



Trichoderma Afroharzianum Ear Rot—A New Disease on Maize in Europe

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Trichoderma species are widespread filamentous fungi in soils, on plant roots and decaying plant residues. Due to their strong competitiveness and mycoparasitic activity against other fungi, particular strains of *Trichoderma* sp. are used in agriculture as biocontrol agents against plant pathogens. Commercial products based on strains of *T. harzianum* or *T. afroharzianum* have been applied to control *Rhizoctonia* spp., *Fusarium* spp., and *Phytophthora* spp. in various crops. In 2018, however, severe infections of *Trichoderma* on maize ears were recorded for the first time in a field in Southern Germany. Infected maize cobs were sampled, the fungus was isolated in pure culture and preliminarily identified microscopically as *T. harzianum*. After silk channel inoculation in the greenhouse, ear rot disease of high severity was observed. In addition to fungal colonization, the dry matter content in cobs was significantly reduced compared to water inoculated cobs. In 2018 and 2019, a total of 13 *T. harzianum* isolates from maize cobs and maize stalks were isolated and tested, for pathogenicity on maize plants in the greenhouse, compared to several reference isolates. Four isolates proved to be highly aggressive, two biocontrol isolates, Trichodex (T39), and strain T12, induced slight infection and eleven isolates were non-pathogenic. After sequencing of the translation elongation factor-1 α (*tef-1 α*) and internal transcribed spacers (*ITS*), the four highly aggressive isolates were reassigned to *T. afroharzianum*, while the commercial biocontrol isolates Trichodex (T39), and T12, as well as the other non-pathogenic strains belonged to *T. harzianum*, *T. atroviride*, or *T. tomentosum*. This, to our knowledge, is the first report on *Trichoderma* sp. as a pathogen causing ear rot disease in maize in Europe with the potential to incite significant yield losses. We therefore propose to name this disease as “*Trichoderma* ear rot on maize”.

Keywords: *Trichoderma harzianum*, *Trichoderma afroharzianum*, pathogenicity, *Trichoderma* ear rot on maize, maize

INTRODUCTION

Members of the genus *Trichoderma* are classified as imperfect fungi in the division Ascomycota and are ubiquitous in various types of soil. Some species of *Trichoderma* have biocontrol potential and can suppress pathogen growth by direct and indirect mechanisms including mycoparasitism, antibiosis, induction of host resistance and competition for nutrients and space (Kubicek et al., 2008; Jaklitsch and Voglmayr, 2015; Samuels and Hebbbar, 2015; Ghazanfar et al., 2018).

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Especially, the ability to detoxify zearalenone protects *Trichoderma* spp. from the chemical defense of *Fusarium* spp. (Popiel et al., 2014). They can thereby control and antagonize a broad range of economically important plant parasitic pathogens (Ferrigo et al., 2014; Gupta et al., 2014; Harman, 2015). In addition, they may increase plant resilience against drought conditions and promote shoot and root growth (Arora et al., 2003). Harman et al. (2004b) reported a significant yield increase in maize due to *Trichoderma* treatments. Apart from the control of root and foliar pathogens, *Trichoderma* spp. enhance nutrient solubilization and uptake as well as root and root hair development (Herrera-Estrella and Chet, 2004; Schuster and Schmoll, 2010).

Trichoderma species have been described as opportunistic, basically avirulent plant symbionts in soil (Harman et al., 2004a); however, a few reports have mentioned *Trichoderma* as ear rot pathogen on maize in the US (Munkvold and White, 2016; Wise et al., 2016; OSU., 2020). *Trichoderma* ear rot infection has been characterized by the occurrence of dark, blue-green layers of conidia on and between the kernels of infected ears causing premature germination of the kernels (Wise et al., 2016). In addition, the dry matter content of ears infected with *Trichoderma* was strongly reduced compared to uninfected ears. The occurrence of *Trichoderma* ear rot was associated with injuries caused by feeding birds or other mechanical damage in Kentucky and Ohio (Vincelli, 2014).

Surprisingly, in 2018 a severe occurrence of *Trichoderma* on the maize cobs was recorded at a field site in Southern Germany. Cobs sampled from 20 maize varieties were overgrown with mycelium producing green layers of conidia between the kernels and on the outside of the husk leaves. Similar disease symptoms have been previously observed in Southern Bavaria, after warm, and dry summers.

