Adaptation of *Fusarium graminearum* to Tebuconazole Yielded Descendants Diverging for Levels of Fitness, Fungicide Resistance, Virulence, and Mycotoxin Production

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ABSTRACT

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Azole fungicides play a prominent role for reliable plant disease management. However, quantitative azole resistance has been shown to develop in fungal pathogens, including *Fusarium graminearum*, the causal agent of Fusarium head blight (FHB). Due to widespread application of azole fungicides, resistance may accumulate to higher degrees in fungal field populations over time. Although azole fungicides are prominent components in FHB control, little effort has been made to investigate azole resistance in *F. graminearum*. We allowed *F. graminearum* strain NRRL 13383 to adapt to an azole fungicide in vitro, applying a strongly growth-reducing but sublethal dose of tebuconazole. Two morphologically distinguishable azole-resistant phenotypes were recovered that differed with regard to levels of fitness, fungicide resistance, virulence, and mycotoxin production. Isolates of the adapted "phenotype 1" exhibited azole-specific cross-resistance, whereas "phenotype 2" isolates displayed the phenomenon of multidrug resistance because the sensitivity to amine fungicides was also affected. Assessment of individual infected spikelets for mycotoxin contents by high-performance liquid chromatography mass spectrometry and for *Fusarium* DNA by quantitative polymerase chain reaction indicated that some of the adapted isolates produced significantly higher levels of nivalenol per fungal biomass than the NRRL 13383 strain.

Additional keywords: DMIs, fenpropimorph, *Gibberella zeae*, prochloraz, prothioconazole, spiroxamine, trichothecene, zearalenone.

The ascomycete *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is a major causal agent of Fusarium head blight (FHB), a destructive floral disease of small grain cereals strongly compromising wheat production in many parts of the world (23). Infection primarily leads to the reduction of yield and quality of the harvested grain and secondarily to seedling blight when infected grains are used for sowing (41). Importantly, *F. graminearum* produces several potent mycotoxins that have detrimental impact on human and animal health (43,53). The most prominent mycotoxins are the trichothecenes deoxynivalenol (DON) and nivalenol (NIV) and the estrogenic polyketide zearalenone (ZEA). Severe outbreaks of human and animal toxicoses in history could be traced back to toxication with *Fusarium* mycotoxins (14). As food safety and consumer protection have gained considerable attention, strict regulations for tolerated levels of *Fusarium* mycotoxins in cereals and products derived thereof have been established (21).

The increase in FHB prevalence and severity during the last decades in many wheat-growing regions of the world has stimulated research on disease mechanisms and control. Although crop rotation, tillage practices, and breeding for FHB resistance have made considerable progress in wheat, fungicide application is an indispensable measure to limit FHB severity and mycotoxin contaminations (3,20,36). Several studies have shown that application of some azole fungicides (e.g., tebuconazole) at anthesis can consistently control the disease.

Azole fungicides are one of the most important classes of systemic site-specific fungicides that have been intensively used since the 1980s against a broad spectrum of plant-pathogenic fungi. They inhibit the cytochrome P450 sterol 14α-demethylase (CYP51), an enzyme that is essential for ergosterol biosynthesis, leading to disturbance of fungal membrane integrity (8). Together with piperazine, pyridine, and pyrimidine fungicides that act at the same target site, the azoles are grouped as demethylation inhibitors (DMIs) (30). In contrast to other site-specific fungicides that were rendered ineffective due to point mutations at the genes encoding the target proteins, the efficacy of DMIs has decreased relatively slowly and gradually (7). Dose-response curves observed with single-spore isolates of fungal field populations exhibited the presence of a continuum of levels of DMI resistance (16), indicating that quantitative fungicide resistance had developed. Several studies have shown that decreased azole sensitivity is due to different mechanisms, such as (i) target-site alterations based on point mutations in the *CYP51* gene (31,51), (ii) overexpression of the target gene (24,44), (iii) alterations in sterol biosynthesis and cellular sterol composition (25,26), (iv) enhanced energy-dependent efflux transport of the toxic compound (38,42), and (v) increased copy numbers of the target gene and a transcriptional regulator of drug efflux pumps (46). Additive effects were also reported among these mechanisms (28,46,49). Strains with reduced sensitivity were found in field populations of, for example, *Blumeria graminis* f. sp. *tritici* (22), *Botrytis cinerea* (48), and *Mycosphaerella graminicola* (35), partially compromising an effective disease control. The fact that azoles

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are the most prominent fungicide class used in cereals today raised significant concerns about the development and accumulation of azole resistance.

Fungicide resistance can be studied by analyzing parameters such as resistance and cross-resistance levels, or mycotoxin chemotype of isolates from fields that have a known history of fungicide treatment. However, field isolates often have an undefined genetic background, which makes it difficult to compare them directly to a reference genotype, for example, by analyzing expression levels of putatively affected genes by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments. This limitation can be overcome by using a characterized reference strain for selecting fungicide-resistant descendants in vitro under controlled experimental conditions. This can be achieved by several methods such as short exposures to UV light or chemical mutagens to introduce mutations. Other methods aim at selecting existing mutations, which can be accomplished by treating large numbers of spores with a fungicide. Alternatively, spontaneous genetic alterations can be selected by continuous exposure of fungal mycelia to a sublethal fungicide concentration, which is referred to as adaptation. The latter approach has been successfully used in previous studies (12,47,49). Reasons for the observed adaptability in the absence of mutagens were discussed (10). Due to the smaller scale of in vitro experiments compared with the selection occurring in the field, such experiments may only uncover some part of the genetic alterations leading to fungicide resistance; however, genetic alterations identified in mycelia continuously exposed to fungicides may be relevant in agronomical settings.

In spite of its agroeconomic importance, little is currently known about azole resistance in *F. graminearum*. A small-scale study on strains isolated from German fields during 1987 to 2004 suggested declining triazole sensitivity (29). Another report described three DMI-resistant isolates that were discovered among 159 field isolates from China (52). In order to study the development of azole resistance in *F. graminearum*, we selected isolates in vitro and under controlled conditions that exhibited reduced levels of sensitivity for tebuconazole. Adapted strains were evaluated for the level of fungicide resistance acquired, general vitality, and virulence. To assess the in planta mycotoxin production per fungal biomass, we took advantage of methods that allowed us to resolve mycotoxin production and DNA quantification at the level of individual spikelets.

MATERIALS AND METHODS

Cultivation and storage of fungi. The *F. graminearum* reference strain NRRL 13383 (kindly provided by Kerry O'Donnell, National Center for Agricultural Utilization Research, United States Department of Agriculture–Agricultural Research Service, Peoria, II), which was originally isolated from corn in Iran, was used as the parental isolate for adaptation. Within the *F. graminearum* species complex, it belongs to lineage 7 (39) that was referred to as *F. graminearum* sensu stricto (40). NRRL 13383 was identified as a strong NIV producer that is highly aggressive to wheat (23). The nonadapted reference strain as well as the adapted isolates generated in this study were stored as conidial suspensions (10^6 conidia/ml) in 20% (vol/vol) glycerol at –70°C. For subsequent cultivation, 20 µl of the freezer stock was transferred to a potato dextrose agar (PDA) (Becton Dickinson GmbH, Heidelberg, Germany) plate and incubated for 5 days at 23°C in darkness.

