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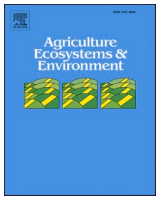

Landuse affects the likelihood of soil colonization by a key plant pathogen

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ABSTRACT

Fencing and other biosecurity measures can help to reduce the spread of soil-borne pathogens, but are often compromised by weather, animals and insects. Once contaminated soil spreads beyond a farm, neighbouring land can either help or hinder pathogen dispersal based on its susceptibility to colonization. Fusarium wilt of banana, caused by the soil-borne pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*), poses a serious threat to global banana production. In the Wet Tropics Region of Queensland, where most of Australia's bananas are grown, we found that banana production land is mostly bordered by rainforest, grassland, and sugarcane production areas. In soil inoculation experiments using quantitative PCR, we found that *Foc* was highly likely to colonize banana soils, moderately likely to colonize sugarcane soils, and unlikely to colonize rainforest or grassland soils, suggesting that rainforest and grassland may act as natural barriers against *Foc* spread. When sterilized soils were inoculated, *Foc* proliferated to high levels regardless of landuse, indicating that biotic factors underpin the differential response of landuses to *Foc* colonization. Differences in the extent and likelihood of *Foc* soil colonization between soils were associated most strongly with the soil fungal and bacterial community composition and fungal:bacterial biomass ratio. Based on our findings, we propose that future work should explore the use of ground covers, soil amendments and other strategies to improve soil suppressiveness to *Foc*. Together, our findings offer valuable insights for land managers and demonstrate the importance of rainforest and grassland soils in limiting *Foc* spread across the landscape.

1. Introduction

The fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is the causal agent of Fusarium wilt of banana – one of the most devasting diseases of *Musa* spp. worldwide, for which there are no effective control measures (Dita et al., 2018). While the development of new chemicals (Cannon et al., 2022) and *Foc*-resistant banana varieties (Chen et al., 2023; Dale et al., 2017) may offer future solutions, it is also important to find ways to minimize the growth and survival of *Foc* in infested soils, and its likelihood of colonizing new areas (Munhoz et al., 2024). In this context, biosecurity measures such as farm access control and sanitation can be invaluable; however, the movement of *Foc* via animals, insects, floods and other factors is almost impossible to avoid (Pegg et al., 2019). Hence, to predict rates of pathogen dispersal across the landscape, it is necessary to consider the factors that contribute to the growth and survival of *Foc* in soils post-introduction, both within banana production areas and in neighbouring landuses.

Current evidence indicates that some soils are disease suppressive. Suppressive soils are those where a pathogen fails to establish or persist; establishes but does not cause disease; or initially causes disease then declines over time (Schlatter et al., 2017). Most studies of *Foc* suppressive soils have focussed on banana production areas in which the disease is already widespread. In these studies, suppression is typically inferred based on low observed disease incidence. For example, potential *Foc* suppression has been studied in soils associated with: 1) non-symptomatic vs. symptomatic plants (Fan et al., 2023; Jamil et al., 2022; Nisrina et al., 2021, Rodríguez-Yzquierdo et al., 2023; Shen et al., 2015; Zhou et al., 2019); 2) fields exhibiting low vs. high disease (Ou et al., 2019; Shen et al., 2015); and 3) experimental treatments that differ in disease incidence (Hong et al., 2020; Ren et al., 2024; Wang et al., 2022). While this approach has identified a range of factors associated with disease incidence, *viz.* microbial diversity, biomass and

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community composition, it often remains unclear whether these factors are causal in suppressing the pathogen or have arisen in response to the pathogen.

A more direct approach to assess the ability of a soil to suppress the establishment or survival of a pathogen is to inoculate a soil with the pathogen and quantify its population over time. For example, by adding *Foc* to different soils, Ou et al. (2019) were able to demonstrate that *Foc* survival was significantly lower in soils exhibiting low disease incidence. This approach has proven effective for the assessment of pathogen growth and survival in soils, and by extension, may be useful to determine the likelihood of *Foc* colonizing new areas.

Most of Australia's banana production occurs in the Wet Tropics of North Queensland (Fig. 1a), which is a UNESCO world heritage-listed biodiversity hotspot (Harvey et al., 2016). At present, the incidence of *Foc* Tropical Race 4 (TR4), which affects the most commercially important banana varieties in the region, is limited to a small number of sites, where diseased plants have been detected early and destroyed. Within the region, banana farms border ancient rainforests, semi-natural grasslands, sugarcane farms and other landuses (DSITI., 2016) (Fig. 1a, b). As the capacity of these soils to harbour or suppress *Foc* is currently unknown, it is not possible to predict areas of vulnerability to the banana industry within the landscape. Therefore, understanding the bio-defensive capacity of landuses adjacent to banana plantations may help to improve disease management. Additionally, identifying the factors that contribute to *Foc* suppression may offer opportunities to manipulate soils to reduce the growth and persistence of *Foc* in soil. Previously, we demonstrated that the biomass and diversity of soil bacterial and fungal communities within this region differ significantly between landuses (Birt et al., 2024), but the consequences of these differences for invasive organisms are unknown.

In this study, we investigated the ability of *Foc* to colonize soils collected from North Queensland banana farms, and from the region's three main neighbouring landuses: rainforest, grassland, and sugarcane. Soils were inoculated with *Foc* or a mock treatment and then the *Foc* load was measured at 28 days post inoculation using quantitative PCR (qPCR). A subset of each soil was also sterilized with gamma (γ) radiation to facilitate comparisons of *Foc* colonization in dead (sterilized) vs. living (non-sterilized) soils. To contextualize our results, we then investigated whether changes in *Foc* load were significantly associated with the biomass, diversity or composition of soil bacterial and fungal communities. Our objectives were to explore the impact of landuse on *Foc* colonization of soil and to identify putative drivers of observed differences in *Foc* colonization between soils. Our results have important implications for land management practices and pathogen dispersal across the landscape.

2. Materials and methods

2.1. Identification and quantification of landuses bordering banana polygons

The digital landuse map of the Wet Tropics Region of Northern Queensland segments the area into landuse polygons based on the Australian Landuse and Management (ALUM) classifications within the Queensland Landuse Mapping Programme (QLUMP). To determine which landuses border banana production areas within the region, the 'Landuse mapping - 1999–2015 - Wet Tropics NRM region' dataset (DSITI., 2016) was downloaded from the Queensland Spatial Catalogue (Queensland Government, 2024). Using the R package *sf* (Pebesma and Bivand., 2023), we created a 100 m buffer zone around the perimeter of each banana polygon and identified the landuses present within these buffer zones. We then calculated the sum of the area occupied by each landuse within all buffer zones and reported the data as total area (ha) and percentage of total area (%). The results from this analysis informed which landuses to investigate for the present study.

2.2. Designation of sampling sites within landuses and soil sample collection

In August 2019, soils were collected from banana farms as well as from the predominant landuses bordering banana polygons, which were rainforest, grassland and sugarcane. Within a 47.5 × 56.4 km zone in the Wet Tropics Region, North Queensland (Fig. 1a, b), eight sites per banana, rainforest, grassland or sugarcane (Table S1) were selected using the create random point tool in ArcGIS Pro 2.4.2 (ESRI Inc. 2019) (Fig. 1c). Within each site, nine sample locations were marked out 1 m apart in a 3 × 3 grid format. At each of these locations, a 0–10 cm depth soil core was collected using an auger and pooled into a bag. The auger was cleaned with bleach and flame sterilized between sites. Soils were transported on ice to a laboratory where they were homogenized into a composite sample for each site, passed through an 8 mm stainless steel sieve, and then split into subsamples for different analyses. Some soil was used for DNA metabarcoding and PLFA analyses, the results of which are reported in our previous study (Birt et al., 2024). The remaining soil was used for the present study as outlined below.

