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Research Papers

Detection and persistence of *Bacillus*- and *Trichoderma*-based biocontrol products on lemon plants

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Summary. Knowledge of the ecology of biological control agents (BCAs), previously reported to be effective against the citrus vascular pathogen *Plenodomus tracheiphilus*, is required to improve scheduling of BCA field applications. Culture-dependent methods (dilution plate and direct plating) and culture-independent qPCR assays were used to determine survival of *Bacillus amyloliquefaciens* QST 713 and *Trichoderma asperellum* ICC 012 + *T. gamsii* ICC 080 on citrus stem and leaf tissues following foliar applications, and root endospheres and rhizospheres following root drenches with these *Trichoderma* BCAs. Viable population levels of *B. amyloliquefaciens* did not change over time on treated stems, whereas these decreased in leaf samples after 14 d. The qPCR assay detected *B. amyloliquefaciens* in all collected samples, with no temporal changes in population levels. Although the qPCR assay detected *T. asperellum* and *T. gamsii* in leaf and stem tissues, these fungi were isolated only from stem tissues, with increased isolations at 21 d post treatment compared with 7 d. Neither qPCR nor culturing detected *Trichoderma* species in citrus vascular root tissues. However, qPCR and culturing detected these fungi in host rhizospheres at 7, 14 and 21 d post inoculation, confirming their rhizosphere competence. This study has provided insights into colonization and survival within citrus plants of *B. amyloliquefaciens* and *T. asperellum* + *T. gamsii* contained in commercial biocontrol products. These indicate that integrating qPCR and culture-dependent approaches is important for detecting and quantifying these BCAs. The endophytic lifestyle of *B. amyloliquefaciens* and *T. asperellum* + *T. gamsii* makes them likely to provide long-term biological control. These results also indicate that the selected *Bacillus* and *Trichoderma* agents could spread and colonize citrus tissues over extended periods, and especially after host pruning or damage, which could promote plant colonization and protection from pathogens.

Keywords. BCA, citrus, endophytic colonization, monitoring, qPCR.

INTRODUCTION

Citrus types are among the most economically important fruit crops, appreciated by consumers for the high amounts they contain of health-promoting nutrients and bioactive compounds, such as flavonoids, phenolic acids, vitamins, carotenoids, pectins, and fatty acids (Liu *et al.,* 2022). Citrus cultivation is distributed in tropical, subtropical and Mediterranean climatic regions, expanded from East Asia to all continents on a total of 12.7 million ha (FAOSTAT, 2024; Istat, 2024). Italy is the second-largest producer of oranges and the third-largest producer of lemons, reaching production of nearly 3.1 million tons (FAOSTAT, 2024; Istat, 2024).

Several abiotic and biotic factors affect citrus production (Timmer *et al.,* 2000). Several Ascomycete fungi, which infect wood through natural openings or pruning wounds and colonize host vascular tissues, are involved in twig, branch, and trunk diseases of citrus. Wind, hail and frost damage, sunscald, overhead irrigation, and mechanical injuries can facilitate citrus infections by fungi (Fawcett 1936; Aiello *et al.,* 2023; Leonardi *et al.,* 2023a). *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*), the causal agent of Mal secco disease, is considered to be the major destructive fungal disease, causing twig and branch wilt and dieback, with serious economic impacts on citrus industry of the Mediterranean and Black Sea regions (Catara and Cutuli, 1972; Solel and Salerno, 2000). *Colletotrichum* spp. are generally recognized as important pathogens in all cultivated *Rutaceae*, causing stem-end rots, and twig and branch dieback (Timmer *et al.,* 2000; Mayorquin *et al.,* 2019; Leonardi *et al.,* 2023a). Species in *Botryosphaeriaceae* and *Diaporthaceae* have also been reported to cause cankers, gummosis, blight and dieback in different citrus-producing countries (Polizzi *et al.,* 2009; Huang *et al.,* 2013; Guarnaccia and Crous, 2017; Gusella *et al.,* 2025), as well as other fungal genera including *Fusarium*, *Neocosmospora*, *Peroneutypa*, and *Phaeoacremonium* (Timmer 2000; Mayorquin *et al.,* 2016; Sandoval-Denis *et al.,* 2018; Espargham *et al.,* 2020; Gusella *et al.,* 2025).

Management of fungal pathogens affecting the vascular systems and woody tissues of plants is difficult, because no effective treatments can protect hosts once pathogens have colonized internal host tissues. Management relies on application of fungicides or on agronomic practices (e.g., sanitation), when varietal resistance cannot be achieved (EFSA PHL, 2014; 2023; Vitale *et al.,* 2021). Frequent use of chemical fungicides in agriculture has raised concerns about phytotoxicity, development of resistant pathogen strains, soil accumulation, and negative effects on environments and human health (European Chemicals Agency, 2018; Piel *et al.,* 2019; Triantafyllidis *et al.,* 2020; Dao *et al.,* 2021; Burandt *et al.,* 2024). Based on scientific assessments of chemical toxicity, the European Union (EU) has approved laws that result in banning or restriction of their use by imposing decreased maximum residue limits (MRLs) (Clark *et al.,* 2002; EFSA 2020; European Commission, 2020). Some chemicals, such as copper compounds authorized for the control of *Alternaria* spp., *Colletotrichum* spp., and *P. tracheiphilus*, have been classified by the EU as candidates for substitution (European Commission, 2018), with the aim of finding alternative substances that are environmentally friendly and cost-effective.

Biological control of plant diseases using endophytic microorganisms able to colonize the same ecological niches as pathogens, and that have antagonist activity, have been widely investigated. Among biological control agents (BCAs), bacteria and fungi that are used to manage plant diseases are reported as rapid colonizers of phyllospheres, endospheres or rhizosphere (Kalai-Grami *et al.,* 2014a, 2014b; Dimaria *et al.,* 2023; Giordano *et al.,* 2023; Nascimento *et al.,* 2023; Tan *et al.,* 2025).

Bacillus and *Trichoderma* spp. have been widely studied for abilities to control citrus plant pathogens, through different biocontrol mechanisms (Weideman and Wehner, 1993; Agostini *et al.,* 2003; Kalai-Grami *et al.,* 2014a, 2014b; Kupper *et al.,* 2011, 2019; Chen *et al.,* 2020; Ferreira *et al.,* 2020; Kalimutu *et al.,* 2020; Ezrari *et al.,* 2021; Silvia, 2021; Aiello *et al.,* 2022; Calcagnile *et al.,* 2022; Khuong *et al.,* 2023; Leonardi *et al.,* 2023b; Phal *et al.,* 2023; Lombardo *et al.,* 2024; Zhou *et al.,* 2024). Activity of these microorganisms is linked to production of biodegradable biological molecules that can inhibit pathogen growth, induce systemic resistance, and/or compete with pathogens for nutrient resources (Lugtenberg and Kamilova, 2009; Ezrari *et al.,* 2021).

Trichoderma and *Bacillus* are part of the microbiota of healthy plant tissues, without causing disease but assisting host plants to defend against biotic and abiotic stresses (Chen *et al.,* 2020; Fontana *et al.,* 2021). For these reasons, several *Trichoderma* and *Bacillus* species have been identified as potential biocontrol agents, and some have been developed as biocontrol agents in commercial formulations. Among these, Serenade®Aso, containing *Bacillus amyloliquefaciens* strain QST 713 (formerly *B. subtilis*), and Remedier®, containing *T. asperellum* isolate ICC 012 and *T. gamsii* isolate ICC 080, are currently registered in Italy (Ministero della Salute, 2025). Recent studies have demonstrated the efficacy of these agents for controlling symptoms of leaf vein chlorosis caused by *P. tracheiphilus* on Volkamer lemon plants in controlled growth conditions (Aiello *et al.,* 2022; Leonardi *et al.,* 2023b; Leonardi *et al.,* 2026).

