Advancing Strain-Specific TaqMan Assays for *Trichoderma asperellum* Detection in Commercial Agricultural Settings

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Abstract

The global agricultural sector is facing significant challenges in achieving higher sustainability, which has increased interest in using biological control agents (BCAs) to manage plant diseases. However, it is essential to ensure that microbial-based products, such as BCAs, are utilised in a manner that does not harm soil quality and fertility while decreasing reliance on synthetic pesticides. To accomplish this, it is crucial to monitor the fate and persistence of bioinoculants in the soil, which is essential for optimising their application over time, as well as for regulatory and commercial purposes and environmental risk assessment. A qPCR detection method utilising TaqMan chemistry is proposed, which has demonstrated high specificity and sensitivity in detecting *Trichoderma asperellum,* a common BCA species, in soil. The primers and probe were designed based on the βtubulin2 gene. The TaqMan-based assay was applied and validated on soils where tomatoes and strawberries were grown after a previous application of *T. asperellum* FC80 strain over three years. The TaqMan-based assay was able to detect the target strain accurately, meeting the stringent requirements for commercial and regulatory applications.

Significance and impact of the study: The TaqMan assay developed here has the potential to impact the agricultural sector significantly. It can be used for regulatory, commercial, and scientific purposes to track, monitor, and determine the presence and fate of *T. asperellum* under field crop conditions, thereby contributing to adopting more sustainable and efficient agricultural practices.

Highlights

- The global agricultural sector increasingly uses biological control agents (BCA) to control plant diseases, particularly soil-borne ones.
- BCAs can/shall preserve soil quality and fertility while reducing reliance on synthetic pesticides.
- Monitoring the fate and persistence of BCAs in the soil is crucial to optimise their application.
- An improved TaqMan assay with high specificity and sensitivity in detecting *Trichoderma asperellum* in soil was developed.
- The assay was successfully applied and validated on soils where tomatoes and strawberries were grown.

Keywords: Fungi *Trichoderma asperellum*, soil, traceability qPCR, biological control agent, TaqMan Assay

Introduction

Natural antagonistic microorganisms have been extensively studied for biological control to control plant diseases. Using biocontrol agents (BCAs) in agriculture is an eco-friendly strategy that can replace synthetic plant protection products (PPPs) for controlling fungal pathogens, particularly soil-borne ones. Their use can reduce the risk of developing fungicide-resistant mutants (Ghorbanpour et al., 2018). However, BCAs shall not affect soil quality and fertility, considering the current challenges and policies for protecting this resource (FAO and ITPS 2015, UN SDG 2030, Jensen et al., 2016; Bruce et al., 2017; Rotolo et al., 2018).

It is essential to monitor the fate and persistence of Biological Control Agents (BCAs) in soil for their registration, as it has ecological and technical implications. This helps to reduce environmental risk and optimise application methods and efficacy (Lacey et al., 2015; Wallis and Sisterson 2024). Monitoring can also support the application technology by avoiding unnecessary BCA application, which can be costly, and by evaluating their efficacy in controlling pest populations. This allows for adjustments to treatments based on local pedo-climatic conditions (Malusà et al., 2021; Vassilev and Malusà, 2021; Ptaszek et al., 2023). However, monitoring and quantifying BCAs in complex matrices such as soil require procedures with high specificity and sensitivity.

Real-time PCR (qPCR) assay is currently the primary approach used to specifically detect and quantify a bioinoculant in soil because of its high sensitivity (Manfredini et al. 2021; Manfredini et al., 2023; Canfora et al. 2016; 2017). Although qPCR is suitable for tracking and quantifying bacteria or fungi introduced in the soil, its applicability depends on specific and discriminant primer pair and probe design (Manfredini et al., 2021). The whole genome of a target microbial species can be exploited to design qPCR primers and probes that allow good discrimination power among different species. It follows that the design of a customised species-specific marker is needed to ensure discriminant detection of the target species in soil. The molecular detection and quantification of different fungal species (Filion et al. 2003; Lievens et al. 2006; López-Mondéjar et al. 2010; Sharma and Salwan 2017) are substituting conventional techniques, such as those based on the evaluation of colony-forming units (CFU) and chemical, biological, and immunological assays (Thornton et al. 1994).