Determination of parameters of fungal fitness. Growth rates of nonadapted and adapted isolates were determined at different temperatures (7, 15, 23, and 30°C). Mycelial plugs (5 mm in diameter) were excised from the margins of 5-day-old colonies and placed upside down in the centers of PDA plates (90 mm in diameter). Growth of the colonies was linear between days 2 and 6, and mycelial growth was measured on the fourth and fifth day in two perpendicular directions. Three replicates were analyzed per isolate and temperature. Averaged colony diameters were used to calculate radial growth.

Conidia were produced in 300-ml flasks filled with 50 ml of mung bean broth (MBB) (4) that was inoculated with five colonized PDA plugs and incubated for 7 days at ambient light and temperature conditions, with shaking (50 rpm). To assess the in vitro sporulation capacity of isolates, spore concentrations were determined using a hematocytometer and five biological repeats. If needed for further experimentation, spores were collected by filtering the broth through Miracloth (Merck KGaA, Darmstadt, Germany) into a 50-ml tube with centrifugation for 10 min at $3{,}000 \times g$. After transfer to a 2-ml tube, spores were washed twice with and finally suspended in 1 ml of sterile 0.01% (vol/vol) Tween 20.

To assess the conidial germination rate, 20 µl of a suspension with 5×10^4 conidia/ml was placed on coverslips and incubated in a glass petri dish at 100% relative humidity (r.h.). After 24 h, germination rates were determined by bright-field microscopy (Nikon Eclipse E600; Nikon GmbH, Düsseldorf, Germany). For each strain, the germination rate was assessed in three biological repeats of 200 conidia.

Adaptation procedure. The triazole tebuconazole (FOLICUR; Bayer CropScience, Monheim, Germany) was used for adaptation. The *F. graminearum* strain NRRL 13383 was grown as a submerged culture in liquid media to ensure that the fungicide permeated the entire mycelium. A 300-ml flask containing 100 ml of potato dextrose broth (PDB) was inoculated with three mycelial plugs (5 mm in diameter) and incubated with shaking at 100 rpm in darkness at 23°C. Two days later, tebuconazole was added to PDB to result in a concentration of 10 mg/liter. Incubation continued for another 33 days with two exchanges of the spent medium with fresh fungicide-amended medium after the 11th and 22nd day. This adaptation procedure was performed in four biological repeats. The mycelia were harvested by filtration through sterile filter papers and then suspended in twice of their estimated volumes of 20% (vol/vol) glycerol. Subsequently, the mycelial suspensions were blended (Ultra Turrax; IKA Labortechnik, Staufen, Germany) for 30 s using speed 5 and stored at –70°C.

To obtain strains that originated from single spores, 100 µl of the freezer stock was used for inoculation of MBB to induce conidiation as described above. Thus, a suspension of conidia was produced that was serially diluted and spread onto PDA amended with tebuconazole at 10 mg/liter. Agar plugs from single colonies were transferred to PDB and MBB to produce mycelial (first generation after adaptation) and conidial suspensions (second generation), respectively, of the resistant strains that were kept as glycerol stocks as described above.

To test for mitotic stability of acquired resistance, isolates underwent further sub-cultivation. The conidial freezer stocks (20 µl, second generation) were transferred to PDA and incubated for 5 days at 23°C in darkness. Subsequently, MBB was inoculated and incubated as above. After 7 days, 10 µl of a conidial suspension was transferred to PDA and the subcultivation procedure was correspondingly repeated until the sixth conidial generation.

Assessment of fungicide sensitivity. Sensitivity to tebuconazole was quantified by measuring radial growth of NRRL 13383 and the derived adapted isolates on PDA adjusted to different tebuconazole concentrations (0, 0.025, 0.05, 0.075, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, and 30.0 mg/liter) in three replicates each. After 6 days of incubation at 23°C in darkness, colony diameters were measured in two perpendicular directions and the original plug diameter was subtracted. Furthermore, the inhibitory effect of the fungicide on conidial germination was assayed. In all, ≈1,000 spores in 20 µl of PDB amended with tebuconazole (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 7.5, or 10.0 mg/liter) were placed on coverslips,

transferred to glass petri dishes, and incubated for 24 h at 100% r.h. and 23°C in darkness. For each strain and each concentration, germination was assessed by bright-field microscopy in three repeats. Because azole fungicides may not directly affect spore germination due to endogenous sterol reserves, spores were counted as germinated only when the length of the germ tube was at least twice the spore length. Effective inhibition of mycelial growth and conidial germination were determined for each fungicide concentration relative to control medium without fungicides. Effective doses leading to a 50 or 90% inhibition (ED_{50} and ED_{90}, respectively) were calculated on the basis of a linear regression analysis of logit-transformed fungicide efficiency and the $\log_{10}$ transformed fungicide concentrations as described (42). These sensitivity assays were not only performed for the second vegetative generation after adaptation but also for mycelia and conidia of the sixth generation.

In addition, a qualitative cross-resistance assay was conducted with prothioconazole (PROLINE) and spiroxamine (PROSPER) (Bayer CropScience GmbH) as well as prochloraz (SPORTAK 45 EW) and fenpropimorph (CORBEL) (BASF SE, Ludwigshafen, Germany). In a preliminary screen for fungal growth on various concentrations of these fungicides, discriminatory doses were determined at which the parental isolate showed no or very little growth. For comparison of the isolates, 2 µl of 10^6 conidia/ml was applied onto PDA amended with prothioconazole at 15 mg/liter, prochloraz at 0.15 mg/liter, spiroxamine at 285 mg/liter, or fenpropimorph at 100 mg/liter and incubated at 23°C in darkness. After 7 days, plates were photographed (Nikon D50; Nikon GmbH).

Plant infection assay. Virulence of nonadapted and adapted *F. graminearum* isolates was tested as previously described (23), with minor modifications. Briefly, FHB-susceptible spring wheat cv. Picolo (Saatzucht Dr. J. Ackermann & Co., Irlbach, Germany) was grown until anthesis in the greenhouse at 26 ± 4°C with 16 h of supplemental light. Three seeds were planted per 14-cm pot containing steamed garden soil. The pots were watered daily, their positions were exchanged randomly once a week, and they were fertilized 28 days after planting by the addition of 4 g of mineral fertilizer Plantosan Compact (NPK[Mg], 15-8-15-[2]) (Spiess-Urania Chemicals GmbH, Hamburg, Germany). At anthesis, 10 µl of a suspension containing 1×10^6 spores/ml (in 0.01% Tween 20) was applied into the ninth spikelet. For assaying infection in the presence of fungicide, conidial suspensions were adjusted to contain a tebuconazole concentration of 10 mg/liter immediately before inoculation. After inoculation, each head was sprayed with 0.5 ml of sterile water and individually enclosed in a moistened plastic bag for 72 h. The inoculated plants were incubated in a controlled growth chamber (Percival Scientific AR-75HIL; Percival Scientific Inc., Perry, IA) that was kept at 27°C, 75% r.h. and a 16-h photoperiod applying a light intensity of 200 µE m^{-2} s^{-1} at soil level. Fourteen days postinoculation (dpi), 10 spikelets (in addition to the inoculated spikelet, 4 lower and 5 upper ones) were scored for disease symptoms (necrosis or bleaching). The disease rate is expressed as percentage of symptomatic spikelets among the 10 scored spikelets. All infection assays were repeated twice. Each repetition included six wheat plants per experimental variant. Results from all 12 plants were used for data analysis.