Because of increased interest in the use of BCAs in integrated management of citrus diseases, knowledge on their colonization and survival within plant systems is important for achievement of disease control, and for scheduling BCA applications (Torsvik and Øvreås, 2002; Timofeeva *et al.,* 2023). Some *Trichoderma* and *Bacillus* species inhabit soil and plants, with ability to colonize rhizospheres and endospheres of several crop plants (Bae *et al.,* 2009; Labiadh *et al.,* 2021; Tseng *et al.,* 2020; Wang *et al.,* 2021). However, few investigations have assessed endophytic colonization ability of *Bacillus amyloliquefaciens* in citrus plants, and these have only used culture-dependent methods (Kalai-Grami *et al.,* 2014a; Aiello *et al.,* 2022). Although time-consuming for field monitoring, culture-dependent methods have been the most widely used for quantifying BCA population levels. Alternative culture-independent methods, including quantitative polymerase chain reactions (qPCRs), have been proposed for more sophisticated detection and quantification of *Bacillus* spp. and *Trichoderma* spp. selected as plant pathogen BCAs (Rubio *et al.,* 2005; Savazzini *et al.,* 2008; Johansson *et al.,* 2014; Mendis *et al.,* 2018; Stummer *et al.,* 2020; Xie *et al.,* 2020; Li *et al.,* 2021; Su *et al.,* 2024). qPCR assays have been validated on grapevine bunches, wood and soil for quantification of *Bacillus* and *Trichoderma* (Rotolo *et al.,* 2016; Gerin *et al.,* 2018).

The aim of the present study was to accurately detect and quantify, over time, the levels of *B. amyloliquefaciens* QST 713 and *T. asperellum* ICC 012 + *T. gamsii* ICC 080 within the vascular tissues of lemon seedlings, after application of these two formulations under controlled growth conditions, using culture-dependent and molecular methods.

MATERIALS AND METHODS

Plant material and growth conditions

Ten-month-old seedlings of Volkamer lemon (*Citrus volkameriana*) were previously grown in a nursery greenhouse in Catania province (Eastern Sicily, Italy), and were maintained in plastic trays (850 mm^2 and 900 mm deep). The plants were obtained by sowing healthy seeds on a commercial substrate (90% blond peat + 10% perlite) to which had been added an organic fertilizer (35–40% KNO_3, 0.3–1% $CuSO_4{\cdot}5H_2O$, 0.1–0.2% BH_3O_3, 0.1–0.2% $ZnSO_4$) at 1 kg m^{-3}), and a micronutrient fertilizer (15% Fe, 2.5% Mn, 0.20% B, 1% Cu, 1% Zn, 0.04% Mo) at 300 g m^{-3}. Two weeks before experiments were carried out, the seedling trays were transferred to a growth chamber at the Department of Agriculture, Food and Environment, University of Catania, which was maintained at 25°C, 80% relative humidity (RH), and with a daily cycle of 16 h light and 8 h darkness.

BCA selection and preparation

To assess the endophytic colonization ability of the BCAs on citrus plants, the commercial BCA formulations Serenade® Aso, containing *B. amyloliquefaciens* (formerly *B. subtilis*) isolate QST 713 at 1.05×10^{12} CFU L^{-1} (Bayer CropScience) and Remedier®, containing *T. asperellum* isolate ICC 012 + *T. gamsii* isolate ICC 080 at 3×10^7 CFU g^{-1} (Gowan Crop Protection), were selected from BCAs that had previously been shown to minimize symptoms caused by *P. tracheiphilus* (Aiello *et al.,* 2022; Leonardi *et al.,* 2023b; Leonardi *et al.,* 2026). Before commencing applications to the plants, the viability of the BCAs was confirmed by plating the respective product suspensions on Potato Dextrose Agar (PDA) (Lickson).

Foliage and root applications

Colonization ability of *B. amyloliquefaciens* into *C. volkameriana* plants was assessed on leaves and stems (from foliar applications), whereas *Trichoderma* spp. colonization was assessed on leaves and stems (foliar applications), into root endospheres and into soil rhizospheres (root applications). Each of these was considered as a distinct experimental treatment, since effectiveness had been previously confirmed through root drench applications (Leonardi *et al.,* 2026). Prior to the foliar BCA applications, wounds were made by pruning plant stem tips to 5 cm length to favour microbial colonization of the vascular systems.

Both commercial products were prepared according to the label rates (167 g hL^{-1} for the *Trichoderma*-based product, and 533 mL hL^{-1} for the *Bacillus*-based product). Inoculations were carried out by spraying 300 mL of each formulation (100 mL per replicate) onto all plant canopy with a manual sprayer. For the foliar treatments, inoculation controls consisted of seedlings pruned and then sprayed with sterile deionized water (SDW). Root applications each consisted of a 30 mL drench of the commercial products at the rates described above. Experimental control treatments were with 30 mL of SDW. The foliar-treated plants were then allowed to dry for around 4 h, and all plants were then transferred into a growth chamber maintained at 25 ± 1°C, 80% relative humidity (RH), with a photoperiod of 16 h light and 8 h darkness. A total of 45 seedlings were used for each treatment (foliar or root), with three replicates per treatment, each including 15 seedlings.

Leaf and stem collections

To evaluate development of *Bacillus* or *Trichoderma* within the inoculated tissues after treatments, three post-treatment collection were made at 7, 14, and 21 d after treatments. At each collection, 15 seedlings (five per replicate) were randomly selected for analyses. From each plant, a 5 cm stem section from the artificial wound was collected, together with three leaves from the same stem portion, so each seedling was divided into stem and leaf tissue. The samples were washed under tap water and surface sterilized using the protocols of Mushtaq *et al.* (2019) for endophyte isolation from citrus leaf vascular tissues, and Araújo *et al.* (2002) for isolation from stems, with some modifications. Leaf samples were sterilized in 1% sodium hypochlorite solutions for 5 min, followed by three washes in SDW. Stem samples were sterilized in 70% ethanol for 5 min, sodium hypochlorite solution (2.5% available Cl^-) for 5 min, and 70% ethanol for 30 s, followed by two washes in SDW. To confirm that leaf and stem samples were disinfested of contaminants, aliquots of the sterile distilled water used in each final rinse were plated on PDA, and the plates were examined for microbial growth after incubation at 25 ± 1°C for 3 to 5 d. After thorough drying, the leaves and stems (after aseptic bark removal), were cut longitudinally. Half of the leaf and stem tissues was then processed for culture-dependent analysis, and the other half for qPCR analysis.

Rhizosphere and root collections

Collection of rhizosphere samples was carried out as described by Xu *et al.* (2022), with modifications. Fine roots (approx. 1 mm diam.) were collected from each plant to a depth of 5-6 cm, and were gently shaken to remove soil not tightly attached to the roots. Adherent soil surrounding the roots was then carefully scraped off with a hairbrush, and collected to obtain 2 g per replicate (each of five plants). One g of rhizosphere soil was immediately stored at -80°C for DNA extraction. The fine roots were used to determine populations of *Trichoderma* in the endospheres.

Culture-dependent detection of Bacillus *from leaf and stem tissues*

A total of 1 g of each leaf or stem tissue sample was homogenized by mortar and pestle in 5 mL of sterile-filtered phosphate-buffered saline (PBS 1X; Thermo Scientific), and serial dilutions (10^{-1} to 10^{-4}) from each sample were plated onto PDA plates. A total of nine plates per replicate were used, with three replicates per time after inoculation. The plates were incubated at 25 ± 1°C for 48 h, and developed bacterial colonies were then subcultured onto Nutrient Agar (NA) and identified based on colony morphology. Bacterial population densities were estimated by counting the colonies, and were expressed as colony-forming units (CFU) per g of sample fresh weight determined from numbers of CFU mL^{-1} of sample plated, and the dilution factors.

Colonies morphologically identified as *Bacillus* were molecularly characterized. Their DNA was extracted and sent to Macrogen Inc. (Seoul, Republic of Korea) for PCR and sequencing. The 16S rDNA was amplified using the primers 27F/1492R (Somerville *et al.,* 2020), and the *gyrA* gene regions were amplified using primers 42f/1066r (Chun and Bae 2000). The DNA sequences generated were assembled with Lasergene SeqMan Pro (DNASTAR), deposited in GenBank (https://www.ncbi.nlm.nih.gov/), and compared with the NCBI GenBank nucleotide database using the standard nucleotide Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Culture-dependent detection of Trichoderma *from leaf and stem tissues*

For fungal isolations, leaf and stem samples were surface sterilized and processed using the methods described above for *Bacillus*-treated plants. Although PDA was used under the same conditions to allow simultaneous recovery of fungi and bacteria, to ensure accurate detection and quantification of *Trichoderma* and to allow for rapid growth of these fungi, isolations from plant samples treated with *Trichoderma* were also carried out on Rose Bengal-chloramphenicol agar (RBA; Biolife), a selective medium for these fungi (Qiao *et al.,* 2018; Zanfaño *et al.,* 2024).