The genetic variability of bacteria and fungi can be leveraged to distinguish species and strains. To identify target microorganisms, specific genetic markers should be utilised to build a collection of sequences and pinpoint unique traits that do not overlap with those of other closely related species. One such marker is the beta-tubulin2 gene, which encodes the structural protein tubulin, a component of microtubules (Dubey et al., 2014; Morello et al., 2019; Ezeonuegbu et al., 2022). This gene is particularly useful for identifying and differentiating various fungal species, such as *Fusarium* spp. and *Trichoderma* spp. Studies have shown that sequences of the beta-tubulin2 gene can elucidate phylogenetic relationships within genera of Ascomycetes that cannot be discerned based on morphology alone, notably in the *Aspergillus* and *Pestalotiopsis* genera (Lesage-Meessen et al., 2011).

Trichoderma, a fungus utilised as a biocontrol agent against various plant pathogens, including nematodes, has several species and strains registered as biocontrol agents. These include, among others, *T. asperellum*, *T. atroviride*, T. *harzianum*, *T. gamsii*, *T. polysporum*, *T. viride* (Malusà 2023). Commercially available strains in Europe include several strains of *T. asperellum* (formerly *T. harzianum*): T25 (formerly *T. viride*), TV1 (formerly *T. harzianum*), SF 04 (URM) 5911, and T34. Trichoderma strains have various modes of action when used in biocontrol, showcasing traits such as strong rhizosphere and soil colonisation, production of antifungal substances, and induction of plant resistance (Pugliese et al., 2008). These soil-inhabiting organisms are also effective colonisers of organic matter, commonly found in tropical and temperate soils, wood and plant materials, as well as in organic fertilisers like compost and re-used substrates for soilless systems.

The differentiation of Trichoderma using morphological characteristics is complicated due to the paucity of specific traits (Błaszczyk et al. 2011; Devi et al. 2012). Therefore, detecting a target species in soil can be challenging. Different qPCR and qRT-PCR assays have been proposed to quantify *T. harzianum* (Rubio et al. 2005; López-Mondéjar et al. 2010; Beaulieu et al. 2011), *T. atroviride* (Cordier et al. 2007; Savazzini et al.

2008; Hilje-Rodrìguez et al. 2020), *Trichoderma* spp. (Hagn et al. 2007; Kim and Knudsen, 2008), and *T. asperellum* (Gerin et al. 2018). These assays rely on specific primers and probes targeting conserved regions in the genomes of different *Trichoderma* species.

The present study introduces a new TaqMan-based assay for tracking and quantifying the *T. asperellum* strain FC80 when used in field conditions, which was validated on commercial tomato and strawberry crops previously treated with it. *T. asperellum* FC80 is a strain isolated from reused perlite and perlite-peat substrates in soilless tomato cultivation. This strain has shown high effectiveness (95%) in reducing *Pythium ultimum,* damping-off on cucumber plants and promoting growth (more than 130% increase compared to the untreated control) (Clematis et al. 2009; Liu et al. 2009). Despite its promising effects, no methods are currently available to monitor its presence after application on target crops.

Materials and methods

1. Microbial isolates for specificity assay

T. asperellum FC80 was isolated from re-used perlite and perlite-peat substrates in soilless tomato cultivation (Clematis *et al.* 2009; Liu *et al.* 2009). This strain's identity was confirmed through the elongation factor 1-alpha-like (tef1) gene, and the corresponding sequence has been deposited in GenBank® under accession number MZ222413.

For the specificity tests, 21 fungal strains and 17 different *T. asperellum* strains were used (Table 1). To achieve optimal growth, the fungal strains were cultured on Potato dextrose agar (PDA) medium (DifcoTM, Detroit, MI, USA) for one week at 25°C.

2. Experimental trials inoculated with *T. asperellum* FC80 and soil sampling

Field trials were carried out at a tomato farm in Moretta (CN, Italy; 44.7823N - 7.5155E) and a strawberry farm in Boves (CN, Italy; 44.3440N - 7.5732E). In both locations, plants were transplanted into mulched soil and irrigated using a drip irrigation system. Standard cultivation practices in the region were followed. Specifically, tomato plants of the "Cuore di bue" type (cv. Meneghino) was planted in April 2021, 2022, and 2023. The trial was conducted under a 540 m^2 plastic tunnel.

The experimental design utilised a randomised block approach with five replicates per treatment, each comprising 40 plants. Eight days before transplantation, the seedlings in trays were treated with FC80 at a dose of 400 mL suspension per tray. Subsequently, a second treatment was administered during planting using 40 mL of the prepared FC80 suspension per plant.

