



Targeting allergenic fungi in agricultural environments aids the identification of major sources and potential risks for human health

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HIGHLIGHTS

- Detection system for outdoor allergenic fungi enabling source monitoring
- Allergenic fungi are ubiquitous in agricultural soils and plant materials.
- Plant residues in fields after harvest are major sources of allergenic fungi.
- Crops at harvest, particularly cover crops, show high allergenic fungal loads.

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ABSTRACT

Fungi are, after pollen, the second most important producers of outdoor airborne allergens. To identify sources of airborne fungal allergens, a workflow for qPCR quantification from environmental samples was developed, thoroughly tested, and finally applied. We concentrated on determining the levels of allergenic fungi belonging to *Alternaria*, *Cladosporium*, *Fusarium*, and *Trichoderma* in plant and soil samples from agricultural fields in which cereals were grown. Our aims were to identify the major sources of allergenic fungi and factors potentially influencing their occurrence. Plant materials were the main source of the tested fungi at and after harvest. Amounts of *A. alternata* and *C. cladosporioides* varied significantly in fields under different management conditions, but absolute levels were very high in all cases. This finding suggests that high numbers of allergenic fungi may be an inevitable side effect of farming in several crops. Applied in large-scale studies, the concept described here may help to explain the high number of sensitization to airborne fungal allergens.

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1. Introduction

Respiratory allergies and associated diseases show a rapidly growing incidence worldwide, with allergic rhinitis alone affecting between 10% and 30% of the population (Pawankar et al., 2011). In recent years, the extensive contribution of fungi to allergies and asthma has received increasing attention (e.g., Denning et al., 2006; Burge and Rogers, 2000). However, some features of fungal allergy still remain elusive such as the complex relations of allergy and allergens in fungi; for example,

the sheer number of allergens, cross-reactivities, poly-sensitizations, or the hampered diagnosis of allergy (Simon-Nobbe et al., 2008). Up to 80% of asthmatics are sensitized to fungal allergens (Lopez and Salvaggio, 1985) and a disease pattern of severe asthma with fungal sensitization (SAFS) has been recently proposed (Denning et al., 2006).

So far, most in-depth environmental studies have focused on the more obvious issues with indoor surroundings such as children's homes (Reponen et al., 2010), office buildings (Pitkäranta et al., 2008), or moisture-damaged building materials (Andersson et al., 1997). Recently, assessments of potentially allergenic fungi in indoor environments have been greatly enhanced because of the use of molecular techniques, such as mold-specific quantitative PCR (MSQPCR, Haugland and Vesper, 2002), which is based upon analyzing house dust as a time-integrated sample of fungal load in indoor air. So far, no corresponding methods for outdoor environments have been available, despite growing evidence that fungi detected in most indoor environments may be

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dominated by the local outdoor airborne community (Amend et al., 2010; Adams et al., 2013).

For monitoring allergenic fungi in the outdoor air, spore traps and other impact samplers, which deliver data over large time scales, have been used (Levetin, 2004). For example, results from spore monitoring indicate that allergenic fungi, such as *Alternaria*, which are naturally associated with plants, follow a seasonal course with a strong peak in summer or early fall (Corden and Millington, 2001). However, allergenic fungi, such as *Cladosporium* spp., are present in high-levels during most of the year (Sautour et al., 2009), suggesting more diverse environmental sources. Propagation of fungi is localized and often triggered by certain preconditions such as suitable temperature and humidity (Katial et al., 1997), thunderstorms (Nasser and Pulimood, 2009), farming operations (Friesen et al., 2001), or crop cultivation in combination with warm and dry weather (Corden et al., 2003). For these reasons, time-integrating methods with poor spatial resolution may not provide insight into cause–effect relations. Therefore, it is highly desirable to have methods, which deliver a more differentiated picture of the presence of potentially allergenic fungi in the environment. These methods would enable studying conditions that favor their development, which are prerequisites to the production and release of allergens. Consequently, the origins of allergenic fungal matter need to be considered. With that aim, we established and rigorously tested a workflow that was compatible with existing probe-based qPCR tests and can be used with varying source materials such as soil or plant residues. For developing the procedure, we focused on samples from an agricultural environment and on four fungal groups: the two most relevant outdoor allergen producers *Alternaria alternata* and *Cladosporium cladosporioides*, with approximately 20% of patients with respiratory allergy alone reacting to *Alternaria* in skin and prick tests (Barta et al., 2009), and *Fusarium culmorum* as well as the *Trichoderma viride/atroviride/koningii* group. The latter two belong to genera frequently known to cause hypersensitivity (e.g., Kurup et al., 2000), although with uncertain prevalence, and they were included because they were frequently found in agricultural environments, including the present study site (Hagn et al., 2003).

After developing a workflow adapted to the relevant sample types in an agricultural context (soil and plant materials), our next aim was to identify the main factors influencing the amounts of allergenic fungi in these environmental samples. Therefore, we analyzed agricultural field samples with the aim of detecting 1) major sources of allergenic fungi; 2) critical time points in the annual cycle from harvest to the next growing season; 3) the influence of spatial heterogeneity within fields; 4) the role of agricultural management; and 5) fungal colonization of different crops at maturity just before harvest. With a thoroughly tested procedure and the first set of applications, the overall aim of the study was to develop a strategy enabling environmental monitoring efforts for elucidating the paths of fungal allergens from the environment to sensitized persons.

2. Material and methods

2.1. Fungal cultures and conidial supplements

Fungal cultures from *A. alternata* (Fr.) Keissler (DSMZ 62006), *C. cladosporioides* (Fresen.) G.A. de Vries (DSMZ 62121), *T. viride* Pers. Ex Gray (DSMZ 63065), *F. culmorum* (W.G. Smith) Sacc. (DSMZ 62191), and *Penicillium roqueforti* Thom (DSMZ 1080) were grown, as recommended by Samson et al. (2010). Accordingly, the media used for sporulation were ‘potato carrot agar’ for *A. alternata*, ‘synthetischer nährstoffarmer agar’ (supplemented with cellulose filters (Ø 3 cm Whatman 1001-329, GE Healthcare, Little Chalfont, UK) as support for mycelia) for *F. culmorum* and ‘20% malt-extract agar’ for the other fungi. Conidia (asexual spore) stocks were prepared by rinsing spore cultures on plates with 0.01% Tween 80, as described by Haugland et al. (2004). Conidia from the reference fungus (*P. roqueforti*) were

used to spike all samples prior to DNA extraction as an internal standard, allowing normalization of possible extraction differences during calculations and screening for PCR inhibition.

‘Calibrator’ samples, which contained only conidia from the reference fungus *P. roqueforti* and one or more target fungi, were prepared and extracted with each batch of environmental samples. These calibrators provided the basis for the comparative quantification according to the MSQPCR principle and served as positive controls for all qPCR runs. The amounts of conidia in calibrators were set to 4×10^5 for *P. roqueforti*, 2×10^5 for *C. cladosporioides* and *T. viride*, and 1×10^5 for *A. alternata* and *F. culmorum*.

To determine an alternative reference fungus, in silico comparisons for amplification of *P. roqueforti* and a *Geotrichum candidum* strain used in the literature (Haugland and Vesper, 2002) have been made beforehand. The results showed a comparable and equally unlikely amplification of both fungi from our test environments i.e., plant residues and soil. A pre-experiment with different soil–plant congeries from our environment had revealed only minimal amplification of *P. roqueforti*. Its very low occurrence in the target samples and its easy handling (nonpathogenic, well sporulating) lead to the choice of *P. roqueforti* as reference fungus. Details in Suppl. 2.

