

Article

Crop Diseases and Mycotoxin Accumulation in Temperate Agroforestry Systems

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Abstract: Background: Temperate agroforestry is regarded as a sustainable alternative to monoculture agriculture due to enhanced provisioning of ecosystem services. Plant health and food safety are crucial requirements for sustainable agriculture; however, studies of fungal diseases and mycotoxin contamination of crops grown under temperate agroforestry are lacking. This study therefore aimed to compare fungal colonization and mycotoxin contamination of crops grown in temperate agroforestry against conventional monoculture. **Methods:** The biomass of plant pathogenic fungi in oilseed rape plants and barley and wheat grain harvested in 2016 to 2018 at four paired agroforestry and monoculture sites was quantified using species-specific real-time PCR. Mycotoxin content of barley and wheat grain was determined by HPLC-MS/MS. **Results:** The colonization of oilseed rape plants with the vascular pathogen *Verticillium longisporum* and wheat grain with the head blight pathogen *Fusarium tricinatum* was lower in agroforestry than in conventional monoculture. Mycotoxin content of barley and wheat grain did not differ between agroforestry and monoculture systems and did not exceed the legal limits of the EU. Remarkably, fumonisin B₁ was detected in wheat grains at two sites in two years, yet the low levels found do not raise food safety concerns. No differences were found between the two production systems with regard to infection of wheat and barley grain with five *Fusarium* species (*F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, and *F. proliferatum*) and oilseed rape with fungal pathogens *Leptosphaeria biglobosa*, *Leptosphaeria maculans*, and *Sclerotinia sclerotiorum*. **Conclusions:** Temperate agroforestry does not negatively affect the infection of wheat, barley and oilseed rape with major fungal pathogens though it may suppress the infection of oilseed rape with *V. longisporum* and wheat grain with *F. tricinatum*. Furthermore, temperate agroforestry does not increase mycotoxin contamination of barley and wheat. Therefore, temperate agroforestry does not negatively affect food safety.

Keywords: temperate agroforestry systems; silvoarable agroforestry; alley cropping; barley; wheat; oilseed rape; *Fusarium* head blight; mycotoxins; *Verticillium longisporum*

1. Introduction

Agroforestry systems are multifunctional plant production systems that gained attention as a sustainable alternative to traditional monoculture agriculture. For example, the integration of trees in temperate agricultural systems in alley cropping patterns has been shown to increase C sequestration, soil nutrient availability, and biodiversity [1–3]. Other beneficial ecosystem services that are enhanced by agroforestry include pollination [1] and reduced nitrate leaching [4,5]. While yield is of key importance for the farmers, few studies addressed the long-term impact of temperate agroforestry on crop yield. Recently, a comprehensive long-term study evaluated the crop yield of winter wheat (*Triticum aestivum* L.) and oilseed rape (*Brassica napus* L.) in a temperate agroforestry

system in Germany [6]. The authors found that crop yield was reduced in the vicinity of trees but temperate agroforestry did not reduce the average long-term yield. Similar results were obtained for winter wheat, barley (*Hordeum vulgare* L.), and maize (*Zea mays* L.) in young alley cropping systems in Belgium [7]. Crop yield is significantly reduced by microbial pathogens with fungal diseases being economically most relevant [8]. The loss potential of wheat due to pathogens has been estimated to be 16% [9].

Diversification of agroecosystems has been shown to suppress plant diseases [10–13]. For example, growing a mixture of varieties reduced the severity of diseases in barley [14], wheat [15], and rice [16] compared to pure stands. Crop rotation, which is a common practice in the temperate-zone, substantially contributes to plant disease control [17,18]. Intercropping systems have been shown to be less susceptible to diseases, too [19,20]. Dilution of host plant density in diversified agroecosystems is considered one of the main mechanisms contributing to the improved control of soil-borne and splash-dispersed pathogens [10,19]. Apart from host dilution, crop diseases can be suppressed by soil microflora that harbor antagonists [21–23] as well as by a balanced plant nutrition [11,24]. Increased biodiversity in agroforestry systems has been hypothesized to reduce the occurrence and severity of plant diseases [3], but this hypothesis has not been tested in temperate-zone plant production systems.

Modern alley cropping systems alter several microsite conditions that may affect the development and severity of crop diseases. For example, the integration of trees alters the microclimate in agroforestry systems [25,26], which in turn can affect crop diseases [19]. Furthermore, trees in agroforestry effectively reduce wind speed [27], diminishing the dissemination of splash-dispersed [28] as well as airborne pathogens and the dispersion of windblown soil carrying inoculum of fungal or bacterial pathogens [29]. If competition for essential resources (e.g., water, soil nutrients, and light) in the vicinity of the trees occurs, reduced crop development could suppress plant resistance to certain pathogens [30]. There is a lack of studies comparing fungal infection and mycotoxin contamination of crops under temperate agroforestry with monoculture systems [19].

This study aimed to investigate the impact of temperate agroforestry on the infection of oilseed rape plants and barley and wheat grain with plant pathogenic fungi and the accumulation of mycotoxins. We hypothesized that (i) agroforestry systems as a whole show either equal or lower fungal infection and mycotoxin concentration compared to conventional monoculture, and (ii) the abundance of pathogenic fungi and the concentration of mycotoxins in crops increases with the distance from the tree row in agroforestry systems.

2. Materials and Methods

2.1. Study Sites

Four sites (Jena, Cottbus, Göttingen, and Braunschweig) at which monoculture cropland was replaced by agroforestry were chosen (Figure A1). The site characteristics are reported in Table 1. The agroforestry systems were established between 2007 and 2011. The aboveground biomasses of the trees in the agroforestry systems were harvested one (Jena, Cottbus, Göttingen) or two harvests (Braunschweig) prior to our first sampling in 2016. Additionally, the trees in the agroforestry system at Cottbus were harvested in winter 2017/18. Adjacent to each agroforestry system, a monoculture cropland served as a reference system. Each paired agroforestry and monoculture system had identical crops, cultivation and harvest methods, fertilization rates, and pesticide treatments. In the agroforestry systems, poplar trees (clone Max1; *Populus nigra* × *P. maximowiczii*) were planted in 12-m wide rows in an alley cropping pattern (Jena, Cottbus, and Braunschweig) or as an adjacent short-rotation coppice (Göttingen) in a north-south direction with 48-m (Jena, Cottbus, and Braunschweig) or 24-m wide (Göttingen) crop rows (Table 2).

Table 1. Study site locations and characteristics.