Analysis of in planta produced mycotoxin levels. Inoculated wheat spikelets were individually harvested, weighed, frozen in liquid nitrogen, and stored at –70°C. Spikelets were individually lyophilized for 20 h (Beta 1-B; Christ GmbH, Osterode, Germany) and ground with four tungsten-carbide beads in a TissueLyser (Qiagen GmbH, Hilden, Germany) for 3 min at maximum speed. Next, 2 ml of 84% (vol/vol) acetonitrile (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added to the powdered samples, which were then horizontally shaken at 23°C for 4 h. After centrifugation for 10 min at 5,000 × *g*, 1.7 ml of the supernatant was transferred to a 15-ml tube. The precipitate was resuspended in 1.7 ml of fresh solvent, extracted for an additional 16 h, and separated from the extract by centrifugation. Both extracts were combined, yielding 3.4 ml of extracts for each of 12 individual spikelets for all experimental variants. Acetonitrile extracts were stored at –20°C until mycotoxin quantification by high-performance liquid chromatograph mass spectrometry (HPLC-MS).

NIV concentration was determined by HPLC with a triple quadrupole detector (Varian GmbH, Darmstadt, Germany) in filtered crude extracts diluted 1:10 with column equilibration buffer. A polar-modified RP-18 column (Polaris C18-Ether, 3 µm, 100 by 2 mm; Varian GmbH) maintained at 40°C was used. The flow rate was set to 0.2 ml/min and the injected volume was 10 µl. A binary gradient with 5% acetonitrile in water as solvent A and methanol as solvent B were used as follows: 0 to 1 min, 20% solvent B; 1 to 8.5 min, 20 to 40% solvent B; 8.5 to 10.5 min, 40 to 98% solvent B; followed by washing with 98% solvent B and reequilibration with 20% solvent B. Tandem mass spectrometry (MS/MS) detection was carried out using a Varian 1200LC triple quadrupole with negative electrospray ionization (Varian GmbH). Gas temperature and pressure were set to 200°C and 18 psi. Needle, shield, and capillary voltage were –3,500, –600, and –40 V, respectively. Fragmentation was performed in a collision cell filled with argon gas (1.3 mTorr). Quantitative determination was performed in single reaction monitoring with a dwell time of 1 s and a width of 0.7 m/z unit, using the transition 311 > 281. Quantification of ZEA by HPLC-MS/MS was performed on filtered, defatted crude extracts diluted 1:1 with the mobile phase as described previously (1). Acetonitrile extracts of healthy spikelets amended with NIV (5 to 1,000 ng/ml) and ZEA (5 to 320 ng/ml) in three repetitions were used for calibration.

Extraction and quantification of fungal DNA in infected spikelets. Ground spikelets that had previously been used for mycotoxin extraction (see above) were dried in a vacuum centrifuge (Univapo100H; UniEquip GmbH, Martinsried, Germany) overnight to remove residual acetonitrile. Extraction of genomic DNA was conducted as reported (6) with modifications as follows. Ground plant samples were blended in 1 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer (10 mM Tris, 20 mM EDTA, 1.4 M NaCl, 2% [wt/vol] CTAB, and 1% [wt/vol] polyvinylpyrrolidone, pH 8.0), 20 µg of proteinase K, and 5 µl of mercaptoethanol. After mixing thoroughly, the samples were incubated for 30 min at 42°C followed by incubation for 30 min at 65°C. Mixing continued intermittently every 10 min. The samples were then shaken vigorously with 800 µl of chloroform-isoamyl alcohol (24:1), incubated on ice for 15 min, and centrifuged at 10.000 × *g* for 10 min. The supernatant (600 µl) was mixed with 200 µl of 30% (vol/vol) PEG 6000 and 100 µl of 5 M NaCl and centrifuged for 15 min at 14,000 × *g*. Pellets were washed twice with 70% ethanol, dried, and resuspended in 50 µl of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH set to 8.0 with HCl). Subsequent species-specific qPCR analysis for *F. graminearum* was performed as described (6).

Statistical analysis. The Student-Newman-Keuls test (33) was used to determine significant variations between all strains. Statistical comparisons between different treatments of the same strain employed the two-sided *t* test. Correlations were evaluated by calculation of Pearson's correlation coefficient (*r*). In all cases, a confidence limit of 95% ($P < 0.05$) was considered significant. All statistical analyses used the XLSTAT program (version 2008; Addinsoft Deutschland, Andernach, Germany).

RESULTS

Adaptation of *F. graminearum* to tebuconazole. We investigated the adaptation of *F. graminearum* to relatively high but sublethal fungicide concentrations without the influence of mutagens in order to determine potential outcomes of selection on

spontaneous genetic alterations. In preliminary experiments, we determined that tebuconazole at 10 mg/liter was a suitable concentration because it restricted mycelial growth of the *F. graminearum* strain NRRL 13383 to 95% (data not shown). After adaptation, the mycelia were transferred to MBB to induce conidiation. Conidia were allowed to grow on PDA amended with tebuconazole at 10 mg/liter. Whereas spores derived from a control culture were unable to form a mycelium, the conidia derived from tebuconazole-adapted cultures efficiently formed new colonies. In two out of four repetitions of this adaptation procedure, two types of distinct colonial morphologies, designated phenotype 1 (P1) and phenotype 2 (P2), were found (Fig. 1A). Colonies of P1 arose at a frequency of 15%, respectively 19% whereas, in the other cultures, only P2 colonies occurred. On unamended medium, P1 isolates exhibited a colony morphology that closely resembled that of the nonadapted parental strain, which is characterized by a dense aerial mycelium. In contrast, P2 isolates produced aerial mycelia that were less dense (Fig. 1B). The P1 isolates grew on fungicide-amended agar with an extensive aerial mycelium, whereas P2 isolates grew flat on the agar with few aerial hyphae (Fig. 1C). For further experiments aiming to characterize these adapted phenotypes in detail, three isolates of each (P1-1, P1-9, and P1-11 and P2-1, P2-4, and P2-8) were chosen arbitrarily.

Evaluation of fungicide sensitivities of NRRL 13383 and adapted isolates. The levels of resistance to tebuconazole were determined quantitatively by measuring radial growth and conidial germination rates of the parental strain NRRL 13383 and adapted isolates at increasing tebuconazole concentrations (Table 1). In both assays, all adapted isolates tested exhibited significantly ($P < 0.05$) increased ED values compared with NRRL 13383 with the exception of the ED_{50} values of the P2 isolates in mycelial growth assays. Resistance factors (RFs) determined by the germination assays varied little between the P1 and P2 isolates. In contrast, the mycelial growth assays revealed consistently higher RF values of the P1 isolates.

To evaluate the mitotic stability of fungicide-adapted phenotypes, the isolates were subjected to additional cycles of mycelial growth and sporulation. For the sixth vegetative generation, mycelial growth and spore germination were assayed as before (Table 1). Except for isolate P1-9, that showed a decrease in ED_{50} in the mycelial growth assay, mostly minor changes were obtained compared with those obtained previously. We conclude that phenotypes P1 and P2 differ not only in morphology but also in the degree of tebuconazole resistance, and that this resistance is mitotically stable.

Cross-resistance in tebuconazole-adapted isolates to additional fungicides was qualitatively assessed by discriminatory dose screens (Fig. 2). We tested the two azole fungicides prothioconazole (subclass triazoles) and prochloraz (subclass imidazoles), as well as the two amine fungicides, spiroxamine (subclass spiroketalamines) and fenpropimorph (subclass morpholines). The strobilurine azoxystrobin was also tested but it was unable to significantly inhibit NRRL 13383 even when applied at a concentration of ≤800 mg/liter (data not shown). For the other fungicides, we determined discriminatory doses as minimal inhibitory concentrations for the parental isolate NRRL 13383. This assay revealed that all adapted isolates exhibited positive cross-resistance to at least one of the two fungicide classes tested. Interestingly, the P2 isolates displayed increased tolerance not only for both azoles but also for the two amine fungicides. Thus, P2 may be referred to as a multidrug-resistance (MDR) phenotype. Positive cross-resistance of the P1 isolates was confined to azoles. Additional screens with the P1 isolates on lower concentrations of spiroxamine and fenpropimorph did not indicate negative cross-resistance effects (data not shown). With respect to resistance to the three azole fungicides tested in this work, P1 isolates grew better than P2 isolates, especially in the presence of the triazoles used. In contrast, the P2 isolates exhibited better growth on the imidazole prochloraz.