In addition to the plate dilution technique, direct plating was also used for *Trichoderma*, because rapid growth and filamentous habit of these fungi can affect colony separation. Stem and leaf sections were cut into 4 to 6 mm pieces, and placed onto RBA amended with 100 mg L^{-1} of streptomycin sulfate (Sigma-Aldrich) to prevent bacterial growth. Plates were then incubated at 25 ± 1°C in the dark for 4 to 6 d or until fungal growth was observed. Population densities were estimated by calculating the isolation frequency (IF) as the percentage of tissue fragments that yielded colonies morphologically resembling *T. asperellum* or *T. gamsii*, out of the total number of fragments plated per replicate (including tissue collected from five plants). Other *Trichoderma*-like colonies were not counted, but representative colonies were chosen and molecularly characterized to confirm their identities. A total of nine plates per replicate, each containing eight leaf or stem fragments, were used for isolations at each collection time. *Trichoderma asperellum* and *T. gamsii* colonies obtained were firstly identified based on culture characteristics (colony appearance, upper and lower surface colours, borders, texture, conidia), and were molecularly characterized.

The collected colonies distinguished based on morphotype were grown on PDA for 5 d, and genomic DNA was then extracted. Mycelium was scraped off and processed using the Wizard Genomic DNA Purification Kit® according to the manufacturer's protocol (Promega Corporation). DNA samples obtained were stored at 4°C until processed. The DNA-directed RNA polymerase II second largest subunit gene (*rpb2*) was amplified with primers RPB2-5F2 (Sung *et al.,* 2007) and fRPB2-7cr (Liu *et al.,* 1999), and the PCR amplification products were estimated visually by electrophoresis on 1% agarose gels and sent to Macrogen Inc. (Seoul, Republic of Korea) for purification and sequencing. The DNA sequences generated were assembled, deposited in GenBank, and compared with reference sequences in the NCBI GenBank nucleotide database, as outlined (above) for *Bacillus.*

Culture-dependent detection of Trichoderma *from roots and rhizospheres*

Root samples were washed with tap water and sterilized using the methods of Trivedi *et al.* (2011), with some modifications. Root segments were immersed in sodium hypochlorite solution (2.5% available Cl^-) for 5 min, and then washed five times in SDW. For a sterility checks, aliquots of each last rinse water were plated onto PDA and RBA plates. Isolation of fungi from roots were carried out using agar dilution and direct plating of wood fragments. Samples (1 g each) were homogenized using a mortar and pestle in 5 mL of sterile PBS, and the homogenized samples were then used to prepare serial dilutions (10^{-1} to 10^{-4}) which were plated on RBA amended with 100 mg L^{-1} of streptomycin sulfate. Roots were also cut into fragments and plated onto RBA medium amended with 100 mg L^{-1} of streptomycin sulfate to prevent bacterial growth. Plates were then incubated at 25 ± 1°C in the dark for 6 d until fungal growth was observed.

Isolations from rhizospheres were carried out using the plate-dilution method of Xu *et al.* (2022). Collected rhizosphere soil (1 g) was suspended in 9 mL of PBS buffer, and then shaken at 250 rpm for 40 min. The resulting suspension was serially diluted at tenfold intervals to 10^{-4} dilution. Aliquots of each dilution were then spread onto three replicate RBA plates.

Trichoderma population densities were estimated by counting fungal colonies on plates, and were expressed as CFU g^{-1} fresh weight of soil or root, and (only for roots), calculating the IF as described above. A total of nine plates per replicate were used for direct plating, each containing eight root fragments, with three replicates per interval. *Trichoderma* colonies obtained from treated and untreated rhizosphere soil were first identified based on culture characteristics, and pure cultures were also molecularly characterized based on the *rpb2* gene, as described above.

Extractions of total bacterial and fungal genomic DNA from plant tissue and rhizosphere samples

Leaf, stem and root tissues, collected at 7, 14 and 21 d, were crushed and ground to powder in liquid nitrogen, and totals of 300 or 600 mg of plant tissue was collected from each replicate and stored at -80°C until DNA extraction. Total bacterial and fungal genomic DNA (gDNA) were extracted using the DNeasy Plant Pro kit (Qiagen), according to the manufacturer's instructions and with slight modifications. To improve the extraction yields, the samples were each twice homogenized with a Precellys Evolution homogenizer (Bertin Technologies) at 4,500 rpm, with each homogenizing separated by a pause on ice. After tissue destruction, the samples were subjected to extraction and purification steps according to the kit procedures.

The same protocol and extraction kit were adopted (with modifications) for DNA isolations from soil samples. Prior to the bead beating step, DNA yield was improved by heating each soil samples (100 mg) to 65°C for 10 min in the presence of lysis solution. After incubation, the steps described above were followed.

For all the samples, the gDNA concentrations were determined with a Qubit 4.0 fluorometer (Invitrogen), and purity was checked with a NanoDrop spectrophotometer (Thermo Scientific). The gDNA templates obtained were stored at -20°C, and were subjected to qPCR detection.

qPCR monitoring of Bacillus amyloliquefaciens

For the molecular detection of *B. amyloliquefaciens* QST 713, species-specific primers were used to detect colonization of the inoculated BCA, at different sampling times. The protocol of Rotolo *et al.* (2016) was used for the qPCR analyses. The reaction mixtures each consisted of 10 μL of QuantiNovaTM SYBR Green PCR

Kit (Qiagen), 10 µM of each primer BS_yndJ-F (AATGACCGTGCTCCATCTGTAA) and BS_yndJ-R (TTCCGATCTTACGGATTGCT), 5 µL of Dnase/Rnase-free water and 3 µL of templated gDNA. The amplification cycles included an initial pre-denaturation step (Hold) at 95°C for 3 min, followed by 40 cycles, each of denaturation at 95°C for 10 s, annealing at 58°C for 45 s, and extension 72°C for 60 s. The melting temperatures were set between 55°C and 95°C. The slope of the regression curve obtained by plotting the logarithm of DNA concentrations against corresponding mean cycle thresholds (Ct values) was used to determine primer efficiency, according to the equation: $E=0.5\ (10(-1(-1\mathit{slope})) \times 100$.

To assess reaction efficiency, gDNA from the *B. amyloliquefaciens* QST 713 isolate (Serenade® Aso) was extracted using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), and was used as a reference sample to generate standard curves. Standard curves were established by serially diluting the QST 713 gDNA to obtain concentrations ranging between 12 and 5 log CFU mL^{-1}. Quantitative PCR assays were carried out using a Rotor-Gene Q thermocycler (Qiagen), and each sample was analyzed in triplicate.

qPCR monitoring of Trichoderma asperellum *and* Trichoderma gamsii

For detections of *T. asperellum* and *T. gamsii*, a Duplex-qPCR was set up, following the protocol of Gerin *et al.* (2018), to monitor the colonization by fungal formulation at different sampling times. The reaction mixtures were each prepared using 10 µL of QuantiNovaTM SYBR Green PCR Kit (Qiagen), 0.25 µM of each of the primers, and 0.15 µM of each of the probes as:
for *T. asperellum*;
Ta_rpb_fw (GGAGGTCGTTGAGTACGAA),
Ta_rpb2_rev_3 (TTGCAATAGGATTTACGACGAGT),
Ta_rpb2_probe (FAM-CGCTGAGGTATCCCCATGCGACA-BHQ1),
and for *T. gamsii*;
Tg_rpb2_fw (GCCACCTGGTTGACCAAGGA),
Tg_rpb2_rev (CGCACCAGCCCTGATCA), and
Tg_rpb2_probe (HEX-CCTCCAGAAGACCCAAGCATGAAGCTC-BHQ1).

Dnase/Rnase-free water (5.4 µL) and 2 µL of templated DNA was included in each reaction. The amplification cycles each included an initial pre-denaturation step (Hold) at 95°C for 2 min, followed by 40 cycles each of denaturation at 95°C for 5 s, annealing at 62°C for 30 s, and extension 65°C for 30 s. The melting temperature was set in the range of 65°C to 95°C. The slopes of the regression curves were calculated as described above.

