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Endophytic Fungal Root Colonization of *Eragrostis tef* in Eroded Croplands of the Ethiopian Highlands Is Limited by Low Spore Density and Fertilisation

Gezahagn Getachew [1,2], Boris Rewald [2,*], Douglas L. Godbold [2] and Hans Sandén [2]

[1] Sirinka Agricultural Research Center, P.O. Box 74, Woldia, Ethiopia; ggezahagn@gmail.com
[2] Institute of Forest Ecology, Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, Vienna (BOKU), Peter-Jordan-Straße 82, 1190 Vienna, Austria; douglas.godbold@boku.ac.at (D.L.G.); hans.sanden@boku.ac.at (H.S.)
* Correspondence: boris.rewald@boku.ac.at; Tel.: +43-1-47654-91219

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Abstract: *Eragrostis tef* (teff) is a (sub-)tropical cereal crop and a staple food in Eastern Africa. As soil erosion has become increasingly worse in the Ethiopian highlands, we test the hypotheses that (1) eroded soils possess low arbuscular mycorrhizal fungi (AMF) spore densities, (2) teff growth is limited by low endophytic fungal root colonization rates and (3) colonization rates and spore densities are additionally reduced by fertilization. A pot experiment was set up to study the effect of cropland soil inoculation using pristine fungal communities (from adjacent forests) or fertilization. AMF spore densities in soil with and without teff and root colonization by AMF and dark septate endophytes (DSE) were related to straw and grain yields. AMF and DSE colonization rates were higher after inoculation, which provides evidence that a low spore density limits teff root colonization in eroded soils. However, teff yields were significantly increased after fertilisation but not inoculation. N-P fertilization further lowered root colonization rates and spore density. We conclude that forest soils serve as a refugium for soil biota in the degraded landscape of the Ethiopian highlands. As both increased AMF and DSE increase the stress resistance of plants, their inoculation potential should be considered when developing sustainable management methods for teff.

Keywords: Arbuscular mycorrhiza; Colonization rates; Dark septate endophytes; *Eragrostis tef* (teff); Erosion; Ethiopian Highlands; N-P fertilizer; Spore density

1. Introduction

Eragrostis tef (teff) is a warm-season, annual cereal crop that is primarily grown in Ethiopia and Eritrea. Teff accounts for about a quarter of total cereal production in Ethiopia [1,2], but it is also increasingly cultivated in Kenya, South Africa, Australia and across the Americas. Teff is a valued forage crop for its high palatability, nutritive value, yield, rapid growth and drought resistance [3]. In the region, it is mainly used for making "Injera", a traditional flat bread, and accounts for more than half of the daily protein intake by Ethiopian people [4]. Teff grains are increasingly popular around the globe because of their gluten-free nature, low glycaemic index and beneficial nutrient composition—holding high fiber, protein, iron and calcium contents [3,5]. Additionally, teff straw is used as an alternative source of forage and is frequently incorporated into traditional construction materials in Ethiopia and the wider eastern African area [6].

Teff can be grown in a wide range of agro-ecological zones, including arid and semi-arid areas that are prone to water stress and heat [3,6,7]. In Ethiopia, agricultural systems are based on natural rainfall. Thus, drought-tolerant teff varieties [8] are widely used over other C4 grasses, such as maize,

in drought-prone areas, although the average grain yield of teff remains low. Its adaptability to cooler climates, but not frost, allows it to grow at altitudinal ranges between 800 and 3200 m a.s.l [9]. The Ethiopian dry lands in general (which account for 67% of the country's total land area) and the agriculture sector in particular have been identified as being vulnerable to climate variability and land degradation [10]. Land degradation is a serious problem in Ethiopia, especially in the Amhara region and other highland areas, as soils are highly susceptible to rainfall erosion, which results in their productivity having been largely diminished over time [11,12]. Church forests, i.e., small patches of relatively undisturbed forests around churches and monasteries, are the only Ethiopian highland areas with organic-rich soils that feature a high biological productivity [13].