	Study Site			
	Jena	Cottbus	Göttingen	Braunschweig
Mean annual air temperature 1981–2010	9.9 ± 0.1 °C ^a	9.6 ± 0.2 °C ^b	9.2 ± 0.1 °C ^c	9.6 ± 0.2 °C ^d
Mean annual air temperature 2016	10.8 °C	10.3 °C	9.8 °C	10.3 °C
Mean annual air temperature 2017	10.9 °C	10.3 °C	9.9 °C	10.3 °C
Mean annual air temperature 2018	11.5 °C	11.2 °C	10.6 °C	11.2 °C
Mean annual precipitation 1981–2010	608 ± 21 mm ^a	568 ± 21 mm ^b	651 ± 24 mm ^c	637 ± 23 mm ^d
Annual precipitation 2016	528 mm	593 mm	544 mm	504 mm
Annual precipitation 2017	648 mm	621 mm	777 mm	819 mm
Annual precipitation 2018	415 mm	429 mm	430 mm	380 mm
Meters above sea level	289 m	67 m	329 m	82 m
Year of agroforestry system establishment	2007	2010	2011	2008
Harvest dates of the aboveground tree biomass of the agroforestry system	winter 2014/15	winter 2014/15, winter 2017/18	winter 2014/15	winter 2013/14
Soil type	Calcaric Phaeozem	Gleyic Cambisol	Eutric Cambisol	Vertic Cambisol

^a mean ± SE during 1981–2010; climate station at Jena (station ID: 2444) of the German Meteorological Service, ^b mean ± SE during 1981–2010; climate station at Cottbus (station ID: 880) of the German Meteorological Service, ^c mean ± SE during 1981–2010; climate station at Göttingen (station ID: 1691) of the German Meteorological Service, ^d mean ± SE during 1981–2010; climate station at Braunschweig (station ID: 662) of the German Meteorological Service.

Table 2. Crop rotation of the agroforestry and monoculture systems from 2015 to 2018.

Study Site	Crop Rotation			
	Crop 2015 ^a	Crop 2016	Crop 2017	Crop 2018
Jena	summer barley	summer barley	winter oilseed rape	winter wheat
Cottbus	maize	winter wheat	winter barley	maize ^b
Göttingen	summer wheat	winter barley	winter oilseed rape	winter wheat ^a
Braunschweig	winter barley	winter oilseed rape ^c	winter wheat	winter wheat

^a no sampling for crop diseases conducted, ^b not analyzed, ^c no determination of the oilseed rape plant yield.

2.2. Experimental Design and Crop Harvest

Four transects were established in each agroforestry system and four sampling areas were set in each monoculture system as replicate plots (Figure A1). In each replicate plot of the agroforestry systems, harvest samples were taken within the crop row at distances of 1 m, 4 m, 7 m, and 24 m (Jena, Cottbus, and Braunschweig) or 1 m, 4 m, and 7 m (Göttingen) from the tree row (Figure A1). In the monoculture systems, harvest samples were taken in each replicate plot. The layout of the replicate plots within the study sites is schematically illustrated in Figure A1. Harvest samples were taken in 2016 to 2018 and include barley and wheat grain, as well as oilseed rape plants.

Barley and wheat grain as well as oilseed rape plant samples were harvested at a width of 1.5 to 2.0 m (orthogonal to the tree row of the agroforestry system) and a length of 10 m (parallel to the tree row of the agroforestry system) at each sampling location of both management systems using a plot combine. Harvest samples were subsequently dried to determine their water content and to calculate the crop yield per area. Harvest and crop yield data were provided by our cooperation partners (see Acknowledgements).

2.3. Quantification and Identification of Phytopathogenic Fungi

Fungal phytopathogens in harvest samples were quantified using species-specific real-time PCR (qPCR). DNA from wheat grains and oilseed rape plants was extracted using a cetyltrimethylammonium bromide (CTAB) protocol according to Brandfass and Karlovsky [31]. Briefly, 30 mg of finely ground material were suspended in 1 ml CTAB-buffer with proteinase K. Following incubation at 42 °C and 65 °C for 10 min each, a chloroform/isoamylalcohol (24:1 (v/v)) extraction was performed and DNA was precipitated with polyethylene glycol (PEG). Precipitated DNA was pelleted by centrifugation and the pellets were washed with 80% (v/v) ethanol twice prior to drying by vacuum-centrifugation. The dried pellets were resuspended in 50 µl TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) and incubated at 42 °C for 2 h to facilitate the dissolution of DNA. DNA from barley grains was extracted using a modified sodium dodecyl sulfate (SDS) extraction protocol from Cristina et al. [32]. 30 mg of finely ground barley grains were suspended in 500 µl lysis buffer (2.5% sorbitol (w/v), 100 mM Tris, 50 mM EDTA, 500 mM NaCl, 2% sodium N-laurylsarcosinate (w/v), 1% sodium dodecylsulfate (SDS) (w/v)). 100 µl potassium acetate (3 M) were added, the samples were thoroughly mixed and centrifuged at 7380× g for 10 min. An aliquot of 400 µl was transferred to a new 1.5 ml Eppendorf tube, 400 µl chloroform/isoamylalcohol (24:1 (v/v)) were added, the mixture was shaken, incubated on ice for 10 min, and centrifuged at 7380× g for 10 min. Following centrifugation, 400 µl of the upper phase was transferred to a new Eppendorf tube containing 100 µl of PEG 6000 (30% (w/v)) and 50 µl of NaCl (5 M). The mixture was incubated at room temperature for 20 min to allow DNA precipitation and centrifuged at 16,000× g for 15 min to pellet the precipitated DNA. DNA pellet was washed with 80% (v/v) ethanol twice prior to drying in vacuum. Dried pellets were resuspended in 50 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and incubated at 42 °C for 2 h to facilitate the dissolution of DNA. DNA extracts from barley and wheat grain were purified using spermine precipitation as described by Reineke et al. [33]. The quantity and quality of DNA in all extracts was assessed by electrophoresis in 0.8% (w/v) agarose gels stained with ethidium bromide.

Barley and wheat grain samples were tested for the presence of DNA of six *Fusarium* species: *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. proliferatum*, and *F. tricinctum*. DNA of oilseed rape plants was examined for the presence of fungal pathogens *Leptosphaeria biglobosa*, *Leptosphaeria maculans*, *Sclerotinia sclerotiorum*, and *Verticillium longisporum*. DNA standards for qPCR assays were obtained from *F. avenaceum* DSM 62161, *F. culmorum* DSM 62191, *F. graminearum* IFA66, *F. poae* F448, *F. proliferatum* DSM 62261, *F. tricinctum* DSM 23357, *L. biglobosa* IPP1560 (provided by A. von Tiedemann, University of Goettingen, Germany), *L. maculans* T12aD34 (provided by A. von Tiedemann, University of Goettingen, Germany), *S. sclerotiorum* IPP 1309 (provided by A. von Tiedemann, University of Goettingen, Germany), and *V. longisporum* VL43 (provided by A. von Tiedemann, University of

Goettingen, Germany). DNA of quantification standards were extracted using a CTAB protocol [31] and quantified by densitometry [34].

PCR analysis was carried out in the CFX 384 Thermocycler (Biorad, Rüdigenheim, Germany) in 384-well microplates. qPCR assays were performed with 1 µl diluted DNA extracts (barley grain: 1:20, wheat grain: 1:50, and oilseed rape plants: 1:100 in double-distilled H₂O (ddH₂O)) in 4 µl reaction volume. The reaction contained mastermix ThermoPol (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton®X-100, pH 8.8 at 25 °C) or standard *Taq* reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3 at 25 °C) (New England Biolabs, Beverly, Massachusetts, USA) (Table A1), containing MgCl₂, deoxyribonucleosides (Bioline, Luckenwalde, Germany) and primers in concentrations given in Tables A1 and A2, 0.1X SYBR Green I (Invitrogen, Karlsruhe, Germany), 1 mg/mL bovine serum albumin, and 0.025 u *Taq* DNA Polymerase or Hot Start *Taq* DNA Polymerase (New England Biolabs, Beverly, Massachusetts, USA) (Table A1). 1 µl template DNA Assays without template DNA served as negative controls. All DNA standards as well as negative controls were amplified in duplicates. Standard curves were generated from 1:3 serial dilution series of 100 pg/µl fungal DNA. The PCR conditions and detection limits are given in Table A3. Following amplification, melting curves were recorded from 55 °C to 95 °C with 0.5 °C increase per step and continuous fluorescence measurement.