Characterization of fitness and virulence. To examine differences in fitness between the fungal isolates, growth rates on fungicide-free PDA were determined at four temperatures (Fig. 3A). All P1 isolates grew rather similarly to NRRL 13383 at all temperatures tested. In contrast, the P2 isolates grew significantly ($P < 0.05$) slower than NRRL 13383 and the P1 isolates at 23 and 30°C. At lower temperatures, the fitness impairment of the P2 isolates was generally lower and almost not apparent at 7°C. Additionally, we assessed two other aspects of fungal fitness (i.e., the capacity to produce macroconidia and their germination rate). The growth-impaired P2 isolates and the P1 isolates produced amounts of macroconidia similar to the parental strain (Fig. 3B). Germination rates of all isolates were very similar, close to 100%.

We further analyzed the effect of in vitro azole adaptation on fungal virulence on wheat heads. NRRL 13383 and its adapted descendants were assayed by inoculation of one central spikelet within individual wheat heads, and virulence was assessed by evaluating the spread of symptoms to the neighboring spikelets. The spread of symptoms has been reported to correlate with fungal spread and reflects virulence (23). As an independent measure, virulence was evaluated by the fresh weight of the inoculated spikelets after harvest. These assays were performed both with and without the application of tebuconazole. For each strain analyzed, fungicide application significantly ($P < 0.05$) reduced the spread of symptoms (Fig. 4A) and the loss of spikelet fresh weight (Fig. 4B). The fresh weights of all inoculated

TABLE 1. Sensitivity of *Fusarium graminearum* NRRL 13383 and tebuconazole-adapted isolates to tebuconazole[y]

	Mycelial growth assay						Spore germination assay					
	Second generation			Sixth generation			Second generation			Sixth generation		
Description, isolate[z]	ED_{50} (mg/liter)	ED_{90} (mg/liter)	RF	ED_{50} (mg/liter)	ED_{90} (mg/liter)	RF	ED_{50} (mg/liter)	ED_{90} (mg/liter)	RF	ED_{50} (mg/liter)	ED_{90} (mg/liter)	RF
Nonadapted												
NRRL 13383	0.14 ± 0.04 a	5.67 ± 0.89 a	n.a.	0.15 ± 0.01 a	5.45 ± 1.35 a	n.a.	0.61 ± 0.02 a	0.95 ± 0.02 a	n.a.	0.61 ± 0.08 a	0.93 ± 0.10 a	n.a.
Adapted P1												
P1-1	0.48 ± 0.03 b	28.32 ± 1.22 d	3.4	0.50 ± 0.02 bc	22.76 ± 2.11 bcd	3.3	2.46 ± 0.12 d	3.29 ± 0.18 d	4.0	2.37 ± 0.04 d	3.12 ± 0.05 e	3.8
P1-9	1.52 ± 0.11 d	35.36 ± 1.56 e	10.9	0.86 ± 0.08 d	30.36 ± 4.15 d	5.7	1.60 ± 0.07 c	2.73 ± 0.19 c	2.6	1.53 ± 0.02 c	2.43 ± 0.02 d	2.5
P1-11	0.73 ± 0.02 c	30.73 ± 1.48 de	5.1	0.61 ± 0.01 c	26.56 ± 1.93 cd	4.1	1.42 ± 0.09 bc	2.19 ± 0.09 b	2.3	1.41 ± 0.07 bc	2.25 ± 0.08 cd	2.3
Adapted P2												
P2-1	0.25 ± 0.01 a	11.88 ± 0.82 b	1.8	0.39 ± 0.03 b	23.26 ± 1.22 bcd	2.6	1.21 ± 0.06 b	1.93 ± 0.11 b	2.0	1.45 ± 0.06 bc	2.21 ± 0.11 cd	2.4
P2-4	0.30 ± 0.01 a	17.15 ± 1.44 c	2.1	0.26 ± 0.01 a	17.77 ± 1.04 bc	1.7	1.24 ± 0.03 b	2.02 ± 0.04 b	2.0	1.25 ± 0.02 b	1.90 ± 0.02 b	2.0
P2-8	0.27 ± 0.02 a	13.42 ± 1.22 bc	1.9	0.27 ± 0.02 a	13.42 ± 1.22 b	1.7	1.34 ± 0.02 b	2.03 ± 0.07 b	2.2	1.33 ± 0.03 bc	2.15 ± 0.04 c	2.2

[y] Second and sixth generations after treatment; the nonadapted parental strain was subcultivated in parallel. Data are given as means ± standard error. ED_{50} and ED_{90} = effective doses leading to a 50 or 90% inhibition, respectively. Means (within a column) designated by the same letter are considered not to be significantly different by the SNK-test at $P < 0.05$. Resistance factor (RF) is the ratio of the ED_{50} value of an adapted strain to ED_{50} of NRRL 13383; n.a. = not applicable.

[z] P1 and P2 = adapted phenotypes 1 and 2, respectively.