To ensure reaction efficiency, total gDNA of the *T. asperellum* and *T. gamsii* mixture isolates was isolated using the QIAamp DNA Mini kit, with some adjustments in the initial steps (below). Mycelium of the two species, grown on agar plates, was collected and dissolved in phosphate buffered saline (PBS, pH 8.0), to allow washing of conidia and removal of extracellular parts. Each solution was centrifuged at 7,500 rpm for 10 min. Subsequently, the resulting pellet was resuspended in 180 µL of 2 mM EDTA solution and incubated in Thermomix for 30 min at 37°C. Following this, 20 µL of proteinase K and 200 µL of lysis buffer (provided with the kit) were added, and the samples were then incubated at 56°C for 30 min and then at 95°C for 15 min. The solutions were then transferred to bead-beater vials for mechanical lysis (9,000 rpm for 2 min with pause on ice). The solutions were transferred to purification columns provided by the kit for the final purification steps, according to the kit manufacturer instructions. qPCR standard curves were each obtained by serially diluting the DNA mixture to concentrations from 1.13×10^{10} to 1.13×10^{3} conidia mL^{-1}. The qPCR reactions were carried out with a Rotor Gene Q instrument (Qiagen). Each sample was assayed in triplicate.

Data analyses

All CFU data from dilution plate isolations and from qPCR assays were log-transformed, and the percentage IFs from direct plating isolations were arcsine (sin^{-1} square root x) transformed, to meet the assumptions of homogeneity of variance, which were determined using Levene's test. A primary analysis of variance (ANOVA) was then carried out for each experimental treatment, by calculating F and the associated P values to evaluate whether effects of single factors (i.e. treatment, time, and sample type) and their interactions, were statistically significant. *Post hoc* comparisons were conducted on significant interactions using Tukey's HSD test ($\alpha = 0.05$). When no significant interaction was detected, the main effect of a single factor was evaluated separately using one-way ANOVA.

RESULTS

Culture-dependent detection of Bacillus amyloliquefaciens *in leaf and stem tissues*

At each collection time, bacterial colonies developed from treated samples as determined using the culture-dependent method (Figures 1 and 2). Developed colonies

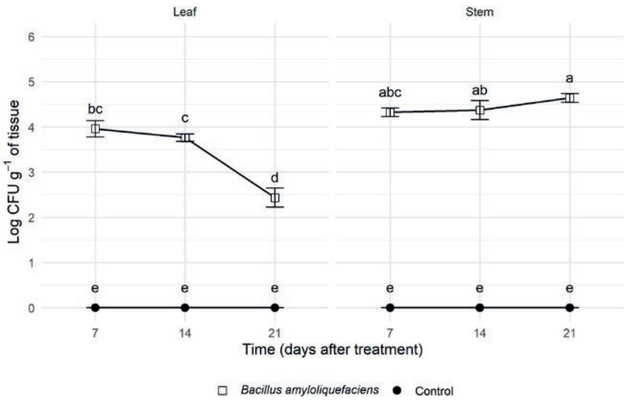

Figure 1. Mean numbers (± standard errors) of *Bacillus amyloliquefaciens* (expressed as Log CFU g^{-1} of tissue) detected in citrus leaf or stem tissues at 7, 14 or 21 d after treatment using a culture-dependent method. Results were from *Bacillus*-treated (open symbols) and control plants (close symbols) using the agar dilution technique. Different letters accompanying symbols indicate differences ($\alpha = 0.05$) among combined factors (treatment × sample × time), according to Tukey's HSD tests.

in the cultures had smooth and sticky surfaces, and were protruding with slightly irregular edges (Figure 2) (Qiao *et al.,* 2024). A culture of a representative strain (BA1) was molecularly characterized, and 16S rDNA (Acc. No. PX559962) and *gyrA* (Acc. No. PX571115) gene sequences of this culture were deposited in GenBank. Comparison of the sequences obtained with those present in the NCBI nucleotide database showed 99.75% similarity for 16S rDNA with different *Bacillus* species isolates (including *B. amyloliquefaciens* isolate D3, Acc. No. KR871014.1), and 99.80% similarity for the *gyrA* gene with different *B. amyloliquefaciens* isolates (including, INH2-4b Acc. No. CP061852). No colonies resembling *Bacillus* were obtained from the controls through using the culture-based method.

Bacillus amyloliquefaciens concentrations from leaf samples were 3.96 log CFU g^{-1} after 7 d, 3.76 log CFU g^{-1} after 14 d, and 2.43 log CFU g^{-1} after 21 d in culture. From stem samples, *B. amyloliquefaciens* concentrations were 4.32 log CFU g^{-1} after 7 d, 4.32 log CFU g^{-1} after 14 d, and 4.64 log CFU g^{-1} after 21 d in culture. Homogeneity of variances was confirmed by Levene's test ($F = 0.9311$, $P = 0.53$). The multifactor ANOVA showed that the single factors, (treatment, sample type, collection time), and the combinations of these factors, influenced the respective *Bacillus* populations ($P < 0.001$). Tukey's *post hoc* test of the combined factors (treatment × sample type × collection time), showed that *Bacillus*-treated samples differed in microbial populations within leaf and stem tissues from those of untreated plants, at each of the three collection times ($P < 0.001$). The populations of *B. amyloliquefaciens* recorded in the leaf sam-

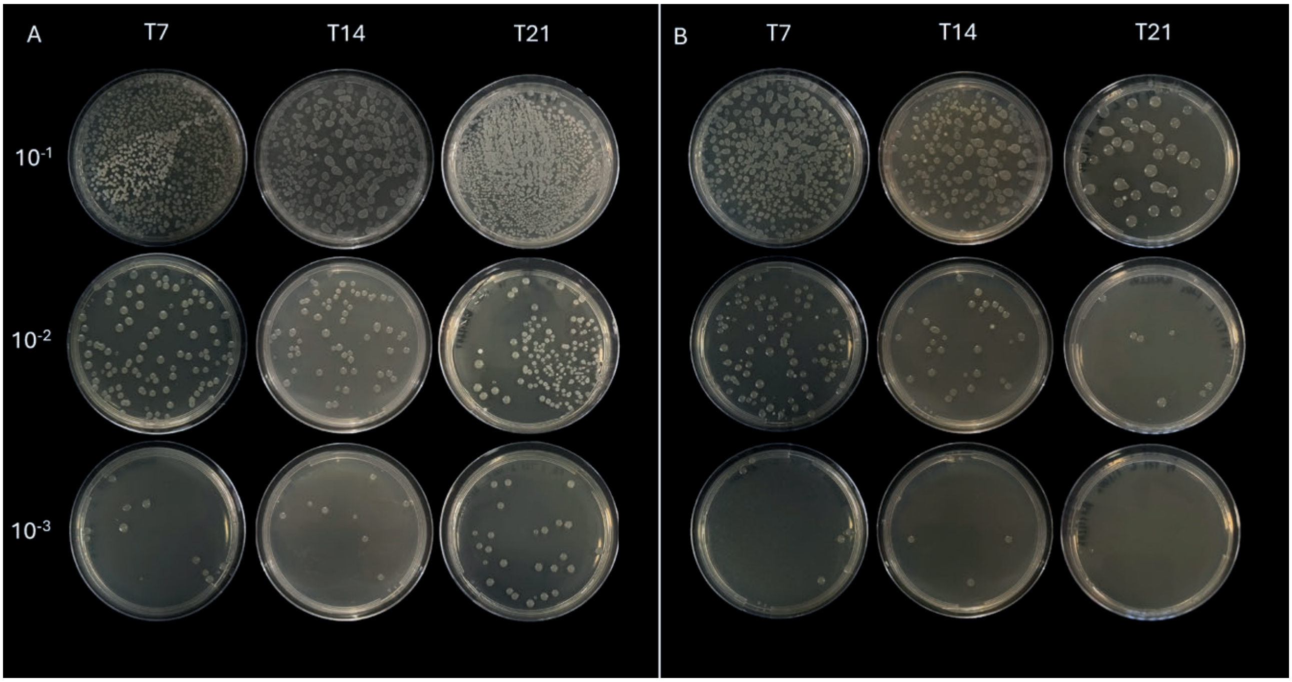

Figure 2. Representative *Bacillus amyloliquefaciens* isolation plates from citrus stem (A) or leaf (B) tissues, at different collection times (T7, T14, or T21 d after treatments), showing colony growth on potato dextrose agar across serial dilutions (from 10-1 to 10-3).

ples using the culture-dependent method did not differ ($P > 0.05$) between 7 and 14 d after culturing, but were reduced after 21 d (Figure 1). Populations of *B. amyloliquefaciens* in the stem tissue samples did not change with time, although a slight increasing population trend was observed from 14 to 21 d. Populations of the bacterium in stem tissues at 14 and 21 d differed significantly from those detected in the leaf tissues at each of the three assessment times.

