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# Evidence for natural resistance towards trifloxystrobin in *Fusarium graminearum*

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**Abstract** A collection of 55 *Fusarium graminearum* (*Gibberella zeae*) strains isolated between 1969 and 2009 in Belgium, Canada, Germany, Italy, Luxembourg, or the USA belonging to the three known chemotypes (3-acetylated deoxynivalenol, 15-acetylated deoxynivalenol and nivalenol) were screened for their sensitivity towards the fungicide trifloxystrobin using a liquid culture assay. None of the isolates was completely inhibited by trifloxystrobin concentrations up to 3 mM. For comparison, prothioconazole completely inhibited fungal growth of a standard isolate at concentrations as low as 0.007 mM. The maximum level of inhibition, which could be obtained by trifloxystrobin, ranged from 14 to 65% among the strains tested and was not significantly affected by the country of origin or by the chemotype. The absence of significant differences in resistance levels between the countries of origin and chemotypes as well as the fact that strains isolated before the market introduction of strobilurins in 1996 also showed a high level of resistance is evidence that this is largely a case of natural resistance and not primarily related to strobilurin use in agriculture.

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## Introduction

*Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is a fungal plant pathogen infecting major crops such as wheat, maize, or rice at anthesis. It is one of the causal agents of Fusarium head blight, a disease of cereals that can cause devastating problems by reducing both yield and quality (Parry et al. 1995). The fungus produces mycotoxins with harmful effects on human health. Mycotoxin contamination of cereals is a permanent concern in food safety, and, therefore, subject of national and European regulations on maximum tolerable limits for mycotoxin concentrations in selected cereals and cereal products (Anonymous 2006). *Fusarium graminearum* strains can be classified according to the mycotoxins they produce. The profile of mycotoxin production expressed by a fungal strain is usually referred to as its chemotype. Three chemotypes can be distinguished in *F. graminearum,* (15-acetylated deoxynivalenol – 15-ADON, 3-acetylated deoxynivalenol – 3-ADON, and nivalenol – NIV). Among chemotypes, differences in fitness, pathogenicity and in the amount of toxin(s) produced were postulated (Ward et al. 2008) and partially verified (von der Ohe et al. 2010).

*Fusarium graminearum* can be controlled by avoiding maize as previous crop, by growing cultivars

with low susceptibility towards *Fusarium* species, or, by removing the plant debris of the previous crops from the surface of the soil for instance by using a moldboard plow (Beyer et al. 2006). However, when infection conditions around the time of wheat anthesis are favourable, these actions are not sufficient to avoid mycotoxin contamination of the grain (Vogelgsang et al. 2011). In this case, fungicides can additionally be applied. At present, chemical control of *Fusarium* species relies largely on demethylation inhibitors (DMIs) such as azoles, which disturb fungal membrane integrity. The response of *F. graminearum* towards selected DMIs was studied (Liu et al. 2010), but sensitivity losses over time were very small, so far (Klix et al. 2007). In contrast to fungicides containing DMIs as active ingredients, the group of strobilurin fungicides was reported to be hardly effective on several occasions (Siranidou and Buchenauer 2001; Gullino et al. 2002) against different *Fusarium* species in field or greenhouse experiments. A natural strobilurin with low light stability was first extracted from *Strobilurus tenacellus*. In most synthetic strobilurin analogs used as fungicides in agriculture, the central double bond was replaced with a phenyl ring structure to increase their light stability. The mode of action of the strobilurins is to block the cytochrome bc1 enzyme complex (sometimes referred to as complex III), thereby inhibiting respiration. Some authors found that strobilurins are particularly effective during the developmental stage of spore germination (Bartlett et al. 2002). Strobilurins were first introduced to the markets in 1996 (Russell 2005).

A fungal population can either be resistant towards a certain fungicide from the very beginning, a phenomenon termed natural resistance (Brent and Hollomon 2007), or it can become resistant towards the fungicide during the course of its use, a phenomenon often referred to as acquired resistance (Dekker 1976). It was the purpose of this study to characterize a *F. graminearum* strain collection isolated between 1969 and 2009 for its (in)sensitivity towards trifloxystrobin and to provide evidence for either acquired or natural resistance. Moreover, given the recent observations of a shift of the *F. graminearum* chemotype composition in different agricultural conditions (Ward et al. 2008; Gale et al. 2007; Zhang et al. 2010) it could be hypothesized that a selective pressure caused by the increased use of fungicides might have favoured a certain chemotype. Because it has been noted that oxidative stress is triggered by fungicides and that chemotypes respond differently to oxidative stress (Ponts et al. 2009), we also tested if the level of sensitivity to strobilurins differed among chemotypes.