In order to prepare the conidia suspension, *T. asperellum* FC80 was grown in a 1000 mL flask with 250 mL of liquid casein hydrolysate medium and kept under static culture conditions at 25°C. After 15 days, the resulting mycelium was transferred into 200 mL of sterile distilled water and homogenised using a rotary hand apparatus. The resulting conidia suspension was adjusted to a concentration of 1 x 10^7 CFU for use in the experimental trials.

Soil samples were gathered during experimental trials conducted in tomato and strawberry fields at specific intervals.

For the tomato trial in 2021, soil sampling occurred 60 (T1) and 130 (T2) days after planting. In 2022, soil samples were collected 30 (T1) and 130 (T2) days after planting. In 2023, soil sampling occurred 30 days (T1) and 130 days (T2) after planting.

The strawberry trial was planted (cv. Elodì) in July 2021 under a 360 m^2 plastic tunnel. The experimental setup included 50 plants for each replicate, with a randomised block arrangement of five replicas per treatment. The treatments with FC80 in the strawberry trial were administered as follows:

I) Root immersion of seedlings at planting using 80 mL of spore suspension per plant.

II) Soil drench conducted 14 days after planting, using 100 mL per plant with soil injector equipment.

The soil samples were gathered from the strawberry trial at specific time intervals: 45 days (T1 – 2021), 90 days (T2 – 2021), 270 days (T3 – 2022), 450 days (T4 – 2022), 600 days (T5-2023), 690 days (T6-2023), and the final sampling at 700 days (FS-2023) from the planting date. The sampling at T6 was conducted before the reapplication of treatment with *T. asperellum* FC80. Approximately 10 grams of soil near the plant's roots were collected during sampling. Additionally, the tomato and strawberry trials included an untreated control group. The chemical-physical characteristics of the soils can be found in Table 2.

3. Design of primers and probes specific for *T. asperellum* FC80 detection

Bt2b region encoding, as per Glass and Donaldson 1995, was sequenced across various Trichoderma species (Bardin and Pugliese, 2020). The beta-tubulin2 sequences were then aligned using Lasergene software package (v.7.1, DNAstar Madison, WI, USA) to find suitable target sequences. Primers and a probe with minor groove binder (MGB) modifications were designed using Beacon Designer™ free edition, considering a primer annealing temperature of 60°C and an amplicon length of less than 109 bp (see Table 3). Nucleotide BLAST was used for in silico amplicon analysis to ensure specificity. Furthermore, the MFOLD web server was utilised to assess target secondary structures and primer/template accessibility with corrections for 50 mM Na+ and 5 mM ionic conditions Mg^{2+} at a folding temperature of 60°C. Finally, the b-tub2 sequence was submitted to NCBI (ID: 2664935) for reference and public access.

4. DNA and RNA isolation from soil samples

The samples collected from the field were treated with a nucleic acids preservation solution according to the manufacturer's protocol (LifeGuard®, Qiagen, Italy). After treatment, all samples were stored at -80°C until nucleic acids extraction. For RNA extraction, the ZymoBIOMICS™ DNA/RNA Miniprep Kit (Zymo Research, Irvine, CA, USA) was used following the manufacturer's protocol. The extracted RNA was then reverse transcribed into cDNA using SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The obtained cDNA was quantified using a Qubit® 2.0 Fluorometer with DNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and following the manufacturer's instructions. The quantified cDNA was diluted to a concentration of 10 ng μL^{-1} and stored at -20°C for subsequent downstream analyses.

5. DNA extraction from Trichoderma and qPCR conditions

The DNA from each Trichoderma strain was extracted using the Quick-DNA Fungal/Bacterial Kits from Zymo Research Corporation (Irvine, CA, USA). Subsequently, the DNA was purified using Amicon® Ultra-0.5 centrifugal filter devices from Merck KGaA (Darmstadt, Germany) as per the user guide. To quantify the DNA, the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was employed with a Qubit 2.0 fluorometer Kit (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the specified protocols. Real-time PCR experiments were carried out on the QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the QuantStudio™ Design and Analysis Software ver. 1.5.1. The qPCR reactions were set up in a total volume of 20 µL, consisting of 2 µL of DNA or cDNA template, 10 µL of TaqMan™ Fast Advanced Master Mix 2x (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 µL of Custom TaqMan™ Gene Expression Assays 20x (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 7 µL of nuclease-free water. The thermal cycling protocol included an initial hold at 50°C for 2 minutes, polymerase activation at 95°C for 2 minutes, and 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 20 seconds.

