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Microscopy, Culture, and Quantitative Real-Time PCR Examination Confirm Internalization of Mycobacteria in Plants

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The environment is a reservoir of nontuberculous mycobacteria and is considered a source of infection for animals and humans. Mycobacteria can persist in different types of environments for a relatively long time. We have studied their possible internalization into plant tissue through intact, as well as damaged, root systems of different types of plants grown *in vitro* and under field conditions. The substrate into which plants were seeded was previously contaminated with different strains of *Mycobacterium avium* (10^8 to 10^{10} cells/g of soil) and feces from animals with paratuberculosis. We detected *M. avium* subsp. *avium*, *hominissuis*, and *paratuberculosis* in the stems and leaves of the plants by both culture and real-time quantitative PCR. The presence of mycobacteria in the plant tissues was confirmed by microscopy. The concentration of mycobacteria found inside plant tissue was several orders of magnitude lower (up to 10^4 cells/g of tissue) than the initial concentration of mycobacteria present in the culture medium or substrate. These findings led us to the hypothesis that plants may play a role in the spread and transmission of mycobacteria to other organisms in the environment.

Nontuberculous mycobacteria cause a wide range of diseases in animals and immunocompromised individuals. Mycobacterial infection is acquired mainly through the respiratory and gastrointestinal tracts. Mycobacteria are ubiquitously distributed, and some are present in high numbers in natural and man-made environments; thus, they pose a constant risk to susceptible species of animals and immunocompromised humans. The diversity of mycobacteria in the environment was studied with a combination of molecular biology methods (1). This enabled qualitative and quantitative analysis and detection of sequences of pathogenic mycobacteria in all types of tested soil. Nontuberculous mycobacteria have been described as causal agents in different types of diseases, most often pulmonary, skin, and soft tissue infections (2). Although members of the *Mycobacterium avium* complex are usually associated with pulmonary disease, colonization and infection of the gastrointestinal tracts of AIDS patients have also been described (3). However, the route of transmission is usually unclear. Water has been proposed as a main reservoir (4), while infection through aerosol from soil has also been described (5).

Little information regarding the possible contamination of plants or food of vegetable origin with mycobacteria is available. Likewise, only a small number of studies have been concerned with food as a source of infection in humans. A study of food as the source of exposure of HIV-positive patients to mycobacteria detected mycobacteria in 7 out of 121 samples examined (6). A later study compared the genotypes of *M. avium* isolates from patients and foods by using PCR-restriction fragment length polymorphism and demonstrated a link between them (7). Nontuberculous mycobacteria were isolated from salads, leeks, lettuce, mushrooms, and other vegetables, as well as apple juice. Twenty-nine isolates were obtained from 46 samples, with the predominantly isolated species being *M. avium* (8).

Studies investigating the contamination of vegetables with mycobacteria have not proven whether mycobacteria can be present inside plant tissue. In a few studies, mycobacteria were identified in or on the surfaces of different plants (9, 10, 11). Zwielehner et al. (11) studied the microbial communities present in the phylosphere of lettuce leaves. After denaturing gradient gel electrophoresis and sequencing analyses, sequences of members of the genus *Mycobacterium* were found on leaves, as well as in soil samples. The sequence obtained from conventionally grown lettuce was most similar to *M. alvei*. Also, *M. avium* subsp. *paratuberculosis* was detected in grass samples by quantitative PCR (qPCR) (12).

Plants in aquatic environments are also known to harbor mycobacteria. *M. avium* was detected in a reed bed sample from a constructed wetland, and plants were also selected as possible reservoirs of *M. ulcerans* in the environment (9, 13).

The penetration of plant tissues by bacteria, namely, *Salmonella* and *Escherichia coli*, has been studied previously (14, 15, 16, 17). It was shown that motile bacteria can enter the plant through roots or even hydathodes on the leaves of tomato plants (18).

The persistence of bacteria inside plant tissue most probably depends on the conditions inside the plant. The survival of *Salmonella* in basil was limited to a few days (17). To the best of our knowledge, there is no record of internalization of mycobacteria through the intact root system of plants and their distribution inside the plant itself.