## Materials and methods

### Fungal strains

Fungal strains were either obtained from culture collections or isolated from blighted wheat heads sampled in Luxembourg. For isolation of fungal strains, wheat spikes with typical Fusarium head blight symptoms were sampled at various locations across Luxembourg in 2009. Plant material was stored at −20°C until further use. After defrosting, spikes were disinfected using first a 2% sodium hypochlorite solution, then a 70% ethanol solution and afterwards rinsed for two times with sterile deionized water. After drying in a sterile workbench, spikes were individually transferred in 50 ml tubes filled with 25 ml PDB (Potato Dextrose Broth) containing an antibiotic mix (100 μg streptomycin·ml$^{-1}$ PDB; 59.9 μg penicillin·ml$^{-1}$ PDB) to suppress growth of bacteria. After 3 days, growing mycelium was transferred to Petri dishes containing DCPA (Dichloran Chloramphenicol Peptone Agar; Andrews and Pitt 1986). Developing colonies appearing like *Fusarium* species were transferred to SNA (Synthetic low-level Nutrient Agar; Nirenberg 1976) plates and were incubated at 30°C until they were completely covered. Spores were washed off with sterile deionized distilled water and three dilutions (1:10, 1:100, 1:1000) were produced. From the 1:100 and 1:1000 dilutions, 1 ml of each was spread on an SNA plate. The colonies developing out of a single spore were transferred to a new SNA plate for spore production. Spores produced on that plate were washed off using sterile deionized water and stored at −80°C until further use. *Fusarium graminearum* strains originating from other locations and collections (Table 1) were also included in the bioassays described below.

Spores from the stock solutions were transferred to SNA plates and the liquid was distributed over the surface of the medium using a sterile triangle-shaped metal spreader rod. After 7 to 15 days at 25±1°C,

**Table 1** Names, years of isolation, countries of origin, chemotype, strain collections and the maximum inhibition (%) obtained by trifloxystrobin concentrations up to 3 mM in liquid cultures of *F. graminearum*

| Strain designation | Year of isolation | Country of origin | Chemotype | Strain collection[d] | Maximum inhibition (%) in response to trifloxystrobin |
|---|---|---|---|---|---|
| MUCL 11946 | 1969 | Belgium | 3-ADON[a] | BCCM/MUCL | 57.44 |
| 45276 | 1987 | Germany | 3-ADON | LfL Freising | 55.32 |
| ITA 202 | 1993 | Italy | 3-ADON[c] | V. Balmas | 51.21 |
| ITA 285 | 1993 | Italy | 3-ADON[c] | V. Balmas | 44.26 |
| ITA 593 | 1994 | Italy | NIV[c] | V. Balmas | 39.85 |
| 37 | 1994 | Germany | 15-ADON | HU Berlin | 36.84 |
| ITA 802 | 1995 | Italy | 15-ADON[c] | V. Balmas | 41.96 |
| ITA 1377 | 1997 | Italy | 3-ADON[c] | V. Balmas | 42.58 |
| 37254 | 1998 | Canada | 3-ADON[b] | ARS | 44.36 |
| ITA 1502 | 1998 | Italy | 15-ADON[c] | V. Balmas | 41.43 |
| 37294 | 2000 | Canada | 3-ADON [b] | ARS | 37.34 |
| MUCL 42825 | 2000 | Belgium | NIV[a] | BCCM/MUCL | 33.25 |
| ITA 1601 | 2001 | Italy | NIV[c] | V. Balmas | 46.22 |
| ITA 1606 | 2001 | Italy | 3-ADON[c] | V. Balmas | 45.12 |
| 37384 | 2001 | Canada | 3-ADON[b] | ARS | 38.75 |
| 37308 | 2001 | Canada | 3-ADON[b] | ARS | 35.99 |
| 38381 | 2003 | USA | 3-ADON[b] | ARS | 64.81 |
| 37404 | 2004 | Canada | 3-ADON[b] | ARS | 50.49 |
| 37523 | 2004 | Canada | 3-ADON[b] | ARS | 48.41 |
| 38995 | 2004 | Canada | 3-ADON[b] | ARS | 35.09 |
| 05 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 42.73 |
| 211 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 30.88 |
| 237 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 44.81 |
| 321 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 46.32 |
| 401 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 40.69 |
| 402 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 41.37 |
| 405 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 35.95 |
| 453 | 2007 | Luxembourg | NIV[a] | CRP-GL | 32.48 |
| 484 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 38.84 |
| 568 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 42.84 |
| 630 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 44.21 |
| 734 | 2007 | Luxembourg | 15-ADON[a] | CRP-GL | 55.71 |
| LT 11-24 | 2008 | Luxembourg | 15-ADON[a] | CRP-GL | 54.96 |
| 16-09 | 2008 | Luxembourg | NIV[a] | CRP-GL | 29.37 |
| 09-5a | 2009 | Luxembourg | 15-ADON | CRP-GL | 49.62 |
| 09-4a | 2009 | Luxembourg | 15-ADON | CRP-GL | 48.95 |
| 09-1a | 2009 | Luxembourg | 15-ADON | CRP-GL | 48.45 |
| 09-21a | 2009 | Luxembourg | 15-ADON | CRP-GL | 45.26 |
| 09-22a | 2009 | Luxembourg | 15-ADON | CRP-GL | 45.19 |
| 09-45b | 2009 | Luxembourg | 15-ADON | CRP-GL | 45.00 |
| 09-25a | 2009 | Luxembourg | 15-ADON | CRP-GL | 44.85 |
| 09-3a | 2009 | Luxembourg | 15-ADON | CRP-GL | 43.84 |
| 09-12a | 2009 | Luxembourg | 15-ADON | CRP-GL | 42.64 |