6. Analytical Specificity and Sensitivity Assays

The TaqMan-based qPCR assay's specificity was evaluated using a diverse panel of microorganisms commonly found in soil fungi and commercial products containing *Trichoderma asperellum* icc012, *Trichoderma gamsii* icc080 (Remedier, ISAGRO, Italy), and *Trichoderma asperellum* TV1 (Xedavir, Xeda, Italy).

The primers and probes were tested against 38 fungal species to assess specificity, using 50 ng of pure genomic DNA in three technical replicates. Each reaction was conducted in ninety-six well-clear plates, with a reaction volume of 20 µL per sample, and performed on the QuantStudio™5 Real-Time PCR System (QuantStudio™ 5, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

For each developed assay, pure genomic DNA extracted from the target species was used as a positive control to validate the specificity of the primer and probe sets. The sensitivity limits for the amplification method were determined by amplifying a 10-fold dilution series of the appropriate nucleic acid extract using primer and probe sets.

7. TaqMan Assay and Standard curves

Relative standards were created by performing an 8-log10 serial dilution (1:10) of *T. asperellum* FC80 DNA. The initial concentration of each DNA stock was measured using the Qubit dsDNA HS (high sensitivity, 0.2 to 100 ng) Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The number of DNA copies in each reaction was estimated, and the mass of a single fungal genomic DNA was calculated using the DNA copy number calculator available at https://www.Thermofisher Scientific.com/it/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html. It was determined that the concentration of the initial stock of fungal DNA was 35.7 ng μL^{-1}, which is equivalent to $3.03E^{+11}$ copies per µL. The DNA was serially diluted from $3.03E^{+11}$ to $3.03E^{+1}$ copies per µL, with eight dilutions loaded in triplicated reactions, ranging from $3.03E^{+7}$ to $3.03E^{+1}$ copies per µL.

The qPCR reaction mix was prepared with a final volume of 20 µL, comprising 10 µL of TaqMan® Fast Advanced Master Mix, 1 µL of TaqMan® Assay (20X), 7 µL of Nuclease-Free Water, and 2 µL of isolated DNA or cDNA. The qPCR analysis conditions involved enzyme activation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 1 second and annealing/extension at 60°C for 20 seconds.

Standard curves generated for each amplification plate were evaluated for consistency between runs, considering the variability of amplification efficiency, slope, Y-intercept, and R^2 for each target. The threshold cycle (Ct), inversely proportional to the starting amount of target genetic material, was used for quantitative measurement. High efficiency and desirable R2 values (>90% and <110%) were observed when quantifying target fungal pathogens from pure genomic DNA, as demonstrated in Figure 1, showing standard curves and Ct values. The efficiency was estimated using data from a ten-fold serial standard calibration curve with seven points in quadruplicates created for the target species using genomic DNA.

The limit of detection (LOD) was determined using the MIQE guidelines (Bustin et al. 2009). The detection limit in the number of genomes was also estimated based on the LOD, genome sizes obtained from NCBI and considering 660 Daltons per base pair.

8. Data analysis and statistical analysis

Statistical analysis was conducted using Origin Lab software (version 9.65) and Microsoft® Excel® for Microsoft 365 MSO (Version 2402 Build 16.0.17328.20124). Differences in the parameters among treatments were compared by conducting a one-way analysis of variance (ANOVA) at the end of each bioassay. This was followed by Duncan's multiple range tests ($P < 0.05$) in Origin Lab software (version 9.65). A value of $P < 0.05$ was considered significant.

Results

1. Specificity of TaqMan assay for *T. asperellum* FC80

Using TaqMan technology, the qPCR assays targeted the beta-tubulin2 gene and produced amplification products for the *T. asperellum* strain FC80. The study also included commercial products (Remdier and XEDAVIR) and other Trichoderma species (see Table 4). DNA extracted from various fungi was analysed

with the same probes (see Table 1); no amplification was observed in these fungal species, including *Trichoderma spp* and other fungi, while amplification was observed exclusively for *T. asperellum* strains. These results show a high level of specificity in the designed assays.