The aims of this study were to investigate whether mycobacteria present in culture medium or in feces from infected cattle can penetrate the intact or damaged tissue of two different plant species through the root system. To this end, we first analyzed the presence of mycobacteria in *in vitro*-grown plants under sterile conditions. Subsequently, we performed a field experiment in which plants were grown in a phytotron. We used beans and tomatoes, as both produce edible parts that are not in contact with contaminated soil. Moreover, both plants can be routinely cultured under laboratory conditions.

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MATERIALS AND METHODS

***In vitro* experiment.** *M. avium* subsp. *hominissuis* (field isolate obtained from infected swine) was grown under laboratory conditions on Middlebrook broth (M7H9) with enrichment (oleic acid-albumin-dextrose-catalase [OADC]; Becton Dickinson) with constant shaking for 2 weeks. *M. avium* subsp. *hominissuis* was chosen for its rapid availability and fast growth *in vitro*. The concentration of IS*1245* was quantified by qPCR (19). To 20 ml of Murashige and Skoog agar (Duchefa, Haarlem, The Netherlands) at a temperature of 50°C, the suspension was added at 10^9 cells/ml. Cultivation was performed in 250-ml cylindrical glass vessels. Surface sterilization of pinto bean (*Phaseolus vulgaris*) seeds was performed by submersion in 3% sodium hypochlorite for 3 min, followed by three subsequent washes in sterile water (for 5 min each). Sterilized seeds were planted on agar and grown under laboratory conditions for 2 weeks. Samples for microscopy, mycobacterial culture, and qPCR were collected from leaves and stems with sterile forceps and scissors.

Field experiment. For the field experiment, we used *M. avium* subsp. *paratuberculosis* (reference strain CAPM 6381) and *M. avium* subsp. *avium* (reference strain CAPM 5889). These subspecies were chosen because of their possible presence in soil after fertilization with feces from infected animals. Both strains were grown on Middlebrook broth (M7H9) with enrichment (OADC; Becton Dickinson) and mycobactin J (Allied Monitor) with constant shaking for 1 month.

Tomato (*Solanum lycopersicum*) seeds were planted in pots containing 1 liter of a commercially available substrate of potting soil. The substrate was tested by qPCR, and it was negative for the presence of *M. avium* subsp. *avium* and *paratuberculosis* prior to inoculation. A total of 24 plants were tested; 9 of them were grown in soil contaminated with *M. avium* subsp. *avium*, 12 were grown in soil contaminated with *M. avium* subsp. *paratuberculosis*, and 3 served as negative controls. Three groups of plants were grown in soil contaminated with *M. avium* subsp. *avium*, and four groups were grown in soil contaminated with *M. avium* subsp. *paratuberculosis*. Each group contained three plants. In the first group, the substrate was contaminated with an *M. avium* subsp. *avium* (10^8 cells/liter of potting soil) or *M. avium* subsp. *paratuberculosis* (10^{10} cells/liter of potting soil) suspension immediately before seeding. Ten milliliters of the suspension was added to the soil, and the soil was mixed with a glass spatula. In the second and third groups, the suspensions were added 2 weeks after seeding, in the proximity of the main roots, with the difference that the roots of the third group were mechanically damaged with a syringe needle. The fourth group consisted of plants seeded in the substrate mixed with feces from a cow clinically ill with paratuberculosis. The concentration of cells in the substrate after the addition of feces was 10^6 cells/liter of potting soil (quantified by qPCR).

The plants were grown in a phytotron with a daytime temperature of 22°C, a humidity of 29%, and a CO_2 concentration of 630 ppm. There were three collection points. Leaf, stem, and fruit samples (where available) were collected 2, 4, and 8 weeks after seeding (for group 1) and after contamination (for groups 2 and 3).