Apart from water scarcity and suboptimal management practices, low teff yields are attributed to nutrient deficiencies, mainly of nitrogen (N) and phosphorous (P) [14,15]. Both nutrients are dramatically reduced in plant availability on fields where the nutrient rich top soil has been eroded [13,16]. Therefore, many teff farmers in Ethiopia use N-P fertilizers, with teff accounting for 54% of the total fertilizer applied in the grain production of Ethiopia, but not always in recommended amounts [17,18]. In general, teff has low nutrient use efficiencies and the nitrogen use efficiency (NUE) for fertilized teff ranges from 17% to 61% [14]. However, soil quality depends not only on physical and chemical soil properties, but also on the diversity and activity of beneficial soil biota [19]. Although physical erosion processes are well documented, only a few studies have attempted to quantify losses of soil biota by erosion [20], which has hampered a better understanding of links between land use, erosion and ecosystem services.

In particular, mycorrhizal fungi are known to be of direct importance for nutrient uptake and drought resistance of many crops [21,22]. Specifically, arbuscular mycorrhizal fungi (AMF) are important for crop plants, e.g., through mobilizing P under limited conditions, and thus, enhancing aboveground biomass and yields across the tropics, including Ethiopia [23,24]. Although AMF inoculation has previously been shown to increase the growth and yield of teff in low pH soils from Scotland [25], information on AMF colonisation rates of teff roots and the growth dependency of teff on AMF colonisation under realistic production conditions, e.g., in eroded Ethiopian soils, is virtually non-existent.

Similar to AMF, dark septate endophytes (DSE; "Class 4 endophytes"), a group of heterogeneous root-associated endophytic fungi characterized by melanized intercellular and intracellular runner hyphae and micro-sclerotia, can form associations with plants and are increasingly observed in the root cortex of other plant species, often in parallel to AMF [26,27]. While much less is known on the characteristics of DSE compared to AMF, they appear to be ubiquitous in occurrence and abundant across various ecosystems, including high-stress environments. Furthermore, there is increasing evidence that (some) DSE facilitate plant growth and survival [28–30]. However, little is still known about the detailed role of DSE and even their occurrence in important (crop) species, such as members of the Poaceae, and tropical ecosystems is not well documented [27,31,32].

In Ethiopia, specifically in the highland areas, severe productivity losses are caused by soil erosion [12]. However, it remains open if (1) the eroded croplands in the Ethiopian highlands are also depleted of AMF spores and other potentially growth-promoting endophytic fungi, such as DSE, and (2) to what magnitude the assumed lack of symbiotic endophytes affects straw and grain yields of teff. We hypothesize that the growth of teff increases significantly if cropland soils are inoculated with soil from adjacent forest stands by increasing beneficial fungal colonization; remnant church forests could thus act as important refugia for soil biota in a highly degraded landscape. We further hypothesize that teff growth increases under recommended N-P fertilisation regimes although this fertilisation will affect colonisation rates of beneficial entophytic fungi and AMF spore densities.

2. Material and Methods

2.1. Plant and Soil Material

Eragrostis tef (Zucc.) Trotter, commonly known as teff, belongs to the family Poaceae, subfamily Eragrostoidae. The seeds of the white-coloured *Eragrostis tef* var. 'Quncho' were obtained from

the Adet Agricultural Research Centre, Adet, Ethiopia. 'Quncho' is a hybrid of two varieties; var. 'DZ-01-974' that has a high yield and the var. 'DZ-01-196', which is popular for its very white seed colour, but has low productivity. Officially released in 2006, the teff var. 'Quncho' is utilized by farmers and seed producers [33] and is currently one of the most popular teff varieties in the region.

Soil was collected manually from three eroded croplands and adjacent pristine forest stands (i.e., "church forests") in the Amhara region, North West Ethiopia, between 13th to 17th July 2015. The three sites were located at Gelawdios (11°38′25″ N, 37°48′55″ E), Tara Gedam (12°8′47″ N, 37°44′45″ E) and Katassi (11°0′05″ N, 36°44′8″ E); Gelawdios and Tara Gedam are situated in South Gondar and Katassi in the Awi administrative zones. Taking the environmental heterogeneity of the Amhara region into account, the three cropland sites were chosen from different agro-ecological zones and possess different soil properties (Table S1). Gelawdios is situated in the highland (Dega) parts while Katassi and Tara Gedam are located in a mid-altitude range (Woina Dega). At each site, croplands and forest stands were chosen adjacent to each other (i.e., 150–180 m distance) for sampling. The detailed information on the effects of land use and deforestation on the soil properties of Gelawdios and Tara Gedam sites can be found in the study by Assefa et al. [13]. At each of the six locations, soil material was sampled from the top soil (5–10 cm) at 12 randomly chosen points, with forest soils sampled at a minimum distance of 50 m from the forest edge. Composite samples per site were prepared by thorough mixing, packed in plastic bags (avoiding compression) and transported to Bahir Dar.