Culture-indipendent detection of Bacillus amyloliquefaciens *in leaf and stem tissues*

Bacillus amyloliquefaciens detection was assessed by qPCR assays of leaf and stem samples. The detection threshold was calculated from standard curves, and ranged from a maximum of 12 log CFU mL^{-1} (cycle threshold (Ct) = 10.6) to a minimum of 5 log CFU mL^{-1} (Ct = 31.7). No DNA copies were detected from the negative controls (Ct = 39.2). The obtained slope value was -3.17308, and the coefficient of determination (R^2) was 99.1%. For the leaf samples, the inoculated bacteria were detected at concentrations of 6.92 log CFU g^{-1} after 7 d, 6.55 log CFU g^{-1} after 14 d, and 6.46 log CFU g^{-1} after 21 d. For the stem samples, the bacteria were detected at concentrations of 8.27 log CFU g^{-1} after 7 d, 7.85 log CFU g^{-1} after 14 d, and 7.72 log CFU g^{-1} after 21 d. The Ct data confirmed the cell densities detected, with Ct values (for the three sampling times) of 25.44, 26.44 and 26.26 for the leaf samples, and 21.62, 23.90 and 23.75 for stem samples (Supplementary Table I).

The qPCR analyses revealed presence of *B. amyloliquefaciens* DNA in the control plants, with concentrations in the leaf tissues of 1.79 log CFU g^{-1} (Ct= 33.43) at 7 d, 1.73 log CFU g^{-1} (Ct = 33.11) at 14 d, and 1.66 log CFU g^{-1} (Ct = 34.05) at 21 d. Control plant populations in the stem tissues were 5.48 log CFU g^{-1} at 7 d (Ct = 31.69), 5.02 log CFU g^{-1} at 14 d (Ct = 32.04), and 5.47 log CFU g^{-1} at 21 d (Ct = 31.88). Homogeneity of variances of DNA concentrations was confirmed by Levene's test (F = 1.03, $P = 0.45$). The multifactor analysis of variance (ANOVA) showed that the single factors (treatment, plant tissue, collection time), and the combined factor treatment × sample influences detection of *Bacillus* ($P < 0.001$). Tukey's *post hoc* test of the combined factors (treatment × sample) showed that *Bacillus*-treated samples differed in microbial concentrations within the leaf and stem tissue samples from those of untreated plants (Figure 3). Although the levels of *B. amyloliquefaciens* detected in stem tissues were greater than in leaf tissues, there were no statistically significant population changes ($P > 0.05$) during 21 d post inoculation in either the leaf or stem tissue samples.

Culture-dependent detection of Trichoderma asperellum *and* Trichoderma gamsii *in leaf and stem tissues*

Trichoderma was isolated from stem tissue samples using direct plating (Figures 4 and 5), but no *Trichoderma*-like colonies were obtained from leaves using plate dilution or direct plating. Two distinct *Trichoderma*-like colonies were consistently obtained from the stems of treated plants, so representative isolates were collected for each of the two *Trichoderma* morphotypes (named TA and TG), which were distinguishable by colony morphology. Comparisons of the *rpb2* sequences of one representative isolate per morphotype, TA1 (Acc. No. PX597589) and TG1 (Acc. No. PX597590), with those present in the NCBI nucleotide database, showed 99.61% similarity of TA1 with *T. asperellum* isolate ICC 012 (Acc. No. MU868004.1), and 98.97% similarity of TG1 with *T. gamsii* isolate T065 (Acc. No. OK813898.1). The overall IFs of the two *Trichoderma* morphotypes from wood fragments of treated plants were 8.5% after 7 d, 10.6% at 14 d, and 55.6% at 21 d after treatment. Although both morphotypes were recovered, TG was consistently less frequently recovered than TA. No colonies were recorded from the untreated control treatments. The assumption of homogeneity of variances for IFs was confirmed by Levene's test (F = 2.91, $P = 0.06$). The two-way analysis of variance (ANOVA) showed that

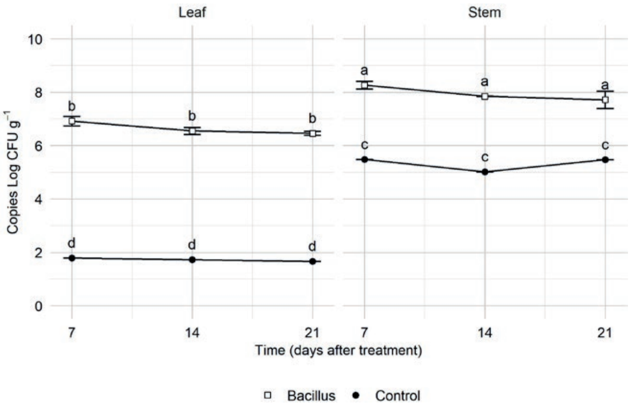

Figure 3. Mean numbers (± standard errors) of *Bacillus amyloliquefaciens* (expressed as copies Log CFU g^{-1} of tissue) detected in citrus leaf or stem tissues detected at 7, 14 or 21 d after treatment using a culture-indipendent method. Results were from *Bacillus*-treated (open symbols) and control plants (close symbols) using the qPCR assay. Different letters accompanying symbols indicate differences ($\alpha = 0.05$) among combined factors (treatment × sample), according to Tukey's HSD tests.

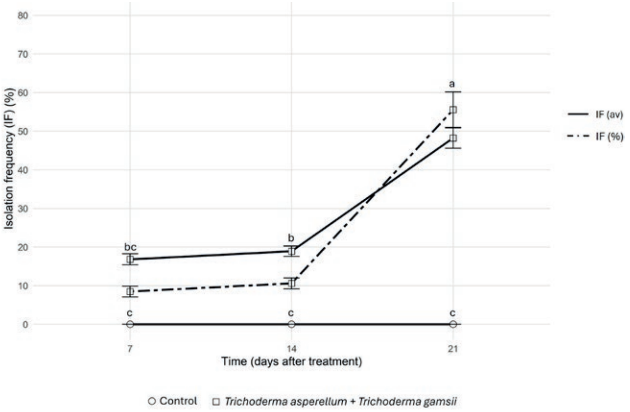

Figure 4. Mean numbers (± standard errors) of *Trichoderma asperellum* + *Trichoderma gamsii* (expressed as isolation frequency, IF) detected in citrus stem tissues, detected at 7, 14 or 21 d after treatment using a culture-dependent method. Results were from *Trichoderma*-treated (square symbols) and control plants (circle symbols). Different letters accompanying symbols indicate differences (α = 0.05) among combined factors (treatment × time), according to Tukey's HSD tests performed on arcsine square-root transformed data (av).