2.4. Determination of Mycotoxins

Concentrations of mycotoxins 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), deoxynivalenol (DON), enniatin A, enniatin A₁, enniatin B, enniatin B₁, fumonisins B₁, fusaric acid, fusarin X, HT-2 toxin, nivalenol (NIV), T-2 toxin, and zearalenone (ZEN) as well as deoxynivalenol-3-glucoside (DON-3-glu), were determined in barley and wheat grain samples. Mycotoxins were extracted from 1 g finely ground dry material into 10 ml acetonitrile/water/acetic acid (84:15:1 (v/v/v)) by shaking at 170 rpm overnight.

The next day, samples were centrifuged for 10 min at 4750× g, 1 ml of the supernatant was transferred into a new 2 ml Eppendorf tube and evaporated using vacuum centrifugation. The dry extracts were stored at −20 °C until analysis. Water purified by an Arium pro ultrapure water system (Sartorius, Goettingen, Germany) and LC-MS grade methanol (Th. Geyer, Hoexter, Germany) were used in the following steps. Dried extracts were dissolved in 1 ml water/methanol (4:1 (v/v)), centrifuged for 10 min at 7380× g to remove undissolved residues and the supernatants were transferred to HPLC-vials.

Blank matrix samples of barley and wheat were spiked at the beginning of the extraction with a mixture of all monitored toxins to a final concentration of 300 µg/l to determine the recovery. Non-spiked blank matrix extracts were also analyzed, to ensure that the matrix was toxin-free.

An Agilent 1290 Infinity II HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6460 QQQ (Agilent Technologies, Waldbronn, Germany) was employed for mycotoxin quantification. Separation was carried out using an Agilent Zorbax Eclipse C18 column with 1.8 µm particle size and 100 × 2.1 mm (Agilent Technologies, Waldbronn, Germany). A 10-point calibration ranging from 1.9 to 800 µg/l was used and after every 10th sample, a quality control standard to monitor the stability of the system and a blank were analyzed. The MS/MS transitions, the LC-gradient, limits of detection (LODs), limits of quantification (LOQs), and the recoveries are listed in the Supplementary File S1.

2.5. Statistical Analysis

Concentrations of fungal DNA below the LOQ but with positive melting curves were set to half of the LOQ [35]. Concentrations of mycotoxins below the LOQ and above the LOD were set to half the LOQ [35]. Mycotoxin concentrations below the LOD were set to zero. Only sites at which at least 10% of all sampling locations across both management systems reported values above the LOQ were

statistically analyzed. Homoscedasticity (Levene's test) and normality of distribution (Shapiro-Wilk test) were assessed for each parameter.

A comparison of fungal infection and mycotoxin level between agroforestry and monoculture systems was achieved by weighting the amounts of fungal DNA and mycotoxins determined at sampling locations within the agroforestry systems by the area of the sampling location and the crop yield for this area. By this procedure, weighted average fungal DNA concentrations and mycotoxin levels were obtained for each replicate plot of each agroforestry system. To assess the statistical significance of differences between agroforestry and monoculture systems, we applied linear mixed effect models. Our model assigned the management type (agroforestry versus monoculture system) as fixed effects and sites nested within a year as random effects. Log- or square-root-transformation of data were performed when normality of distribution or homoscedasticity were not met. In addition, *t*-tests were performed which yielded similar results. Here, we reported the results obtained from the linear mixed effect models. In cases when the management types within only one site and sampling date were compared, independent *t*-test or Mann-Whitney *U* test was conducted. Statistical significance was considered at $p \leq 0.05$.

Unweighted fungal DNA concentrations or mycotoxin concentrations among sampling locations within each agroforestry system were compared using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test. When homoscedasticity or normality of distribution was not met, Kruskal-Wallis test with multiple comparison extension was used instead. Statistical significance was considered at $p \leq 0.05$. All statistical analyses were performed in R version 3.4.3 [36]. The datasets of the pairwise comparison of agroforestry versus monoculture systems that were generated during this study are available in the BonaRes Data Centre repository, [<https://doi.org/10.20387/BonaRes-XPWF-EY08>].

3. Results

3.1. Occurrence and Abundance of Phytopathogenic Fungi

3.1.1. Oilseed Rape Plants

All investigated pathogens of oilseed rape (*V. longisporum*, *L. biglobosa*, *L. maculans*, and *S. sclerotiorum*) were detected at all three sites that grew oilseed rape, except for *S. sclerotiorum*. The latter pathogen was only detected in Braunschweig in 2016, which was the only site for which no data on oilseed rape plant yield are available. The abundance of *V. longisporum* was lower in the agroforestry compared to the monoculture systems ($p = 0.008$) (Figure 1A). No differences between the two management systems were found for *L. maculans* and *L. biglobosa* (Figure A2A,B). At different distances from the tree rows within the crop rows of the agroforestry systems, DNA concentrations of *L. biglobosa* increased from 1 m distance to 24 m distance to the tree row at Braunschweig in 2016 ($p = 0.029$) (Figure A3). No differences in the abundance of the other oilseed rape pathogens among samples collected at different distances from the tree row within the crop row were found at any other site and year.

3.1.2. Barley and Wheat Grain

F. tricinctum was the most abundant *Fusarium* species detected in both barley and wheat grain. The second most abundant species in both grains was *F. graminearum*. In barley, *F. avenaceum* was detected in nine out of 56 samples, and in wheat grain, in six out of 80 samples. No *F. culmorum*, *F. poae*, and *F. proliferatum* DNA were found in our samples. Higher abundance of *F. tricinctum* in barley grain was found in the agroforestry systems compared to monoculture systems ($p = 0.001$) (Figure 1C). In contrast, the abundance of *F. tricinctum* in wheat grain was lower in the agroforestry than monoculture systems ($p = 0.021$) (Figure 1B). The amount of *F. tricinctum* DNA in wheat grain was about 20 times higher than in barley (cf. Figure 1B,C). The abundance of *F. avenaceum* and *F. graminearum* was

not affected by agroforestry practice (Figure A2C–E). No differences in the DNA concentration of any *Fusarium* species in barley or wheat at any site and year were observed at different distances from the tree rows within the crop rows of the agroforestry systems.