**Table 1** (continued)

| Strain designation | Year of isolation | Country of origin | Chemotype | Strain collection[d] | Maximum inhibition (%) in response to trifloxystrobin |
|---|---|---|---|---|---|
| 09-52b | 2009 | Luxembourg | 15-ADON | CRP-GL | 41.84 |
| 09-14a | 2009 | Luxembourg | 15-ADON | CRP-GL | 40.01 |
| 09-13a | 2009 | Luxembourg | 15-ADON | CRP-GL | 36.84 |
| 09-51b | 2009 | Luxembourg | 15-ADON | CRP-GL | 36.36 |
| 09-2a | 2009 | Luxembourg | 15-ADON | CRP-GL | 35.90 |
| 09-6a | 2009 | Luxembourg | 15-ADON | CRP-GL | 34.18 |
| 09-53b | 2009 | Luxembourg | 15-ADON | CRP-GL | 31.86 |
| 09-42b | 2009 | Luxembourg | 15-ADON | CRP-GL | 13.96 |
| PH-1 | unknown | USA | 15-ADON[b] | USDA | 46.83 |
| UMW 00-706 | unknown | USA | 15-ADON[c] | L. Gale | 42.64 |
| 28336 | unknown | USA | 3-ADON[b] | ARS | 41.66 |
| UMW 00-531 | unknown | USA | 15-ADON[c] | L. Gale | 22.97 |

LfL Freising = Bayerische Landesanstalt für Landwirtschaft (Germany)

V. Balmas = Strains kindly provided by Virgilo Balmas (Dipartimento di Protezione delle Piante, Università degli Studi di Sassari Italy)

L. Gale = Strains kindly provided by Liane Gale (Department of Plant Pathology, University of Minnesota USA)

HU Berlin = Humboldt University Berlin (Germany)

ARS = Agriculture Research Service (USA), kindly provided by Kerry O'Donnell

CRP-GL = Centre de Recherche Public-Gabriel Lippmann (Luxembourg)

USDA = United States Department of Agriculture (USA), kindly provided by Kerry O'Donnell

[a] From Pasquali et al. 2010

[b] From Ward et al. 2008

[c] From Gale et al. 2007

[d] BCCM/MUCL = Belgium Coordinated Collections of Micro-organisms/Mycothèque de l'Université Catholique de Louvain (Belgium)

propagation of the spores resulted in spore clusters that were washed off using sterile deionized water.