2.2. Experimental Set up

The research was conducted at the Amhara Regional Agricultural Research Institute (ARARI), Bahir Dar, Ethiopia in an insect-free net house (0.425 mm mesh size) under natural climatic conditions. The average air temperature in the experimental period (July 23 to November 07, 2015) was 22 °C; the min./max. air temperature was 10 °C/28 °C; and the relative humidity was 59–72%. The amount of precipitation was 396 mm in July, 375 mm in August, 211 mm in September, 87 mm in October and 12 mm in November 2015.

Three soil treatments were established per site: (1) Cropland soil (Control), (2) Cropland soil mixed with forest soil (Inoculated) and (3) N-P fertilized cropland soil (Fertilized). Forest soils were used as a source of AMF and other soil organisms for inoculation, with cropland soils blended with the corresponding forest soils at a rate of 5% (vol/vol). Fertilizer application followed recommendations for teff production in the region [3], i.e., 130 kg ha^{-1} of di-Ammonium phosphate (DAP), applied at the day of sowing, and 36 kg ha^{-1} of Urea fertilizer, applied at tiller emergence. Thus, N was added at a rate of 40 kg ha^{-1} rate and P at a rate of 26 kg ha^{-1} rate to the fertilized treatment. The 3-L pots were filled with soil at a natural bulk density of 1.2 g cm^{-3}, with holes at the bottom of the pots allowed for free drainage. Fifteen pots were established per site (3) and treatment (3), of which 5 pots were sown with teff at a seeding rate of 5 kg ha^{-1} (0.02 g per pot) on July 23. The plants were germinated on July 30, 2015. One week after germination started, plants were thinned in order to ensure an equal and realistic plant density of 30 plants pot^{-1}. Five pots per site were left unplanted ("bare soil") to study the effect of teff plants on AMF spore densities. All pots were watered by natural precipitation until September when additional irrigation water was homogeneously applied as needed. The pots were controlled for pests and weeded on a daily base. In total, 90 pots were arranged randomly with a distance of 10 cm between pots.

2.3. Harvest and Soil Sampling

On November 7, 2015, a harvest was conducted to determine straw (stem and leaves) and grain yield, AMF and DSE root colonization rates (45 pots) and AMF spore densities in the soil (90 pots; 45 pots with teff and 45 bare pots). To gather root material and soil samples, the substrate was carefully removed from the pots and placed separately on plastic sheets. Root systems were subsequently collected by hand (from the pots with teff), rinsed carefully with tap water and preserved in 70% (v/v)

ethanol until further analysis. The soil was air dried before being sieved (2 mm) for homogenization and to remove remaining root fragments. Subsequently, approximately 50 g of the soil pot^{-1} was subsampled, packed in paper bags and transported to Austria. To determine straw and grain yields, the shoots were packed in paper bags by pot, dried (70 °C, 48 h), separated into straw (stems and leaves) and grains and subsequently weighed (± 0.001 g).

2.4. AMF and DSE Colonization Rates and AMF Spore Density

The method of Vierheilig et al. [34,35] was used to stain AMF and DSE, which involved using ink with acetic acid. The effectiveness of this staining method compared to the Trypan blue technique [36] was determined in a pre-test. Six segments, which had a length of 2 cm, were randomly selected and dissected from each root sample, keeping a minimum distance of 2 cm to the tip. A total of 270 teff root segments were used for staining. The stained roots were mounted on a microscope slide using polyvinyl-lacto-glycerol and screened for structures of arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSE) using a microscope at 400–1000× magnification (Axiophot, Carl Zeiss, Oberkochen, Germany). Visual criteria used to differentiate AMF and DSE in teff roots are shown in Figure 1. The colonization rate of AMF (i.e., percentage of arbuscules, + % vesicles, + % AM hyphae) and DSE (percentage of micro-sclerotia, + % dark septate hyphae) per unit root length was determined by using a magnified intersection method without any modifications [37]. Total colonization was calculated as the sum of colonization percentage of the different morphological structures (Figure 1) and therefore, colonization rates >100% were calculated in a few (3) cases when the structures were overlapping.