2.2. Field sampling of soil and plant residues

Samples were collected at the Scheyern Research Station, 40 km north of Munich (48.50°N, 11.45°E, 450–490 m asl) with a mean annual precipitation of 803 mm and mean annual temperature 8.4 °C. Two farm management systems (organic and conventional) have been used on the farm since 1992 with different crop rotations and use of synthetic fertilizers and pesticides, respectively (Schröder et al., 2008). Field samples were used for the following two major targets: 1) establishing a standardized workflow for outdoor environmental samples and 2) identifying major sources of target fungi and environmental factors associated with allergenic fungal growth. The major focus of the study was on wheat (*Triticum aestivum* L.) as the major crop cultivated under organic and conventional farming at the research station. In addition to wheat, other crops were taken into account for the study of fungal loads at the time of harvest (see below).

For a postharvest time series, eight wheat fields (four organic, four conventional) were sampled after wheat harvest in 2011. Samples were collected at least 2 m from the margins of each field. Soils on the farm are cambisols and luvisols. Four different sample types were collected, each composed of 10 subsamples per field: (1) decaying stems and (2) roots from 1 to 10 cm soil depth with the adhering soil shaken off manually but without further cleaning, (3) soil directly attached to and shaken off of decaying roots: ‘root/soil’, and (4) bulk soil from 1 to 4 cm depth. Ten subsamples were collected on each field at least 10 m distant from one another in the six larger fields, and at least 5 m distant in the two smaller fields. For each subsample, four blocks within 1 m² were separated with a spade. From each block one source material was collected. Equal weights but at least 10 g of each source material were gathered per subsample and the subsamples of a source material were merged to one sample per field. Time points were one week after harvest, after tillage, in late autumn, and before the next sowing to cover a time series of crop residue decay after harvest (Table 1). For the development of the procedure and for sensitivity tests, plant residues and soil samples from the late-autumn date were used. Within-field heterogeneity was studied in one of the fields, which was chosen for its heterogeneous soil conditions.

The field was divided into four sections of 0.7 ha, gradually descending from a hillside of 490 m asl (section 1) to a depression of 470 m asl (section 4) with soil conditions changing from sandy-to-loamy partially stagnic cambisol with high-stone content (section 1) to cambisol with colluvic material (section 4). In each section, three independent replicate samples were used for reproducibility testing. Each replicate sample consisted of 10 merged subsamples each of bulk soil and decaying

Table 1
Overview of the field samplings.

Experiment	Sampling dates	Plot sizes (plot n)	Plot type	Source materials	Subsamples per source and plot
Accuracy of different calculation approaches	10/25/11	Spot samples (4)	Harvested wheat fields	Bulk soil [spiked with conidia]	12 [conidia dilution series of each fungus]
Reproducibility of whole procedure	10/25/11	Spot sample (1)	Harvested wheat fields	Bulk soil, decaying roots, decaying shoots	6 [3 spiked with conidia of target fungi]
Postharvest time series	08/17/11 09/19/11 10/25/11 03/27/12	0.29 ha–3.6 ha (8)	Harvested wheat fields ^a	Bulk soil, 'root/soil', decaying roots, decaying shoots	10 [merged to 'field sample']
Within-field heterogeneity	10/25/11	0.7 ha (4)	Sections of 1 field ^b	Bulk soil, decaying shoots	10 [merged to a 'field-section sample'] ^c
Preharvest test of individual plants	07/23/14	0.01 ha (1) ^d	Fields with mature crops ^d	Lower part of plants, upper part of plants, seed heads	4 plants [individuals not merged]
Preharvest field trial	07/23/14	0.014 ha (39)	Field trial plots: mature crops ^e	Lower part of plants, seed heads	25 [merged to a 'plot-sample']

^a 4 organic, 4 conventional.

^b Quarters of the most heterogeneous field from the postharvest time series.

^c Divided into 3 replicates per subsample before merging to test reproducibility.

^d Different fields for each species: wheat, rye, barley, oat, pea, field bean.

^e Plots for organic wheat (n = 15), organic rye (n = 15), conventional wheat (n = 9).

shoot samples, respectively. Samples were collected anew for the heterogeneity test and in the same manner as for the postharvest time series.

After plant materials were identified as a major source of the tested allergenic fungi, a series of second sampling (preharvest) was performed immediately before harvest, a time point representing farming operations critical for release of potentially high-amounts of fungal-colonized material. Wheat and rye (*Secale cereale* L.) plants were collected from 15 organic plots each, omitting a 1 m border-strip. Because rye had not been a part of the crop rotation, only wheat was collected from 9 conventional plots of the field trial. From each plot, 25 randomly sampled plants were divided into two parts (stems 0–30 cm above soil and seed heads), which were separately analyzed.

Additional plant samples were collected at the same date at a 5-km distance from the research station, i.e., field bean (*Vicia faba* L.), oat (*Avena sativa* L.), pea (*Pisum sativum* L.), winter barley (*Hordeum vulgare* L.), winter rye, and winter wheat, in four replicates (10 m distant from each other) per plant; these samples were divided into three sections (0–30 cm above the soil, >30 cm above the soil, and seed heads), which were separately analyzed.

All samples were frozen onsite on dry ice and stored at –20 °C until further processing. An overview over all samplings is given in Table 1.

2.3. Extraction of DNA

The optimal DNA extraction method was determined in pre-experiments and showed that the PowerSoil-htp 96 Well Soil DNA Isolation Kit (Mo-Bio Laboratories Inc., Carlsbad, USA) was most suitable in comparison with the suitability of other extraction protocols with respect to DNA quality, PCR functionality, handling, and cost (Suppl. 1). Soil samples were treated according to the manufacturer's recommendations for spores in soils, except for sample processing after centrifugation following the mechanical lysis step. Here, for each extraction plate, the lowest volume of a sample was determined as the volume to be transferred in the next step for all extracts on the plate. To avoid high plate to plate variability, only calibrator samples from the same extraction plate as the respective samples were used later for calculation.

Plant residues and plant–soil mixtures were homogenized as follows: a stainless steel grinder (UM 620, Karcher AG, Birkenfeld, Germany) was precooled with dry ice, loaded with 4–6 g of frozen sample and approximately 10 g of pelleted dry ice, and ground to a particle diameter of <2 mm. The resulting composites could be handled like soil samples after evaporation of dry ice. Input weights for the extractions were approximately 0.25 g after the manufacturer's recommendations for the kit, except for very light homogenized materials, which only

allowed input of >0.15 g. The exact input of fresh-weight was noted for later calculations and dry-weights were determined separately.

2.4. qPCR assays

Prior to qPCR, in silico tests were conducted to choose a suitable TaqMan® assay for the calibrator (Suppl. 2). The following assays (original names) were used: Proqu (*P. roqueforti*), C.clad1 (*C. cladosporioides*), Aaltr (*A. alternata*), Tviri (*T. viride/atroviride/koningii*) from Haugland and Vesper (2002), and the assay of *F. culmorum* from Cullen et al. (2005). Oligonucleotides were synthesized by MWG-Eurofins GmbH (Ebersberg, Germany). Reactions were carried out in optical-grade 96-well reaction plates from Applied Biosystems (Foster City, CA, USA) with 5 µl undiluted DNA-template, reagents, concentrations, and PCR conditions according to Haugland and Vesper (2002). PCR was performed on an Applied Biosystems 7300 system (Foster City, CA, USA).