2.3. Experimental setup

The experimental setup is summarized in Fig. 2. Soil was sampled from four landuses, with eight sites per landuse, four experimental treatments per site, and two timepoints per experimental treatment. For

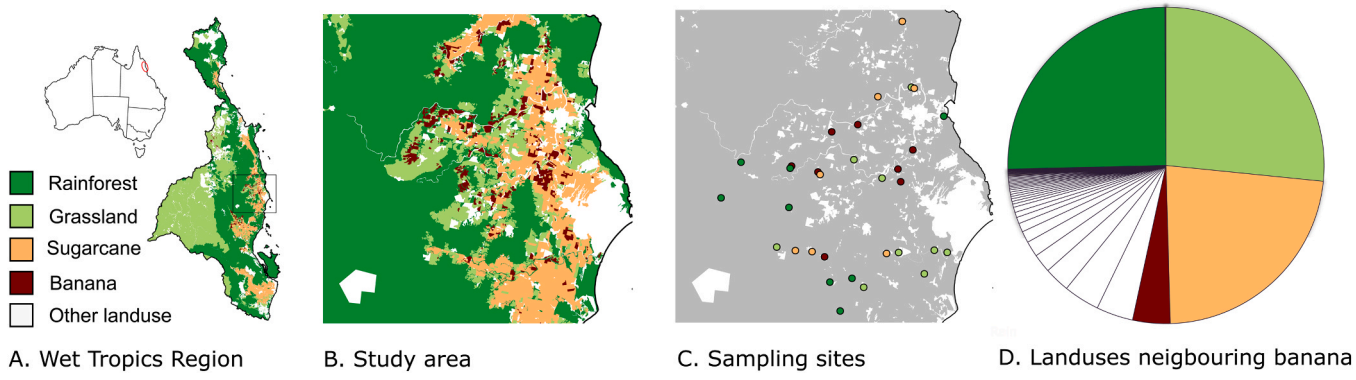

Fig. 1. Study area, landuse distribution, sampling sites and banana-bordering landuses in the Wet Tropics Region of Queensland. (A) The location of The Wet Tropics region is circled in red on the map of Australia. The map of the Wet Tropics Region shows the distribution of polygons classified as rainforest, grassland, sugarcane or banana landuses, as defined in Table S1. The black square indicates the study area. (B) Enlargement of the study area showing the distribution of the four landuse polygons. (C) The sample sites allocated by random stratified sampling for each landuse in the study area. (D) The proportion of area assigned to each landuse within a 100 m perimeter of each banana polygon in the Wet Tropics Region. A breakdown of all landuses bordering banana polygons is listed in Table S2.

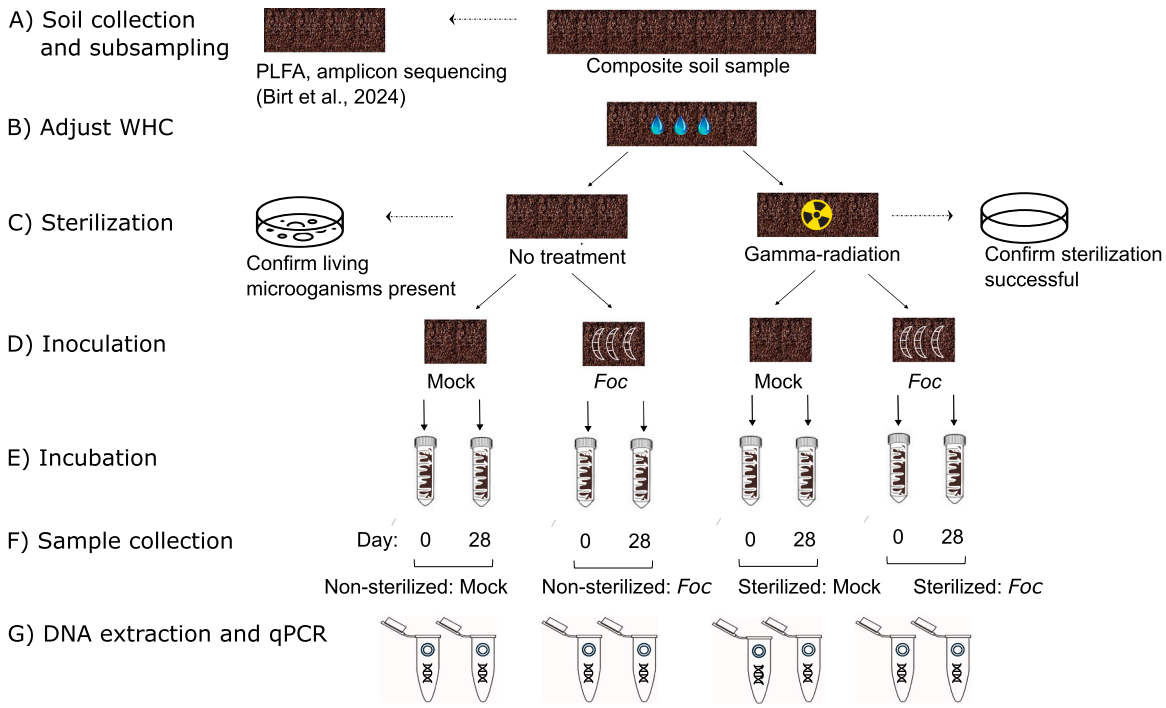

Fig. 2. Summary of experimental methods. (A) For each study site, soil cores from nine subsites were pooled, homogenized and sieved to < 8 mm to form a composite sample. The composite sample was split into two, with one part used for phospholipid fatty acid analysis (PLFA) and DNA metabarcoding (16S and ITS) as published previously (Birt et al., 2024) and (B) the other part adjusted to 60 % water holding capacity (WHC). (C) The WHC-adjusted sample was then split into two, with one half subjected to gamma (γ) radiation and the other half left untreated. Sterilization and non-sterilization status of the irradiated or untreated soils were validated by confirming a lack or presence of microbial growth, respectively, on agar plates at 7 days post incubation. (D) Soils were then inoculated with water (mock) or a *Foc*R1 spore suspension (*Foc*) and (E) immediately transferred to falcon tubes covered with pierced parafilm where they were incubated at 25ºC in the dark. (F) At 30 mins post inoculation, the Day 0 sample was collected and stored at −80 ºC. At 28 days post incubation, the Day 28 sample was collected, frozen at −80 ºC and then all samples were lyophilized and homogenized. (G) DNA was extracted from 500 mg of each soil, with an internal plasmid control added to the DNA extraction buffer to correct for DNA extraction efficiency and PCR inhibition differences. Quantitative PCR (qPCR) assays were performed on each treatment to quantify the *Foc*R1 copy number normalized to the DNA recovery of the internal plasmid control.

each site, the soil, which was adjusted to 60 % water holding capacity, was divided into two subsamples. One subsample was gamma irradiated to sterilize the soil microbiome, while the other was left untreated. This approach aimed to compare the effects of a dead (sterilized) microbiome versus a living (non-sterilized) microbiome on *Foc* colonization. The sterilized and non-sterilized soils were then further split into two and inoculated with either *Foc* or a mock treatment. The four treatments for each site included: non-sterilized mock inoculated soil, non-sterilized *Foc*-inoculated soil, sterilized mock inoculated soil and sterilized *Foc*-inoculated soil. Soils were harvested at 30 min and 28 days post inoculation and subjected to qPCR.

2.3.1. Soil sterilization

Soils were sterilized by gamma irradiation (35 kGY over a 3.5-day period) (McNamara et al., 2003). To test the efficacy of the sterilization, 1 g of sterilized soil was combined with 10 mL of sterile water and 200 µL of this solution was spread on a 1/10 tryptic soy agar (TSA) plate. No colonies were observed at one week following incubation, confirming that the sterilization treatment was effective.

2.3.2. Inoculation

A *Foc* R1 BRIP43996 mycelial agar plug was transferred to potato dextrose broth (PDB) and incubated at 25°C on a shaker at 130 rpm for four days. The culture was filtered through sterile cheesecloth (muslin) and the filtered solution was centrifuged at 1900 rcf for 10 min. The pellet was rinsed and resuspended in sterile water, then adjusted to 1250 spores / mL using a haemocytometer. Soils (sterilized and non-sterilized) were inoculated with either *Foc* (200 µL spore suspension g soil^{-1}) or for the mock treatment, water only (200 µL g soil^{-1}). The inoculated soils were placed in 50 mL falcon tubes covered with pierced parafilm and maintained at 25°C in the dark (Fig. 2).

