	16.7	30.6	38.9	41.7	34.7	16.7	19.5	37.5	29.2	23.6
Alternaria alternata (Fr.: Fr.) Keissl.	47.2	22.2	11.1	12.5	19.4	52.8	36.1	26.4	26.4	19.4
Alternaria tenuissima (Kunze: Fr.) Wiltshire	–	2.8	4.2	–	–	–	2.8	–	–	2.8
Aspergillus sp. div.	–	–	–	2.8	5.6	–	–	2.8	–	–
Bipolaris bicolor (Mitra) Shoemaker	1.39	–	–	–	–	–	–	–	–	–
Bipolaris spicifera (Bainier) Subramanian	1.39	–	–	–	–	–	–	–	–	–
Cladosporium cladosporioides (Fresen.) G. A. de Vries	36.1	34.7	23.6	43.1	36.1	59.7	29.2	37.5	37.5	50.0
Cladosporium herbarum (Pers.: Fr.) Link	–	–	–	7.0	2.8	–	–	–	–	5.6
Cladosporium sphaerospermum Penz.	7.0	8.3	7.0	15.3	8.3	4.2	8.3	9.7	13.9	12.5
Clonostachys rosea f. *catenulata* (J. C. Gilman and E. V. Abbott) Schroers	–	–	–	–	–	–	–	1.39	–	–
Coniothyrium sporulosum (W. Gams and Domsch) Aa	–	–	–	–	–	–	–	–	1.39	–
Curvularia eragrostidis (Henn.) J. A. Mey.	–	1.39	–	–	–	–	–	–	–	–
Epicoccum nigrum Link	19.5	2.8	5.6	9.7	15.3	45.8	27.8	22.2	25.0	18.1
Fusarium sp. div.	37.5	68.1	62.5	66.7	80.6	27.8	69.4	72.2	69.5	86.1
Geotrichum candidum Link: Fr.	–	–	–	–	–	–	–	–	1.39	–
Gonatobotrys simplex Corda	–	–	–	–	–	1.39	–	–	–	1.39
Harzia acremonioides (Harz) Cost.	–	–	–	1.39	–	–	–	–	–	–
Humicola fuscoatra Traaen var. *fuscoatra*	–	–	–	–	–	5.6	–	–	–	–
Lecanicillium cf. *longisporum* (Petch) Zare and W. Gams	2.8	4.2	11.1	–	–	2.8	4.2	–	–	–
Lecanicillium muscarium (Petch) Zare and W. Gams	16.7	2.8	4.2	8.3	8.3	9.7	11.1	11.1	11.1	8.3
Mucor circinelloides f. *circinelloides* Tiegh.	20.8	15.3	9.7	31.9	22.2	27.8	47.2	25.0	66.7	30.6
Mucor circinelloides f. *janssenii* (Lendn.) Schipper	–	–	–	–	–	1.39	–	–	–	–
Mucor circinelloides f. *lusitanicus* (Bruderlein) Schipper	–	–	–	–	–	–	2.8	–	–	2.8
Mucor hiemalis f. *corticolus* (Hagem) Schipper	–	–	–	–	–	–	–	–	–	1.39
Mucor hiemalis f. *hiemalis* Wehmer	–	5.6	–	2.8	2.8	–	2.8	–	7.0	7.0
Mucor hiemalis f. *luteus* (Linnem.) Schipper	–	2.8	–	–	2.8	2.8	2.8	–	5.6	–
Penicillium sp. div.	5.6	5.6	2.8	13.9	6.9	5.6	5.6	2.8	12.5	6.9
Pithomyces chartarum (Berk. and Curtis) M.B.Ellis	–	–	–	–	2.8	–	2.8	2.8	–	2.8
Rhizopus oryzae Went and Prins. Geerl.	–	2.8	–	–	–	2.8	–	2.8	–	2.8
Rhizopus stolonifer (Ehrenb.) Vuill.	2.8	2.8	–	–	2.8	2.8	2.8	–	2.8	5.6
Ulocladium chartarum (Preuss) E. G. Simmons	–	–	–	–	–	1.39	–	–	–	–
Total microfungi	**13**	**16**	**11**	**13**	**15**	**17**	**16**	**13**	**14**	**18**