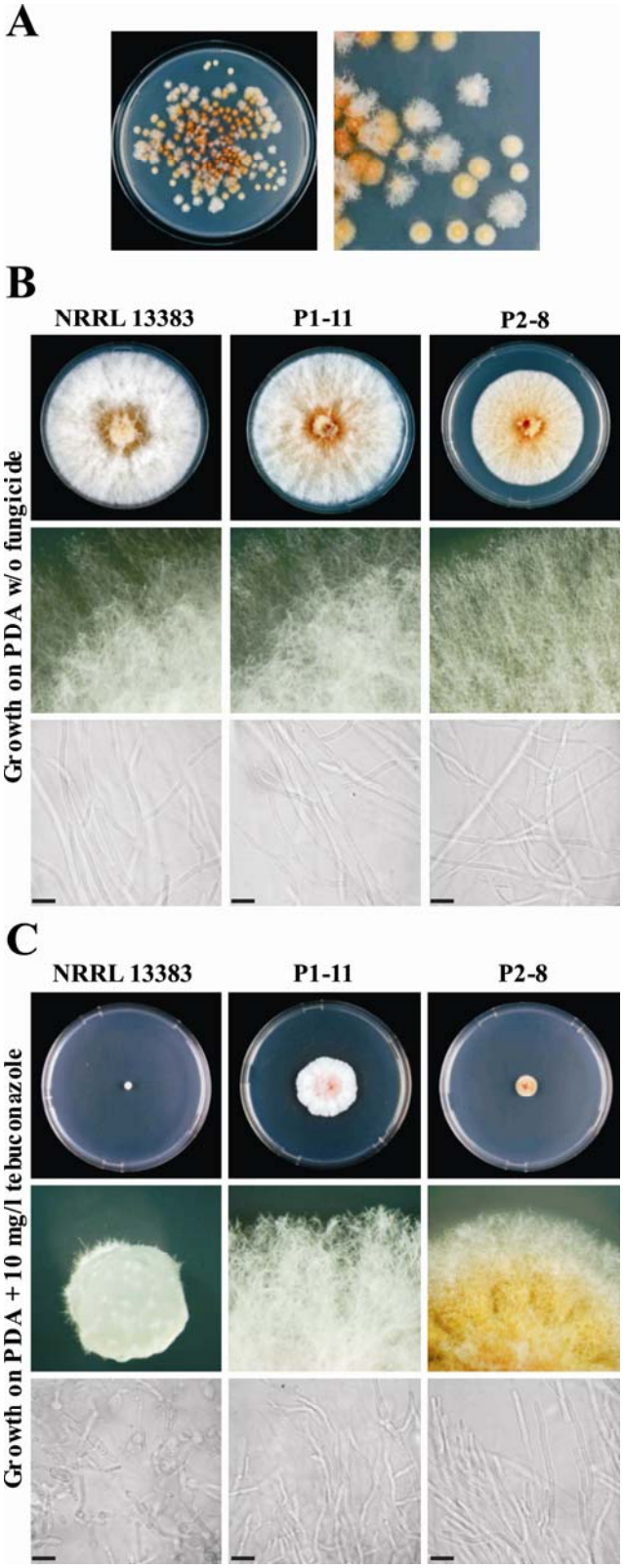

Fig. 1. Colonial and hyphal morphologies of *Fusarium graminearum* NRRL 13383 and descendants obtained after adaptation to tebuconazole at 10 mg/liter. **A,** Morphologically distinct fungal colonies arose after adaptation. A conidial suspension (200 conidia in 100 µl) that was derived from an adapted mycelium was streaked onto potato dextrose agar (PDA) plates (145 mm in diameter) amended with tebuconazole at 10 mg/liter and incubated at 23°C for 9 days. **B** and **C,** Mycelia of NRRL 13383, P1-11, and P2-8 grown on PDA plates **B,** without and **C,** with tebuconazole at 10 mg/liter. Plates were centrally inoculated with an agar plug and grown at 23°C for 7 days. Photographs were taken from mycelia (upper two rows) and from hyphal fronts (lower rows). Magnification was 3× (middle rows) and 600× (lower rows). Black bars represent 20 µm.

variants differed significantly from the noninoculated control (Fig. 4B). NRLL 13383 and the P1 and P2 isolates did not differ significantly ($P > 0.05$) in their ability to spread disease symptoms below and above the point of inoculation, although the P2 isolates appeared less virulent when the fungicide was omitted (Fig. 4A). Furthermore, fresh weight reductions caused by the P2 strains were often significantly less severe than those caused by NRRL 13383 and the P1 strains when the fungicide was omitted (Fig. 4B). The same trend was also evident in the presence of tebuconazole, even though not supported by statistics for most comparisons.

In summary, P1 and P2 isolates differed not only in morphology but also with respect to fitness and virulence. Whereas P1 strains exhibited only minor variations from strain NRRL 13383, the P2 strains showed growth deficits and reduced virulence.

Analysis of mycotoxin accumulation in planta. Whether the adaptation to fungicides influences mycotoxin production is of major interest for agronomy. To address this question, the inoculated spikelets assessed in the virulence assays were individually used to extract and analyze mycotoxins by HPLC-MS. Additionally, after mycotoxin determination, the same spikelets were also used to extract total DNA to quantify *F. graminearum* by qPCR. This allowed relating the amount of mycotoxins found in each spikelet analyzed to (i) its fresh weight (Fig. 4C and E) and (ii) the amount of *Fusarium* DNA, reflecting fungal biomass (Fig. 4D and F). Importantly, this experimental design allowed direct comparison of the effects of fungicide treatments of adapted and nonadapted strains on mycotoxin production in planta.

In all variants analyzed, considerable levels of the trichothecene NIV were detected. In all spikelets inoculated in the absence of the fungicide, the adapted P1 isolates showed higher NIV levels than their parent NRRL 13383, whereas infections with P2 isolates yielded lower NIV levels. Although, when expressed as absolute NIV contents, such differences could statistically not be proven ($P > 0.05$) (Fig. 4C), some data were more distinct when expressed as relative NIV productivity (i.e., mycotoxin production per unit of fungal DNA) (Fig. 4D). Isolate P1-9 produced significantly more NIV per fungal biomass than NRRL 13383. Moreover, the comparison of P1 and P2 isolates showed significant differences for NIV productivity in several cases. Fungicide application significantly ($P < 0.05$) reduced NIV contents in the inoculated spikelets for each individual strain when compared with the corresponding variants without the fungicide (Fig. 4C). However, for assessments of NIV productivity (Fig. 4D), tebuconazole application resulted in a significant reduction only for NRRL 13383 ($P = 0.045$) and P1-9 ($P = 0.019$). When comparing the fungicide-treated isolates with each other, P1 again exhibited higher NIV contents (Fig. 4C) and higher NIV productivity (Fig. 4D) than NRRL 13383 and the P2 isolates. Isolate P1-11 had significantly higher NIV productivity compared with the parental strain but also with P2-4 and P2-8.

In addition to the trichothecene NIV, the polyketide mycotoxin ZEA was detected in 56.5% of all samples analyzed. ZEA amounts exhibited a high variability between different spikelets that were infected by the same fungal strain, rendering statistical evaluation of differences among strains and treatments impossible (Fig. 4E and F). Interestingly, the average ZEA quantity of all fungicide-treated samples was higher (174.8 ± 440.6 ng) compared with the untreated samples (78.6 ± 306.1 ng) ($P = 0.103$).

Taken together, the mycotoxin analyses revealed further differences between the two adapted phenotypes. P1 strains tended to produce more and P2 strains produced less NIV than their parent NRRL 13383. ZEA levels tended to be generally higher in samples treated with the fungicide.

Correlation analyses. The association of the fresh weight of the inoculated spikelet with virulence, fungal biomass, and mycotoxin contents was assessed by scatter plots that used the averaged data from each individual isolate. Virulence, expressed as symp-

toms that had spread from a single inoculated spikelet, showed a highly significant negative correlation with the losses of fresh weight (Fig. 5A). This supported the suitability of fresh weight measurements for assessing virulence of *Fusarium* strains in the performed infection assay. Both with and without tebuconazole application, the P1 isolates always clustered with NRRL 13383, whereas the P2 isolates always formed a distinct cluster. Furthermore, the effectiveness of the fungicide to limit symptom spread was evident because the clusters of the treated and untreated isolates were clearly separated. To test whether the reduction of spikelet fresh weight depended more on mycotoxin contents than solely on fungal biomass, we assessed further correlations. We observed a highly significant negative correlation of spikelet fresh weight to NIV contents (Fig. 5C) but no correlation to fungal biomass (Fig. 5B). There was no indication that ZEA contents may have affected the fresh weight of the inoculated spikelets (correlation not significant) (Fig. 5D).

DISCUSSION

Due to the prevalence of azoles in the control of fungal pathogens, it is important to assess the risk of the emergence of and to investigate the mechanisms leading to azole resistance. In this work, we addressed the potential to generate in vitro acquired resistance in *F. graminearum* NRRL 13383 and studied the effects of adaptation in the obtained strains. Exposure to tebuconazole at 10 mg/liter for 33 days resulted in the emergence of two morphologically distinct phenotypes differing in the degree of resistance to tebuconazole. In addition, these phenotypes also differed in general fitness, cross-resistance against other fungicides, and virulence and mycotoxin levels in wheat.

Laboratory experiments are well suited to investigate mechanisms of the evolution of fungicide resistance, because it is possible to easily replicate them, to control the strength of selection, and to use defined reference strains (11). Fungicide-resistant strains can be generated in vitro by several approaches, such as short exposures to UV light or chemical mutagens to introduce mutations. Furthermore, selection of spontaneous genetic alterations can be achieved by continuous exposure of fungal mycelia to sublethal fungicide concentration. This approach, which is referred to as adaptation, was used in this study.