The aim of the present study was to identify and verify *Trichoderma* as a new pathogen causing ear rot disease on maize. Therefore, *Trichoderma*-infected cobs from four locations in Germany and France were sampled, cultured, and microscopically examined as well as analyzed by sequencing the gene for translation elongation factor-1 α (*tef-1 α*) and internal transcribed spacers (*ITS*). Furthermore, pathogenicity of *Trichoderma* isolates and the impact of infection on dry matter content of maize cobs were tested after artificial inoculation at flowering in the greenhouse.

MATERIALS AND METHODS

Fungal Isolation and Cultivation

Maize cobs and stalks were collected from naturally infected silage and grain maize in Germany in 2018 and 2019. Thirty randomly chosen kernels from each cob were surface sterilized for 10 min with 0.1% silver nitrate and placed on potato dextrose agar (PDA). The stalk samples were cut in nine slices, surface sterilized as described above and placed on PDA plates. After 2 days, mycelium outgrown from the sample was transferred to PDA plates. Single conidia cultures were produced, and isolates were stored on synthetic low nutrition agar (SNA) plates at 4°C.

Inoculation Procedure

Spores from single spore cultures were transferred to PDA plates containing antibiotics (400 μ g/ml streptomycin, Duchefa Biochemie, Haarlem, The Netherlands; 30 μ g/ml rifampicin, AppliChem, Darmstadt, Germany) and incubated under Near-UV-light ($\lambda = 340\text{--}400$ nm) at 23°C in a growth chamber. After 2 weeks, sterile water was added to plates and conidia were scraped off with a microscope slide. The conidia suspension was then filtered through gauze and cell density was measured with a Thoma haemocytometer and adjusted to 1×10^6 conidia per ml. Primary ears of maize plants were inoculated seven days after silk channel emergence (BBCH 65). For this purpose, 1 ml of conidia suspension was injected with a syringe (Braun, Melsungen, Germany) into the silk channel between the cob tip and the point where silks emerge from the husk.

Plant Cultivation and Pathogenicity Assessment on Maize Ears

Maize seeds of two varieties were sown in a mixture of soil (potting soil/compost/sand mixture of 1:2:1) in 20 cm diameter pots. Pots were placed in the greenhouse at 23°C under a seasonal day-/night light cycle. Five plants per isolate were inoculated by silk channel injection and five additional plants were inoculated with water, as control. Thirteen *Trichoderma* isolates originally isolated from maize cobs in the field were compared to four reference strains of *T. harzianum* (IPP0318, IPP0319, IPP0320, T12), one type strain of *T. afroharzianum* (CBS 124620) and one strain of *T. atroviride* (IPP0316) obtained from different fungal collections (see **Table 1**). In addition, one commercial biocontrol isolate, T39 (Trichodex), and one isolate, T12, with potential biocontrol activity (kindly provided by the Department for Phytopathology of Justus-Liebig-University, Gießen, Germany) were tested. Four weeks (28 dpi) after inoculation, husk leaves of inoculated and control ears were removed, and disease severity was assessed visually according to the EPPO guidelines (European Mediterranean Plant Protection Organization., 2015) as the percentage (0–100%) of the cob surface covered with fungal mycelium. Finally, cobs were weighed and dried for 5 days at 60°C to assess the dry matter content. In addition, isolates were re-isolated and cultured on PDA to confirm the Koch's Postulate.

DNA Extraction and Phylogenetic Analysis

Total DNA was extracted from lyophilized mycelium of single spore cultures by using a CTAB-based method described by Brandfass and Karlovsky (2008). Quality and quantity of the extracted DNA was assessed by agarose gel electrophoresis (60 min at 4.6 V/cm) and stained with ethidium bromide. Partial translation elongation factor-1 α (*tef-1 α*) and internal transcribed spacer (*ITS*) were used to differentiate within the *Trichoderma harzianum* complex. Amplification was performed in a peqSTAR96 universal gradient thermocycler (PEQLAB, Erlangen, Germany) using 1:100 dilution of DNA extract in a total reaction volume of 25 μ l. Marker genes *tef-1 α* and *ITS* were amplified with the primers EF1 (ATGGGTAAGGARGACAAGAC) and EF2 (GGARGTACCAGTSATCATGTT) (O'Donnell et al.,

TABLE 1 | Host, geographic locations, and year of isolation of *Trichoderma* isolates used in this study.