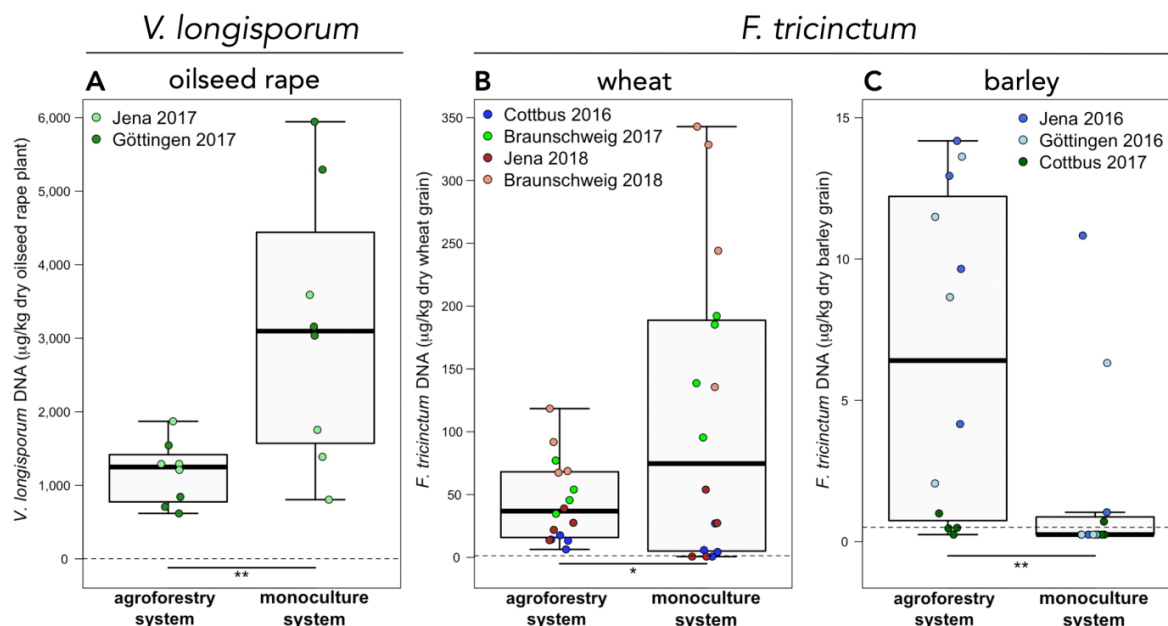


Figure 1. Abundance of (A) *Verticillium longisporum* in oilseed rape plants and *Fusarium tricinctum* in (B) wheat and (C) barley grain of temperate agroforestry and monoculture systems. $n = 4$ replicate plots per management system and study site and year. Fungal DNA concentration of each replicate plot in the agroforestry systems are obtained from three (Göttingen) or four (Jena, Cottbus, and Braunschweig) sampling locations within the crop rows of the agroforestry systems and are weighted averages by area and yield. Dashed lines represent the limit of quantification (LOQ) of the respective real-time PCR assay. Samples below LOQ and above the limit of detection were assigned to half the LOQ. Asterisks indicate significant differences between agroforestry and monoculture systems (linear mixed effect model at * $p \leq 0.05$ and ** $p \leq 0.01$).

3.2. Mycotoxin Concentrations in Barley and Wheat Grain in Agroforestry versus Monoculture Systems

Mycotoxin levels were below the EU defined legal limits in all of our samples. DON was frequently observed in barley and wheat grain but agroforestry practice did not affect DON levels (Figure 2A,B). NIV was exclusively and consistently observed in wheat grain samples from Cottbus in 2016. At this site, no effect of agroforestry on NIV accumulation was observed (Figure 2C). Fusaric acid was detected at all sites, years, and crops, except for wheat grain samples from Braunschweig in 2017. Concentrations of fusaric acid in barley and wheat grain did not differ between the two management systems (Figure 2D,E). Fumonisin B₁ was only detected in wheat at two sites (Cottbus in 2016 and Braunschweig in 2017). At each site, fumonisin B₁ was found in two samples; fumonisin levels in Braunschweig in 2016 have not exceeded the LOQ. Compared to monocultural management, agroforestry did not influence the accumulation of fumonisin B₁ in wheat grain (Figure 2F). Enniatin B was found more frequently and in higher concentrations than enniatin B₁ in both barley and wheat grain, whereby the amounts of enniatin B₁ in wheat grain were always below LOQ. Compared to the monocultural management system, no effect of agroforestry on enniatin B and enniatin B₁ accumulation in barley and wheat grain was found (Figure 2G–I). Enniatin A and enniatin A₁ were exclusively found at one site in barley grain (Göttingen in 2016), whereby enniatin A was only detected in a single sample at a level below LOQ. For enniatin A₁, no difference between agroforestry and monoculture system was detected (Figure 2J). 3-ADON, 15-ADON, DON-3-glu, DAS, fusarin X, T-2, and ZEN were not detected in our samples. HT-2 was found only in two samples below LOQ.

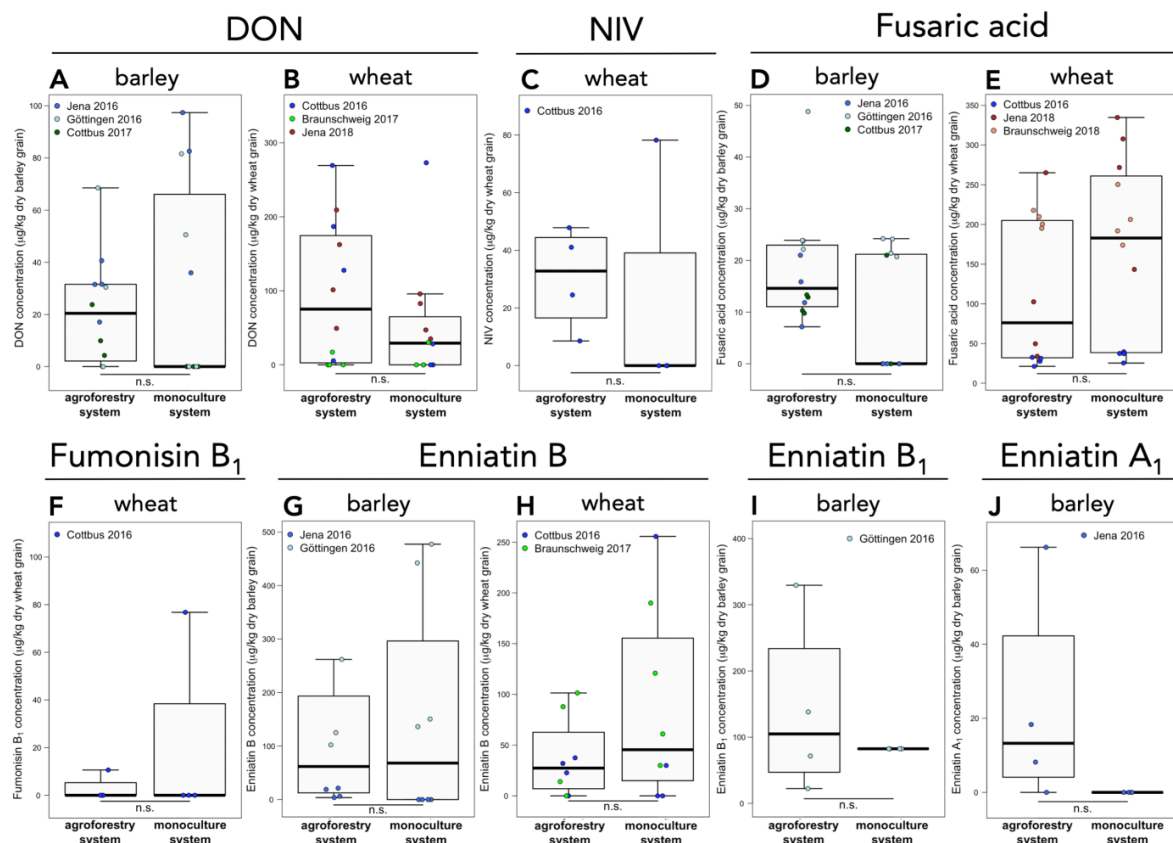


Figure 2. Mycotoxin concentrations in barley and wheat grain samples of temperate agroforestry and monoculture systems. Deoxynivalenol (DON) in (A) barley and (B) wheat, (C) nivalenol (NIV) in wheat, fusaric acid in (D) barley and (E) wheat, (F) fumonisin B₁ in wheat, enniatin B in (G) barley and (H) wheat, (I) enniatin B₁ in barley, and (J) enniatin A₁ in barley grain (J). *n* = 4 replicate plots per management system and study site and year. Mycotoxin concentrations of each replicate plot in the agroforestry systems are obtained from three (Göttingen) or four (Jena, Cottbus, and Braunschweig) sampling locations within the crop rows of the agroforestry systems and are weighted averages by area and yield. Samples below LOQ and above the limit of detection were assigned to half the LOQ, samples below the limit of detection to zero. n.s. indicates no significant differences between agroforestry and monoculture systems (linear mixed effect model or Mann-Whitney *U* test).