2.5. Calculation of conidial equivalents

The amount of fungal DNA in unknown samples was estimated on the basis of the amount of DNA extracted from known numbers of conidia (determined by microscopic counting) and expressed as conidial equivalents (CE). To quantify the target fungus in samples, two variants of the comparative CT (cycle threshold) method exist (Haugland and Vesper, 2002). Both rely on calibrator-samples consisting of known amounts of extracted conidia instead on standard curves. The first, directly associates the CT of a test sample with the CT of a corresponding calibrator sample, using an assay of only the target fungus (Eq. (i)) and the second, includes normalization via reference fungus (here: *P. roqueforti*) assay (Eq. (ii)):

$$N = N_0 \times E^{-\Delta CT} \quad i$$

$$N = N_0 \times E^{-\Delta\Delta CT} \quad ii$$

where N_0 = the number of target fungus conidia in the calibrator; $\Delta CT = CT_{\text{sample, target fungus}} - CT_{\text{calibrator, target fungus}}$; $\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{calibrator}}$; $\Delta CT_{\text{sample}} = CT_{\text{sample, target fungus}} - CT_{\text{sample, reference fungus}}$; $\Delta CT_{\text{calibrator}} = CT_{\text{calibrator, target fungus}} - CT_{\text{calibrator, reference fungus}}$; and E = efficiency of the target assay. The calculated N therefore is the number of target fungus conidial equivalents (CE) in the PCR-reaction of the respective sample.

Results are shown as CE/g (dry-weight of sample). The $E^{-\Delta CT}$ approach was used for all experiments after comparisons of both.

Reaction efficiencies (E) were calculated using CTs from dilution series (4-fold steps) of the calibrators, via the slopes of log-transformed dilution vs. CT plots. The final calculation results were means from comparative calculations with CTs from the first three dilutions of the calibrator, as similarly described by Pitkäranta et al. (2008). The dilution series were therefore only used to get mean values from different PCR-reactions, thus increasing the accuracy of calculation. For that and the determination of E, only dilutions in the linear range (similar ΔCT between dilution steps) were used. With our dilution steps, the linear range usually ended around 0.00098x (e.g., resembling the equivalent of 5 conidia of *A. alternata* in the PCR reaction of a diluted calibrator). The theoretical detection limit is not coupled to a standard-curve for MSQPCR and similar approaches. However, if no $CT_{\text{sample,target fungus}}$ is available, Eq. (i) or (ii) is incomplete. With our qPCR setup, we reached that calculated theoretical limit at <10 CE/g for the assay on *C. cladosporioides* and at <1 CE/g for the other assays. For routine analyses, a lower detection limit of 5×10^2 CE/g is recommended (see Discussion).

2.6. Tests for accuracy and sensitivity

The accuracies of different calculation approaches in field samples were compared using soil samples from four different fields. These soils were previously identified to be having low-levels of test fungus DNA (not shown). Subsamples of each soil were spiked with different amounts of conidia from *C. cladosporioides* (2×10^3 – 2×10^6) and *T. viride* (2×10^3 – 2×10^5) as well as fixed amounts of *P. roqueforti* (4×10^5) and subsequently extracted.

Accuracies of different calculation approaches near the detection limit were assessed with six extractions from an artificial conidial mixture, containing 1×10^3 conidia of *A. alternata* and *F. culmorum*, 2×10^3 of *C. cladosporioides* and *T. viride*, and 4×10^5 conidia of *P. roqueforti* per extraction.

Possible amplification bias in the PCR reactions between calibrators (low-total DNA) and field samples (high-total DNA) was accounted for by supplementing three of the six replicates with 2 μ g of salmon sperm DNA (Carl Roth GmbH, Karlsruhe, Germany) as nontemplate DNA (ntDNA) prior to extraction.

Reproducibility of the whole procedure, including DNA extraction, was tested by dividing one sample each of bulk soil, wheat stems, and wheat roots into six subsamples. For each material, three of the subsamples were spiked with conidia from all target fungi. DNA from the subsamples was then individually extracted and analyzed by qPCR. Replicate extractions from the within-field heterogeneity test were used to assess reproducibility in native field conditions.

2.7. Statistical analysis

The following factors potentially contributing to allergenic fungal presence in the environment were taken into account: a) major reservoirs (soils and decaying plant residues), b) time after harvest until spring, c) agricultural management (organic vs. conventional), and d) occurrence in crop species at harvest. The postharvest experiment was analyzed using one fitted ANOVA model (with type III sum of squares) for the results of each fungus. In addition, results for each fungal group and sampling material were treated as effects and analyzed using the Adonis function (PERMANOVA) in the vegan package (version 2.0-10) for R (R Core Team, 2013) with 10,000 permutations and Bray–Curtis dissimilarities. This analysis enabled evaluation of the overall effects of sampling time and management or individual field characteristics for the whole population of tested allergenic fungi. For fitting ANOVA with preharvest results, those results were grouped into the following three sets of comparable data: (1) all conventional plots, (2) all organic plots, and (3) all wheat plots. Input data were augmented by 2 and transformed to base-10 logarithms for all ANOVA. For Adonis,

original values were used. The R programming environment was used for all computation.

3. Results

3.1. Outdoor fungal detection system

All qPCR assays were successfully applied to the following sample types: soil, soil–plant mixtures, and different plant constituents (Suppl. 3). Accuracies of different calculation approaches were first compared using spiked soil samples (Fig. 1A) and secondly using diluted conidial suspensions (Fig. 1B) to evaluate performance at low-conidia levels. Overall, both calculation approaches well represented the numbers of added conidia. The simplified calculation setup ($E^{-\Delta CT}$) exhibited a tendency to underestimate conidial counts for large supplements (max.: 0.33-fold for 2×10^6 *C. cladosporioides* conidia from microscopical counts). The $E^{-\Delta\Delta CT}$ setup exhibited a tendency to overestimate conidial counts for small supplements (max.: 1.99-fold for 2×10^3 counted conidia of *C. cladosporioides*). The ratios of calculated to counted conidia for individual extracts were between 0.28–1.16 ($E^{-\Delta CT}$, median: 0.62, mean 0.63) and 0.82–2.5 ($E^{-\Delta\Delta CT}$, median: 1.26, mean 1.36). If the samples spiked with 2×10^3 conidia are ignored because of the chance of interference with small amounts of conidia native to the soils, the ratios were between 0.28–0.83 ($E^{-\Delta CT}$) and 0.82–1.96 ($E^{-\Delta\Delta CT}$). Using diluted conidia, both approaches appeared equally precise at low-conidia levels (Fig. 1B). In the same experiment, three of six low-conidia samples were supplemented with an excess of nontemplate DNA prior to extraction. Results for supplemented and nonsupplemented extracts were similarly heterogeneous. However, variability was significantly reduced in samples complemented with ntDNA ($p < 0.05$, t tests with $E^{-\Delta CT}$ results of all fungal test assays).

The reproducibility of the entire detection system was high within all fungal assays and sample types, which were first tested using replicated DNA extractions of spiked environmental samples (standard deviations (s.d.): 4.5%–24.9% for bulk soil, 2.6%–11.5% for root material, 7.3%–14.4% for shoots) and secondly tested using replicated sampling ($n = 3$) plus DNA extraction from four subplots of one wheat field (s.d. <31% for results > 10^4 CE; Fig. 2).

3.2. Quantity of allergenic fungi in environmental samples

In the postharvest period, the major factor determining quantity of each fungus was sample type (ANOVA $p < 0.001$, except *Trichoderma*: $p = 0.03$). Decaying shoots were the major source of the tested fungi, with the exception of the *Trichoderma* group. For *C. cladosporioides*, high-numbers were also detected in decaying roots (Fig. 3). Fungal abundances decreased in the order of shoot > root > immediate root vicinity > bulk soil for all allergenic fungi, except for the *Trichoderma* group, which was in most cases found at low-levels and without a clear pattern.

Time point was a significant factor for *C. cladosporioides* and *Trichoderma* (ANOVA $p < 0.001$) with highest numbers of these fungi found in shoots at the late-autumn sampling date. Interaction effects of sample type with sampling time were found for *C. cladosporioides*, *A. alternata*, and *F. culmorum* (ANOVA $p < 0.001$). Amounts of *C. cladosporioides* and *F. culmorum* in decaying shoots remained substantial until the following spring (Fig. 3).