Isolates	Species	Year	Location	Source
Tri1	<i>Trichoderma afroharzianum</i>	2018	Croix de Pardies (F)	<i>Zea mays</i> (cob)!
Tri2	<i>Trichoderma afroharzianum</i>	2018	Kuenzing (D)	<i>Z. mays</i> (cob) !
Tri3	<i>Trichoderma afroharzianum</i>	2018	Pocking (D)	<i>Z. mays</i> (cob)!
Tri4	<i>Trichoderma tomentosum</i>	2018	Altoetting (D)	<i>Z. mays</i> (cob)!
Tri5	<i>Trichoderma afroharzianum</i>	2019	Bernburg (D)	<i>Z. mays</i> (cob)!
IPP0316	<i>Trichoderma atroviride</i>	1976	–	Baby food
IPP0318	<i>Trichoderma harzianum</i>	1992	Mae Hia (T)	Soil
IPP0319	<i>Trichoderma harzianum</i>	1992	Chiang Mai (T)	Soil
IPP0320	<i>Trichoderma harzianum</i>	1992	Mae Hia (T)	Soil
Tri6	<i>Trichoderma harzianum</i>	2019	Kleinwanzleben (D)	<i>Z. mays</i> (cob)
Tri7	<i>Trichoderma harzianum</i>	2019	Grucking (D)	<i>Z. mays</i> (cob)
Tri8	<i>Trichoderma harzianum</i>	2019	Loeningen (D)	<i>Z. mays</i> (cob)
Tri9	<i>Trichoderma harzianum</i>	2019	Loeningen (D)	<i>Z. mays</i> (cob)
Tri10	<i>Trichoderma harzianum</i>	2019	Großumstadt (D)	<i>Z. mays</i> (cob)
Tri11	<i>Trichoderma harzianum</i>	2019	Pfaffenhofen (D)	<i>Z. mays</i> (cob)
Tri12	<i>Trichoderma harzianum</i>	2019	Pfaffenhofen (D)	<i>Z. mays</i> (stalk)
Tri14	<i>Trichoderma harzianum</i>	2019	Pfaffenhofen (D)	<i>Z. mays</i> (stalk)
T12*	<i>Trichoderma harzianum</i>	–	–	–
T39 (Trichodex)	<i>Trichoderma harzianum</i>	–	Tel Aviv (ISR)	–
CBS 124620	<i>Trichoderma afroharzianum</i>	–	Peru	<i>T. cacao</i>

F, France; D, Germany; T, Thailand; ISR, Israel; IPP-fungal collection of the Plant Pathology Division, University of Goettingen; CBS-fungal collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, NL; *provided by the Department of Phytopathology of Justus-Liebig-University, Gießen, Germany; ! (exclamation mark) indicating samples with *Trichoderma* disease symptoms.

1998) and ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al., 1990), respectively. Reactions were carried out in a mixture of standard Taq reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3 at 25°C; NEB), 100 μM of each deoxyribonucleoside triphosphate, 0.3 μM of each primer, 0.62 U HotStart-polymerase (New England Biolabs), and 1 μL template DNA solution. The final MgCl₂ concentration was adjusted to 2 mM. The PCR cycling conditions for amplification of *tef-1α* included an initial denaturation for 30 s at 95°C; 30 cycles consisting of 30 s at 94°C, 30 s at 58°C, and 1 min at 68°C; and final extension for 5 min at 68°C. The PCR cycling conditions for amplification of *ITS* included an initial denaturation for 30 s at 95°C; 10 cycles consisting of 30 s at 94°C, a gradual decrease from 62°C to 53°C (–1°C/cycle) for 40 s, and 1 min at 68°C; 30 cycles of 30 s at 94°C, 40 s at 56°C, and 1 min at 68°C; and final extension for 5 min at 68°C. Species were identified by multiple alignment of each sequence with reference sequences using ClustalW (Thompson et al., 1994) in MEGA Version 7.0.2 (Kumar et al., 2016).

Statistical Analysis

Statistical analysis was conducted with STATISTICA version 13 (Statistica GmbH, Germany). Differences between means of disease severity was analyzed using the non-parametric Kruskal-Wallis ANOVA by 5% probability. Analysis of variance (ANOVA) for dry matter content was carried out, followed by Tukey's-HSD-test at the 5% probability level.