As determined by mycelial growth assays, the P1 and P2 isolates differ in the RF to tebuconazole that had increased through adaptation up to 10.9- and 2.1-fold, respectively, compared with the base level in *F. graminearum* NRRL 13383 (Table 1). Similar levels of resistance had been described for UV-induced mutants of *Nectria haematococca* var. *cucurbitae* to the DMIs fenarimol (RF 2.0 to 4.0), triadimenol (RF 5.1 to 8.0), and imazalil (RF 2.0 to 4.0) (28) and, in another report using the same experimental system, to tebuconazole (RF 4.0 to 15.7), flusilazole (RF 1.8 to 11.7), penconazole (3.3 to 5.7), imazalil (RF 2.0 to 6.5), and prochloraz (RF 2.0 to 9.4) (2). In *Pseudocercosporella herpotrichoides*, UV mutagenesis allowed for the recovery of strains with RF values to prochloraz of 1.5 to 19.8 (27). From large numbers of spores of *Penicillium italicum*, fenarimol-resistant strains were obtained with RF values of 15 to 50 (17).

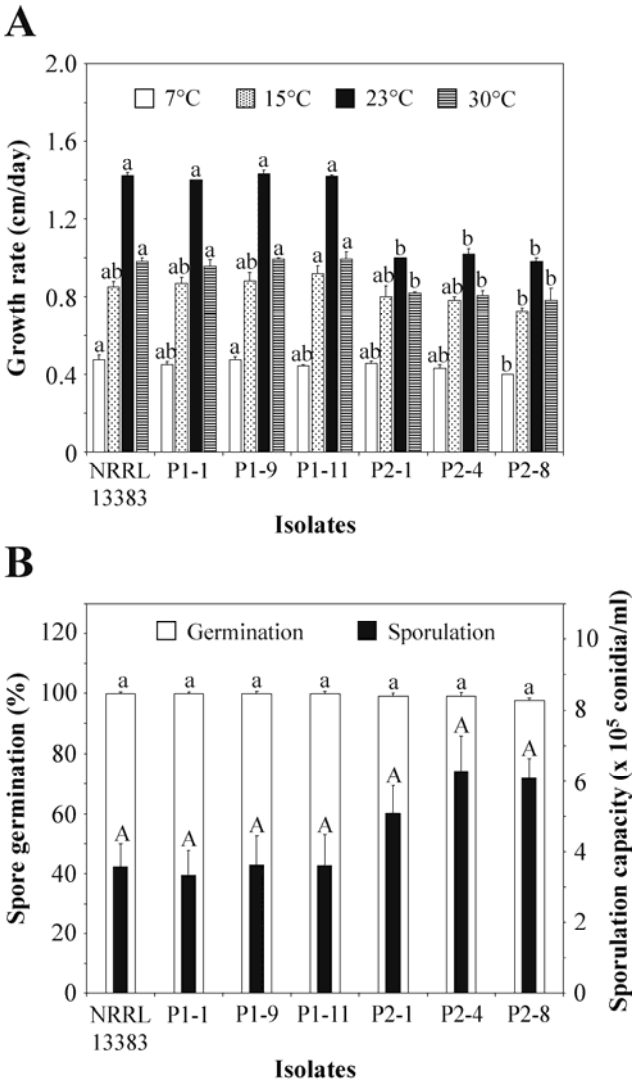

Fig. 3. Fitness parameters of *Fusarium graminearum* NRRL 13383 and adapted descendants. **A,** Growth rates on potato dextrose agar at indicated temperatures. **B,** In vitro spore formation capacity (black bars) and spore germination frequency (white bars). Columns represent the means of five and three replicates, respectively. The error bars indicate standard errors. For comparison of the isolates within one treatment, letters give groups of means that were determined by multiple comparisons using the Student-Newman-Keuls test. Different letters indicate significant differences at $P < 0.05$.

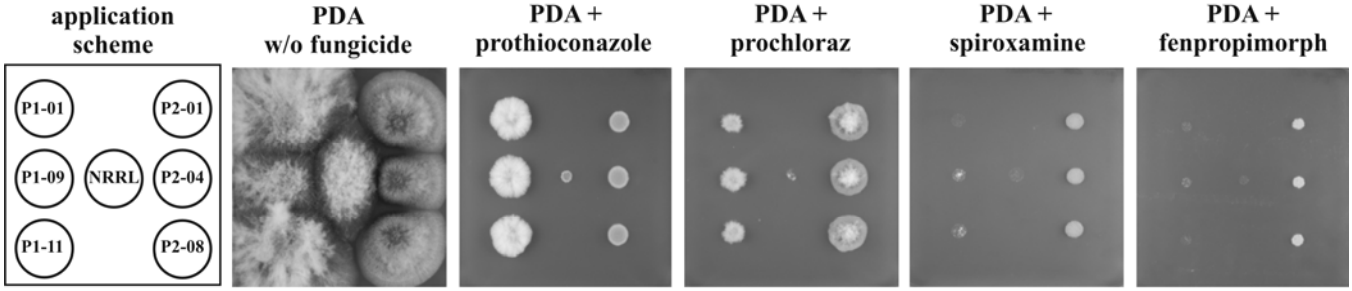

Fig. 2. Evaluation of cross-resistance of the adapted isolates. Conidial suspensions (2,000 conidia in 2 µl) were applied on potato dextrose agar (PDA) plates amended with concentrations of fungicides previously optimized for discrimination (i.e., prochloraz at 0.15 mg/liter, prothioconazole at 15 mg/liter, spiroxamine at 285 mg/liter, and fenpropimorph at 100 mg/liter). Plates were incubated at 23°C for 7 days.

Previous adaptation experiments on *Colletotrichum graminicola* to tebuconazole (47) and *Ustilago maydis* to triadimefon (49) started with low doses that were gradually increased, which yielded isolates with RF values of up to 7.2 and 42, respectively. For *U. maydis,* a correlation was observed between the number of the gradually intensified fungicide treatments and the degree of the acquired resistance (49). Thus, resistance levels against azole fungicides cover some range and vary with the