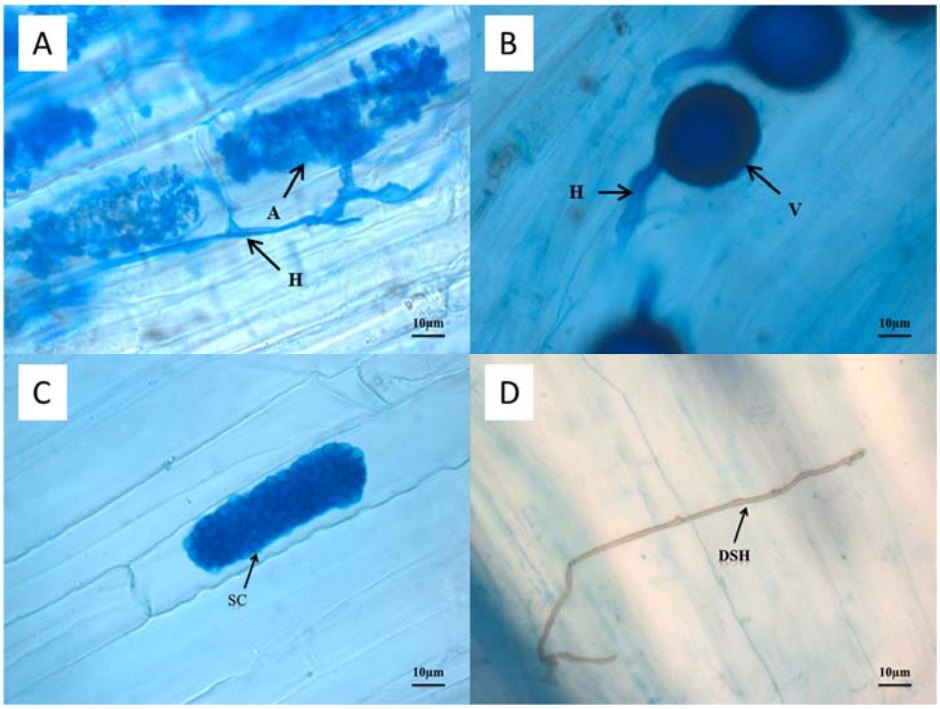

Figure 1. Arbuscular mycorrhizal fungi (AMF; **A**,**B**) and dark septate endophyte (DSE; **C**,**D**) structures used for identification and differentiation of both fungal types in *Eragrostis tef* roots. Labels: arbuscules (A), vesicles (V), AMF hyphae (H); Micro-Sclerotium (SC) and DSE hyphae (DSH). Size bars are 10 µm.

Arbuscular mycorrhizal fungal spores were extracted from approximately 5 g of the soil following Brundrett et al. [38] and counted under a stereo microscope at 10–23× magnification (Stemi 2000CS with Axiocam ERC5S, Zeiss, Germany). AMF spore density was calculated as the number of spores per g dry soil^{-1}. Spores were sorted by morphological criteria and photographed for subsequent, approximate taxa determination.

2.5. Statistical Analysis

All statistical analyses were conducted using R 3.2.2 Software [39]. As no significant differences were found between parameters in each of the three sites/soil types × three treatments (two-way ANOVA, data not shown), the data are subsequently presented as the mean of the three sites by treatment (n = 15). The Shapiro-Wilk test was used to test for normality. One-way ANOVA followed by a posthoc Tukey HSD test ($p < 0.05$) was conducted to determine significant treatment effect. A two-way ANOVA was used to test for general treatment effects on spore density in pots with and without teff. A non-linear correlation was used to relate AMF colonization rates and AMF spore densities. Means and standard errors (mean ± SE) are presented throughout the manuscript.

3. Results

3.1. AMF Spore Density in Soil

Arbuscular mycorrhizal fungal (AMF) spore density was highest in the inoculated treatment and lowest in the fertilized pots (two-way ANOVA; $p < 0.001$). Thus, the spore density increased with inoculation and decreased with fertilization relative to the control (Figure 2). In general, AMF spore densities were greater in pots with teff plants compared to pots with bare soil. The largest increase inf spore densities with teff plants, compared to bare soil, occurred in the inoculated pots (31%). The most abundant spore taxa across all treatments were *Glomus* sp., *Gigaspora* sp. and *Entrophospora* sp. (data not shown). No significant differences of spore densities were found between the three cropland sites (data not shown).