## Fungal characterization

Fungal cultures were grown and DNA was extracted according to the protocol described in Giraud et al. (2010). The identity of the strains isolated from symptomatic wheat heads was confirmed by sequencing of the elongation factor 1α (EF-1α), using primers and PCR conditions as described in O'Donnell et al. (1998). The sequences were then blasted using both the *Fusarium* database (http://isolate.fusariumdb.org/blast.php) and the NCBI (National Center for Biotechnology Information) Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome), in order to determine the species. The chemotypes were determined according to the procedure described by Pasquali et al. (2010).

## Fungicides

Trifloxystrobin and prothioconazole were purchased from Fluka, Fluka Chemie AG, Switzerland. The chemical purity was ≥98%. Trifloxystrobin was introduced to the markets in 1998 and prothioconazole in 2002 (Russell 2005).

## Bioassays

Fungicides were solved in ethanol to reach the concentration of 3.061 mM for trifloxystrobin and 7.117 mM for prothioconazole. Fungicides were transferred into the wells of sterile, clear, flat bottom 96 microtiter plates resulting in concentrations of 100, 50, 10, 5, 1, 0.1, 0.01, and 0% of the stock solution. Solvents were allowed to evaporate overnight. Finally, 100 µl of spore suspension (treatment) or 100 µl water (control) and 100 µl of PDB medium were transferred

into each well. All experiments were conducted with three replicates for each strain. A control plate was produced using prothioconazole on the strain PH-1. For detailed information on the strains see Table 1. The gap between microtiter plates and lids was sealed with Parafilm and plates were incubated on an orbital shaker at 120 rpm, 22°C, in the dark for 5 days. Afterwards, 10 μl of resazurin (0.6 mg $ml^{-1}$), an indicator for cell viability, was added to each well. An orange staining is an indication of living tissue with active respiration, whereas a pink staining indicates the absence of respiring tissue (Fig. 1). After 24 h of incubation on an orbital shaker at 120 rpm, 22°C in the dark, the optical density of the spore suspensions and the respective control liquids was determined using an absorbance reader (Fluorescence, absorbance and luminescence reader, model Genios, Tecan Group Ltd., Männedorf, Switzerland). A preliminary experiment established that the difference in optical density between stained living fungal material and stained dead fungal material was the largest at 405 nm within the range of available wavelengths. Hence, this wavelength was used as a default setting of the absorbance reader whenever scanning stained cultures.

Initially, the concentration of spores was determined with a Fuchs Rosenthal haemocytometer and a light microscope and the optical density of the same unstained solutions was assessed using the absorbance reader. A standard curve was produced first, linking the optical density at 280 nm of eight dilutions of a spore suspension, with the number of spores per litre. The regression equation was: Spore concentration (spores $l^{-1}$)=$2.77\times10^7/(1 + \exp(-(\text{optical density}_{280nm}-2.48\times10^3)/33.8))$, $r^2=0.99$. Spore concentrations were subsequently estimated from optical densities using the latter relationship. The measurements of the optical density were carried out in 96-well standard microplates with clear bottom (Perkin Elmer Inc., Waltham, MA), on a volume of 200 μl of spore suspension. The effect of different spore densities ($5\times10^8$, $2.5\times10^8$, $1.25\times10^8$, $5\times10^7$, $5\times10^6$ spores $l^{-1}$) used for inoculation on the optical density of fungal cultures growing in the standard trifloxystrobin concentration series under standard conditions was then tested. There was no significant effect of spore density ($P$=0.182) within the range of $5\times10^7$ – $5\times10^8$ spores $l^{-1}$, but optical densities were slightly lower when starting the experiment with $5\times10^6$ spores $l^{-1}$. Hence, spore densities of at least $5\times10^7$ spores $l^{-1}$ were subsequently used.