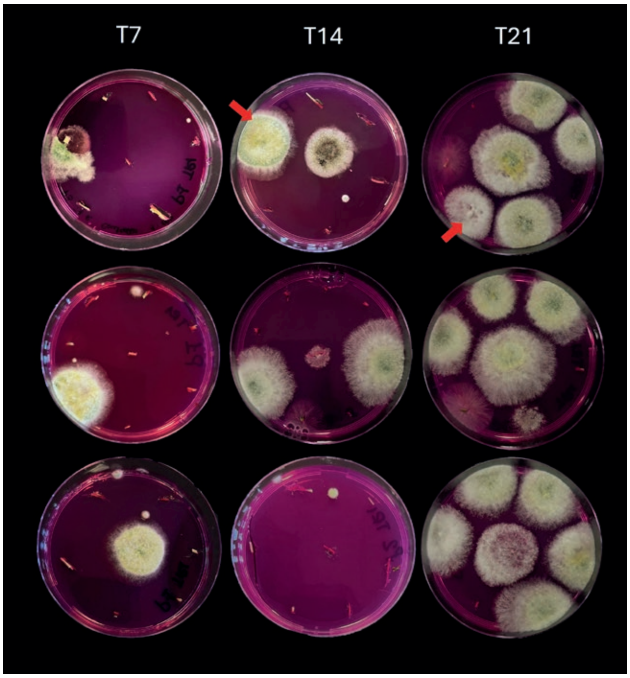

Figure 5. Representative isolation plates of the two *Trichoderma* morphotypes (red arrows) from plants treated with *Trichoderma asperellum* ICC 012 + *Trichoderma gamsii* ICC 080 at different collection times (T7, T14, or T21 d after treatments) on Rose Bengal-chloramphenicol Agar (RBA) medium.

the single factors, treatment (F = 223.60, $P < 0.001$) and collection time (F = 85.23, $P < 0.001$), and the combined factors (treatment × collection time) (F = 85.23, $P < 0.001$), all influenced detection of *Trichoderma* (angular-transformed value). Tukey's *post hoc* test of the combined factors showed that *Trichoderma* from treated plants differed significantly in IFs from those from untreated plants at each of the three collection times. Incidence of the two *Trichoderma* species was greater after 21 d than that recorded at 7 or 14 d ($P < 0.001$) (Figures 4 and 5).

Culture-independent detection of Trichoderma asperellum *and* Trichoderma gamsii *in leaf and stem tissues*

The culture-independent approach was used to determine trends of *Trichoderma* spp. in leaf and stem samples. Detection thresholds were calculated from standard curves, and ranged from a maximum of 10 log CFU g^{-1} (Ct = 16.29) to a minimum of 3 log CFU g^{-1}, (Ct = 34.68). The slope was -2.87841 and the coefficient of determination (R^2) was 99.2%. The negative experimental control gave Ct values of 39 to 40, with no DNA copies detected. *Trichoderma* spp. were detected in leave tissues at concentrations of 5.33 copies log CFU g^{-1} after 7 d culture, 5.69 copies log CFU g^{-1} after 14 d, and 4.87 copies log CFU g^{-1} after 21 d, and in stem tissues at 5.72 copies log CFU g^{-1} after 7 d, 5.70 copies log CFU g^{-1} after 14 d, and 5.66 copies log CFU g^{-1} after 21 d. The Ct values for leaf samples were 32.01 after 7 d, 32.09 after 14 d, and 34.14 after 21 d, and for stem samples were 31.97 after 7 d, 31.16 after 14 d, and 31.83 after 21 d (Supplementary Table II). No amplification was detected from the control samples. Homogeneity of variances of data was confirmed by Levene's test (F = 1.70, $P = 0.13$). The ANOVA showed that only treatment influenced the survivability of *Trichoderma* in leaf and stem tissues (F = 1442.1, $P < 0.001$). Tukey's *post hoc* test (α = 0.05) showed differences in detection of *Trichoderma* spp. at each collection time in treated and control leaf and stem tissue samples ($P < 0.001$). However, the qPCR assays did not detect statistically significant differences in *T. asperellum* and *T. gamsii* populations between the leaf and stem samples (Figure 6), with no statistically significant decreases up to 21 d after treatment.

Culture-dependent detection of Trichoderma asperellum *and* Trichoderma gamsii *from roots and soil-rhizospheres*

Culture-based methods (dilution and direct plating techniques) did not detect *T. asperellum* and *T. gamsii* from vascular root tissues. For rhizospheres, *Trichoderma* colonies were isolated from treated plants using the

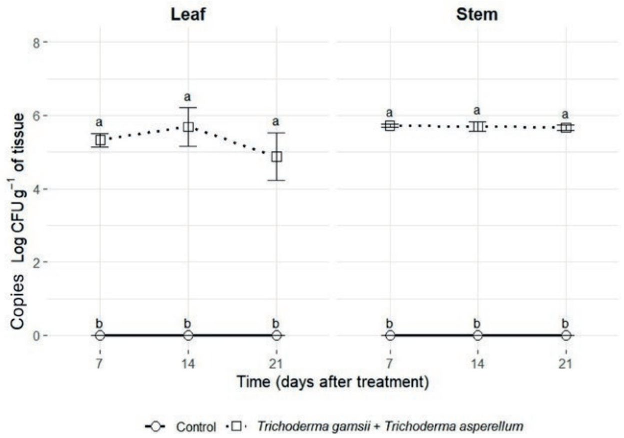

Figure 6. Mean numbers (± standard errors) of *Trichoderma asperellum* + *Trichoderma gamsii* (expressed as copies Log CFU g^{-1} of tissue) detected in citrus stem and leaf tissues, detected at 7, 14 or 21 d after treatment using a qPCR assay. Results were from *Trichoderma*-treated (square symbols) and control plants (circle symbols). Different letters accompanying symbols indicate differences ($\alpha = 0.05$) according to Tukey's HSD tests.

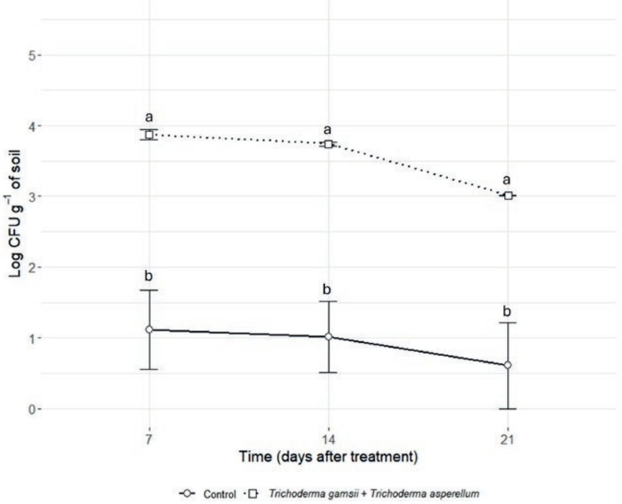

Figure 7. Mean numbers (± standard errors) of *Trichoderma asperellum* + *Trichoderma gamsii* (expressed as Log CFU g^{-1} of tissue) detected in the soil rhizosphere, detected at 7, 14 or 21 d after treatment using the agar dilution technique. Results were from *Trichoderma*-treated (square symbols) and control plants (circle symbols). Different letters accompanying symbols indicate differences ($\alpha = 0.05$) according to Tukey's HSD tests.

dilution plating (Figures 7A and 8A). However, some *Trichoderma*-like colonies were isolated from the rhizospheres of untreated plants (Figures 7 B and 8 B). Some of these colonies were morphologically different from those collected from treated plants (Figure 8 B). Based on the *rpb2* gene region, the representative *Trichoderma* isolates S-TA (Acc. No. PX597587) and S-TG (Acc. No. PX597588) collected from rhizospheres of treated plants gave 100% of identity with *T. asperellum* ICC 012 (Acc. No. MU868004.1) and 100% identity with several *T. gamsii* isolates, including *T. gamsii* strain PPRI 14668 (Acc. No. MF043068.1). In contrast, the four representative isolates obtained from experimental controls were identified, based on their *rpb2* gene regions, as *T. virens* for S1 (Acc. No. PX571116) and for S3 (Acc. No. PX571117), with 99.72% similarity and 99.91% of identity with *T. virens* strain TRA1-50 (Acc. No. MW325767.1), for S4 *T. harzianum* (Acc. No. PX597586) having 98.93% similarity with *T. harzianum* strain Vimi-17.0073 (Acc. No. MZ675885.1), and for S2 *Trichoderma asperellum* (S2 Acc. No. PX597585), which showed 99.91% of identity with *T. asperellum* strain CGMCC 6422 (Acc. No. KF425755.1), but only 97.34% similarity with *T. asperellum* ICC 012 (Acc. No. MU868004.1). *Trichoderma asperellum* and *T. gamsii* were isolated from treated samples at concentrations of 4.73 log CFU g^{-1} after 7 d, 4.63 log CFU g^{-1} after 14 d, and 4.37 log CFU g^{-1} after 21 d (Figure 7 A). Since control plates yielded colonies of different *Trichoderma* species, only colonies resembling *T. asperellum* were included in the plate count data. These showed overall concentrations of *T. asperellum* colonies in the control plates as 1.12 log CFU g^{-1} after 7 d, 1.02 log CFU g^{-1} after 14 d, and 0.61 log CFU g^{-1} after 21 d. Homogeneity of variances was confirmed by Levene's test (F = 0.62, $P = 0.68$), and ANOVA showed that only the treatment significantly influenced the survivability of *Trichoderma* spp. in soil rhizospheres (F = 65.68, $P < 0.001$). A one-way ANOVA showed differences (F = 67.32, $P < 0.001$) in the amounts of *T. gamsii* + *T. asperellum* in the soil rhizospheres of the treated and untreated plants. Although not statistically significant, small decreases, over time, in populations of these fungi were detected in the control and treated samples (Figure 7).