Within each agroforestry system and year, no differences in mycotoxin concentrations in wheat grain among the different distances to the tree row were detected. In barley grain at Jena in 2016, DON concentrations were lower at 1 m distance than 24 m distance to the tree row ($p = 0.023$) (Figure 3A). Fusaric acid concentrations in barley grain at Jena in 2016 were higher at 24 m distance compared to 4 ($p = 0.046$) (Figure 3B) and 7 m distance from the tree row ($p = 0.029$) (Figure 3B). No further differences in mycotoxin concentrations among the different distances from the tree row within each agroforestry system were found at any other site and year.

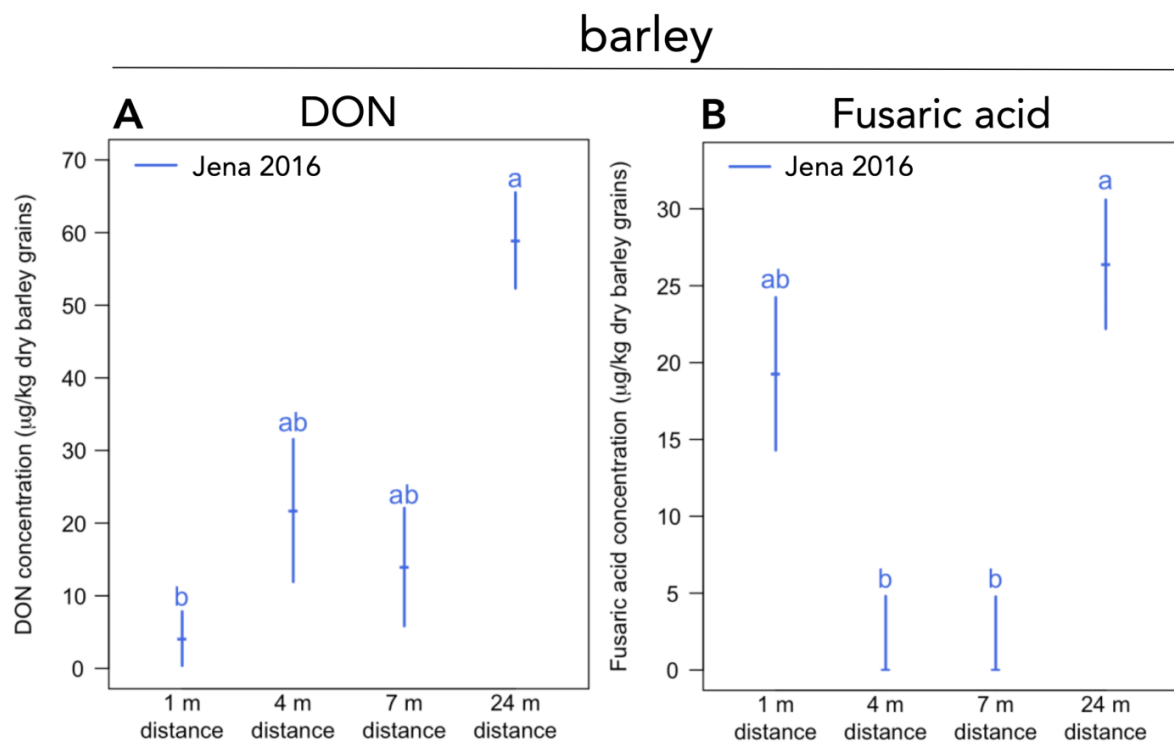


Figure 3. (A) Deoxynivalenol (DON) and (B) fusaric acid concentrations in barley grain samples within the temperate agroforestry system at Jena in 2016. Medians with standard error ($n = 4$ per replicate plot). Distances (1 m, 4 m, 7 m, and 24 m) are distances from the tree row within the crop row of the agroforestry system. Samples below the limit of quantification (LOQ) were assigned to half the LOQ, samples below the limit of detection to zero. Different lowercase letters indicate significant differences among distances from the tree row within the crop row of the agroforestry system (Kruskal-Wallis test with multiple comparison extension at $p \leq 0.05$).

4. Discussion

Agricultural systems are shaped by interactions of numerous abiotic and biotic factors. Similarly, colonization of crops by plant pathogenic fungi and accumulation of mycotoxins in cereal grains is influenced by environmental factors. The integration of trees into agricultural systems represents a new factor that may affect crop health and mycotoxin contamination.

In German agroforestry systems with short-rotation poplar trees in particular, recent studies have shown that winter wheat yields were either positively affected or not substantially affected by agroforestry [6,26]. Long-term yields of oilseed rape were not substantially reduced under agroforestry practice [6]. For both crops, however, yield depression was observed close to the interface between the trees and crops, presumably caused by competition for resources [6,26]. Apart from reducing yield, competition for water, nutrients, and light is likely to affect crop health [11,24]. For instance, coverage of seedlings by leaf litter derived from the trees may suppress seedling development [37], particularly for winter crops. Other factors that may influence the susceptibility of crops to fungal pathogens include altered microclimatic conditions (temperature, humidity, wind, evaporation) [25–27], soil microbial communities [38–41], and the dilution of the host crop by the trees.

4.1. Suppression of *V. Longisporum* in Oilseed Rape under Agroforestry

Our results indicate that *V. longisporum* in oilseed rape plants was suppressed in agroforestry compared to monoculture systems (Figure 1A). *Verticillium* affiliates are soil-borne fungal pathogens with a cosmopolitan distribution which cause vascular plant diseases [42]. *V. longisporum* is widely spread in Europe, causing premature senescence of oilseed rape and leading to significant economic

losses [43]. Despite crop rotation, *V. longisporum* poses a major threat to oilseed rape cultivation, as microsclerotia of the pathogen can survive for over 10 years in soil [44]. Their germination is stimulated by root exudates from the host, resulting in hyphal growth towards the host root [45]. A recent study on oilseed rape and *V. longisporum* showed that drought had no impact on disease severity [46]. Thus, abiotic stress might not be among the factors determining the extent of *V. longisporum*-induced vascular disease in oilseed rape.

Microbial antagonism may contribute to the suppression of *V. longisporum* under agroforestry management. *Pseudomonas* species have been shown to suppress the viability of *V. longisporum* microsclerotia *in vitro* [47]. Similarly, bacterial isolates that can colonize the rhizosphere of oilseed rape like *Burkholderia cepacia*, *Flavobacterium indologenes*, *P. chlororaphis*, *Serratia rubidaea*, and *Serratia plymuthica* have been described as antagonists of *V. longisporum* [48,49]. A recent study investigating soil bacterial communities in temperate alley cropping systems reported alterations of the community structure in response to agroforestry practices [39]. Furthermore, diversification of soil microbial communities under agroforestry is likely to occur [38,40,41]. Soil microbial diversification as well as increasing plant diversity is believed to suppress soil-borne pathogens through enhanced microbial antagonism [50,51]. Therefore, we hypothesize that belowground diversification in agroforestry systems enhances the suppression of soil-borne pathogen *V. longisporum*.