RESULTS

Geographic Origin of Samples

In 2018, four isolates from four locations (Altoetting, Pocking, Kuenzing and Croix de Pardies) were obtained from maize cobs in Germany and France and subjected to further identification. In 2019, ten cobs from six locations, mainly from southern Germany, especially Bavaria, and along the Rhine valley (Bernburg, Kleinwanzleben, Grucking, Loeningen, Großumstadt, and Pfaffenhofen), were examined. In addition, two isolates from maize stalks from a single location (Pfaffenhofen) were obtained. Isolates Tri1, Tri2, Tri3, and Tri5 were isolated from maize cobs displaying strong *Trichoderma* infection, whereas the other *Trichoderma* isolates were obtained from cobs or stalks that did not induce any visual disease symptoms.

Species Identification

A molecular phylogenetic analysis was performed with thirteen *Trichoderma* isolates from maize cobs and six isolates obtained from fungal culture collections, based on partial *tef1α* (Figure 1) and *ITS* (Supplementary Figure 1). Sequences of 17 species in the *T. harzianum* complex (Chaverri et al., 2015) were selected from GenBank (Clark et al., 2015). Multiple gene alignments assigned eight isolates (Tri6-Tri12, Tri14) to *T. harzianum*, four isolates (Tri1-Tri3, Tri5) to the clade *T. afroharzianum*, and one isolate to *T. tomentosum*. Strains IPP0318-0320 were verified as *T. harzianum* and IPP0316 as *T. atroviride*. Isolates designated as *T. harzianum* clustered in two separate groups highly supported



FIGURE 1 | Molecular phylogenetic analysis of translation elongation factor 1 alpha (*tef-1 α*) by maximum likelihood method (1,000 bootstrap replicates) (Tamura et al., 2013). The analysis was performed with MEGA version 7.0.26 (Kumar et al., 2016) with partial *tef-1 α* sequences. The strains are listed in **Table 1**; GeneBank accession numbers of the sequences are shown in brackets. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values are presented next to the nodes. Individual accession numbers are presented in **Supplementary Table 1**. Sequence alignment is available at TreeBASE (<http://purl.org/phylo/treebase/phylows/study/TB2:S26786>).

by bootstrap values. This result was largely confirmed by analysis of *ITS* sequences. Isolates Tri6-Tri12 and Tri14 showed high similarity to the selected references of *T. harzianum*, however, two isolates (T12 and T39) formed a separate clade, together with the reference sequence of *T. harzianum* CBS 226.95 (AY605833). All obtained sequences of *T. afroharzianum* clustered in one phylogenetic group highly supported by bootstrapping and showed 99.4% (523 out of 526) nucleotide similarity to the *tef-1 α* sequence of *T. afroharzianum* CBS124620 (FJ463301). Newly obtained sequences were deposited at Genbank under the accession numbers MT793725 to MT793743 for *tef-1 α* and

MT793744 to MT793762 for *ITS*, and sequence alignments were lodged at TreeBASE (<http://purl.org/phylo/treebase/phylows/study/TB2:S26786>).

Disease Symptoms and Severity on Maize Ears

Trichoderma strains obtained from maize cobs displayed typical characteristics of this genus on PDA plates, such as initial growth of white mycelium, soon turning into green, and gray-green colonies, while the reverse side of the culture plates stayed uncoloured or light yellow. *Trichoderma* ear rot infection is