Culture-independent detection of Trichoderma asperellum *and* Trichoderma gamsii *in roots and rhizosphere soil*

The same qPCR reaction protocol used for leaf and stem samples was applied for the detection of *Trichoderma* spp. in the endospheres of roots and rhizosphere samples. The qPCR assay did not detect *T. asperellum* or *T. gamsii* from root vascular tissues. For rhizospheres, the qPCR assay detected these fungi at concentrations of 5.79 log CFU g^{-1} (Ct = 31.92) after 7 d, 5.93 log CFU g^{-1} (Ct = 31.47) after 14 d, and 6.01 log CFU g^{-1} (Ct = 31.21) after 21 d (Figure 9). Homogeneity of variances was confirmed by Levene's test (F = 1.76, $P = 0.19$), and the two-

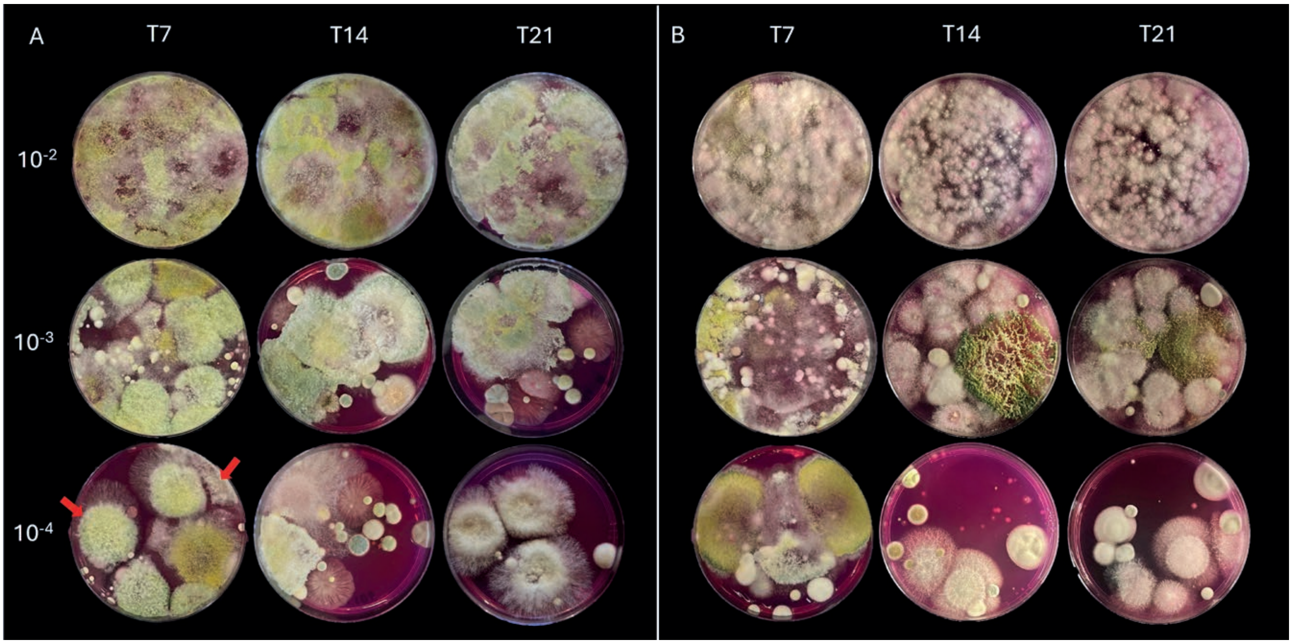

Figure 8. Representative isolation plates of *Trichoderma* spp. from rhizosphere treated with *Trichoderma asperellum* ICC 012 + *Trichoderma gamsii* ICC 080 (A) (red arrows) and controls (B) at different collection times (T7, T14, or T21 d after treatments), showing colony growth across serial dilution (from 10^{-2} to 10^{-4}) on Rose Bengal-chloramphenicol Agar (RBA) medium.

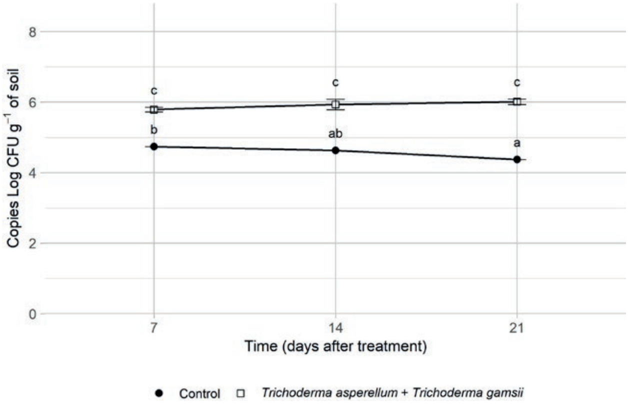

Figure 9. Mean numbers (± standard errors) of *Trichoderma asperellum* + *Trichoderma gamsii* (expressed as copies Log CFU g^{-1} of tissue) detected in the soil rhizosphere, detected at 7, 14 or 21 d after treatment using the qPCR assay. Results were from *Trichoderma*-treated (square symbols) and control plants (circle symbols). Different letters accompanying symbols indicate differences (α = 0.05) according to Tukey's HSD tests.

way ANOVA showed that the treatment affected population dynamics of *T. asperellum* and *T. gamsii* in the soil rhizospheres (F =15.37, $P < 0.001$) (Figure 9). The Tukey post hoc analysis ($\alpha = 0.05$) showed differences in population levels detected through the qPCR assays among the treated and control samples ($P < 0.01$). However, no statistically significant differences were detected due to collection time.

DISCUSSION

Bacillus amyloliquefaciens isolate QST 713 and *T. asperellum* ICC 012 + *T. gamsii* ICC 080 are beneficial microorganisms that can have important roles in management of citrus Mal secco disease (Aiello *et al.,* 2022; Leonardi *et al.,* 2023b; Leonardi *et al.,* 2026). Accurate quantification of BCAs over time, within plant vascular tissues and in soil rhizospheres, is important for improving effectiveness these agents, and for improved rating and scheduling their applications (Mathre *et al.,* 1999; McGuire, 2000; Whipps, 2001; Ojiambo and Scherm, 2006). The levels of colonization and persistence of BCAs in niches of pathogens are key factors underlying their biocontrol activity (Mohandoss and Suryanarayanan, 2009; Rodriguez *et al.,* 2009; Lahlali *et al.,* 2022), and to be effective BCAs must also densely colonize the host sites where pathogens are likely to infect (Card *et al.,* 2016). The present study assessed, using culture-dependent and culture -independent methods, quantitative differences in the endophytic colonization by *Bacillus* and *Trichoderma* in different citrus tissues. A qPCR methodology was developed and validated for the quantitative and specific detection of the biocontrol agents *B. amyloliquefaciens* and *T. asperellum* + *T. gamsii* on grapevine berries, wood and associated soil (Rotolo *et al.,* 2016 Gerin *et al.,* 2018). The present used qPCR to investigate the population dynamics of the same BCAs within vascular tissues of *C. volkameriana*. This citrus species, previously used to assess the efficacy of BCAs against *P. tracheiphilus* (Aiello *et al.,* 2022; Leonardi *et al.,* 2023b; Leonardi *et al.,* 2026), is a widely used rootstock in citrus-producing countries, including Italy (Bowman and Joubert, 2020).