2.3.3. Sample harvesting

At 30 mins (Day 0) and 28 days (Day 28) post-inoculation, soils from each treatment were collected, freeze-dried and homogenized in the TissueLyser II (Qiagen) at 30 Hz for 5 mins.

2.4. DNA extraction and qPCR

2.4.1. DNA extraction

DNA was extracted from all samples as previously described (Deng et al., 2010), albeit with the addition of a plasmid containing an artificial 16S rRNA gene sequence (Daniell et al., 2012) which acted as a standard to facilitate estimation of DNA extraction efficiency and PCR inhibition. Briefly, 500 mg dry soil was added to 1 mL extraction buffer (0.12 M Na_2HPO_4, 1 % SDS) supplemented with 3 × 109 copies of the artificial reference plasmid, which we refer to as pMUT16S (Daniell et al., 2012). After homogenization using a TissueLyser II (Qiagen) at 30 Hz for 2 min, the homogenate was centrifuged at 16,000 rcf for 1 min to remove debris. DNA was purified with phenol: chloroform:IAA followed by chloroform:IAA. To precipitate the DNA, 0.1×3 M Na-acetate and 1x isopropanol was added to the aqueous phase, incubated at −20°C for 20 min and centrifuged at 16,000 rcf for 5 min. The DNA pellet was then rinsed with 75 % ethanol and resuspended in 100 µL water. To remove inhibitors, the resuspended DNA was purified using a QIAquick Gel extraction kit (Qiagen) according to the manufacturer's instructions and eluted in 50 µL water. DNA diluted 1 in 10 was used as the template for qPCR.

2.4.2. Plasmid purification

Escherichia coli cultures transformed with either pMUT16S (Daniell et al., 2012) or pGEM-T plasmid carrying the *Foc*-specific DNA directed RNA polymerase subunit III gene (Orr et al., 2022) were grown in Luria broth (LB) supplemented with ampicillin (100 μg/mL) and shaken at 37°C for 15 h. DNA was extracted with an ISOLATE II Plasmid Mini Kit (Bioline) and then quantified using a Qubit fluorometer (Thermo Fisher), according to the respective manufacturer's instructions.

2.4.3. Quantitative polymerase chain reaction (qPCR)

The primers used to amplify pMUT16S were MUT4-F (CCTACGGGAGGCACGTC) and MUT4-R (ATTACCGCGGCTGGACC) (Tim Daniell, pers. comm); and those used to amplify *Foc* R1 were RTLinVI_F3 (5'-GACATTTGACGACTTTCTGA-3') and *Foc*LinVI-R (5'-GTGTCACTTGGTCCTCGTAT-3') (Matthews et al., 2020). All qPCRs were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Each qPCR consisted of 2 μL template, 1X SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and 0.3 μM (MUT16S) or 0.5 μM (*Foc* R1) of each primer made up to a total volume of 10 μL with molecular biology grade water. Thermocycling conditions for the qPCRs were as follows: an initial denaturing step at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 66°C s for 20 sec. The melt curve cycle was as follows: 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. Standard curves comprised 10-fold serial dilutions of the standards from 10^8 to 10^1 copies (Mut16S) or 10^6 to 10^1 copies (*Foc*R1). Three technical replicates were performed for all qPCRs using a Viia7 Instrument (Applied Biosystems) and quantified against the standard curves using QuantStudio™ Real-Time PCR Software, Version 1.2. Amplicon specificity was confirmed via inspection of the melt curves. *Foc* R1 copy number was then converted to copies g^{-1} dry soil and corrected for DNA recovery as follows:

DNA recovery = pMUT16S copies detected by qPCR / pMUT16S copies input

Normalized *Foc*R1 copies = *Foc*R1 copies g^{-1} dry soil / DNA recovery

2.5. Bacterial and fungal biomass and diversity indices

To test hypotheses regarding the influence of soil bacterial and fungal biomass, diversity and community composition on *Foc* soil colonization, we used data previously collected from the same soils (Birt et al., 2024). Briefly, the biomass of bacterial and fungal communities was estimated using phospholipid fatty acid analysis (PLFA), where bacterial biomass was inferred from the sum of c.14.0, c.15.0, c.17.0, c.15.0_iso, c.15.0_anteiso, c.16.0_iso, c.17.0_anteiso, c.17.0_iso, c.14.0_2OH, c.16.1_w7c, c.17.1_iso_w9c, c.18.1_w7c, c.18.1_w5c, c.16.0_10.methyl, c.17.1_w7c_10.methyl, c.18.0_10.methyl, and fungal biomass was inferred from the sum of c.18.2_w6c, c.18.1_w9c and c.16.1_w5c (Buyer and Sasser, 2012). The diversity and composition of bacterial and fungal communities were characterised using 16S rRNA and ITS2 gene amplicon sequencing, respectively. Sequence data were processed using a modified USEARCH pipeline and then 97 % operational taxonomic unit (OTU) tables were rarefied to 11,000 bacterial and 3500 fungal reads per sample for further analyses (Birt et al., 2024). Bacterial and fungal OTUs were assigned Greengenes 2.0 (McDonald et al., 2023) and Unite v8.3 (Abarenkov et al., 2024) taxonomy, respectively. To provide a measure of bacterial and fungal alpha diversity, we calculated the numbers of observed OTUs in each sample. As measures of bacterial and fungal beta diversity, we performed distance-based principal coordinate analyses (db-PCA) using Hellinger transformed OTU relative abundances. The site scores along the first and second db-PCA axes were then used as our beta diversity metrics.

2.6. Statistical analyses

Firstly, using R package *lmerTest* (Kuznetsova et al., 2017), linear mixed effects models were implemented to determine whether the main and interactive effects of treatment: 'inoculation' (*Foc* or mock), 'sterility' (sterilized or non-sterilized), 'time' (Day 0 or Day 28) and/or 'landuse' (rainforest, grassland, sugarcane, banana) were significantly associated with *Foc* copy number. In these models, the response variable was log-transformed *Foc* R1 copies g^{-1} dry soil, the fixed effects were 'inoculation', 'sterility, 'time' and 'landuse', with a random effect for 'site'. Three-way linear mixed effects models were then performed on subsets of the data sorted according to time (Day 0 or Day 28), inoculation (*Foc* or mock) or sterility (sterilized or non-sterilized) to determine the main and interactive effects of the remaining variables. Next, we focussed on Δ*Foc* (*Foc* R1 marker gene copies at Day 28 minus those at Day 0) as the response variable. Generalized linear models (GLM) were performed using a gaussian error distribution to determine whether Δ*Foc* differed significantly between landuses within subsets of data sorted according to sterility and inoculation, i.e.: 'Non-sterilized: Mock', 'Non-sterilized: *Foc*', 'Sterilized: Mock', 'Sterilized: *Foc*'. To calculate a likelihood of *Foc* colonizing soil, we also performed GLMs with a binomial distribution after converting the Δ*Foc* values to zeros or ones (Δ*Foc* $\leq$ 0 copies = 0; Δ*Foc* > 0 copies = 1). The effects of univariate soil biotic factors on Δ*Foc*, and of landuse on univariate soil biotic factors were assessed using GLMs (gaussian and binomial). *Post hoc* lettering was generated using Tukey's Honest Significance Tests with R package *eemeans* (Lenth et al., 2020). To predict the chance of soil colonization based on the univariate soil biotic variables in each landuse, the logit link function of the logistical model equation was rearranged to allow prediction of the soil colonization probability from the predictor variable as follows:

$$p = \exp(mx+c) / (\exp(mx+c) + 1)$$

where: p = probability of soil colonization; m = slope, x = predictor variable; c = intercept.

Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001), as implemented in *vegan* (Oksanen et al., 2022) was used to determine whether the composition of bacterial and fungal communities differed between landuses. Prior to analysis, OTU relative abundances were Hellinger transformed. We then generated distance-based principal component analysis (db-PCA) ordinations for the Hellinger transformed bacterial and fungal OTU tables and extracted the scores along the primary and secondary axes for each the site. These were used as univariate metrics representing bacterial and fungal beta diversity. The influence of community composition on Δ*Foc* was then assessed using GLMs (gaussian and binomial). To identify OTUs that were correlated with suppression, OTUs were sorted based on their db-PC1 score. Lastly, a correlation matrix containing biotic parameters and Δ*Foc* was generated in R (corrplot) and p values were generated with Pearson's correlation analysis. To compare the strength of the biotic parameters in predicting Δ*Foc,* parameters were z-score standardized to a mean of zero and standard deviation of one. A linear regression was then performed using Δ*Foc* as the response variable and the standardized biotic parameters as the predictors. The slope of the linear regression was used to predict the value of Δ*Foc* based on an increase of one standard deviation in the biotic variable.

3. Results

3.1. Banana production areas in the Wet Tropics are bordered predominantly by rainforest, grassland and sugarcane production landuses

To determine which landuses border banana production areas in the Wet Tropics Region of Northern Queensland, we asked which QLUMP categories occupy the perimeter area (100 m buffered) bordering banana polygons. The main landuses within the perimeter area were rainforest (25.3 %), grassland (26.6 %), sugarcane (23 %), or banana (3.8 %) with the remaining (21.3 %) comprising many other landuses (Fig. 1d, Table S2).

3.2. Baseline Foc levels

At the start of the experiment (Day 0), qPCR indicated the presence of low background levels of *Foc* in non-sterilized, mock-inoculated samples (Fig. 3a). These values did not differ between landuses (Table S3). Furthermore, while a lack of growth on culture plates indicated that gamma-radiation successfully sterilized the soils, *Foc* qPCR counts at Day 0 did not differ significantly between the mock-treated soils, irrespective of sterility (Fig. 3a, b; Table S3). Indeed, the only treatment that significantly influenced *Foc* counts at the beginning of the experiment was the addition of *Foc*, which as expected, led to an increase in *Foc* counts (Fig. 3 and S1, Table S3).

3.3. Foc surged in sterilized-inoculated soils but not in other treatments

Foc counts increased significantly over time in the sterilized, *Foc*-inoculated soils, and by Day 28 were orders of magnitude larger than those in other treatments (Fig. 3d). In contrast, there was no significant change in *Foc* counts over time in the sterilized, mock-treated soils (Fig. 3b, Table S3). In sterilized soils at Day 0, the *Foc*-inoculated samples contained 7.7-fold more *Foc* than mock-treated samples. By Day 28, however, this difference had surged to 33,249-fold. In all sterilized soils there were no significant differences in *Foc* counts between landuses, irrespective of time and inoculation (Table S3).

In non-sterilized soils, our results indicate that the *Foc* count was significantly influenced by the interactive effects of landuse, inoculation and time (Table S3). By Day 28, *Foc*-inoculated banana soils had more *Foc* than those from other landuses (Fig. 3c). Background *Foc* levels in mock-treated banana soils, however, were also larger than in other landuses at Day 28 (Fig. 3a), albeit not significantly.

3.4. Changes in Foc over time were associated with landuse in non-sterilized soils

To better understand the dynamics of *Foc* colonization, we focussed on the non-sterilized *Foc*-inoculated soils, for which we calculated the difference in *Foc* count between the start and the end of the experiment (ΔFoc = *Foc* copies at Day 28 – *Foc* copies at Day 0) for each site. These values indicated that *Foc* copies decreased over time in rainforest and grassland soils, remained similar in sugarcane soils, but increased for banana soils (Fig. 4a). The differences between landuses were significant ($P < 0.05$, Table S4), with Tukey *post hoc* analyses indicating that banana differed from the other landuses (Fig. 4a). Similarly, logistic regression highlighted a significant difference between landuses, with the probability of colonization ($\Delta Foc > 0$) being c. 90 % in banana soils, c. 25 % in sugarcane, and negligible in rainforest and grassland soils (Fig. 4b). In contrast, there were no significant differences between landuses in *Foc* colonization extent or probability when sterilized *Foc*-inoculated samples were compared (Fig. S2).

3.5. Associations between Foc colonization, soil microbial biomass and diversity

As differences in *Foc* colonization between landuses were observed in non-sterilized but not in sterilized soils (Table S4), our results indicate that differences in *Foc* colonization are strongly associated with biotic factors. To explore this further, we characterized the biomass and diversity of soil bacterial and fungal communities using PLFA analysis and

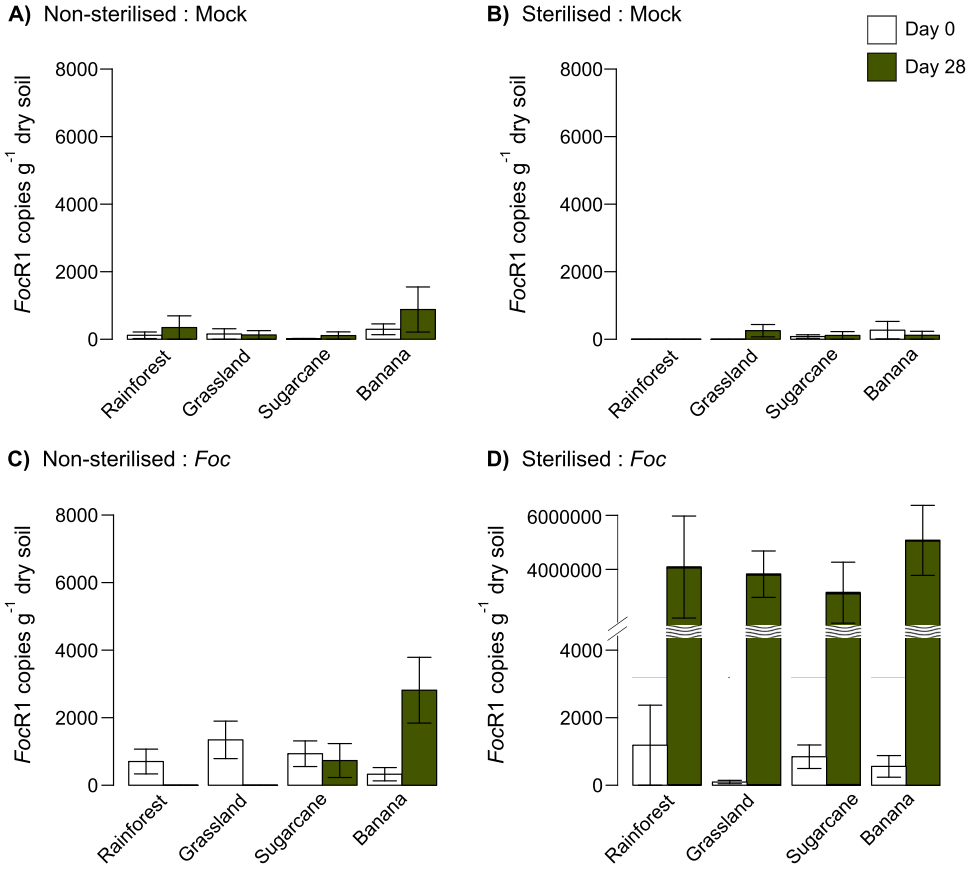

Fig. 3. Quantitative PCR measurements of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) Race 1 marker gene copies per gram of dry soil in (A) non-sterilized mock-treated soils, (B) sterilized mock-treated soils, (C) non-sterilized *Foc* R1-inoculated soils and (D) sterilized *Foc* R1-inoculated soils at the beginning (Day 0) and end (Day 28) of the experiment. Data shown are the mean and standard error of eight sites within each landuse for each treatment. For sterilized *Foc*-inoculated soils (D), the y axis is broken to facilitate representation of the extremely high *Foc* copies detected at 28 days relative to those in other treatments.