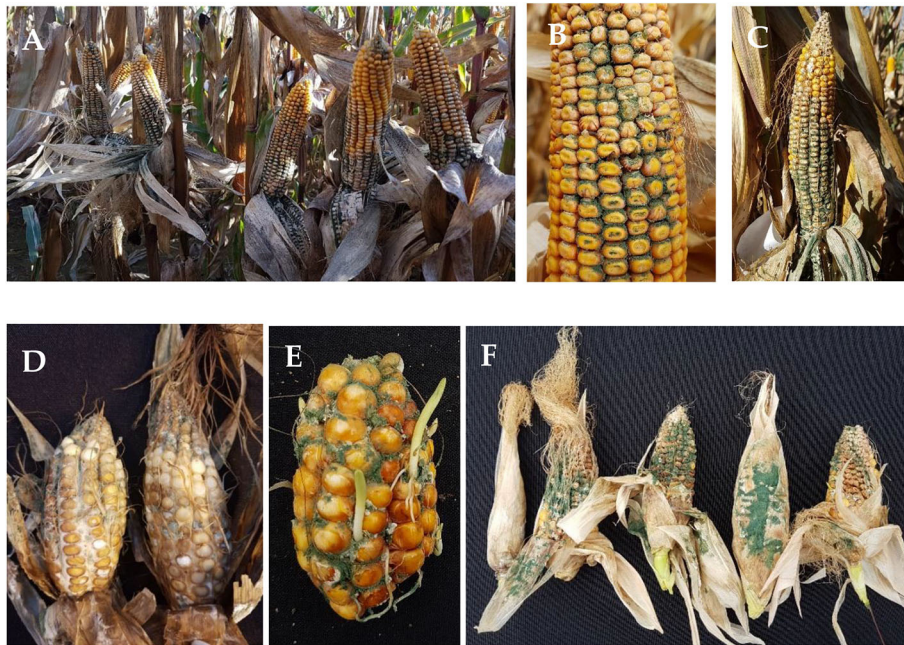


FIGURE 2 | Disease symptoms of natural infection with *Trichoderma* sp. on maize cobs in the field, (A) at Künzing, (B) at Bernburg, (C) and at Altoetting; (A–C); and after silk channel inoculation in the greenhouse (D–F).

characterized by white mycelium growing between the kernels and on the husk leaves with massive production of green to gray-green conidia. Under natural infection in the field, symptoms occurred from the base to the middle part of the cob, covering all kernels, and all layers of husk leaves (Figures 2A–C). No mechanical damage or injuries by birds or insects were observed on infected cobs. After inoculation of ears in the greenhouse, the whole cob, inside and outside of the husk leaves was covered with mycelium, and a green layer of conidia (Figures 2D–F). Some infected cobs in the field as well as in the greenhouse showed premature ripening of the kernels (Figures 2C,E).

After artificial inoculation of the cob in the greenhouse, six isolates were pathogenic on maize ears and ten isolates did not induce any infection. The highest disease severity was observed with *T. afroharzianum* isolates Tri5 (96.0%), Tri1 (94.0%), Tri2 (91.1%), and Tri3 (78.0%), which overgrew the kernels and husk leaves (Table 2). Isolates T12 and T39 (Trichodex) lead to greenish discoloration only at the tip of the cob. The reference strains of *T. afroharzianum* (CBS 124620), *T. harzianum* (IPP0320, IPP0318) and the two remaining strains (IPP0316 and Tri4) as well as the *T. harzianum* isolates from maize cobs (Tri6, Tri7, Tri8, Tri10, and Tri11) were non-pathogenic on maize. Non-pathogenic strains did not cause any significant reduction in dry matter content compared to water treated, non-infected control cobs. However, the three most aggressive strains Tri2, Tri1, and Tri5 of *T. afroharzianum* caused significant losses in dry matter content of cobs 28 days after inoculation (Table 1).

DISCUSSION

Although *Trichoderma* is known as a plant symbiont or antagonist of fungal phytopathogens, our findings support previous observations in the US (Munkvold and White, 2016; Wise et al., 2016) that *Trichoderma* can infect maize cobs and cause ear rot diseases. Molecular-phylogenetic analysis of partial translation elongation factor-1 α (*tef-1 α*) and internal transcribed spacer (*ITS*) genes revealed pathogenic isolates as *T. afroharzianum*. To the best of our knowledge this is the first report on *T. afroharzianum* as an ear rot pathogen in Europe. The disease symptoms described in this study are in agreement with the observations by Wise et al. (2016) indicating that infection of *Trichoderma* appeared as white mold associated with massive production of green or gray-green conidia between the kernels and husk leaves, often involving the entire ear and causing premature ripening of the kernels. In contrast to the report by Munkvold and White (2016), *T. afroharzianum* infection, in the present study, did not require any previous damage on husk leaves and was not associated with injuries from birds or insects. Furthermore, artificial inoculation studies in the greenhouse confirmed that high disease severity occurred without any mechanical wounding after silk channel injection. Therefore, we conclude that *Trichoderma* ear rot infection is not a result of, or promoted by mechanical injuries. This new pathogen cannot be regarded as a wound or opportunistic pathogen, as it is clearly able to infect kernel and husk leaf tissue and cause disease without any previous damages. We therefore propose to use the name “*Trichoderma* ear rot on maize” for this disease.