2. Sensitivity of TaqMan assay for *T. asperellum* FC80

The "analytical sensitivity" or "limit of detection" of an assay refers to its ability to detect low concentrations of a specific substance in a biological specimen (Burd, 2010). In this study, the limit of detection (LoD95%) was determined for a purified *T. asperellum* FC80 sample, and the assay showed excellent sensitivity, with a LoD95% value of 3.03E+4 copies per µL.

3. Diagnostic application: detection of *T. asperellum* FC80 in the soil of tomato and strawberry plants inoculated with *T. asperellum* FC80

T. asperellum FC80 was only detected in soil samples from field trials conducted in 2021, 2022, and 2023 that were treated with the BCA, which were expected to be positive. The untreated control samples consistently produced negative results, confirming the high specificity of the TaqMan assay.

To establish the standard curve for the *T. asperellum* FC80 TaqMan assay, we used DNA concentrations ranging from 10^2 to 10^8, starting with a pure culture of *T. asperellum* FC80. The standard curve showed a well-fitted linear regression curve ($R^2 = 0.9976$) with a slope coefficient of -3.321 and an efficiency percentage (E%) of 100%. The qPCR assay demonstrates high efficiency and accuracy for quantification purposes. Table 5 presents the total RNA yields and cDNA amounts, providing further information on the obtained results for the samples.

The experimental tomato fields consistently showed an increase in the population of active *T. asperellum* FC80 in the soil three months after treatment with the biological control agent (BCA) over the course of three years (Figure 2A). The gene copy number of *T. asperellum* FC80 was significantly lower in 2022 but increased in 2023.

In the strawberry trials, the persistence and activity of T. asperellum FC80 remained stable after the initial treatment in 2021, except during the flowering stage in 2022 (Figure 2B). Even after a single application to strawberries, *T. asperellum* FC80 was consistently detected in 2022, indicating its persistence in the soil of the treated plants, likely due to pathogen presence and environmental conditions. Although a notable decline in gene copies was observed in the strawberry trial, a growth trend was noted during the final sampling after the fungus was reintroduced.

Discussion

Trichoderma is a commonly found genus in soils and litter, which makes it difficult to detect in the complex matrix of soil. Accurately measuring the presence of a bioinoculant in the soil is crucial for environmental risk assessment and diagnostic purposes (Bonaterra et al., 2012). This risk assessment must include information on the BCA's colonisation ability, persistence, spread, and possible dispersal routes in the soil under typical environmental conditions (Longa et al., 2009). It is also essential to monitor the population dynamics of beneficial microorganisms to understand the long-term success of their application. Indeed, changing ecological conditions, such as alterations in the soil's physical or chemical characteristics due to agricultural practices, can result in shifts in the microbial community structure and functionality (Meena, 2023). These changes can have implications for soil health, fertility, and quality. To properly evaluate the impact of BCA on soil functions, it is thus also necessary to detect the fate and the persistence of those microbial-based inoculants in the soil. The specificity of the primers and probes plays a critical role in the success of detection (Manfredini et al. 2021; Manfredini et al. 2023), and the TaqMan-qPCR method utilised in this study enhances the specificity of detection (Taparia et al. 2020).

Considering that the effectiveness of BCAs in controlling plant pathogens depends significantly on the dosage applied (Bonaterra et al., 2022), methods allowing their monitoring, such as that presented in this work, can support fine-tuning their effectiveness and survival after application under diverse field conditions

*(*Ambethgar, 2009; Lopes et al., 2011*).* The method developed in the study utilised a specific trait of *T. asperellum*, the beta-tubulin2 gene encoding the tubulin chain, to create a novel TaqMan-based PCR assay. This assay was developed to detect and quantify the *T. asperellum* FC80 strain in soil, providing results that reflect active population quantification. The TaqMan-based assay is an innovative method for the absolute quantification of *T. asperellum* FC80 in soil using quantitative reverse transcription PCR (qRT-PCR) (Anderson and Parkin, 2007; Beaulieu et al. 2011). PCR has been widely used to detect and quantify microorganisms in diverse environmental matrices, including soil, by targeting various microbial genes (Nesme et al. 1995; Barns et al. 2005; Degrange and Bardin 1995; Canfora et al. 2016). The assay's specificity was confirmed by testing the probe against a panel of fungi commonly used as BCAs.