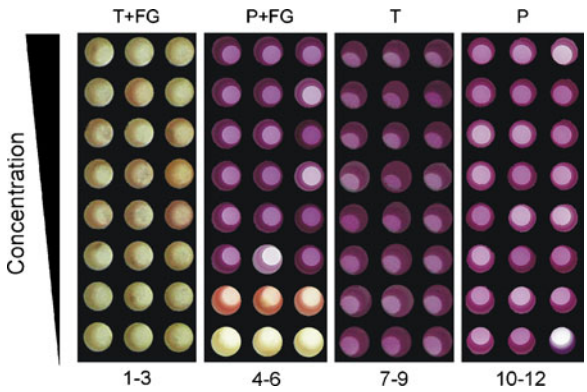


**Fig. 1** Microtiter plate showing cell viability. Stain: resazurin (orange = presence of living tissue; pink = absence of living tissue). Columns 1–3: strain PH-1 and trifloxystrobin; columns 4–6: strain PH-1 and prothioconazole; columns 7–9: trifloxystrobin, columns 10–12: prothioconazole. Trifloxystrobin concentrations: line 1: 3.061 mM; 2: 1.5305 mM; 3: 0.3061 mM; 4: 0.15305 mM; 5: 30.61 μM; 6: 3.061 μM; 7: 0.3061 μM; 8: 0 μM. Prothioconazole concentrations: line 1: 7.117 mM; 2: 3.5585 mM; 3: 0.7117 mM; 4: 0.35585 mM; 5: 71.17 μM; 6: 7.117 μM; 7: 0.7117 μM; 8: 0 μM

## Microscopy

Five μl of trifloxystrobin solved in ethanol (0.015 mM) were transferred to microscopy slides under a sterile workbench. Pure ethanol served as control. Ethanol was allowed to evaporate. Fungal spores were washed off SNA plates with sterile deionized water and 5 μl of spore suspension + 10 μl of liquid PDB medium were transferred to the position of the microscope slide, where the ethanol droplet was located before evaporation. The droplet was covered with a cover slip and the slide was incubated above deionized water in a closed Petri dish to minimize evaporation for 24 h at 22°C. Spore germination was checked under a microscope (model DMR, Leica, Solm, Germany) at 400×.

## Statistics

Optical densities were corrected for the absorbance of the microtiter plates, the medium, and the intrinsic colour of the fungicide. Data were expressed relative to the optical density of the untreated control. Means and standard errors (SE) of 3 replicates were calculated for each individual isolate and plotted against fungicide concentrations. A 4-parameter logistic curve model (y =

min + (max – min)/(1 + (x/$EC_{50}$)$^{Hillslope}$, software package Sigmaplot 10.0, Systat Software GmbH, Erkrath, Germany) was used to describe the effect of fungicide concentration on the fungal growth. "min" is the estimate for the minimum optical density expressed in % of the untreated control which was approximately constant between trifloxystrobin concentrations ranging from 0.03 mM and 3 mM (Fig. 2). The term (100-min) is subsequently used to describe the level of sensitivity of the fungal strains towards trifloxystrobin and is referred to as "maximum inhibition". An analysis of covariance with the maximum inhibition (%) as dependent variable, the country of origin of the fungal strains as independent variable and the year of isolation of the fungal strains as covariate was conducted using SPSS version 16 (SPSS Inc. Chicago, Illinois, USA). The non-parametric Kruskall-Wallis test (SPSS) was used to test whether the maximum inhibition differs among chemotypes. Trifloxystrobin concentrations inhibiting fungal growth by 20% ($EC_{20}$s) were calculated for each strain by setting y to 80% and solving the regression equation given above for x.

## Results

### Fungal characterization

In previous studies, some isolates used here were subjected to genetic chemotyping (Gale et al. 2007; Ward et al. 2008; Pasquali et al. 2009, 2010). The 17 isolates from Luxembourg in 2009 as well as two German isolates (45276 and 37) were characterized here. The EF-1α sequences of the Luxembourgish isolates were deposited in the NCBI gene bank with accession numbers HQ 149051 to HQ 149067. All of these isolates belonged to the 15-ADON chemotype, except one of the German isolates (45276), that belonged to the 3-ADON chemotype (Table 1).

### Fungicide resistance

Trifloxystrobin concentrations ≤0.0003 mM did not inhibit *F. graminearum* significantly as compared to the untreated control (Fig. 2). Concentrations between 0.03 mM and 3 mM resulted in an approximately constant average level of inhibition of about 40% (Fig. 2). All of the 55 isolates tested grew in presence of trifloxystrobin at a concentration of 3 mM.