Management had a significant influence for all fungi [ANOVA $p < 0.001$ (*C. cladosporioides*, *Trichoderma*), $p = 0.002$ (*F. culmorum*), $p = 0.018$ (*A. alternata*)]. The multivariate approach estimated the effect of individual fields as significant (Adonis-PERMANOVA $p < 0.001$) on the whole population of tested allergenic fungi and in the same magnitude as the coupled effect of management (details in Suppl. 4).

Within-field variations (Fig. 2) were more pronounced for bulk soil than for decaying shoots, less substantial for *C. cladosporioides* (soil: 13-fold, shoots: 5-fold) and *A. alternata* (soil: 2-fold, shoots: 4-fold)

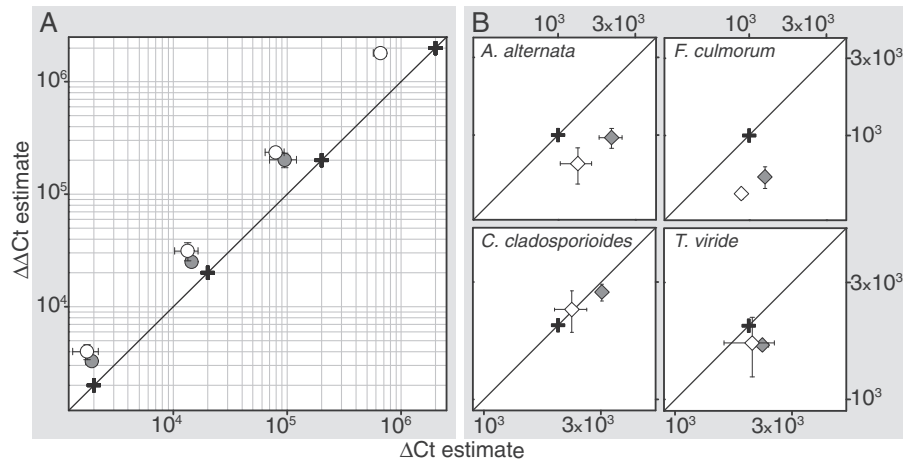


Fig. 1. Estimates of conidial equivalents by $E^{-\Delta CT}$ vs. $E^{-\Delta\Delta CT}$ calculations (means $\pm$ standard deviation). The diagonal line indicates equal results for both calculations and crosses indicate numbers of added conidia, as determined by spore counts; Fig. 1A: results from different soil samples ($n = 4$) spiked with 2×10^3 – 2×10^6 target conidia of *C. cladosporioides* (white circles) and 2×10^3 – 2×10^5 target conidia of *T. viride* (gray circles); Fig. 1B: results from DNA extracts of conidial suspensions with (gray rhombs) or without (white rhombs) addition of ntDNA ($n = 3$, each).

compared to *F. culmorum* (soil: no detection in two sections of the field, shoots: 38-fold) and the *Trichoderma* group (soil: 200-fold, shoots: 8-fold) and in the same range as between-field variations. In most of the 150 preharvest samples of different crops and plant parts, *C. cladosporioides* and *A. alternata* were detected in substantial amounts (99% of samples $> 10^6$ CE/g for *C. cladosporioides* and 95% of samples $> 5 \times 10^3$ CE/g for *A. alternata*). Quantities of *C. cladosporioides* were commonly 10–100 times higher than those of *A. alternata* (Figs. 4 and 5). *F. culmorum* and the *Trichoderma* group were less frequently found (48% and 31% of samples if no detection limit was applied, or 30% and 27% of samples if a lower detection limit of 5×10^2 CE/g was set) and typically in lower quantities than the other two fungi (Fig. 5).

Out of all the crops collected at harvest at separate locations, samples of field bean and pea had very high-amounts of *C. cladosporioides* ($> 10^8$ /g) and *A. alternata* ($> 10^5$ /g) over all plant compartments. Levels for cereals (wheat, rye, oat, and barley) were lower in most cases (Fig. 4), yet at substantial levels ($> 10^7$ /g or $> 10^4$ /g, respectively). Differences between plant compartments were significant for *C. cladosporioides* (ANOVA $p < 0.001$) and *A. alternata* (ANOVA $p = 0.002$). Differences between levels for legumes and cereals were significant (ANOVA $p < 0.001$) for *C. cladosporioides*, *A. alternata* and *F. culmorum*.

Detailed sampling of 15 organic and 9 conventional plots revealed nearly identical fungal loads for rye and wheat on organic plots

(Fig. 5), which were significantly higher than those for wheat on conventional plots (ANOVA $p < 0.001$ for *C. cladosporioides* and *A. alternata*). A significant influence (ANOVA $p < 0.015$ for *C. cladosporioides* and *A. alternata*) of the sampled plant section was found with higher fungal loads on seed heads than on lower stems. Other factors (tillage, fertilizer, and interactions) had only minimal influence on one of the three compared plot types (conventional, organic, or wheat plots; details in Suppl. 4).

4. Discussion

4.1. Evaluation of the outdoor fungal detection system

Accuracies of the assays under the protocols adapted in this study matched that of MSQPCR, with the ratios of calculated to counted conidia falling in a small range when compared, for example, with the ratios from 0.1 to 2.25 (median: 0.70) in Haugland et al. (2004) for similar assays and was consistent with the range of both calculation approaches of this study. Integration of further assays for the fungi of interest may be carried out without extensive re-evaluations.

$E^{-\Delta CT}$ calculations tended to underestimate large amounts of conidia and $E^{-\Delta\Delta CT}$ calculations tended to overestimate small amounts, but in general, both methods were in good agreement and always approximated the magnitude of conidia counts with the hemacytometer. By referring to Haugland and Vesper (2002), such a resemblance should be verified before the $E^{-\Delta\Delta CT}$ approach may be discontinued in favor of the $E^{-\Delta CT}$ method. Consequently, we recommend utilizing the cheaper and faster $E^{-\Delta CT}$ method, provided that similar sample types and analogous qPCR assays are used. Nonetheless, addition of reference fungus conidia is advisable when PCR inhibition cannot be excluded, as mentioned by Roe et al. (2001). This addition may also serve as an internal positive control for DNA extraction, such as for very heterogeneously structured samples. *P. roqueforti* proved useful as a reference fungus, as it will not amplify from most terrestrial environmental sources as readily as *Geotrichum candidum*, which was used as reference by MSQPCR. Examining potential reference fungi may be done through checking primer-specificity and possible PCR products against nucleotide-databases (in silico PCR), if environmental sequences are available.

The study disproved a bias because of sharply differing DNA amounts between samples and calibrators. However, supplying calibrators with ntDNA prior to extraction significantly reduced disparity between replicate extractions and can therefore be recommended in future experiments. For all assays, a detection threshold was defined

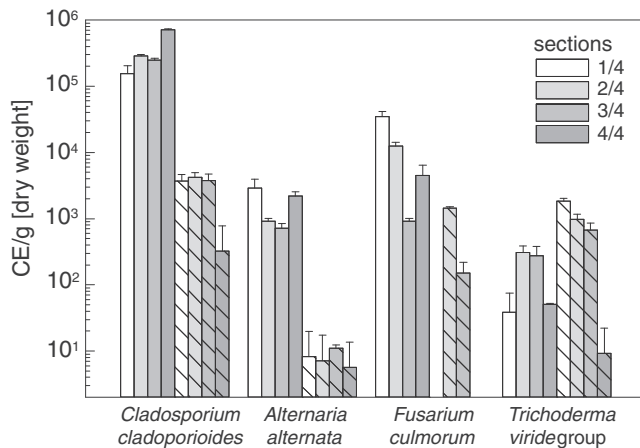


Fig. 2. Within-field variability (in conidial equivalents, CE) of the tested allergenic fungi in late autumn. Four sections of a heterogeneous field sampled in replicates with standard deviation ($n = 3$); open: shoots; hatched: bulk soil.