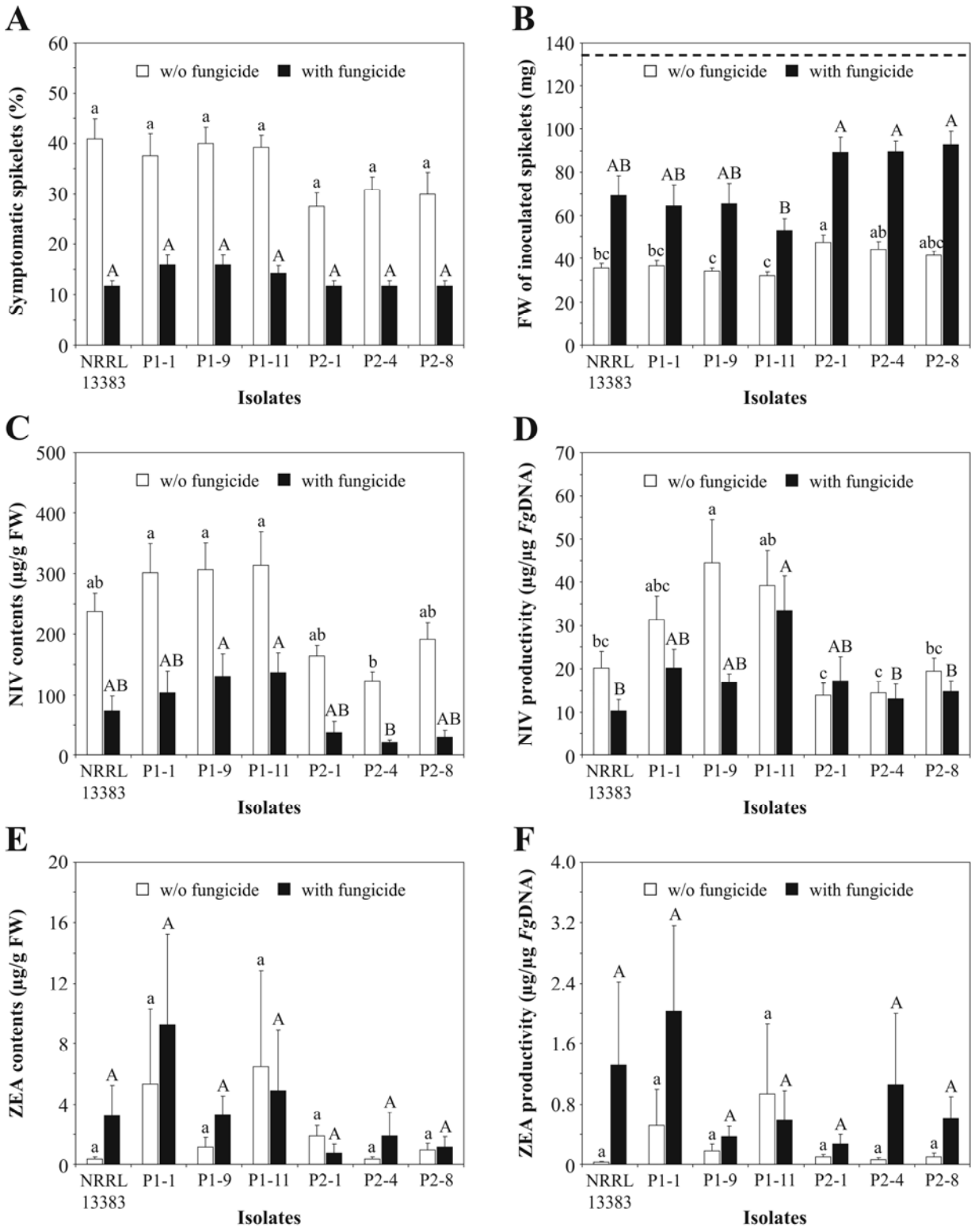

Fig. 4. Virulence and mycotoxin production of *Fusarium graminearum* NRRL 13383 and adapted descendants. Individual central spikelets were point inoculated with 10^4 conidia applied either without or with tebuconazole at a concentration of 10 mg/liter per infection droplet (10 µl). Data were assessed 14 days after inoculation. **A,** Spread of Fusarium head blight symptoms from the inoculated to the neighboring spikelets; **B,** fresh weights of inoculated spikelets; **C and E,** nivalenol (NIV) or zearalenone (ZEA) contents in inoculated spikelets (in relation to fresh weights); **D and F,** NIV and ZEA productivity (in relation to fungal DNA). Columns represent the means of 12 replicates and the error bars indicate standard errors. For comparison of the isolates within one treatment, letters represent groups of means that were determined by multiple comparisons using the Student-Newman-Keuls test. Different letters indicate significant differences at $P < 0.05$. The dashed line gives the average fresh weight of uninoculated controls.

species, the fungicide, and the approach employed to select for resistance.

Whereas the P1 isolates grow in vitro as well and are as virulent on spring wheat as NRRL 13383, the P2 isolates grow more slowly at higher temperatures and appeared less virulent. Although we have determined significant differences in fungicide sensitivity of the P1 and P2 strains and their parent in vitro, the differences observed for virulence on spring wheat treated with tebuconazole were statistically not supported. Nevertheless, P1 isolates appeared somewhat more virulent and the P2 isolates appeared less virulent than NRRL 13383. This discrepancy between the results of in vitro and in planta fungicide treatments could be due to several reasons. In planta, the inhibitory effect of tebuconazole may be more critical because the fungus has to cope not only with the fungicide but also with a reduced supply of nutrients and with host defense reactions. These additional stress factors may overrule the benefits of fungicide resistance and, thus, may mask the differences in fungicide sensitivity between NRRL 13383 and the adapted strains. For comparison, mutants of *P. italicum* (19) and *Pseudocercosporella herpotrichoides* (27) that showed high resistance levels in vitro were similarly resistant against the fungicide when this was applied onto the infected hosts (i.e., orange or wheat). However, in both cases, the fungicide was applied curatively 24 h or 4 days after inoculation. Therefore, the rather minor resistance effects we have observed in planta could also be a consequence of the inoculation procedure. In our experiments, tebuconazole was applied together with the conidia directly into the spikelets and, therefore, was already effective during conidial germination. Because the ED_{90} values were consistently higher in the mycelial growth assays than in the spore germination assays, it is possible that germinating spores were subjected to a high level of stress in the spikelet. In the field, a scenario of the concurrent arrival of conidia and the fungicide at the spikelet may appear rarely and, therefore, practical resistance levels of our adapted isolates may be underestimated. Nevertheless, the inoculum and the fungicide were applied concomitantly to ensure reproducibility in the subsequent mycotoxin and qPCR analyses.

Another difference between the tebuconazole-adapted P1 and P2 isolates was discovered for cross-resistance against additional fungicides. The P1 isolates exhibited positive cross-resistance only to the azole fungicides prothioconazole and prochloraz but not to the amine fungicides fenpropimorph and spiroxamine. Amines, like the azoles, target sterol-biosynthesis but inhibit different enzymes (i.e., sterol Δ14-reductase or sterol Δ8→Δ7-isomerase) (13). In contrast, P2 isolates acquired resistance to all azoles and both amines tested. In azole-resistant strains analyzed in other species, cross-resistance to amine fungicides was only sometimes observed. Some azole-resistant mutants of *U. maydis* were also resistant against fenpropimorph (50), whereas others (5), as well as mutants of *Penicillium italicum* (17,18) and *N. haematococca* (2,28), displayed no cross-resistance or were even more sensitive.

We propose that the P1 isolates may have developed mechanisms mediating resistance to azoles, whereas the P2 isolates may employ rather nonspecific mechanisms that allow tolerating fungicides by different modes of action. MDR found in P2 isolates of *F. graminearum* is well documented in other plant-pathogenic fungi. Typically, MDR is based on enhanced activity of efflux transporters (15,42). Based on several studies, it was assumed that impairment of fitness and virulence of resistant strains could be attributed to increased energy demand for active fungicide efflux (8). However, studies on energy-dependent efflux-mediated fungicide resistance in *N. haematococca* rendered varying results. Whereas the mutants that were resistant to fenarimol showed reduced growth rates and virulence (28), those resistant to tebuconazole were not impaired (2). Furthermore, fitness defects could not be detected in fungicide-resistant *P. italicum* strains with enhanced fungicide efflux activity (17,18). In this context, it should be considered that the P2 isolates showed growth retardation only at higher temperatures, which might indicate that the sterol composition could be modified. Interest-