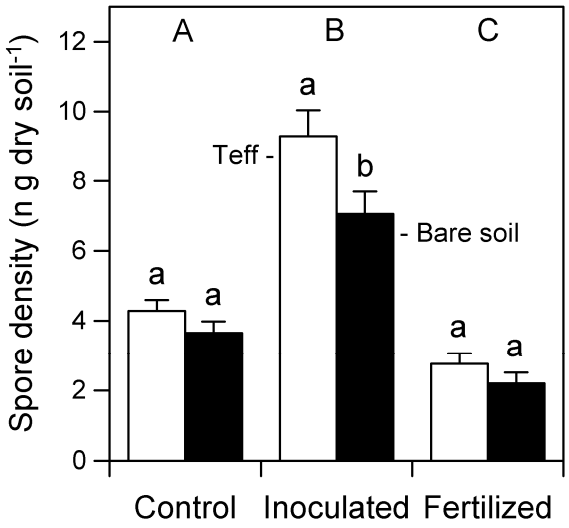

Figure 2. Density of arbuscular mycorrhizal fungal (AMF) spores (n per g dry soil^{-1}) in cropland soil (Control), cropland soil inoculated with forest soil (Inoculated) and N-P fertilisation (Fertilized) with *Eragrostis tef* plants (open bars) and without plants (i.e., bare soil; filled bars). Different capital letters indicate significant differences between treatments (two-way ANOVA, $p < 0.05$) while different small letters indicate significant differences between pots with teff or bare soil within the same treatment (TukeyHSD, $p < 0.05$); values are the average of three soil types of five replicates each (n = 15; mean + SE).

3.2. AMF and DSE Root Colonization Rates

The arbuscular mycorrhizal fungal (AMF) colonisation rate of teff roots was about 92 ± 13% in the inoculated cropland soil, which was significantly greater compared to both the fertilized (26 ± 6%) and the control treatments (71 ± 11%; Figure 3). The application of N-P fertilizer significantly reduced the AMF colonization compared to the control treatment by 63%. The dark septate endophyte

(DSE) colonisation rates of teff roots were lower than AMF colonisation rates although they were still significantly greater in the inoculated soil compared to both fertilised and control treatments. No significant differences were found between the fertilized and control treatments (Figure 3). No significant differences for both AMF and DSE colonisation rates were found between sites (data not shown).

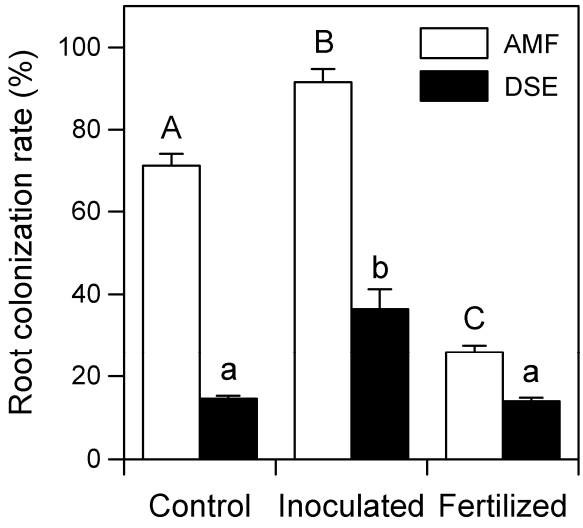

Figure 3. Colonization rates (%) of arbuscular mycorrhizal fungi (AMF, open bars) and dark septate endophyte (DSE, filled bars) on *Eragrostis tef* roots in cropland soil (Control), cropland soil inoculated with forest soil (Inoculated) and after N-P fertilisation (Fertilized). Different capital/small letters indicate significant differences between treatments; values are the average of three soil types of five replicates each (TukeyHSD, $p < 0.05$, n = 15; mean + SE).

The AMF colonization rate was positively and significantly correlated with the spore density (Figure 4). The colonization rate reached about 100% at a spore density of 8–10 spores per g dry soil.