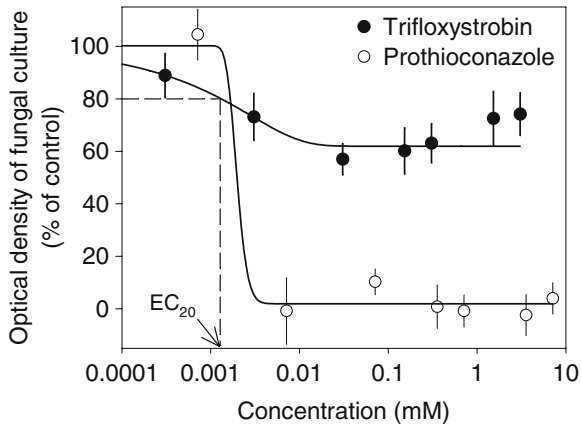


**Fig. 2** Optical densities of *Fusarium graminearum* liquid cultures (200 µl) after incubation at 22°C for 5 days in darkness as affected by trifloxystrobin and prothioconazole concentration. Data for trifloxystrobin represent medians ± SEs of 55 isolates, data for prothioconazole medians ± SEs for isolate PH-1. The number of replicates per isolate was $n$=3

Prothioconazole was ineffective at concentrations ≤0.0007 mM, but concentrations ≥0.007 mM inhibited fungal growth completely (Fig. 2). Spore germination was observed in presence and absence of trifloxystrobin (Fig. 3).

Since trifloxystrobin was unable to reduce fungal growth to 50% for the vast majority of strains, concentrations inhibiting fungal growth by 50% ($EC_{50}$) could not be calculated. Instead, the sensitivity of individual strains was characterized (1) by the maximum level of inhibition, which could be obtained with increasing fungicide concentration and (2) by the concentration reducing fungal growth by 20% ($EC_{20}$).

The maximum level of inhibition was about 56± 7% for the strains isolated in 1969 and 1987 and decreased to 42±1% afterwards (Fig. 4a). One outlier, a strain from the USA isolated in 2003, was detected, which was characterized by a maximum inhibition level of about 60% (Fig. 4a) and an unusual low $EC_{20}$ (Fig. 4b). The effect of the year of isolation was significant at $P$=0.031 in an analysis of covariance, where the maximum level of inhibition was the dependent variable, the country of origin was the independent variable, and the year of isolation of the fungal strains the covariate. The maximum level of inhibition ranged from 14 to 65% (Table 1) and was not significantly affected by the country of origin ($P$= 0.104). $EC_{20}$ values were about $1.4\times10^{-4}$ mM in 1969 and 1987 and increased afterwards with a high

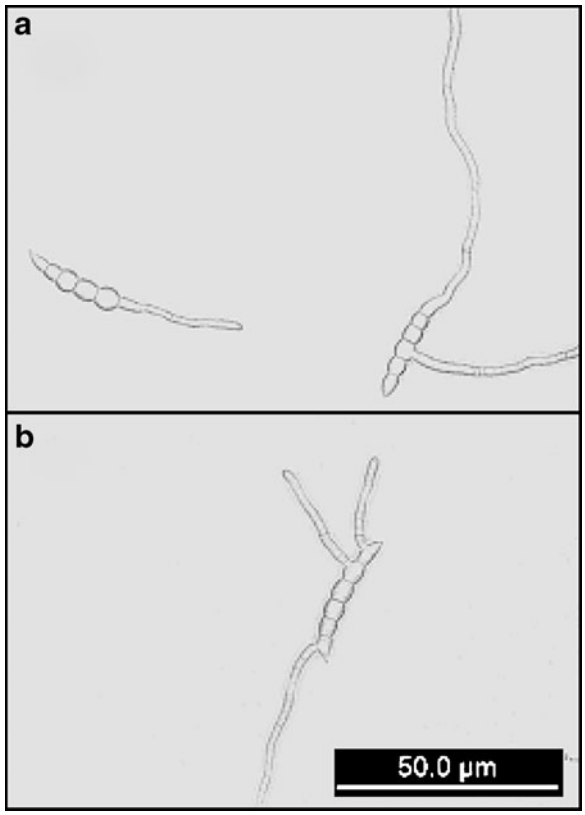


**Fig. 3** Macroconidial germination of *Fusarium graminearum* isolate 74b (Klix et al. 2007) in absence (**a**) or presence (**b**) of trifloxystrobin (0.005 mM)

variability within and between years (Fig. 4b). No significant differences were detected between the chemotypes as far as their maximum inhibition in response to trifloxystrobin was concerned ($P$=0.317).