Results from the present study confirmed that *B. amyloliquefaciens* persisted in grapevine stem vascular tissues. Good agreement was usually found between culture-dependent detection methods and qPCR results for stationary trends over time, except for leaf samples. From these, detections of *Bacillus* decreased after 14 d post-treatment. Endophytic colonization ability of *Bacillus* in vascular tissues of plants has been previously reported (Gagne *et al.,* 1987), and attributed to the large gene cluster responsible for the secretion of antibiotics and siderophores that promote *Bacillus* competitive ability in the root environments, and colonization of host plants (Chen *et al.,* 2020; Compant *et al.,* 2010). Kalai-Grami *et al.* (2014a) recovered endophytic *B. amyloliquefacies* TEB1 from treated *Citrus aurantium* seedlings after 30 d, reporting greater levels of endophytic colonization in stem and root samples than leaves. In a preliminary study, Aiello *et al.* (2022) isolated *B. amyloliquefaciens* QST 713 at 10^3 to 10^4 CFU g^{-1} (3 to 4 log CFU g^{-1}) from internal woody tissues of Volkamer lemon, 50 d after two treatments. Although Aiello *et al.* (2022) used a similar isolation protocol from stem tissue for detecting *B. amyloliquefaciens*, differences in number of treatments and the stem wounds could have influenced bacterial colonization and persistence. In the present study *B. amyloliquefaciens* was detected in leaf and stem samples from untreated citrus plants only using qPCR, while no cells were isolated on nutrient media, confirming the high sensitivity of qPCR and allowing detection of low bacterial populations in non-treated experimental control leaves.

The greater persistence of *B. amyloliquefaciens* recorded with qPCR assays than determined from culture-dependent methods in all the samples analyzed (stem, leaf, and soil), could have been because qPCR detects total DNA, including that from non-viable or viable but non-culturable (VBNC) cells, while culture-dependent methods only detect actively growing populations (Postollec *et al.,* 2011; Gorsuch *et al.,* 2019). Isolation methods could show false negative detections of VBNC bacteria which remain metabolically active but below detection levels (Morawska and Kuipers, 2022; Pinto *et al.,* 2015). Different stress factors for bacteria, such as microbial competition, nutrient-limited conditions, high salt or low pH environments, may lead to bacterial dormancy, which make these organisms undetectable by isolation on nutrient media (Foster, 1999; Xu *et al.,* 2008a, 2008b; Wang *et al.,* 2011; Guo *et al.,* 2019). Therefore, consistent with the literature (Chen *et al.,* 2020; Kalai-Grami *et al.,* 2014a), *Bacillus* may be a natural endophyte in citrus plants, although in the present study it was below the culturing detection limit. Similarly, Aiello *et al.* (2022) reported that no cells were isolated from untreated plants using a culture-dependent method.

Although different strains of *Trichoderma* spp. have been reported to be endophytes (Jaklitsch *et al.,* 2006; Gazis *et al.,* 2011; Muñoz-Guerrero *et al.,* 2021), and some have shown promising activity against pathogens of citrus (de Lima *et al.,* 2017; Ferreira *et al.,* 2020; Choudhary *et al.,* 2021; Muñoz-Guerrero *et al.,* 2021; Garzón *et al.,* 2022; Leonardi *et al.,* 2023b, 2026; Phal *et al.,* 2023), colonization and survival of these fungi in citrus plants and in soil has been little investigated (Ohr *et al.,* 1972; Camprubí *et al.,* 1995; Nemec *et al.,* 1996). In the present study, *T. asperellum* was recovered from wounded stem tissues at 21 d post-treatment only with direct plating, whereas no growth was observed from leaf or root tissues using two culture-dependent techniques. However, from the molecular data, *Trichoderma* was detected from citrus leaves and stems, so for *Trichoderma*-treated leaf samples, there was no agreement between isolation and qPCR results. For root samples, however, the qPCR accurately reflected the results from culturing methods, showing that *Trichoderma* did not colonize the citrus internal vascular tissues. Comparison of results from qPCR and culturing also highlighted a discrepancy in the population dynamics observed over time. While qPCR results showed that DNA levels of *Trichoderma* in stem tissues remained stable across all collection times, culture isolations showed increases in colony IFs 21 d post-inoculation. Greater PCR detection of *Trichoderma* in leaves qPCR than from culturing could be attributed to several factors, including different sensitivities of the two methods, presence of non-viable cells, the types of plant tissue, and presence of stem wounds that permit internal colonization by fungi (Carro-Huerga *et al.,* 2022; Bretträger *et al.,* 2022).

The qPCR assay can detect nucleic acid from damaged, stressed, or non-viable cells, whereas the traditional isolations rely on recovery of actively viable propagules above minimum detection thresholds. Xu *et al.,* (2025) reported that DNA quantification of *T. asperellum* strain T34 from live strawberry leaf, flower or root cells using the PMA-qPCR was, in some cases, less than the total DNA quantified using the qPCR. This indicated that normal qPCR possibly leads to over-estimation of viable population sizes of specific microorganisms. However, Xu *et al.,* (2025) found that dynamics of total DNA and PMA-qPCR DNA did not differ over time, suggesting that conventional qPCR techniques can be used to estimate changes in viable population sizes.

Plant tissues can contain different levels of phenolics, flavonoids, or terpenes, with defense functions that can reduce *Trichoderma* viability, allowing DNA to remain detectable without corresponding *in vitro* isolations. Scott *et al.* (2023) found that endophytic *Trichoderma* isolates had greater diversity of biosynthetic gene clusters (BGC) and degradative gene clusters (DGC) than non-endophytic isolates, allowing colonization of plant rhizospheres or phyllospheres. Plant metabolites in these habitats may be toxic or restrictive for colonization of non-endophytic *Trichoderma*. Several studies have highlighted ability of *Trichoderma* to colonize roots and rhizospheres, but presence of these fungi within phyllospheres is little understood (Guzmán-Guzmán *et al.,* 2025). Genes with potential roles in endophytic colonization by the *Trichoderma* isolates used in the present study have not been identified, but this study has indicated that they can be detected within citrus stem vascular tissues, but not in these tissues of leaves or roots. These results indicate that *T. asperellum* and *T. gamsii* may only be able to superficially colonize these plant tissues, as has been reported elsewhere for other crop plants (Sarrocco *et al.,* 2021). For rhizospheres, the detection level of *T. asperellum* + *T. gamsii* assessed using qPCR gave similar patterns to those from the culture-dependent method. The rhizosphere samples showed persistence of the fungi after 21 d, through plate counts and qPCR assays, confirming rhizosphere competence of *Trichoderma* (Woo *et al.,* 2023).

Although the present study did not track individual BCA isolates within citrus tissues, it provides the first insights into the endophytic ability of *B. amyloliquefaciens*, and of *T. asperellum* + *T. gamsii* in these plants after foliar and root applications. The study was based on species-level identifications, indicating that the qPCR primers and probe assessed can be used for monitoring these BCAs in citrus orchards, without appreciable interference from closely related microorganisms. Although the culture-dependent approach is laborious, time-consuming and requires well-trained personnel, combination of qPCR and culturing methods is important for detection and quantification of BCAs within plant tissues. This will facilitate improved scheduling of BCA field applications. The present study results also indicate that high colonization rates by *Trichoderma* spp. and *Bacillus* sp. could be achieved after long periods, and that application of these BCAs after pruning or damage could protect citrus plants from pathogen infections, as has been reported for other host crops (John *et al.,* 2005; Halleen *et al.,* 2010; Mutawila *et al.,* 2011; Kotze *et al.,* 2011). Results from the present study showed that the recovered isolates (identified at species level) from treated plants matched with the applied BCAs, supporting the inference of their persistence within plant tissues.

Endophytic colonization allows microorganisms to remain protected from environmental influences and fluctuations that could threaten their survival and reduce biocontrol efficacy (Card *et al.,* 2016). This enables *Bacillus* and *Trichoderma* to provide more stable biocontrol effects, and are also ideal for biological control. Nevertheless, as the present study used particular experimental conditions, deeper insights are required into endophyte functioning under varied experimental conditions and with different plant genotypes. The endophytic behaviour of these BCAs in healthy plants may change when host plants are grown under unfavourable or stress conditions (Hardoim *et al.,* 2015). Further research should assess BCA applicability in the field, since it is well-known that UV light, lack of nutrients, and other environmental factors can reduce colonization of above-ground plant surfaces, and that only adapted microorganisms can survive and enter host plants via stomata, wounds, and hydathodes (Hallmann 2001; Compant *et al.,* 2010; Carro-Huerga *et al.,* 2022).

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