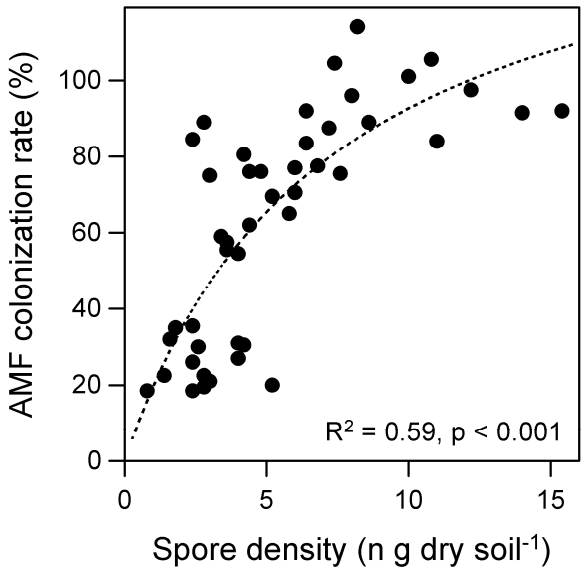

Figure 4. Correlation between AMF colonization rates (%) of *Eragrostis tef* roots and AMF spore densities (n per g dry soil^{-1}) across three treatments (i.e., cropland soil, cropland soil inoculated with forest soil and after N-P fertilisation). Colonization rates >100% are caused by overlapping of different morphological structures (see Materials and Methods).

3.3. *Teff* Biomass and Grain Yield

The grain and straw yield of teff was significantly greater in fertilized soils compared to the inoculated and control treatments. Both parameters were not statistically significant between inoculated and control treatments (Figure 5). No significant differences were found between sites (data not shown).

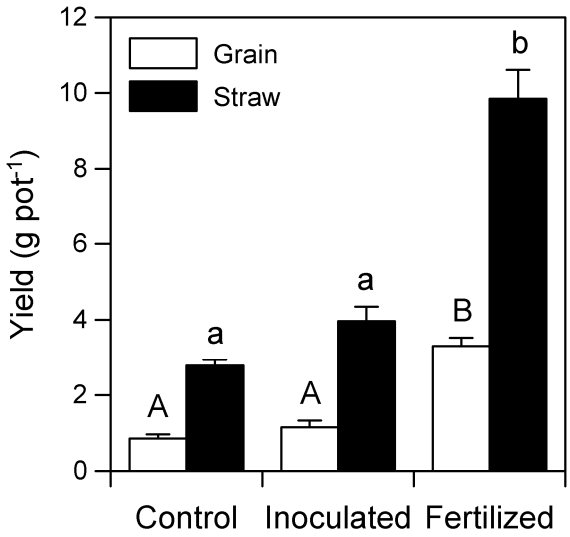

Figure 5. Grain (open bars) and straw yield (g pot^{-1}; filled bars) of *Eragrostis tef* (30 plants pot^{-1}) in cropland soil (Control), cropland soil inoculated with forest soil (Inoculated) and N-P fertilisation (Fertilized). Different capital/small letters indicate significant differences between treatments; values are the average of three soil types of five replicates each (TukeyHSD, $p < 0.05$, n = 15; mean ± SE).

4. Discussion

The soil of Ethiopian church forests contained a high number of AMF spores as evidenced by the almost doubled spore density in cropland soils inoculated with a small amount of forest soil (5% v/v) and not planted with teff (Inoculated, bare) compared to the cropland soil (Control, bare) (Figure 2). Consequently, the sampled forest soil contained almost 20 times more AMF spores per volume than the adjacent agricultural soil. Thus, church forests can be considered to be important refugia for AMF spores in the highly degraded landscape of the Ethiopian highlands. A similar, but less distinct, positive effect of (older age) exclosures on AMF spore densities has been reported recently for soils of the Tigray region, Ethiopia [40]. The high(er) AMF spore content in forest/exclosure soils is likely the result of a more (persistent) vegetation cover and a greater plant diversity in combination with less soil disturbance and greater carbon contents in the top layer [13,40]. The top layer that is rich in organic matter has been eroded in the adjacent cropland, exposing the much poorer subsoil. For Katassi and Gelawdios croplands, Sr/Ca- and Ba/Ca-ratios suggest that >30 cm of the top soil has been eroded [13]. Although we did not find any differences in the relative proportion of dominant AMF spore taxa—spores of *Glomus* sp., *Gigaspora* sp. and *Entrophospora* sp. occurred most frequently across treatments (data not shown), further (genetic) analyses of the soil fungal community composition are needed.