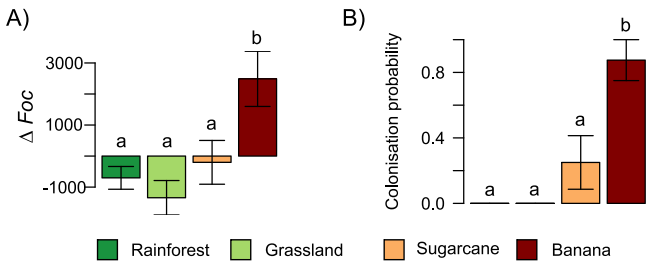

Fig. 4. Impact of landuse on *Fusarium oxysporum* f. sp. *cubense* (*Foc*) soil colonization in non-sterilized soils**.** (A) Differences in *Foc* Race 1 marker gene copy number between the start and end of the experiment (Δ *Foc* = *Foc* R1 marker gene copies at Day 28 minus those at Day 0) for each landuse; (B) The probability of *Foc* colonization (Δ*Foc* > 0) for each landuse. All results are for non-sterilized *Foc*-inoculated soils. Data shown are the mean and standard error of eight sites within each landuse for each treatment. The letters above the bars represent the results of Tukey's Honest Significant Difference *post hoc* tests, where treatments with the same letter cannot be shown to differ.

metabarcoding, respectively. We then evaluated whether these biotic characteristics were significantly associated with differences in *Foc* colonization between the non-sterilized soils and compared these characteristics between landuses.

The biomass of soil bacteria and fungi differed significantly between landuses (Table 1), with banana and sugarcane soils containing approximately half as much fungal biomass as rainforest and grassland soils. *Foc* colonization was significantly less likely with increasing fungal biomass (Fig. 5a). This association was also reflected by a significant negative interaction between *Foc* colonization and the ratio of fungal: bacterial (F:B) biomass (Table 1, Fig. 5b). While bacterial biomass was lower in rainforest and grassland soils than sugarcane and banana soils, it did not significantly impact *Foc* soil colonization (Table 1). Banana soils had significantly higher bacterial diversity (numbers of observed bacterial OTUs (Sobs)) than the grassland soils, but fungal diversity (Sobs), was unchanged between landuses (Table 1). Despite this, *Foc* colonization was significantly negatively associated with fungal alpha diversity (Table 1, Fig. 5c), whereas bacterial alpha diversity did not significantly predict *Foc* soil colonization (Table 1).

Finally, the composition of microbial communities differed significantly between landuses, with those in banana soils being the most distinct (Fig. 6). Importantly, the composition of bacterial and fungal communities, as represented by the primary, but not secondary ordination axis site scores, were significantly associated with *Foc* colonization (Table 1, Fig. 6). *Foc* colonization was positively associated with the relative abundances of bacterial and fungal OTUs predominating in banana soils and negatively associated with those predominating in grassland soils (Fig. 6; Table S5). The top 10 bacterial and fungal OTUs ranked by PC1 loadings (i.e., those negatively correlated with *Foc* colonization), were dominated by members of the Acidobacteriota and poorly characterized members of the Ascomycota and Basidiomycota (Table S5). In contrast, the 10 lowest ranking bacterial and fungal OTUs on PC1 (i.e., those positively correlated with *Foc* colonization), comprised relatively diverse bacteria with no representatives of the Acidobacteriota, and were dominated by a *Fusarium* population and members of the *Nectriaceae* (Table S5).

3.6. Strength and directionality of associations between biotic variables and ΔFoc

Foc colonization was observed to be significantly associated with multiple soil biotic parameters (Table 1); however, many of these were covariable and the strength of their associations differed. Hence, we generated a matrix of Pearson's correlations to highlight significant associations between variables and their direction (Fig. 7). To estimate how strongly each biotic variable was associated with Δ*Foc*, the slope

Table 1
Summary of biotic parameters in each landuse (mean ± standard deviation (SD)), followed by results from ANOVA models summarising whether each biotic parameter (as response variables) was influenced by landuse, and lastly results from generalised linear models (GLM, 'gaussian') indicating which biotic parameters (as predictor variables) were significantly associated with Δ*Foc* (i.e., *Fusarium oxysporum* f. sp. *cubense* (*Foc*) Race 1 marker gene copies at Day 28 minus those at Day 0). The letters next to the means and SDs represent the results of Tukey's Honest Significant Difference *post hoc* tests applied to the ANOVAs investigating the impacts of landuse on each biotic parameter. Treatments with the same letter cannot be shown to differ and comparisons should only be made within each biotic parameter. Asterisks represent the significance of model terms where: $p < 0.001$***; $p < 0.01$**; $p < 0.05$*.

Response / predictor		Rainforest		Grassland		Sugarcane		Banana		Landuse		ΔFoc	
										F val	*P* val	*t* val	*P* val
Bacteria	Biomass	0.030 ± 0.010[a]		0.028 ± 0.006[a]		0.012 ± 0.004[b]		0.016 ± 0.007[b]		13.4	< 0.001 ***	−1.4	0.159
	Alpha diversity (Sobs)	1188.5 ± 141.9[ab]		1151.8 ± 120.2[a]		1209.3 ± 151.1[ab]		1363.4 ± 121.9[b]		3.8	0.021 *	1.9	0.061 .
	Beta diversity (PC1)	0.214 ± 0.181[a]		0.215 ± 0.103[a]		0.051 ± 0.165[a]		−0.480 ± 0.141[b]		38.3	< 0.001 ***	−4.3	<0.001 ***
	Beta diversity (PC2)	−0.370 ± 0.101[a]		0.016 ± 0.163[b]		0.441 ± 0.098[c]		−0.087 ± 0.163[b]		49.6	< 0.001 ***	−0.4	0.724
Fungi	Biomass	0.007 ± 0.001[a]		0.007 ± 0.001[a]		0.003 ± 0.001[b]		0.003 ± 0.001[b]		25.6	< 0.001 ***	−2.2	0.037 *
	Alpha diversity (Sobs)	256.3 ± 75.0		287.4 ± 55.9		198.8 ± 60.1		217.0 ± 79.1		2.7	0.064	−2.1	0.042 *
	Beta diversity (PC1)	0.242 ± 0.134[a]		0.241 ± 0.170[a]		0.171 ± 0.153[a]		−0.654 ± 0.100[b]		76.4	< 0.001 ***	−4.6	<0.001 ***
	Beta diversity (PC2)	−0.587 ± 0.102[a]		0.135 ± 0.142[c]		0.485 ± 0.109[d]		−0.033 ± 0.085[b]		128.5	< 0.001 ***	−0.1	0.941
Other	Fungi: Bacteria (biomass)	0.242 ± 0.042[ab]		0.247 ± 0.033[a]		0.202 ± 0.029[ab]		0.199 ± 0.028[b]		4.7	0.009 **	−2.8	0.009 **