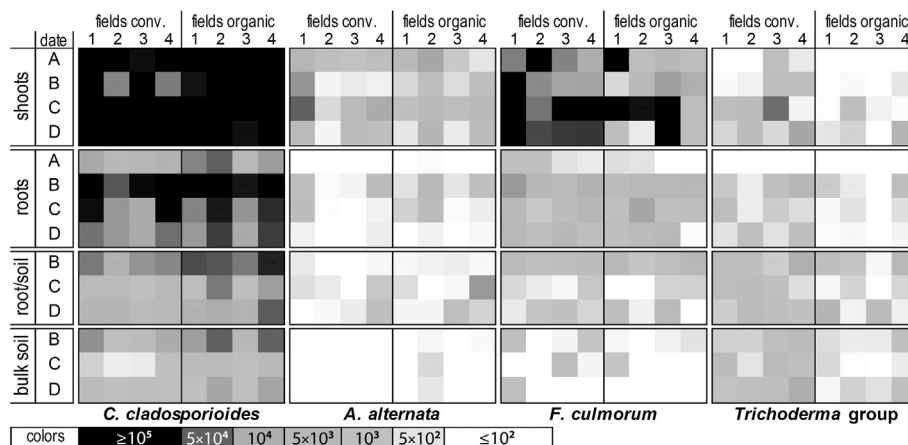


Fig. 3. Postharvest time series. Fungal concentrations as conidial equivalents (CE) per gram (g) of material (dry-weight) coded in grayscale for each fungal qPCR assay. Each row represents one time point for one source material, and each column represents one fungal assay for one individual field. Dates: (A) after harvest in late summer, (B) pretillage in autumn, (C) late autumn, and (D) before sowing in spring; CE: conidial equivalents. The exact values underlying each square are available in Suppl. 5.

and was recommended as a convenient routine for future applications, for several reasons. Meaningful results near the theoretical detection limit demand appropriate replicates to overcome stochastic effects (Peccoud and Jacob, 1996). Yet, replication conflicts with the need for high-throughput along with low-cost, which is necessary for large-scale surveillance. Additionally, limited false positive signals were provoked in a strongly artificial setup with huge overloads of DNA from a single related fungus. A similar behavior cannot be ruled out for other environmental samples. However, limited detection suggests low-abundance, which renders an allergenic response less likely in most sensitized individuals, considering the dose dependence of allergenic immune reactions (Arts et al., 2006), yet, thresholds for respiratory allergens are still not conclusively defined (Ward et al., 2010). Until the immunological relations are better understood, a conservative detection limit of 5×10^2 CE/g is a straightforward strategy to overcome such issues. It could be used in analogy to the threshold estimated for air samples with 1×10^2 spores of *Alternaria* per m^3 air and 3×10^3 spores of *Cladosporium* per m^3 air (Gravesen, 1979).

An important finding for large-scale applications was the high-reproducibility of results in artificial setups as well as in all environmental samples. This finding implies that our mode of sample preparation is generally appropriate without the need of extraction replicates. This situation can facilitate the processing of large sample numbers and enables a rapid indication of the allergenic potential of a given outdoor environment. Similarly, technical replicates of the qPCRs can be reduced to a

minimum, as suggested by Kitchen et al. (2010) and Bengtsson et al. (2008), among others.

4.2. Quantifying allergenic fungi in environmental samples

The results from the postharvest period clearly demonstrate that decomposing plant debris is a larger reservoir of the allergenic fungi *A. alternata*, *C. cladosporioides* and *F. culmorum* than the soils we studied. This result suggests a focus on sampling decomposing plant constituents during this period. Bulk soil in the winter half-year is of negligible importance for fungal allergenicity, with the possible exception of *C. cladosporioides*. In contrast, fields with exposed plant residues, such as those, which are often seen with cover crops or green manure, may be of high-concern as a reservoir for local dispersal of fungal allergens or allergen producers and require focused studies. The species-specific decline in levels of test fungi from decaying shoots to bulk soil reflects the quality of the nutrient source available. The strong decline of *A. alternata* on nearing the winter season in comparison with its high-prevalence at harvest can be explained by its mesophilic physiology and growth as an endophyte in a wide range of plants. Analogous results of a decline during winter and a peak around harvest time were obtained by airborne *Alternaria* spore monitoring (Fernandez-Rodriguez et al., 2014), demonstrating the link between the two steps (along the route taken by outdoor fungal allergens to arrive in the human body) of growth of allergenic fungi and dispersal in the air. However,

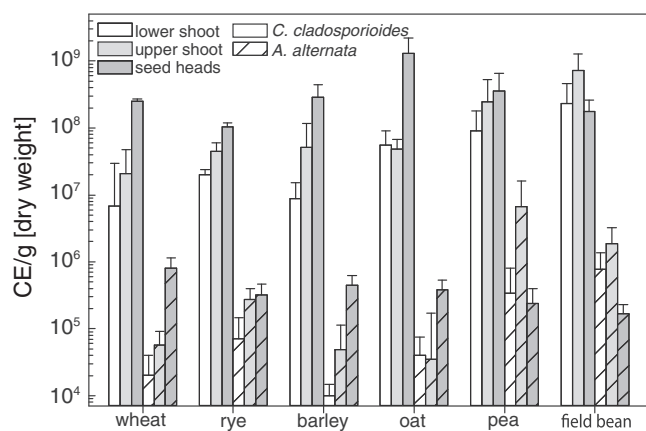


Fig. 4. Amounts (in conidial equivalents, CE) of *C. cladosporioides* (solid columns) and *A. alternata* (hatched columns) in samples of various crops at maturity. The mean of four plants $\pm$ standard deviation.

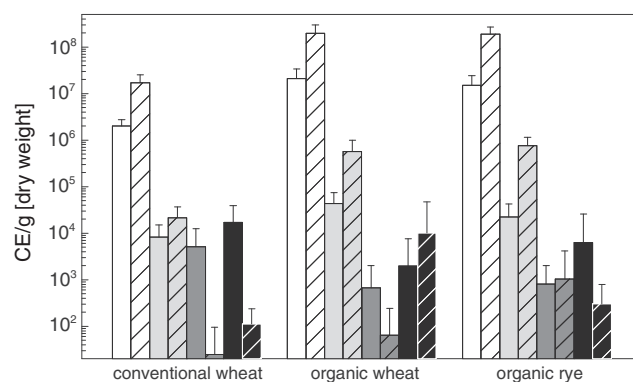


Fig. 5. Quantities (in conidial equivalents, CE) of allergenic fungi on cereal crops cultivated in conventional and organic fields at maturity of the crops; white: *C. cladosporioides*, light gray: *A. alternata*, dark gray: *Trichoderma* group, and black: *F. culmorum*; solid bars: lower shoots (0–30 cm) and diagonally lined bars: ears; $n = 9$ (conventional), $n = 15$ (organic wheat), and $n = 15$ (organic rye); error bars: standard deviation.