The fertilized cropland soil contained significantly less spores than the control at the end of the experiment (two-way ANOVA) (Figure 2). A lower spore density after fertilizer application has been reported frequently [41–44] and is usually associated with a lower spore production rate as mycorrhizal endosymbionts receive less C when nutrient availability is higher for plants [45,46]. However, in our study, the AMF spore density tended to also decrease in fertilized pots without teff plants (Fertilized, bare) compared to the control without teff (Control, bare). This indicates that

the spores degraded/decomposed faster with fertilization, further decreasing the AMF inoculation potential of the cropland soils in addition to a lower spore production rate.

Mamo and Killham [25] previously showed that the AMF root colonization rates of *Eragrostis tef* ranged between 30–60% in Scottish soil depending on pH levels. While this range is similar to our findings in control and fertilized cropland soil, the AMF colonization rate of teff roots increased significantly after inoculation (Figure 3). The colonization rate was positively correlated to the spore density (and *vice versa*) and this approached 100% at a spore density of about 8–10 spores per g soil (Figure 4). This corresponds to a spore density without teff that was about 7–8 spores per g soil (Figure 2). In the eroded cropland soil (Control), the spore density was thus only about half of the density needed to enable a 100% colonization rate of teff roots. The low spore density in the cropland was probably caused by fertilizing, soil disturbance and reduced soil organic matter contents by erosion and ploughing as well as longer periods without vegetation cover [47,48]. The AMF spore densities found in this study (2–9 spores per g dry soil) are comparable to other studies from dry tropical ecosystems, although the reported values vary largely due to methodological uncertainties (sampling time etc.) and site-specific factors, such as land use history, soil properties and plant community composition. For example, AMF spore density was reported as 0.05–64.0 per g soil in a savannah valley of the Chinese dry tropics [49] and 40–130 per g soil for another cropland site of the Amhara region, Ethiopia [50].

While considerable uncertainties exist regarding the reproduction biology of dark septate endophyte (DSE) [27,31], the colonization rate of roots DSE increased with inoculation (Figure 3). For example, this indicates that the frequencies of DSE mycelia fragments or conidial spores may limit DSE colonization in the studied cropland soils. However, it is interesting that no negative effect of fertilizer application on the colonization rate by DSE was found in contrast to AMF. We hypothesize that the carbon allocation to DSE is less affected by the increase in nutrient availability for the plant. However, further studies are urgently needed to clarify the involvement of different DSE species in plant C dynamics.

N-P fertilization strongly increased biomass (straw) and yield (grains) of teff plants, indicating that teff has limited nutrient when growing in the eroded cropland soils of the Amhara region (Figure 5). In contrast to our hypotheses, inoculation and subsequently increased AMF (and DSE) root colonization rates did not have a significant effect on aboveground biomass production or yield. However, a greater colonization by mycorrhiza (>25%) and 3× greater spore production rates are accompanied by higher C costs for plants. On average, AMF symbionts use about 10% of the host plants' C budget in addition to greater root respiration rates [51]. However, higher mycorrhiza colonization rates have been frequently shown to increase plant resistance to stressors, such as drought and heavy metals [52], as well as pathogens and herbivores [53]. As teff plants under the given experimental conditions grew under relatively well watered conditions in a weed and herbivore free environment, an increased stress resistance could be an advantage in situ only. This might be similar for DSE where increased colonization rates are increasingly related to stress resistances and sustained growth [54–56]. Field experiments are needed to test these hypotheses. We do not suggest that forest soil material should be transferred to croplands on a large scale as the few remaining church forests are very limited. However, as high spore densities and colonization rates of endophytic fungi seem desirable for improving the stress tolerance of teff, future measures to develop sustainable methods for teff production should take artificial inoculation into account after soil erosion has been minimized.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/2/73/s1, Table S1 Environmental and soil parameters of the three sampled cropland sites at Gelawdios, Tara Gedam and Katassi in the Ethiopian highlands.

Author Contributions: Conceptualization, G.G., B.R., H.S. and D.L.G.; Formal analysis, G.G. and H.S.; Funding acquisition, D.L.G.; Methodology, G.G. and H.S.; Visualization, B.R.; Writing—original draft, G.G., B.R. and H.S.; Writing—review & editing, B.R., G.G., H.S. and D.L.G.

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Conflicts of Interest: The authors declare no conflict of interest.

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