DISCUSSION

Many studies have shown that the most frequent microfungi isolated from maize kernels before harvest are species of *Alternaria*, *Cladosporium*, *Epicoccum* and *Fusarium* (Fisher et al. 1992, Gonzalez et al. 2002, Lacey and Magan 1991, Ono et al. 1999, Ono et al. 2002, Pitt et al. 1998, Pitt and Samson 1993), *Bipolaris*, *Mucor*, *Rhizopus* (Broggi et al. 2002), *Coniothyrium sporulosum*, *Pithomyces chartarum* (Krivobok et al. 1995) and *Acremonium strictum* (Jesenská 1993). Our observations (Tab. 1) on kernels of pre-harvested maize agree with these findings.

Most studies of Bt-maize have compared the damage of maize plants in relation to ECB (Magg et al. 2001). Gatch and Munkvold (2002) showed lower incidence of *Fusarium* spp. on Bt-maize than on non-transgenic hybrids. However, the entire spectrum of microfungi on Bt-maize has not yet been investigated. Our data suggest that fungus communities on Bt-maize are the most unique of all tested treatments. Our data confirmed the lower incidence of potentially toxinogenic and phytopathogenic *Fusarium* spp. We found a similar effect of Bt-maize on *Acremonium strictum* as well. These fungi apparently occur less frequently on Bt-maize than on other treatments, both on intact and ECB damaged kernels. As such, their lower incidence is not attributable to the absence of transmission by ECB. Perhaps they are endophytic fungi whose growth is inhibited by the Bt-toxin host in green tissues. The effects of the Bt-toxin on fungi, which is only synthesised in the green parts of plants, have not been determined.

The lower incidence of *Fusarium* spp. on Bt-maize is correlated with lower amounts of *Fusarium* mycotoxins (Slezáková, unpublished results). This indicates that the safety of maize for human and animal consumption may be increased by genetic engineering for insect resistance. *Alternaria alternata* and *Penicillium* species, all mycotoxin-producing fungi, were frequently associated with Bt-maize (Kendrick 1992, Samson et al. 1996). Thus, the effect of Bt protection to the overall contamination of Bt-maize is unclear.

Other studies have shown the relationship between the kinds and abundance of microfungi isolated from maize kernels and fungus isolation techniques. Adebajo et al. (1994) used $HgCl_2$ surface sterilisation followed by a distilled water wash, whereas Almeida et al. (2002) and Pitt et al. (1998) mentioned surface sterilisation with NaOCl followed by sterile washes. We surface-washed healthy and damage kernels. Such washing removes most of the spores from the surface of kernels and as such this method detects epiphytes. Our results showed that, in healthy and damaged kernels, the same microfungi were detected, but with different frequency. Direct isolation from damaged kernels revealed that many fungi were living epiphytically on maize kernels. *Acremonium strictum*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Rhizopus stolonifer*, *Fusarium* spp., *Lecanicillium muscarium* and *L. longisporum* have

been routinely isolated from maize as endophytes (Fisher et al. 1992, Gonzalez et al. 2002, Jesenská 1993). As shown here, *Alternaria alternata* and *Epicoccum nigrum*, known endophytes, probably represent fungi which can effectively live as saprophytes on damaged kernels. Thus, the observed difference between the mycoflora of both types of kernel results from the abilities of native fungi to grow on damaged tissue, rather than by the transmission of insect vectors, air or splashing water.

In conclusion, we showed both positive and also possible negative effects of Bt-maize on the quality of corn kernels. To decrease the occurrence of microfungi on Bt-maize, it is also necessary to follow conventional integrated cultivation procedures.

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