## Discussion

Our study showed that isolates of *F. graminearum* differing in their country of origin, year of isolation, and chemotype express a similar level of resistance towards trifloxystrobin, suggesting that *F. graminearum* is naturally resistant towards this strobilurin. *Fusarium graminearum* is not listed among the plant pathogenic organisms resistant to disease control agents, so far (FRAC 2009).

The products currently registered against *Fusarium* species for instance in Germany contain strobilurins at concentrations ranging from 66.7 to 133 g $l^{-1}$ (VZP 2010). The approved application rate is 1.5 l $ha^{-1}$ diluted in 200 to 400 l of water. Molar masses of the strobilurins range from 313.8 g $mol^{-1}$ (kresoxim methyl) to 458.84 g $mol^{-1}$ (fluoxastrobin). This scenario results in molar concentrations ranging from 0.00055 to 0.003 M, which is well covered by the range of concentrations used in our study. Since strobilurins share the same mode of action, it might be speculated that *F. graminearum* is also resistant to other strobilurins. Audenaert et al. (2010) as well as Becher et al. (2010) reported that azoxystrobin failed to inhibit two different *F. graminearum* strains at concentrations ≤800 mg·$l^{-1}$, supporting this assumption. The highest concentrations included in our standard assay were above the water solubility level (0.15 mM

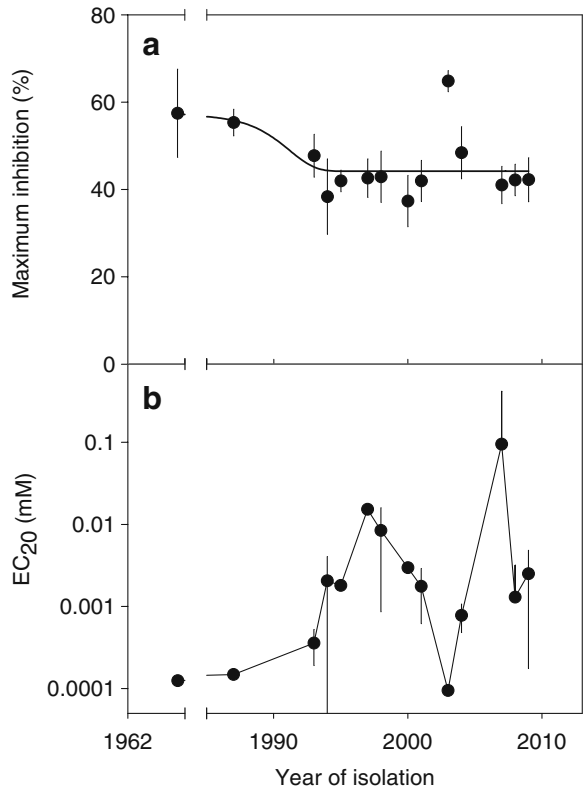


**Fig. 4** The maximum inhibition expressed as percent compared to an untreated control obtained with trifloxystrobin (concentration range 0–3 mM) in liquid cultures of *Fusarium graminearum* (**a**) and trifloxystrobin concentrations inhibiting fungal growth by 20% (**b**) as related to the year of fungal strain isolation. A total of 55 strains from six countries were tested. The number of replicates per isolate was $n$=3. Plot symbols and error bars represent medians and standard errors, respectively. Whenever no error bars are visible, they were smaller than the plot symbol. Asymmetric error bars in (**b**) result from the logarithmic scaling of the y-axis. Strains with unknown date of isolation (bottom of Table 1) were omitted here

at 25°C, calculated using the data by de Melo Abreua et al. 2006) of trifloxystrobin. Typical $EC_{50}$ values of fungal strains being sensitive towards QoIs are very low (eg about $3.7\times10^{-5}$ mM, Reuveni and Sheglov 2002). We tested concentrations being five orders of magnitude larger, without finding complete inhibition. Due to the limited solubility of trifloxystrobin in aqueous media, a further inhibition as consequence of higher concentrations can hardly be expected without additives. Using additives would have introduced fungicidal effects of the additive, and of fungicide x additive interaction into the test.