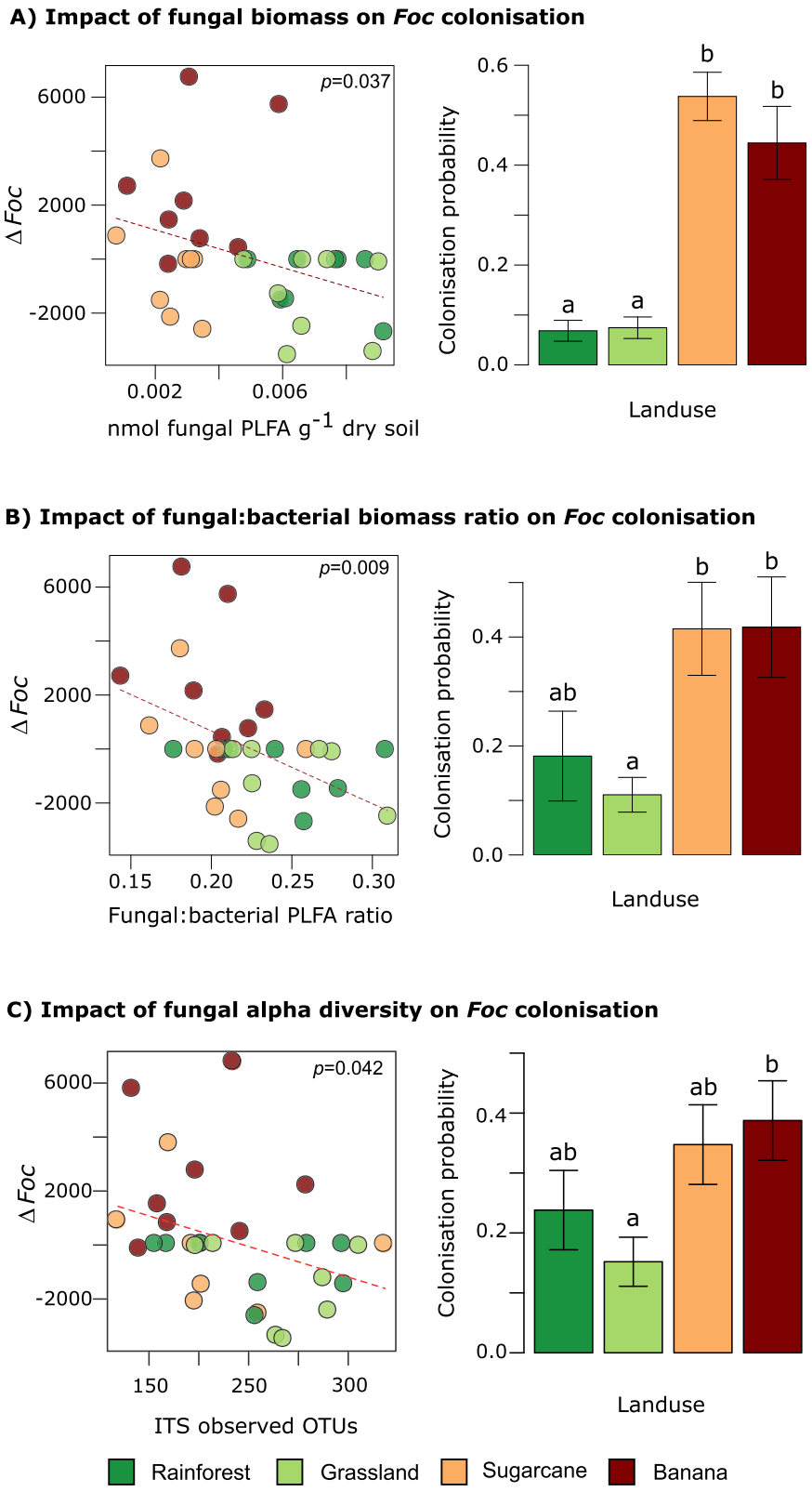

Fig. 5. Significant predictors of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) soil colonization and their impact on the extent and likelihood of *Foc* soil colonization. Scatterplots show the relationship between Δ *Foc* (Δ *Foc* = *Foc* R1 marker gene copies at Day 28 minus those at Day 0) and (A) soil fungal biomass, (B) fungal: bacterial (F:B) biomass ratio, (C) fungal alpha diversity. Regression lines were generated using generalized linear models (GLM, 'gaussian'). Asterisks represent the significance of model terms where: $p < 0.001$***; $p < 0.01$**; $p < 0.05$*. Bar graphs show the predicted probability of *Foc* soil colonization given values of (A) soil fungal biomass, (B) F:B biomass ratio, or (C) fungal alpha diversity for each landuse. The letters above the bars represent the results of a Tukey's Honest Significant Difference *post hoc* test applied to a one-way ANOVA investigating the effect of landuse on the predicted probability of *Foc* soil colonization. Treatments with the same letter cannot be shown to differ and comparisons should only be made within each biotic parameter.

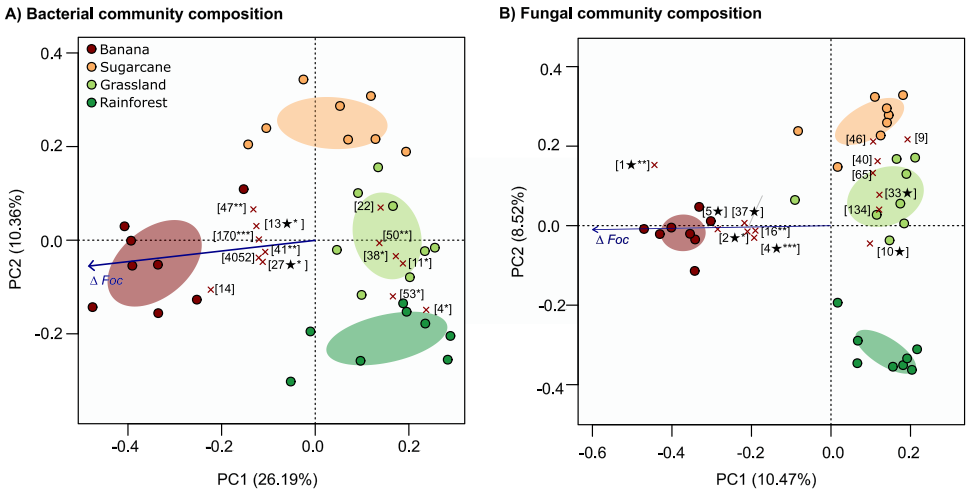

Fig. 6. Principle component analyses (PCA) highlighting the relationship between landuse, *Foc* soil colonization and (A) bacterial or (B) fungal community composition. The ellipses represent standard deviations of the landuse centroids and blue arrows indicate increasing Δ *Foc* (Δ *Foc* = *Foc* R1 marker gene copies at Day 28 minus those at Day 0). Operational taxonomic units (OTUs) that were (A) eight or six standard deviations above or below the PC1 mean, respectively, or (B) four or eight standard deviations above or below the PC1 mean, respectively, are indicated by red crosses which are labelled with the OTU number. OTUs labelled with ★ indicate that the OTU shares > 95 % similarity with a member of the banana core microbiome (Birt et al., 2022, 2023); asterisks indicate a significant correlation between OTU relative abundance and Δ*Foc* as indicated by Pearson's correlation analysis where: $p < 0.001$***; $p < 0.01$**; $p < 0.05$*. Refer to Table S5 for full taxonomic annotation of OTUs and correlation analyses.

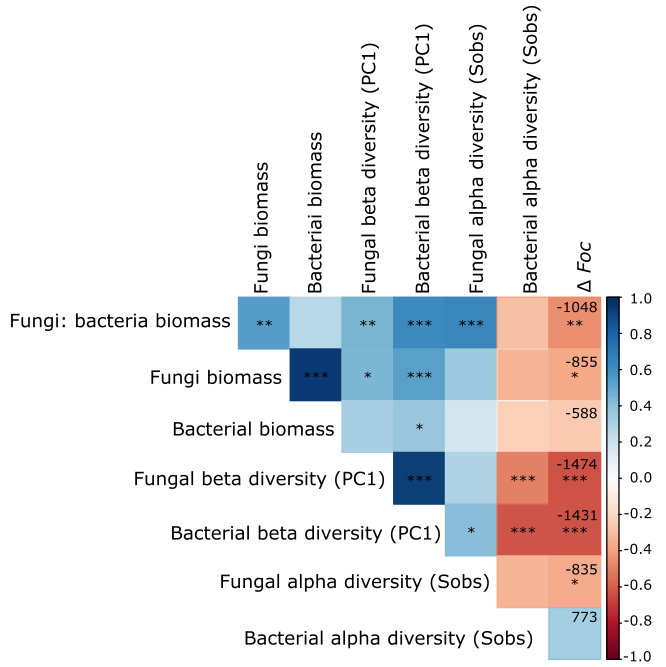

Fig. 7. Correlation matrix of biotic parameters and Δ*Foc* (Δ*Foc* = *Foc* R1 marker gene copies at Day 28 minus those at Day 0). Asterisks represent the significance of Pearson's correlation between variables where: $p < 0.001$***; $p < 0.01$**; $p < 0.05$*. z score-transformed biotic predictors were subjected to a generalized linear model (GLM, 'gaussian') using Δ*Foc* as the response variable to predict the impact of increasing each biotic parameter by one standard deviation on Δ*Foc*. The Δ*Foc* values predicted given an increase of one standard deviation from the mean are shown in the Δ*Foc* column.

coefficient was extracted from linear regression models in which Δ*Foc* was the response, and the z-score normalized biotic variable was the predictor. Based on these analyses, we determined that changes in the composition of fungal and bacterial communities were associated with the largest changes in Δ*Foc,* which for each standard deviation increase was associated with a 1474 or 1431 *Foc* marker gene copy decrease, respectively (Fig. 7). This was followed closely by the ratio of fungal to bacterial biomass, which for each standard deviation increase was associated with a 1048 marker gene copy decrease. Fungal biomass and fungal alpha diversity were both negatively associated with Δ*Foc*, with single standard deviation increases in these variables expected to decrease *Foc* colonization by c. 850 marker gene copies (Fig. 7).