The findings presented showed the different behaviour of *T. asperellum* FC80 on the two crops studied. On the tomato crop (an annual crop), the population of *T. asperellum* FC80 generally increased three months after the initial application. In contrast, on the strawberry fields (which host a two-year cropping period), the population of *T. asperellum* FC80 remained stable for an extended duration, spanning approximately 450 days. In a field experiment with *T. atroviride* strain SCI, Stummer et al. (2020) demonstrated the prolonged persistence of a *Trichoderma* species up to 18 weeks after inoculation. Little information is available about the interaction between *Trichoderma* spp. and root exudates. However, it is worth inferring that the root exudates secreted by the plant in recruiting beneficial microorganisms might also drive the capacity of a BCA to colonise the soil over multiple years. Fernandèz et al., 2017) observed that pathogens and BCAs modify how root exudates are secreted. Indeed, on strawberries, a decline in the population of *T. asperellum* FC80 was observed after approximately 270 days (T3 – 2022), corresponding to the autumn-winter period. This decrease in population aligns with the reduction in soil temperatures and the dormant stage of plants, which can limit the development and survival of Trichoderma. Most *Trichoderma* species are mesophilic, and low temperatures can challenge their biological activity (Kredics et al. 2003)

The effectiveness of these agents can be influenced by various factors, such as environmental conditions, farming practices, application methods, and the biological properties of the microorganisms (Bardin and Pugliese, 2020). The varying amounts of *T. asperellum* FC80 observed on tomatoes at one and three months after the application indicated that soil characteristics and environmental conditions, such as temperature and humidity, might have influenced the abundance of the inoculant (Talley et al. 2002; Wang et al. 2018). However, this extended persistence would allow the farmer to plan only one inoculation within the season, aligning with good agricultural practices that recommend avoiding the need for re-application (depending on the crop*)*. These observations underscore the practical implications of monitoring the population dynamics of biocontrol agents, as they can inform farmers' decisions and optimise the efficacy of biological control strategies.

When tested against other fungal species also used as BCAs, the TaqMan assay showed high discriminant and diagnostic specificity. Validation and compliance were assessed following the guidelines established by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009) and a previously established protocol (Manfredini et al., 2023). The results of the validation procedure were confirmed by the excellent detection specificity in the two separate experiments conducted on tomatoes and strawberries over three years, notwithstanding the complexity of the soil matrix and the presence of autochthonous *Trichoderma* species.

It is important to note that farmers' adoption of BCAs relies on ensuring their reliability and stability in efficacy (Nicot et al., 2011). Knowledge about how monitoring the persistence of a BCA in soil over time, after application, is crucial in determining the best formulations and the application timeframe designed to have persistent, long-term control. Indeed, the study successfully detected and measured the presence of active *T. asperellum* FC80 in the soil near the roots of tomato and strawberry plants using RNA reverse transcription. RNA is a crucial molecule found in actively functioning cells and degrades quickly upon cell death, making it a reliable indicator of living and active microorganisms (Berg et al., 2020). The reverse-transcriptase quantitative PCR (RT-qPCR) method employed in this study enabled the amplification of mRNA, which represents transcribed genetic information, facilitating the quantification of gene expression in viable cells.

Conclusions

The TaqMan assay based on beta-tubulin2 has demonstrated exceptional specificity and sensitivity in detecting *T. asperellum* FC80 in soil samples. This method holds great promise for monitoring the presence of *T. asperellum* strains across diverse environmental conditions. Its precision enables accurate quantification of *T. asperellum* FC80 in soil, rendering it well-suited for regulatory purposes and for optimising its application method.

This is the first report of qPCR assays being used to measure *T. asperellum* FC80 inoculants in tomato and strawberry soil. The assay is versatile and can do more than detect; it can also track the population dynamics and survival rates of *T. asperellum* FC80 under different environmental conditions. By monitoring changes in population over time, researchers can gain insights into the effectiveness and behaviour of this biological control agent in various soil environments. We are currently using this data to understand the agroecological factors influencing the establishment and persistence of the inoculant and the disease-suppressive effects of Trichoderma inoculant in tomato and strawberry cropping systems.

Overall, the comprehensive approach, rigorous validation, and successful application in multiple experiments strongly support the TaqMan assay's specificity in detecting and quantifying *T. asperellum* FC80 in soil samples.

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535 **Conflict-of-interest statement**

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the manuscript's contents, and there is no financial interest to report.

Author contribution statement

Conceptualisation, design of the lab experiment, data analysis and writing A.M., L.C.; design of field experiment P.V. and M.P.; L.C., A.M., P.V. and M.P. wrote the. Drafting review and editing all authors. All authors have read and agreed to the published version of the manuscript.

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