C. cladosporioides showed at most a marginal decline in the environmental sample materials with time. Thus, all tested sample types may serve as a reservoir for the species and lead to a high-abundance found in airborne spore monitoring (Fernandez-Rodriguez et al., 2014), and, in the present study, in plant samples at harvest. Furthermore, results from the postharvest sampling suggest that during the winter period, effects of different managements were strongly superposed by individual characteristics of the fields. Similarly, Schneider et al. (2010) and Hagn et al. (2003) found only minor influences of conventional and organic farming systems on soil fungal communities and identified the sampling time as the most influential factor of community structure. The high-levels of *C. cladosporioides* and *A. alternata* in all plant materials before harvest indicate that their establishment is inevitable. This confirms Burge's (2002) proposal that plant materials are the primary source of airborne fungi. Interestingly, an absolute difference between *C. cladosporioides* and *A. alternata* of a similar magnitude, as in our study, has also been found for airborne spores (Fernandez-Rodriguez et al., 2014). This finding suggests that sampling source materials of allergens could serve as an alternative to air sampling for measuring environmental fungal allergenic potential. Such sampling would obviate the installation and maintenance of impact-sampling devices, a measure strongly limiting temporal and spatial resolution owing to its cost.

The pre-harvest results showing differences between fungal loads of wheat under organic and conventional farming suggest that agricultural practice affects the amounts of allergenic fungi growing on fields. In our case, management differences comprised several factors, such as different tillage systems, fertilizers, pesticides, and crop rotation, precluding general conclusions about fungal allergens in organic or conventional systems. The influence of specific management practices on allergenic fungal growth and dispersal awaits more detailed study. The high-numbers of *A. alternata* and *C. cladosporioides* in air samples and their broad host ranges were reflected in our results. We found identical fungal colonization of rye and wheat under identical farming practices. Along with the results from other cereals (oat and barley) at a different location, this result suggests that spot sampling, for example, on grids or gradients at a defined spatial scale, would be a sufficient strategy for larger surveys. Strongly elevated amounts of fungi in pea and field beans highlight the importance of a focused observation of cover crops, as many of them are legumes and might increase the number of fungal airborne allergens during winter.

Our pilot tests provide a means of planning monitoring programs. Two of the assays used here (*A. alternata* and *C. cladosporioides*), represent a core set of outdoor allergenic fungi (e.g., Simon-Nobbe et al., 2008) and should be complemented with additional assays, according to the environment sampled. In our example, the additions were *F. culmorum* and the *T. viride* group, known as minor allergenic fungi often reported from the sampled environment.

The present study may serve as a template for using or developing comparably built assays for individual needs as well as for extensive surveillance. Together with the MSQPCR application for indoor dust and air-sampling applications (e.g., Meklin et al., 2007), this study contributes to a comprehensive quantitative picture of the route leading from allergen-producing sources to dispersal and human contact.

5. Conclusion

The standardized protocol and suggested line of action allow rapid indication of the actual allergenic fungal potentials in any plant or soil sample. Our pilot experiments provide strong evidence for the ubiquity and quantity of major fungal allergen producers on a range of agricultural plants. The results also suggest a focus on decaying plant debris for sampling allergenic fungi during winter and the inclusion of cover crops along with a range of other crops to evaluate the abundance of outdoor allergenic fungi in monitoring programs during the growing season.

6. Outlook

Quantifying allergenic fungi is a first step toward actually defining outdoor fungal allergenic potentials. The next step forward is to collect data from different environments and to connect the presence of fungi with probabilities of their release into the air. In due course, this measure will enable developing the estimates for the risk of contact of fungal environmental allergens with human immune systems. It will thereby support recommendations for personal avoidance strategies as well as approaches to source control (Burge and Rogers, 2000) or mitigation of release by farming operations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.scitotenv.2015.05.056>.

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Supplementary Information

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SI.1 Choice of DNA-extraction method

Four techniques for soil DNA extraction were evaluated with bulk soil from twelve different plots of the Scheyern research site. Bulk soil was presumed to have the highest concentration of PCR-inhibitors (e.g. humic acids) and lowest amount of fungal DNA. Methods PCS (Töwe et al., 2011) and SV (Sagova-Mareckova et al., 2008) are phenol-chloroform based extractions optimized for soil-samples, whereas method PS and MV root on the PowerSoil DNA Isolation Kit (Mo-Bio Laboratories Inc., Carlsbad CA, USA), PS according to the manufacturer's guidelines and MV with a pretreatment of CaCO_3 to the soil (Sagova-Mareckova et al., 2008). Based on the data shown in suppl. table 1, method PS was chosen for all further analyses.

Method	DNA / μg [0,5 g dry soil]	Absorbance 260 nm / 280 nm	Absorbance 260 nm / 230 nm	Fragmentation DNA	PCR* from undiluted DNA	Handling	Costs	Total score
PCS	37.2 (59) 3	1.39 (8) 2	1.94 (7) 1	6 - 3 kbp 2	weak 1	2	4	15
SV	158 (43) 5	1.74 (4) 4	2.07 (24) 1	8 - 3 kbp 3	strong 3	2	4	22
PS	83.2 (42) 4	1.74 (3) 4	-1.00 (57) 4	8 - 6 kbp 4	overload 5	4	2	27
MV	38.1 (15) 3	1.60 (5) 3	-0.18 (16) 4	> 10 kbp 5	overload 5	2	2	24

Suppl. table 1: Evaluation of soil-DNA extraction methods. n [soil-DNA-extractions] = 12; brackets: % standard deviation; italics: score (5 = excellent, 0 = fail); *ITS1/ITS4 (White et al., 1990)

SI.2 Review and choice of qPCR assays

Primer-probe combinations for fungal groups relevant for the study were found in existing literature (Suppl. table 2). To find the necessary reference fungus for outdoor agricultural environments, oligo-sequences from candidate assays were aligned with ModelInspector (release 5.6.8.3, Genomatix Software GmbH, Munich) against GenBank database (release 184 for invertebrates and plants). Numbers of templates showing in silico amplification for the tested assays were 21 for “Geo” an assay on *Geotrichum candidum* strain UAMH7863, which is the reference fungus for MSQPCR (Haugland and Vesper, 2002), 21 for “Gcand2” on another assay on *G. candidum* (Haugland and Vesper, 2002) and 35 for “Proqu” an assay on *P. roqueforti* (Haugland and Vesper, 2002). The scarce environmental information on the hits in the searches lead us to assume an approximately equally low chance of native growth in our sampling materials soil and plant debris. For its easy handling, we selected *P. roqueforti* as reference fungus for the study.

Suitability of *P. roqueforti* as reference fungus was further tested by applying the corresponding assay on non-spiked samples (soil-root and soil-stem congeries) from 8 different wheat-plots. In agreement with the in silico results, *P. roqueforti* was not detected to considerable extents: 7 of 16 independent samples showed minimal amplifications of 180 ± 57 CE / g, being without consequences for regular conidial supplements of 4×10^5 conidia / sample.

Target organism	original name of assay	Oligo-sequences 5'-3': F (forward), R (reverse), P (probe)
<i>Alternaria alternata</i>	Aaltr*	F GCGGGCTGGAACCTC R GCAATTACAAAAGGTTTATGTTTGTCTGTA P TTACAGCCTTGCTGAATTATTACCCCTTGCTTT
<i>Cladosporium cladosporioides</i> svar.1	Cclad1*	F CATTACAAGTGACCCCGGTCTAAC R CCGGGAGGCAACAGAG P CCGGGATGTTTCATAACCCTTTGTTGTCC
<i>Trichoderma viride/atroviride/koningi</i>	Tviri*	F CCCAAACCAATGTGAACCA R TCCGCGAGGGGACTACAG P CCAAACCTGTTGCCTCGGCGGG
<i>Fusarium culmorum</i>	Fcul**	F TTGGTGTGGGAGCTGCA R CTATGGAAGCTCGACGTGACC P CCTGCTGCACTCCCAAATACATTGG
<i>Penicillium roqueforti</i>	Proqu*	F CGGGCCCGCCTTAAC R TTAAATAATTTATATTTGTTCTCAGACTGCAT P CGCGCCCGCCGAAGACA
Fungi	5.8*	F AACTTTCAACAACGGATCTCTTGG R GCGTTCAAAGACTCGATGATTAC P CATCGATGAAGAACGCAGCGAAATGC

Suppl. table 2: qPCR assays. Probes were labeled according to respective reference (5-FAM, 3-TAMRA); *Haugland and Vesper (2002), **Cullen et al. (2005), assay renamed

SI.3 performance of the selected assays

All qPCR assays were reproducibly deployed on the various sample types as shown in suppl. table 3.