4.2. Effect of Agroforestry on *L. Maculans* and *L. Biglobosa* in Oilseed Rape

Our results suggest that agroforestry systems do not affect the abundance of *L. maculans* and *L. biglobosa* in oilseed rape as compared to monoculture systems (Figure A2A,B). *L. maculans* and *L. biglobosa* cause blackleg disease in oilseed rape, though *L. biglobosa* is considered weakly aggressive [52]. Symptoms of blackleg include premature senescence, lodging of the plant and even seed loss [53], making it an economically important disease in the production of oilseed rape. Similar to the antagonists of *V. longisporum*, different microorganisms such as *Bacillus* species, *Gliocladium catenulatum*, and *Serratia plymuthica* were identified as antagonists acting against blackleg disease in oilseed rape [54,55]. Both *Leptosphaeria* species can co-occur on the same plant and spread via splash-dispersal of inoculum or wind-dispersal of ascospores [56,57]. Although our data did not reveal differences between agroforestry and monoculture systems for the investigated *Leptosphaeria* species, it is reasonable to assume that reduced wind speed [27] and the physical barrier of the trees (host dilution effect) may reduce the extent to which splash-dispersed pathogens spread. This assumption is supported by the lower abundance of *L. biglobosa* closer to the tree row within one of our investigated agroforestry systems in 2016 (Braunschweig) (Figure A3).

4.3. Effect of Agroforestry on *F. Tricinctum* in Barley and Wheat Grain

Our data suggests a higher susceptibility of barley to *F. tricinctum* in agroforestry as compared to monoculture systems (Figure 1C), whereas the opposite applies to wheat (Figure 1B). However, although quantities of *F. tricinctum* DNA in barley grain were higher in agroforestry than monoculture systems, DNA concentrations of *F. tricinctum* in agroforestry systems were negligibly low ($<15 \mu\text{g kg}^{-1}$; on average about 20-times lower than in wheat grain) (cf. Figure 1B,C). We assume that the low colonization of barley with *F. tricinctum* under agroforestry does not exert a substantial impact on crop health and mycotoxin contamination.

F. tricinctum is frequently recovered from wheat grain and can be regarded as one of major *Fusarium* species causing *Fusarium* head blight of wheat [58–61]. Strains of *F. tricinctum* have been shown to produce enniatins, fusarin C, and moniliformin [62–64].

Similar to *V. longisporum* in oilseed rape, the suppression of *F. tricinctum* in wheat grain under agroforestry may have been caused by microbial antagonism. For example, a recent study proved the antagonistic effects of isolates of the bacterial genera *Bacillus* and *Sphingomonas* against *F. tricinctum* [65]. Inhibition of other *Fusarium* species like *F. graminearum* by microbial antagonists has been frequently reported [66–68], particularly by *Bacillus* affiliates [69]. Microbial antagonism has repeatedly been

reported to amplify with agricultural diversification [21–23,50,70]. Similarly, a recent study from central Sweden found that the abundance of *F. tricinctum* in winter wheat kernels was positively linked to increasing agricultural intensification [59]. Therefore, enhanced microbiological control of *F. tricinctum* in wheat grain due to diversification may occur under agroforestry practice. Additionally, altered microclimatic conditions may suppress infection of wheat with *F. tricinctum* in agroforestry systems.

Our results further revealed that within each agroforestry system and year, the abundance of investigated *Fusarium* species in barley and wheat grain was not affected by the distance from the tree row, although yield depressions due to resource competition close to the tree rows are well known [6,26]. Therefore, competition for resources at the interface of crops and trees in temperate agroforestry systems does not seem to affect the abundance of *Fusarium* species in barley and wheat grain. It is conceivable that, although yield depressions occur, negative effects of resource competition may be compensated by an enhanced microclimate and antagonism at the crop-tree interface.

4.4. Mycotoxin Accumulation in Barley and Wheat Grain

Over our three-year sampling period (2016 to 2018), temperate agroforestry systems did not affect the accumulation of DON, 3-ADON, 15-ADON, DON-3-glu, DAS, fusarin X, fumonisin B₁, NIV, ZEN, T-2, HT-2, fusaric acid, enniatin A, enniatin A₁, enniatin B, and enniatin B₁ in barley and wheat grain compared to monoculture systems (Figure 2). However, within one of our agroforestry systems (Jena in 2016), the distance from the tree row influenced the concentration of DON and fusaric acid in barley grain (Figure 3A,B). Since this is the first study investigating mycotoxins in cereal grain in temperate agroforestry versus monoculture systems, no comparison can be drawn with other studies.

The production of DON by *Fusarium* species (mainly *F. graminearum* and *F. culmorum*) has been shown to be dependent on temperature, whereby optimum temperatures were found to be species-dependent [71–74]. Furthermore, temperature cycles of certain ranges and durations can significantly affect DON production by *F. graminearum* [75]. Likewise, particularly in interaction with temperature, water activity is an important environmental factor determining DON production by *F. culmorum* and *graminearum* [71–73]. Thus, the lower concentrations of DON in barley grain at 1 m distance from the tree row within the crop row compared to the center of the crop row (24 m distance from the tree row) in the agroforestry at Jena in 2016 (Figure 3A) may have resulted from altered microclimatic conditions in the vicinity of the trees, particularly during anthesis. Fusaric acid is a fungal metabolite produced by a broad spectrum of *Fusarium* species and has repeatedly been reported in barley grain [76–78]. Fusaric acid shows pharmacological activity [79] and synergetic toxic effects in co-occurrence with other mycotoxins like fumonisin B₁ [80] and DON as well as other trichothecenes [81,82]. Studies on mammals (swine and rats) revealed that fusaric acid exhibits neurotoxic effects [83,84]. While the toxicity of fusaric acid to mammals is low [85], its strong phytotoxicity was already described in 1934 [86]. Furthermore, fusaric acid acts as a virulence factor for *F. oxysporum* [87,88], a soil-borne plant pathogenic fungus that causes vascular diseases. In the 1980s, efforts were undertaken to select for fusaric resistance in barley [77]. Despite this, there is a lack of studies investigating the effect of controlling environmental factors (e.g., temperature and water activity) on fusaric acid production. We assume that if, similar to other *Fusarium* mycotoxins, the production of fusaric acid is affected by temperature and water activity, altered microclimatic conditions may be responsible for the lower fusaric acid accumulation in barley grain close the tree row (at 4 and 7 m distance) compared to the center of the crop row (24 m distance from the tree row) at Jena in 2016 (Figure 3B).

Fumonisin is a mycotoxin known from mainly maize and asparagus. Recently, fumonisins have been detected in wheat in South Europe [89] and artificial seed-borne infection of wheat with *F. proliferatum* was demonstrated to lead to fumonisin accumulation in grains [90]. In our study, fumonisins in wheat grain were detected for the first time in Northern Europe. Despite the low levels of fumonisin B₁ observed, the occurrence of this mycotoxin in wheat grains harvested at two locations

in two years indicates a potential new threat to food safety. Therefore, we suggest that fumonisins be included in routine monitoring programs of wheat grain for mycotoxin contamination.