TABLE 2 | Disease severity (%) and dry matter content (%) of maize cobs, 28 days after inoculation.

Isolate	Disease severity [%]	Dry matter content [%]
Water		
Control	0.0 ± 0.0 ^a	47.9 ± 4.6 ^a
T. harzianum		
IPP0320	0.0 ± 0.0 ^a	48.5 ± 2.5 ^a
Tri6	0.0 ± 0.0 ^a	46.5 ± 13.5 ^a
IPP0318	0.0 ± 0.0 ^a	46.2 ± 6.7 ^a
Tri7	0.0 ± 0.0 ^a	41.6 ± 4.7 ^a
T39	11.0 ± 6.5 ^b	40.4 ± 12.4 ^a
Tri11	0.0 ± 0.0 ^a	39.1 ± 12.9 ^a
Tri8	0.0 ± 0.0 ^a	37.2 ± 6.1 ^a
Tri10	0.0 ± 0.0 ^a	36.7 ± 7.9 ^a
T12	14.0 ± 10.8 ^b	36.7 ± 5.8 ^a
T. afroharzianum		
CBS 124620	0.0 ± 0.0 ^a	39.7 ± 6.0 ^a
Tri1	94.0 ± 5.4 ^d	26.6 ± 4.1 ^b
Tri2	91.1 ± 10.5 ^d	20.9 ± 7.7 ^b
Tri3	78.0 ± 22.8 ^c	34.3 ± 6.8 ^{ab}
Tri5	96.0 ± 8.9 ^d	20.3 ± 6.0 ^a
Others		
IPP0316 (<i>T. atroviride</i>)	0.0 ± 0.0 ^a	49.9 ± 15.0 ^a
Tri4 (<i>T. tomentosum</i>)	0.0 ± 0.0 ^a	45.7 ± 13.2 ^a

Means are given with standard deviation. Letters a,b,c,d, ab indicate significant differences between the isolates ($\alpha \leq 0.05$).

Highest disease severity and highest losses of dry matter content were observed after inoculation with three *T. afroharzianum* (Tri1, Tri2, Tri3, and Tri5) strains; however, the reference type strain of *T. afroharzianum* CBS 124620 did not induce any disease symptoms on maize but may have lost its pathogenicity during cultivation for over 15 years. Otherwise, this may indicate a phylogenetic separation on the species or subspecies level of pathogenic and non-pathogenic strains. Further research is required to clarify the genetic differences between these strains within the *T. afroharzianum* cluster. Surprisingly, a low-level pathogenicity of the two *T. harzianum* strains which were considered for use as biocontrol agents was recorded. Although these strains are usually applied to the soil for control of soilborne diseases, the pathogenicity found in our greenhouse experiment with cob inoculation cannot be ignored with regard to risk analysis in the registration of *Trichoderma* strains as biocontrol agents. The question whether beneficial *Trichoderma* strains could mutate into aggressive plant pathogens is difficult to determine at this stage and requires further research.

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Besides these findings, several epidemiological aspects about *Trichoderma* ear rot remain to be elucidated. Firstly, it is not known how the conidia of *Trichoderma* reach and infect the maize ears under natural conditions in the field, which sources of inoculum exist, and whether there are any alternative hosts. Secondly, there are no reports so far about the effect of weather conditions or agronomic practices possibly favoring infection with *Trichoderma*. The aggressive strains of *T. afroharzianum* were restricted to warmer regions with enhanced maize production in Southern Germany and along the Rhine valley. This is consistent with our previous observations indicating enhanced *Trichoderma* infection in the field in years with high mean temperatures and low precipitation such as in 2018 and 2019 in Germany. Furthermore, the potential production of mycotoxins by aggressive strains of *Trichoderma* awaits examination. Finally, field monitoring to explore the spread of the disease in Europe and yield loss analyses under field conditions are required to assess the economic significance of *Trichoderma* ear rot disease in maize production.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AP: conceptualization, resources, and writing—original draft preparation. AP and SS: methodology, validation, investigation, data curation, and visualization. AT, PK, and SS: writing—review and editing. AT: supervision. AT and PK: project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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