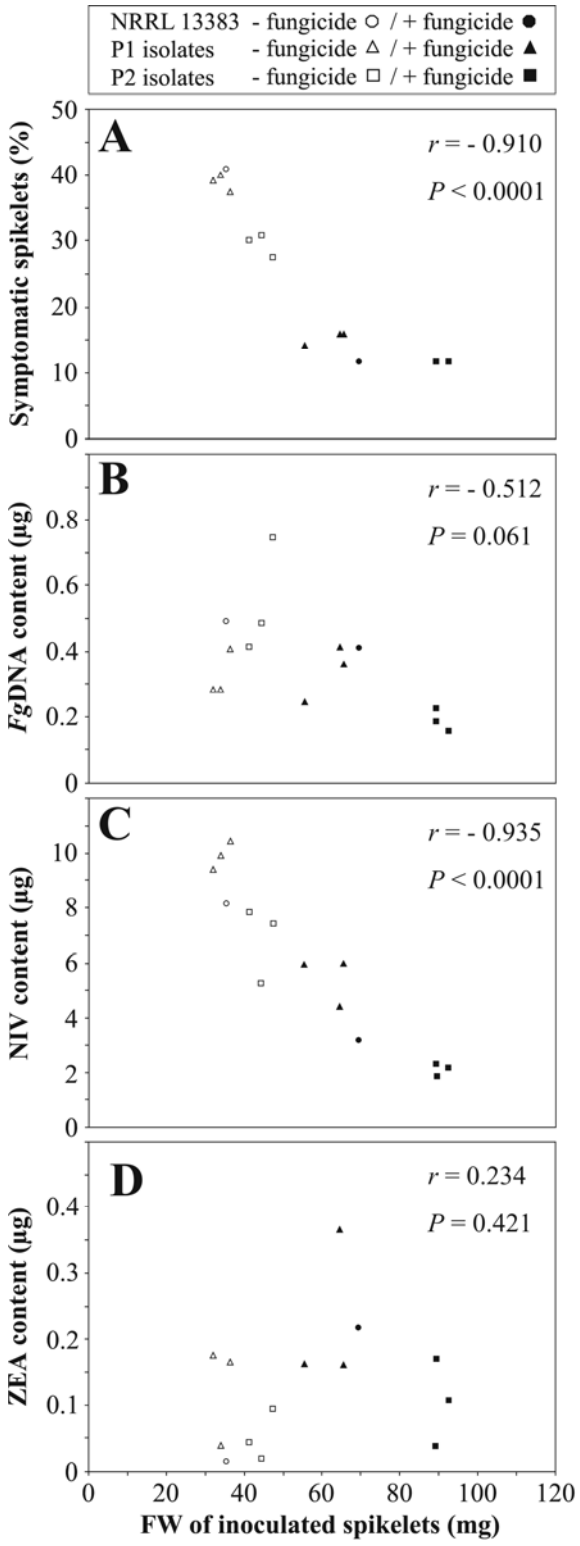

Fig. 5. Scatter plots showing the correlation between the mean fresh weights (FW) of the inoculated spikelets and the means of the **A,** percentage of symptomatic spikelets; **B,** amount of fungal DNA in the spikelets; **C,** nivalenol (NIV) content of the spikelets; and **D,** zearalenone (ZEA) content of the spikelets. Data were assessed 14 days after inoculation. Calculated Pearson's correlation coefficient (*r*) and the respective probability (*P*) for correlation are given.

ingly, an azole-resistant field isolate of *M. graminicola* showed altered membrane sterol composition, which may result in reduced fungicide accumulation and growth rates (25). Furthermore, it has been shown that membrane sterol composition affects localization and function of efflux transporters (37).

Because of their azole-specific resistance, we presumed, for the P1 isolates, amino acid exchanges in CYP51. Various studies on different fungal species have shown that several point mutations in the *CYP51* gene may mediate azole resistance (31,51). The genome of *F. graminearum* harbors three genes putatively encoding sterol 14α-demethylase (FGSG_01000, FGSG_04092, and FGSG_11024). Sequencing of PCR fragments that were amplified from all three genes in the strains P1-11 and P2-8 (and *F. graminearum* NRRL 13383, which was included for comparison) did not reveal any mutation in any of the three genes in the two adapted isolates (data not shown). Therefore, the cause for the resistance in these isolates remains unclear. Likewise, sequence analysis of two of the three *CYP51* genes in Chinese field isolates that were found resistant to tebuconazole and prochloraz did not indicate any aberration associated with DMI resistance (52). However, one advantage of the in vitro approach taken to generate strains with acquired fungicide resistance is that these strains are isogenic descendants of a characterized reference strain which allows for a direct comparison. This will allow gaining deeper insights into the genetics of resistance development for example by microarray analysis.

In addition to the differences in the level of resistance to tebuconazole, fitness, cross-resistance, and virulence, the P1 and P2 isolates also differ in their potential to produce mycotoxins in infected wheat. The point inoculation used allowed us to determine the mycotoxin contents in reproducibly infected, individual spikelets (23). Furthermore, quantification of *F. graminearum* DNA from the inoculated spikelet by qPCR (6) allowed us to assess the potential of mycotoxin production based on fungal biomass at increased spatial resolution. Spikelets inoculated with the adapted P1 isolates exhibited higher NIV contents than their parent NRRL 13383 in either the absence or the presence of tebuconazole, which was accompanied by higher NIV productivity per fungal biomass. Moreover, the negative correlation of spikelet fresh weight to NIV content underlines the contribution of NIV to virulence of *F. graminearum* in wheat (34). Although the P2 isolates have acquired resistance to tebuconazole, they produce lower NIV contents compared with NRRL 13383, even in the presence of the fungicide. In contrast, the NIV productivity per fungal biomass of P2 strains was not diminished by the fungicide. Thus, it appears that tebuconazole may reduce development of the P2 isolates in planta but not their NIV production. ZEA levels appeared to be higher in fungicide-treated spikelets infected with either the nonadapted strain NRRL 13383 or most of the adapted isolates. ZEA did not affect the fresh weight of the inoculated spikelets, which supported previous reports from barley that a loss of ZEA production did not impede fungal infection and symptom development (32).

Furthermore, our study provides additional insights into processes that may occur in fungicide-treated fields, where selection for general fitness is effective in parallel to selection for higher fungicide resistance. Although P1 isolates were less frequently recovered in vitro than P2 isolates, fitness advantages could promote the emergence of P1 in field populations. In contrast, although P2 generally appears to be less competitive, it could emerge and survive in field populations when azole and amine fungicides are repeatedly applied as a mixture. Such conditions may prevent the emergence of P1. The contrasting features observed for the P1 and P2 isolates in this study suggest that not only the amount of azoles sprayed onto cereals but also the combination with other fungicides could influence the composition of *F. graminearum* field populations. Therefore, detailed analyses of isolates recovered from field experiments, which were set up to test for this hypothesis, will be interesting. Highlighting another potential risk, it has been shown that sexual recombination occurred with respect to carbendazim resistance in field populations of *F. graminearum* (9). Thus, even if the P1 and P2 traits may arise independently in different individuals in the field, they could be combined by sexual recombination, rendering progeny that do not suffer fitness penalties and that have high resistance to azoles and cross-resistance to amines. Additionally, it might be possible that compensatory mutations could restore fitness in P2 strains without losing resistance, as has been previously described for fungicide-resistant *Aspergillus nidulans* strains (45).

In conclusion, our work suggests that *F. graminearum* is able to adapt to azole fungicides. Our study shows that certain adapted isolates produced increased NIV levels in grains under defined conditions. Therefore, it is essential to analyze whether a correlation exists between acquired azole resistance and increased mycotoxin levels not only in laboratory strains but also in field populations. Thus, monitoring of *F. graminearum* field populations with respect to fungicide resistance and mycotoxin production is important.

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