5. Conclusions

Compared to monoculture cropland, production of oilseed rape, wheat, and barley in temperate agroforestry does not pose an increased risk to plant health or food safety. The results of our three-year study indicate that temperate agroforestry may suppress *V. longisporum* infection of oilseed rape and *F. tricinctum* colonization of wheat grain but further studies are needed to confirm these effects. It can further be concluded that temperate agroforestry systems with barley or wheat pose no risk of increased mycotoxin contamination and therefore represent an alternative to monoculture agriculture which maintains food safety.

Supplementary Materials: Supplementary File S1 are available online at <http://www.mdpi.com/2071-1050/11/10/2925/s1>.

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Appendix A

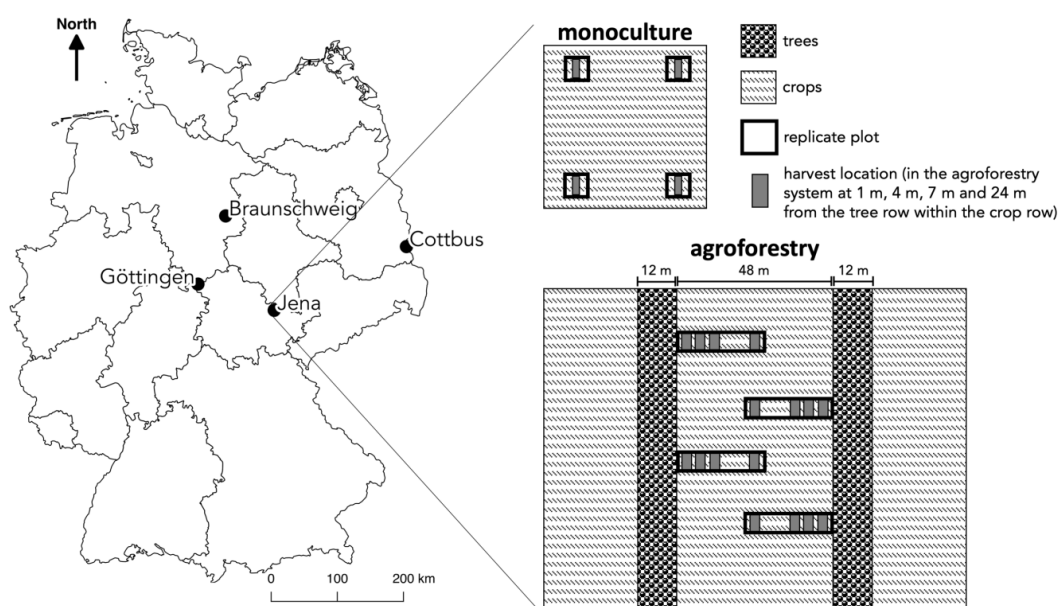


Figure A1. Map of the study sites in Germany and schematic illustration of the experimental design. Borders of federal states are represented by black lines. Dots represent study sites.

Table A1. Mastermix composition for real-time PCR assays.

Target Organism	Final MgCl ₂ Concentration (mM)	Concentration of Each Primer (μM)	Concentration of Each dNTP ¹ (μM)	Choice of DNA Polymerase ²	Choice of Reaction Buffer ²
<i>Fusarium avenaceum</i>	2.5	0.4	100	Hot Start <i>Taq</i>	Standard <i>Taq</i>
<i>Fusarium culmorum</i>	4.0	0.3	200	<i>Taq</i>	ThermoPol®
<i>Fusarium graminearum</i>	2.5	0.3	200	Hot Start <i>Taq</i>	Standard <i>Taq</i>
<i>Fusarium poae</i>	2.0	0.3	100	<i>Taq</i>	ThermoPol®
<i>Fusarium proliferatum</i>	2.0	0.3	125	<i>Taq</i>	ThermoPol®
<i>Fusarium tricinctum</i>	2.5	0.4	100	Hot Start <i>Taq</i>	Standard <i>Taq</i>
<i>Leptosphaeria maculans</i>	2.0	0.3	100	<i>Taq</i>	ThermoPol®
<i>Leptosphaeria biglobosa</i>	2.0	0.3	100	<i>Taq</i>	ThermoPol®
<i>Sclerotinia sclerotiorum</i>	2.0	0.3	100	<i>Taq</i>	ThermoPol®
<i>Verticillium longisporum</i>	3.0	0.3	200	<i>Taq</i>	ThermoPol®

¹ deoxyribonucleoside, ² purchased from New England Biolabs, Beverly, Massachusetts, USA; ThermoPol® reaction buffer (20 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1% Triton®X-100, pH 8.8 at 25 °C); Standard *Taq* reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3 at 25 °C).

Table A2. Primer, primer sequences and product size of the different real-time PCR assays.

Target Organism	Primer Pair	Primer Sequence (5'-3')	Product Size (bp)	Reference
<i>Fusarium avenaceum</i>	JIAf JIAr	GCTAATTCTTAAGTTACTAGGGGCC CTGTAATAGGTTATTTACATGGGCG	220	[91]
<i>Fusarium culmorum</i>	OPT18 F OPT18 R	GATGCCAGACCAAGACGAAG GATGCCAGACGCACTAAGAT	472	[92]
<i>Fusarium graminearum</i>	Fg16N F Fg16N R	ACAGATGACAAGATTCAGGCACA TTCTTTGACATCTGTTCAACCCA	280	[93]
<i>Fusarium poae</i>	Fp82F Fp82R	CAAGCAAACAGGCTCTTCACC TGTTCCACCTCAGTGACAGGT	220	[94]
<i>Fusarium proliferatum</i>	Fp3-F Fp4-R	CGGCCACCAGAGGATGTG CAACACGAATCGCTTCCTGAC	230	[95]
<i>Fusarium tricinctum</i>	Tri1 Tri1	CGTGTCCCTCTGTACAGCTTTGA GTGGTTACCTCCCGATACTCTA	215	[96]
<i>Leptosphaeria maculans</i>	LmacF LmacR	CTTGCCACCAATTGGATCCCCTA GCAAATGTGCTGCGCTCCAGG	331	[97]
<i>Leptosphaeria biglobosa</i>	LbigF LmacR	ATCAGGGGATTGGTGTGTCAGCAGTTGA GCAAATGTGCTGCGCTCCAGG	444	[97]
<i>Sclerotinia sclerotiorum</i>	SsF SsR	AGTCGAGGGACGGGTACTAA CTTGTCCTCATTGCCGTTT	225	[98]
<i>Verticillium longisporum</i>	OLG 70 OLG 71	CAGCGAAACGCGATATGTAG GGCTTGTAGGGGGTTTAGA	261	[99]

Table A3. Real-time PCR conditions and detection limit of the different assays.