4. Discussion

Understanding the factors that determine the likelihood of *Foc* colonizing soil post-introduction is important for land managers seeking to avoid the disease and its spread. While on-farm biosecurity protocols and barriers help to prevent the dispersal of *Foc* in banana production areas, soil microbial communities can also provide significant biological defences that inhibit the spread of *Foc* across landscapes. The 'disease triangle' paradigm states that for a disease to occur, there must be: i) a susceptible host, ii) conducive environmental conditions, and iii) the presence of the pathogen. Recently, this paradigm has been revised to a 'disease pyramid', with the inclusion of the host microbiome (Bernardo-Cravo et al., 2020). In this study, we focused on the 'pathogen' and its interaction with the soil microbiome. Ultimately, the less frequently a pathogen is in the environment, the less often it can interact with a compatible host. To examine how landuse influences the accumulation of *Foc* in soils across a banana-growing landscape, we identified the landuses bordering banana plantations and assessed their potential for *Foc* colonization. We then investigated which soil biotic factors are most strongly associated with *Foc* soil colonization and considered how these variables may be leveraged to decrease the likelihood of *Foc* soil colonization in banana production areas.

4.1. Banana soils are more conducive to Foc colonization than soils from other landuses

Fusarium wilt disease management comprises three phases: 1) prevention at national, regional and farm scales; 2) containment of *Foc* nationally and on-farm; and 3) disease management once *Foc* is present (Viljoen et al., 2020). Australian banana production areas have strict biosecurity protocols to prevent the transfer of soil between farms. However, soil can still be spread by wind, flooding, insects or animals (Pegg et al., 2019) and as such, the *Foc* soil contamination risk is likely to

be influenced by *Foc* inoculum levels in neighbouring soils.

Our results demonstrate that rainforest, grassland, and sugarcane account for c. 75 % of the land bordering banana production areas in the Wet Tropics region of Queensland, Australia. We found that the likelihood of *Foc* colonizing soils differed significantly between these landuses, with banana soils being by far the most conducive to *Foc* soil colonization. Our results indicate that *Foc* failed to colonize all rainforest and grassland soils, had limited success (c. 25 %) in sugarcane soils, but colonized c. 90 % of banana soils. These results suggest that areas of rainforest and grassland surrounding banana farms may provide significant biological defences against the introduction of *Foc*, thereby bolstering biosecurity fences and other measures. In contrast, areas of sugarcane production surrounding banana farms may facilitate *Foc* dispersal, albeit to a much lesser extent than neighbouring banana farms, which represent a significant risk. Thus, our results highlight that in addition to biosecurity, it is important for banana producers to consider the extent to which the landuses neighbouring their properties can buffer or suppress the spread of *Foc*.

It should be noted that for privacy reasons the government landuse dataset that we have used in our study does not differentiate between farms within polygons of banana landuse. Hence, our calculations of landuses bordering banana are based on contiguous areas of banana production rather than individual farms or fields. For this reason, we were not able to identify individual properties that should prioritise biosecurity and improve soil defences due to their surrounding landuse profile. Our general recommendations would be that growers avoid clearing any areas of rainforest and consider introducing grass cover between banana farms and fields.

4.2. Putative drivers of Foc soil colonization

To examine the influence of the living soil biome in mediating *Foc* soil colonization, we firstly compared the *Foc* colonization of sterilized vs non-sterilized soils. In agreement with previous studies (Ou et al., 2019), we observed extremely strong *Foc* colonization of sterilized soils relative to non-sterilized soils. This result was observed regardless of landuse, suggesting that living competitors play a major role in suppressing *Foc* soil colonization.

Having established the importance of living competitors in mediating *Foc* colonization in soil, we sought to determine which biotic factors accounted most strongly for the differential response to *Foc* inoculation within the living (non-sterilized) soils. To achieve this, we related the extent and likelihood of *Foc* colonization to soil bacterial and fungal amplicon sequencing and PLFA data we had previously generated (Birt et al., 2024) for each site tested. Our results are in broad agreement with a range of studies indicating that microbial biomass, diversity and community composition contribute significantly to pathogen suppression (Schlatter et al., 2017; Singh et al., 2025). Based on the strength of their associations, we identified the composition of soil fungal and bacterial communities, as well as the ratio of F:B biomass as the strongest putative drivers of *Foc* soil colonization. Increasing fungal biomass and alpha diversity also promoted *Foc* soil suppression, but to a weaker extent. Of these variables, fungal community composition in particular, has been associated with differences in *Foc*-diseased vs healthy banana soils (Wang et al., 2022;Chen et al., 2024), however, it is unclear whether these differences actively contribute to *Foc* suppression or are a response to *Foc* colonization. We identified several OTUs that were significantly associated with *Foc* suppression (Table S5). These included members of the Acidobacteriota, which are often abundant in forest soils (Kim et al., 2021) and have been implicated in both *Foc*-suppressive and *Foc*-conducive soils (Jamil et al., 2022; Hong et al., 2020; Fan et al., 2023; Nisrina et al., 2021). The largest changes in community composition relating to *Foc* soil colonization were resolved along the PC1 axis of the PCA. However, these differences were confounded by landuse, making it difficult to uncouple associative OTUs (i.e. OTUs inherently overabundant in banana soils and associated with *Foc* soil colonization by default) from causal OTUs (i.e. suppressors or promoters of soil colonization regardless of their association with landuse).

Based on our data, it is not possible to definitively establish the causal mechanisms underlying the differential response of landuses to *Foc* soil colonization. Nonetheless, four of the five putative drivers of *Foc* suppression (bacterial and fungal community composition, F:B biomass and fungal biomass) differed significantly between landuses, suggesting that they are likely to be major contributors to the differences observed between landuses. The putative drivers of *Foc* soil colonisation are likely to be influenced by multiple factors. These may include the application of pesticides, herbicides and fertilizers, as well as physical land disturbance, all of which are common in agricultural systems but much less prevalent in forest or grassland ecosystems. Such practices can disrupt microbial networks, reduce overall biomass, and decrease microbial diversity (Steiner et al., 2024), thereby weaking mechanisms such as niche competition and antibiosis that contribute to pathogen suppression (Singh et al., 2025). These hypotheses warrant further investigation. Likewise, the causal nature of the OTUs associated with *Foc* suppression or colonisation would need to be validated in repeated experiments under field conditions.

4.3. Leveraging the drivers of Foc soil colonization to enhance suppressiveness in banana production areas

Manipulating the putative drivers of *Foc* soil suppression may help land managers to reduce soil susceptibility to *Foc* colonization. Such efforts could focus on engineering the soil microbiome to increase the relative abundance of *Foc*-suppressive consortia and increase the F:B ratio along with fungal abundance and diversity. Strategies to achieve these outcomes may include targeted approaches, such as the direct application of organisms (bioinoculants) associated with *Foc* suppression to the soil, or broader interventions like the use of soil organic amendments, reduced land disturbance or the establishment of ground covers. Below we explore these strategies and suggest that increasing the F:B ratio may represent the most feasible approach to leverage some control over the soil microbiome.