False positives were detected in non-template controls and highly concentrated non-target DNA samples in late cycles (suppl. Table 3).

Assay	E ($R2 \geq 0,998$)	Independent runs of assay	Highest provoked false positives [CE / extract]
Aaltr	1.76 ± 0.02	12	66
Cclad1	1.92 ± 0.02	8	17
Tviri	$1.84 \pm 0,01$	5	277
Fcul	1.87 ± 0.00	7	50
Proqu	1.69 ± 0.03	27	29 (unprovoked: non-template control)
Uni	1.93 ± 0.02	7	not applicable

Suppl. table 3: general properties of the used assays during validation of the qPCR assays

SI.4 Statistical analyses

SI.4 A: Post-harvest time series

1. Univariate analyses of the post-harvest data

Outputs of fitted ANOVA models calculated with R (R Core Team, 2013) in summarized form; Input data were augmented by 2 and transformed to base-10 logarithms.

C. cladosporioides

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
timepoint	3	5.36	1.786	26.320	3.04e-12	***
material	3	58.10	19.367	285.422	< 2e-16	***
manag.	1	2.67	2.671	39.357	1.29e-08	***
plot	6	6.45	1.076	15.852	2.89e-12	***
timepoint:material	7	7.33	1.047	15.434	4.97e-13	***
material:manag.	3	0.70	0.234	3.451	0.0199	*
Residuals	88	5.97	0.068			

A. alternata

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
material	3	60.46	20.155	57.377	< 2e-16	***
manag.	1	2.03	2.031	5.783	0.0183	*
plot	6	14.52	2.421	6.891	5.07e-06	***
material:timepoint	10	15.39	1.539	4.381	5.45e-05	***
material:manag.	3	2.35	0.785	2.234	0.0899	.
Residuals	88	30.91	0.351			

F. culmorum

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
material	3	81.23	27.078	70.725	< 2e-16	***
manag.	1	3.92	3.924	10.248	0.0019	**
plot	6	5.74	0.956	2.497	0.0281	*
material:timepoint	10	18.36	1.836	4.796	1.74e-05	***
material:manag.	3	3.72	1.241	3.242	0.0258	*
Residuals	88	33.69	0.383			

Trichoderma viride/atroviride/koningii

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
timepoint	3	19.872	6.624	29.613	1.03e-13	***
material	3	2.017	0.672	3.005	0.0340	*
manag.	1	9.113	9.113	40.742	5.80e-09	***
plot	6	3.686	0.614	2.746	0.0165	*
Residuals	98	21.921	0.224			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

2. Multivariate analyses of the post-harvest data

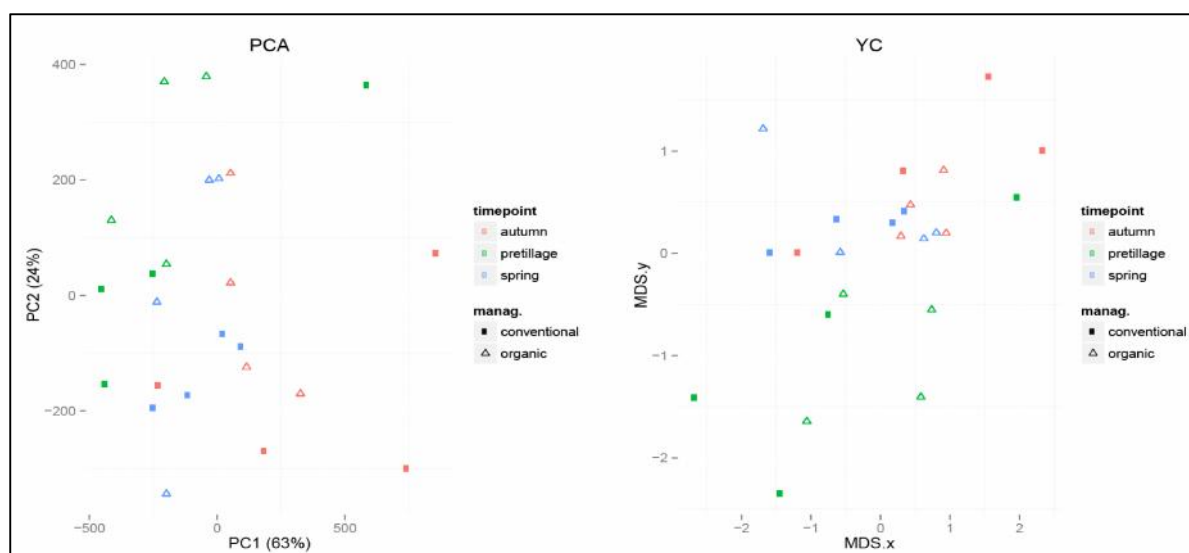
QPCR results were ordered in a way that allowed the fungal group and sampling material to be treated as effect-variables and analyzed with the Adonis function (Permutational Multivariate Analysis of Variance Using Distance Matrices) using Bray-Curtis dissimilarities and 10000 permutations both implemented in the vegan-package (Oksanen et al., 2013) for R.

Adonis results (below) and visualizations via dimension-reductions (suppl. figure 1) highlight the importance of the sampling time in relation to the likewise significant factors individual field, respectively agricultural management.