Target Organism	Initial Denaturation	Denaturation	Annealing	Extension	No. of Cycles	Limit of Quantification (g DNA μL^{-1} Template)
<i>Fusarium avenaceum</i>	95 °C, 120 s	94 °C, 15 s	60 °C, 15 s	68 °C, 25 s	38	1.24×10^{-12}
<i>Fusarium culmorum</i>	95 °C, 120 s	94 °C, 20 s	62 °C, 40 s	68 °C, 45 s	35	1.24×10^{-12}
<i>Fusarium graminearum</i>	95 °C, 120 s	94 °C, 30 s	61 °C, 30 s	68 °C, 30 s	35	1.37×10^{-13}
<i>Fusarium poae</i>	95 °C, 120 s	94 °C, 30 s	62.5 °C, 30 s	68 °C, 35 s	35	1.37×10^{-13}
<i>Fusarium proliferatum</i>	95 °C, 120 s	94 °C, 35 s	64 °C, 30 s	68 °C, 35 s	35	1.37×10^{-13}
<i>Fusarium tricinctum</i>	95 °C, 120 s	94 °C, 20 s	65 °C, 20 s	68 °C, 18 s	38	1.52×10^{-14}
<i>Leptosphaeria biglobosa</i>	95 °C, 120 s	94 °C, 30 s	68 °C, 35 s ¹		40	1.52×10^{-14}
<i>Leptosphaeria maculans</i>	95 °C, 120 s	94 °C, 30 s	68 °C, 35 s ¹		40	4.57×10^{-14}
<i>Sclerotinia sclerotiorum</i>	95 °C, 120 s	94 °C, 30 s	56 °C, 30 s	68 °C, 20 s	40	4.57×10^{-14}
<i>Verticillium longisporum</i>	95 °C, 120 s	94 °C, 10 s	60 °C, 15 s	68 °C, 15 s	40	1.52×10^{-14}

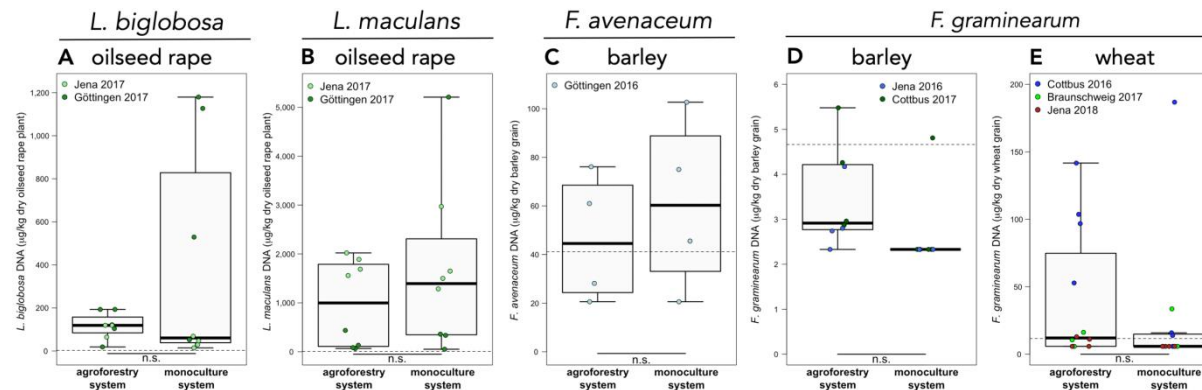
¹ performed as a two-step PCR.

Figure A2. Abundance of (A) *Leptosphaeria biglobosa* and (B) *L. maculans* in oilseed rape plants, (C) *Fusarium avenaceum* in barley grain, and *F. graminearum* in (D) barley and (E) wheat grain of temperate agroforestry and monoculture systems. $n = 4$ replicate plots per management system and study site and year. Fungal DNA concentration of each replicate plot in the agroforestry systems are obtained from three (Göttingen) or four (Jena, Cottbus, and Braunschweig) sampling locations within the crop rows of the agroforestry systems and are weighted averages by area and yield. Dashed lines represent the limit of quantification (LOQ) of the respective real-time PCR assay. Samples below the LOQ were assigned to half the LOQ. n.s. indicates no significant differences between agroforestry and monoculture systems.

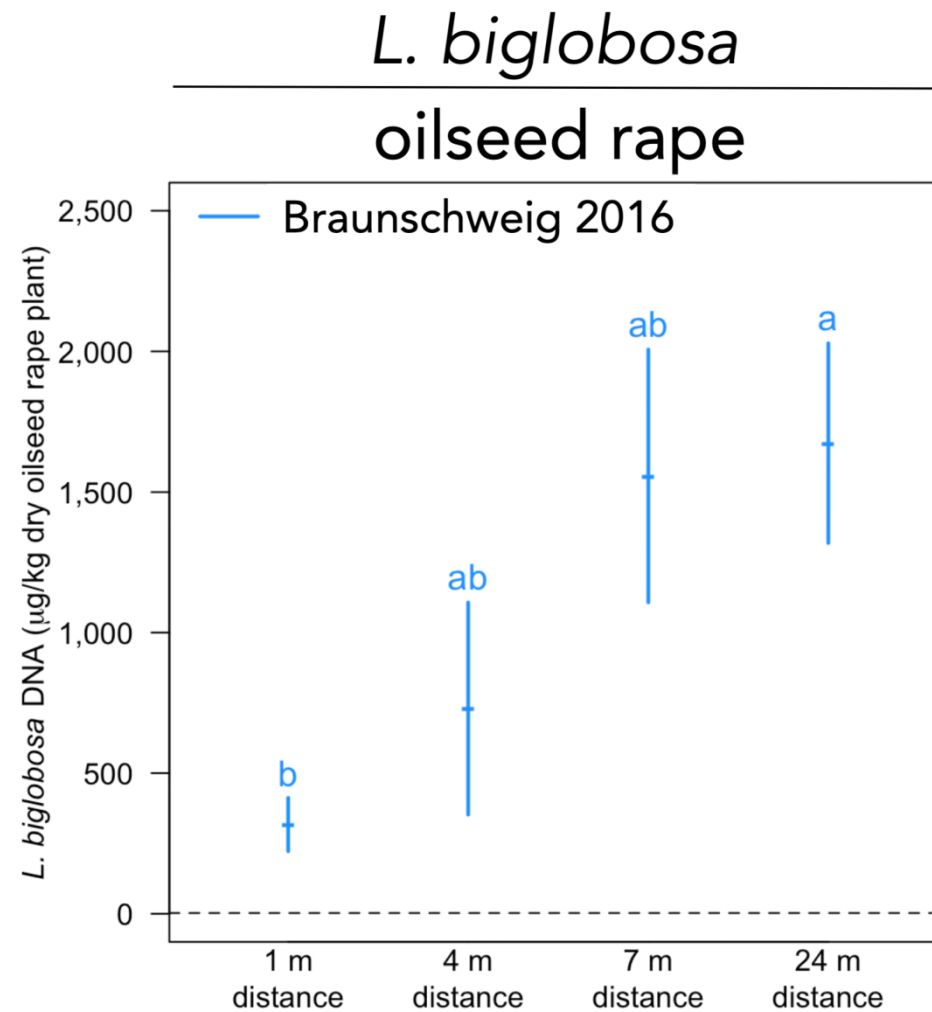


Figure A3. Abundance of *Leptosphaeria biglobosa* in barley grain samples within the temperate agroforestry system at Braunschweig in 2016. Medians with standard error ($n = 4$ per replicate plot). Dashed lines represent the limit of quantification. Distances (1 m, 4 m, 7 m, and 24 m) are distances from the tree row within the crop row of the agroforestry system. Different lowercase letters indicate significant differences among distances from the tree row within the crop row of the agroforestry system (Kruskal-Wallis test with multiple comparison extension at $p \leq 0.05$).

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