Adding bioinoculants, either as single communities or synthetic microbial consortia directly to the soil theoretically holds promise for achieving targeted changes in a soil microbiome. Numerous putative biological control agents of Fusarium wilt and OTUs associated with *Foc*-suppressive banana soils have been identified (Bubici et al., 2019; Fan et al., 2023; Lv et al., 2024), and some have been experimentally validated under controlled conditions (Lv et al., 2024; Fan et al., 2023). Microbial taxa consistently associated with both the host plant and soil suppressiveness, such as OTU77 from the VAZQ01 genus within *Xanthobacteraceae*, a core component of the banana microbiome (Birt et al., 2022), may be particularly promising bioinoculant candidates. However, modifying soil microbial communities in a targeted and persistent manner remains challenging, with few studies reproducibly altering the soil microbiome or demonstrating long-term persistence of bioinoculants (Jansson et al., 2023; Singh et al., 2025). Soil ecosystems are complex, with native microbial communities that may outcompete (Raaijmakers and Mazzola, 2016) or be adversely affected by introduced bioinoculant(s) (Manfredini et al., 2021). The outcomes and persistence of introduced microbes are often location-specific due to variability in indigenous microbial communities. Moreover, the culturable fraction of soil microbes is limited (Torsvik and Øvreås, 2002), restricting the available pool for inoculant development. Although synthetic consortia are an emerging area of research, bioinoculation remains a relatively high-risk and uncertain method to gain control of the soil microbiome.

By contrast, the F:B biomass ratio emerges as a relatively achievable univariate parameter to target in order to leverage a degree of control over the soil microbiome. High carbon-to-nitrogen (C:N) ratios in soils have been correlated with high F:B biomass ratios (Fierer et al., 2009; Carney et al., 2007), as fungi are more efficient than bacteria at decomposing carbon-rich materials such as cellulose and lignin. The F:B

biomass ratio in soils can be increased by the application of high C:N organic matter such as composts or biochar with a high cellulose content (Hannula and Morriën, 2022, Heck et al., 2019; Yuan et al., 2021). Organic amendments have also been associated with untargeted shifts in the soil microbiome toward greater disease suppressiveness across various pathosystems (De Corato, 2020), including *Fusarium* spp (Bonanomi et al., 2007). Cellulose-rich amendments stimulate the growth of saprotrophic fungi, such as members of the *Chaetosphaeriaceae* which can suppress soil-borne pathogenic fungi (Clocchiatti et al., 2021). However, such amendments may also promote the growth of pathogenic fungi like *Foc*, which is a facultative saprotroph. Since the composition of the organic amendments influences the response of the soil microbial community (Ng et al., 2014), the form and application strategy of different amendments must be experimentally optimized to promote suppressive rather than pathogenic organisms. Bananas require high levels of nitrogen fertilisation to achieve optimum yield (Rasiah et al., 2009), which can favour bacterial over fungal growth (Treseder, 2008). Partially substituting inorganic nitrogen fertilisers with organic nitrogen fertilisers, which are often richer in complex carbon compounds, has been shown to enhance banana seedling growth (Huang et al., 2022). This practice may increase fungal biomass and the F:B ratio without compromising yield, although its effects on microbial communities requires further study.

Tillage, which is typically performed every few years in Queensland banana production systems (Rasiah et al., 2009), is predicted to damage fungal hyphae and disrupt fungal networks, while having a comparatively smaller impact on bacterial populations, thereby potentially reducing fungal biomass and the F:B ratio (Strickland and Rousk, 2010; Sünnemann et al., 2021). One study showed that no-tillage banana farming increased total soil microbial biomass (Dorel et al., 2010), however the microbial community composition was not assessed. The use of ground covers or cover crops can also suppress weeds commonly associated with minimum till systems while increasing organic matter inputs and the F:B ratio (Muhammad et al., 2021; Xiang et al., 2023). Crop rotation (Yang et al., 2023), intercropping (Ren et al., 2024) and the use of ground covers (Pattison et al., 2014; Rames et al., 2018) have been reported to reduce *Foc* disease incidence and/or soil colonization, while cover crops promoted fungal diversity in banana soils (Wang et al., 2023). Allowing naturalized or indigenous vegetation from bordering rainforest and grassland soils to grow as ground covers in banana plantations emerge as promising strategies to increase *Foc* soil suppressiveness in banana plantations by increasing the F:B biomass and shifting the soil microbiome towards that of neighbouring, less disturbed, grasslands. However, as with organic amendments, care needs to be taken in the choice of ground cover species, as several non-banana species have been identified as potential alternative hosts for pathogenic *Foc,* potentially serving as inoculum reservoirs (Catambacan and Cumagun, 2022; Dita et al., 2018; Pegg et al., 2019; Ploetz, 2015).

To achieve optimal yields and ensure a lasting suppressive effect on soil for the future, it is essential to balance the use of ground cover, organic amendments and low intensity practices with other agricultural practices such as fertilization and liming. Further studies are needed to evaluate how soils might respond to soil-borne pathogen colonization in response to the manipulation practices discussed to determine their effectiveness.

4.4. Sanitising biosecurity protocols risk Foc soil accumulation upon reinfestation

Protocols used to reduce soil-borne pathogen inoculum by chemical or disinfection treatments have often proved counter-productive in the long-term due to unintended negative effects on the soil microbiome (Gullino et al., 2022). Biosecurity protocols such as urea treatments that are used to destruct *Foc*-infected plants and surrounding soil (Pegg et al., 2019) are non-targeted and the consequences for *Foc* soil suppressiveness are unclear. Given that elimination of the living soil microbiome by sterilization allowed *Foc* to proliferate to huge levels relative to the non-sterilized living soil in this experiment, it is likely that such protocols will facilitate increased soil colonization by *Foc* upon reinfestation.

5. Conclusions

In conclusion, we found that rainforest and grassland soils, which represent > 50 % of the land bordering banana production in our study area are unlikely to be colonized by *Foc* and may therefore provide natural defences against the spread of *Foc* through the landscape. In other words, if soils from rainforests or grasslands enter banana farms via flooding, wind, animals, or other means, they are less likely to introduce significant amounts of *Foc*. In contrast, soil movement between banana farms poses a greater risk, as such soils are more likely to contain high levels of *Foc* inoculum. Conversely, if soil from banana farms is transported into rainforest or grassland areas, the spread of *Foc* may be hindered, as the pathogen is unlikely to build up in these more suppressive soil environments. These findings underscore the importance of maintaining existing natural and semi-natural landuses near agricultural soils that have the potential to be infested by soil-borne pathogens. The surge of *Foc* growth observed in sterilized soils lacking a living microbiome i) demonstrates the major role the biota in plays in suppressing *Foc* establishment and ii) highlights the potential risk of increased *Foc* accumulation if *Foc* recolonizes an area post biosecurity (sanitation) treatment. We asked which factors were responsible for the differences in *Foc* soil colonization between soils and found that that soil fungal and bacterial community composition and the soil fungal: bacterial biomass ratio most strongly influenced *Foc* soil colonization. As such, use of cellulose rich soil amendments and grassland ground covers may enhance soil suppressiveness in banana production systems by increasing the fungal biomass and promoting the growth of suppressive organisms.

Author contributions

PGD and ABP secured funding and designed the study. HWGB, PGD, HRLG, and ABP collected samples. RL, HWGB, and ABC performed experiments and generated data. RL and PGD analysed data. RL and PGD wrote the paper with input from all authors.

CRediT authorship contribution statement

Lyons Rebecca Louise: Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna-Belle C. Clarke:** Writing – review & editing, Methodology. **Hazel R. Lapis-Gaza:** Writing – review & editing, Methodology. **Jiarui Sun:** Writing – review & editing, Formal analysis. **Birt Henry W. G.:** Writing – review & editing, Methodology. **Anthony B. Pattison:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Paul G. Dennis:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2025.109814.

Data availability

Data will be made available on request.

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