Summarized Adonis outputs:

```
      Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
timepoint  2    1.0200 0.51001  4.3775 0.28865 4e-05 ***
manag.     1    0.1837 0.18367  1.5765 0.05197 4e-05 ***
Residuals 20    2.3301 0.11651          0.65938
Total      23    3.5338          1.00000
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

      Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
timepoint  2    1.0200 0.51001  6.1999 0.28865 4e-05 ***
field      7    1.3621 0.19459  2.3655 0.38546 4e-05 ***
Residuals 14    1.1517 0.08226          0.32590
Total      23    3.5338          1.00000
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```



Suppl. figure 1:

Two visualizations of the combined variance in abundance of the tested fungi between the fields at the sampling dates: Principal Component Analysis (PCA) and Yue Clayton similarity (YC) after Kruskal's Non-metric Multidimensional Scaling. PCA using R function prcomp (stats-package 2.15.3) with square-root transformation and YC calculated as in (<http://www.mothur.org/wiki/Thetayc>, April 2014) with non-metric multidimensional scaling using isoMDS (MASS package 7.3-23).

1. Overview of pre-harvest ANOVA analyses

assay	factor	n / category (n with N = 0)	Df	mean SSq	F value	P
conventional plots						
<i>C. cladosporioides</i>	plant-section	9	1	3.781	106.797	< 0.001
<i>A. alternata</i> .	plant-section	9	1	1.152	7.756	0.015
	tillage	6	2	0.563	3.788	0.048
<i>Trichoderma</i> group	plant-section	9 (12)	1	13.035	6.15	0.027
<i>F. culmorum</i>	plant-section	9 (4)	1	17.739	17.56	< 0.001
	tillage	6 (4)	2	3.871	3.832	0.047
organically managed plots						
<i>C. cladosporioides</i>	plant-section	30	1	17.995	230.270	< 0.001
<i>A. alternata</i>	plant-section	30	1	29.059	280.932	< 0.001
	fertilizer	> 24	1	0.502	4.849	0.032
	IA plant-section:crop	30	1	0.381	3.682	0.032
wheat plots						
<i>C. cladosporioides</i>	management	> 18	1	11.046	152.99	< 0.001
	plant-section	24	1	12.018	166.46	< 0.001
<i>A. alternata</i>	management	> 18	1	14.172	102.337	< 0.001
	plant-section	24	1	10.253	74.039	< 0.001
	IA management:plant-section	> 18	1	1.2604	9.102	0.004
<i>Trichoderma</i> group	plant-section	24 (35)	1	13.052	8.105	0.007
<i>F. culmorum</i>	management	> 18 (21)	1	14.037	8.082	0.007
	IA management:plant-section	> 18 (21)	1	9.521	5.482	0.004

Suppl. table 4:

Overview of pre-harvest ANOVA analyses. Significant ($p < 0.05$) factors as seen in fitted ANOVA models (below) on log-transformed values. Df: degrees of freedom, SSq: sums of squares, IA: interaction component

2. Outputs of fitted ANOVA models

Calculated with R, presented in summarized form; input data were augmented by 2 and transformed to base-10 logarithms. As organic and conventional treatments have different plot designs, the plots were grouped into three sets for separate ANOVA, resembling comparable treatments: (1) all conventional plots, (2) all organic plots and (3) all wheat plots.

C. cladosporioides (1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
plant_section	1	3.7808	3.7808	106.7974	6.207e-08	***
tillage	2	0.0869	0.0435	1.2279	0.3226	
Residuals	14	0.4956	0.0354			

A. alternata (1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
plant_section	1	1.1518	1.15183	7.7559	0.01461	*
tillage	2	1.1250	0.56250	3.7876	0.04844	*
Residuals	14	2.0791	0.14851			

T. viride/atroviride/koningii (1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
plant_section	1	13.0345	13.0345	6.1502	0.02647	*
tillage	2	1.8319	0.9160	0.4322	0.65746	
Residuals	14	29.6712	2.1194			

F. culmorum (1)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
plant_section	1	17.7392	17.7392	17.5601	0.0009072	***
tillage	2	7.7423	3.8712	3.8321	0.0470662	*
Residuals	14	14.1428	1.0102			

C. cladosporioides (2)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
plant_section	1	17.995	17.995	230.270	<2e-16	***
fertilizer	1	0.229	0.229	2.929	0.0925	.
Residuals	56	4.376	0.078			

A. alternata (2)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
plant_section	1	29.0588	29.0588	280.9320	< 2e-16	***
fertilizer	1	0.5015	0.5015	4.8488	0.03188	*
plant_section:crop	2	0.7617	0.3809	3.6822	0.03157	*
Residuals	55	5.6891	0.1034			

T. viride/atroviride/koningii (2)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
fertilizer	1	4.280	4.2805	2.8279	0.09852	.
tillage:crop	5	16.136	3.2273	2.1321	0.07577	.
Residuals	53	80.223	1.5136			

F. culmorum (2)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
tillage	2	11.979	5.9895	3.1197	0.05179	.

Residuals 57 109.434 1.9199

C. cladosporioides (3)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
management	1	11.0461	11.0461	152.99	6.427e-16 ***
plant_section	1	12.0183	12.0183	166.46	< 2.2e-16 ***
Residuals	44	3.1768	0.0722		

A. alternata (3)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
management	1	14.1718	14.1718	102.3365	7.939e-13 ***
plant_section	1	10.2531	10.2531	74.0390	8.155e-11 ***
tillage	2	0.8889	0.4445	3.2095	0.050454 .
management:plant_section	1	1.2604	1.2604	9.1015	0.004325 **

T. viride/atroviride/koningii (3)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
plant_section	1	13.052	13.0520	8.1051	0.006627 **
management	1	2.194	2.1943	1.3626	0.249228
Residuals	45	72.465	1.6103		

F. culmorum (3)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
management	1	14.037	14.0369	8.0817	0.006875 **
tillage	2	9.789	4.8944	2.8179	0.071060 .
management:plant_section	2	19.043	9.5214	5.4819	0.007668 **
Residuals	42	72.949	1.7369		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

SI.5 Supplementary table 4: qPCR measurements used for Figure 3

Values in conidial equivalents (CE) per gram (dry material).

C. cladosporioides

	env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A		221931	189596	92529	208114	689661	1029114	187376	266025
	B		1472541	31582	189434	36047	89757	253110	236146	475972
	C		1985546	166855	493060	1082916	725535	453460	518064	692371
	D		483323	126022	253562	433416	254127	625904	91385	553759
roots	A		12481	5248	6182	7367	31598	50620	4936	19375
	B		365418	54958	95696	153298	142270	440342	88190	330342
	C		93646	22022	11857	98243	32056	86610	23835	77318
	D		40810	21943	11689	42958	20404	68723	12025	68794
root/soil	B		37775	8300	23584	30223	60772	55538	36941	81454
	C		3593	3592	1366	4729	2552	37292	2322	17701
	D		6424	7006	3804	4309	8552	10718	7335	52636
bulk soil	B		25061	1384	3229	11275	18507	50987	5195	49944
	C		764	361	417	1249	4052	3782	1143	4436
	D		3104	1497	2361	1031	1444	13463	1498	14722

A. alternata

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	5925	1520	971	2374	4256	13607	874	489
	B	22471	257	410	313	659	1675	611	1801
	C	50705	646	4102	11007	1243	4558	2290	4408
	D	4032	264	2565	2251	460	6541	415	3028
roots	A	43	18	10	17	38	179	102	27
	B	2156	170	268	3339	528	4143	504	1137
	C	2683	154	27	310	765	4009	175	368
	D	317	26	14	370	21	315	49	199
root/soil	B	431	31	59	229	225	305	372	221
	C	569	395	0	901	612	0	212	20704
	D	151	203	400	1947	260	264	1362	744
bulk soil	B	137	0	21	107	106	243	20	185
	C	0	0	0	0	0	656	0	115
	D	0	0	0	64	0	472	0	77

F. culmorum

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	34210	219738	31613	10144	105792	1924	5496	1682
	B	173921	28102	13583	15358	668	4462	18048	9713
	C	372274	39838	256401	1012017	291853	90782	121335	1936
	D	120241	62446	70344	71972	1593	415	187757	2003
roots	A	1421	1009	528	405	571	520	10	9
	B	20304	5951	3430	7806	3389	4760	5044	5387
	C	4122	897	2987	5992	1031	13697	1379	2227
	D	4762	1587	5291	1582	2059	3468	4774	183
root/soil	B	3080	1101	2086	3000	5035	920	1271	5294
	C	666	420	234	906	82	94	726	770
	D	431	930	989	705	1522	337	3910	54
bulk soil	B	1491	104	264	934	245	46	308	516
	C	33	0	2473	257	923	7	138	20
	D	1127	0	101	0	130	52	134	80

Trichoderma group

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	104	82	1122	423	10	87	55	0
	B	194	314	2172	2554	66	194	108	485
	C	1864	3311	43469	269	145	1520	297	158
	D	714	3035	673	13455	492	756	102	6873
roots	A	82	18	69	112	44	28	0	87
	B	885	389	1025	5907	854	412	108	2226
	C	1532	366	831	616	204	525	33	577
	D	535	1508	538	1537	158	224	89	268
root/soil	B	2136	1890	807	9803	1794	928	244	4897
	C	2236	2566	1487	589	243	731	145	699
	D	826	4013	1660	993	6956	289	4039	330
bulk soil	B	765	771	1148	3776	594	392	411	1287
	C	2925	505	3234	1462	656	147	61	405
	D	964	1085	1625	9815	252	774	539	1012

SI.6 Supplementary References

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