Trifloxystrobin concentrations up to 3 mM did not completely inhibit fungal growth of 55 *F. graminearum* isolates. For comparison, concentrations ≥0.007 mM prothioconazole inhibited fungal growth of strain PH-1 completely. The country of origin did not significantly affect the level of resistance. Seven strains of the collection were isolated before the introduction of strobilurin fungicides to the markets in 1996 (Russell 2005). The absence of significant differences in resistance levels between the countries of origin and the fact that strains isolated before the market introduction of strobilurins also showed a high level of resistance are clear evidence that the resistance of *F. graminearum* towards trifloxystrobin is a case of natural resistance as defined by Brent and Hollomon (2007). The slightly significant effect of the year of isolation on the maximum inhibition observed in the present study can either be interpreted as a small loss of vitality of the fungal strains with storage time, or, as evidence for a total resistance effect composed of (1) a high level of natural resistance plus (2) a low level of resistance acquired around 1993. The optical densities of the control cultures were 0.95±0.25 and 0.64±0.10 for the strains isolated before 1993 and after 1993, respectively, suggesting that the older isolates did not lose vitality due to the longer storage period. Isolates obtained from collections were mainly stored as freeze dried mycelium there, while the strains isolated in Luxembourg were stored as spore suspension with (2007, 2008) or without (2009) glycerol at −80°C. Since the maximum inhibition did not differ significantly between strains from collections isolated later than 1987 and our strains ($P$=0.507), the storage conditions used did not seem to be a factor. The small difference in response of *F. graminearum* towards trifloxystrobin over time was caused by only two old isolates (Fig. 4a), hence, this effect should be interpreted with caution. Further studies are required to clarify, which of the two potential explanations outlined above stand critical testing.

It has been suggested that the population shifts in chemotype composition of *Fusarium graminearum* (Ward et al. 2008; Starkey et al. 2007) might have been induced also by the changes in the use of fungicides (Gale et al. 2007). Strobilurins induce oxidative stress responses in *Fusarium* species (Audenaert et al. 2010). This pathway plays a primary role in the control of toxin synthesis (Reverberi et al. 2010). Because physiological differences linked to oxidative stress responses were found among chemotypes (Ponts et al. 2009), the behavior of the three different chemotypes of *F. graminearum* was investigated. From our study it seems that no significant difference among chemotypes could be found regarding the sensitivity towards trifloxystrobin, suggesting that no particular chemotype was favored by trifloxystrobin use due to a lower sensitivity level. It should be noted that we did not test whether the chemotypes differ with respect to the amount of toxins they produce *in vivo*. If a certain chemotype would produce significantly more toxin in response to trifloxystrobin than the others, a selective effect of trifloxystrobin application could still be observed, even though trifloxystrobin sensitivity of the strains does not differ. Because it is the amount rather than the type of trichothecene produced that functions as a major determinant of aggressiveness on wheat (Goswami and Kistler 2005), the most productive chemotype would have an advantage on moderately resistant cultivars (von der Ohe et al. 2010). Further studies are warranted to elucidate this hypothesis.

In many fungal species, resistance towards strobilurins is caused by mutations in the gene coding for the strobilurin target protein cytochrome b (Torriani et al. 2009). Mutations affecting the amino acid positions 129 and 143 were described to be particularly critical (Gisi et al. 2002; Kim et al. 2003). No modifications can be found at the amino acid positions 129 and 143 in *F. graminearum* strain PH-1 (FCD 2010) even though this isolate was resistant towards trifloxystrobin. Hence, resistance must be caused by factors other than the already known target mutations, at least in this isolate. A recent study by Kaneko and Ishii (2009) carried out with two Japanese strains suggests that alternative oxidase, the enzyme responsible for alternative respiration, is involved in the resistance of *F. graminearum* towards azoxystrobin. The inhibitors of

alternative oxidase salicylhydroxamic acid (SHAM) and n-propyl gallate both inhibited growth of *F. graminearum* with an $EC_{50}$ of 2.1 mM (Beyer et al. 2010), but the effect of the interaction between SHAM and trifloxystrobin concentrations was non-significant at $P=0.68$, indicating that addition of sub-lethal SHAM concentrations did not significantly affect the response of *F. graminearum* towards trifloxystrobin. The understanding of how natural resistance evolved can be helpful for predicting the range of applications of new antibiotics, in particular if they are derived from already known compounds.

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