Additionally, we performed distribution analysis of two selected plants, one grown on *M. avium* subsp. *avium*-contaminated soil and the other grown on *M. avium* subsp. *paratuberculosis*-contaminated soil. After 8 weeks, we analyzed stems and leaves taken from the plants at 5-cm height intervals, as well as root, fruit, and pollen samples (pollen samples were collected from several plants during the flowering phase).

Sample examination. (i) Light and fluorescence microscopy. Microscopy of histological sections (15 to 20 μm thick, made with a cryostat) of stem, root, and leaf tissues was performed after staining by the Ziehl-Neelsen method. Slides were analyzed at a magnification of ×1,000 with an Olympus BX41 microscope. Slides were prepared for fluorescence microscopy with a primary polyclonal rabbit anti-*Mycobacterium* antibody and a Cy3-labeled anti-rabbit secondary antibody (ExBio, Czech Republic). Briefly, the protocol included fixation in acetone, followed by incubation with a blocking solution (Dako). The primary antibody was diluted to 10 μg/ml, and incubation was performed overnight (12 to 16 h) at 4°C. After three washes in phosphate-buffered saline (PBS), the secondary antibody was applied for 1 h at room temperature. After the final wash in PBS, slides were mounted with mounting medium and analyzed at a magnification of ×1,000 with an Olympus BX41 microscope (Olympus, Japan). For the samples stained with fluorescent antibody, the 510- to 550-nm filter was used.

(ii) Electron microscopy. Ultrathin sections of plant tissue were fixed in 3% glutaraldehyde in cacodylate buffer; postfixed in 1% OsO_4 solution in cacodylate buffer; dehydrated in 50, 70, 90, and 100% acetone; and embedded in an Epon 812 (Serva, Germany)-Durcupan (ACM Fluka, Switzerland) mixture. The sections were stained with 2% uranyl acetate and 2% lead citrate and observed at 80 kV in a Philips EM 208 transmission electron microscope (Philips, The Netherlands).

Cultivation of mycobacteria. Samples from stems and leaves were washed in 3% sodium hypochlorite for 3 min and sterile water (three times for 5 min each time) prior to culture to avoid surface contamination. Samples were cut from tomatoes aseptically, and the inside part of the fruit was cultured. Cultivation of 1 g of each sample (homogenized in 2 ml of PBS) without any decontamination and an additional 1 g with decontamination was performed according to Fischer et al. (20). Briefly, the homogenized sample was treated with 1 M HCl for 20 min and subsequently neutralized with 2 M NaOH. The material was inoculated onto four different culture media (Table 1), Herrold egg yolk medium, Leslie medium, and Middlebrook M7H11 with a PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) antibiotic mixture or without antibiotics. Middlebrook M7H11 was obtained from BD Diagnostics (Denmark). The rest of the media were prepared in our laboratory as described previously (21). When culturing the samples from the tomato plants, it was necessary to perform a decontamination step because of the high rate of contamination.

DNA isolation and qPCR. Several commercially available kits for DNA isolation from plant material were tested for efficiency. Because of the expected low number of bacterial cells in the plant tissue, we attempted to use as much starting material as possible. The best results for 0.25 g of tissue were achieved with the commercially available PowerFood Microbial DNA isolation kit (MoBio, USA) with certain modifications of the original protocol. Initial homogenization of the samples was done in a MagnaLyser (Roche, Germany) at 6,400 rpm for 2 min after the addition of four 3.2-mm chrome steel beads (Biospec, USA), as well as the beads provided in the kit. An increased volume of lysis buffer (700 μl) was used, and an additional step of heating at 65°C for 10 min with shaking at 1,400 rpm was included. The remaining steps were performed according to the manufacturer's recommendations. DNA was eluted in 100 μl of preheated Tris-EDTA buffer (Amresco, USA) and used subsequently in qPCR assays.

Triplex qPCR for simultaneous detection of IS*1245* and IS*901* was performed for every sample in duplicate for the plants contaminated with *M. avium* subsp. *avium* and *hominissuis*, according to Slana et al. (19). For the tomato plants contaminated with *M. avium* subsp. *paratuberculosis*, qPCR for detection of IS*900* was performed as described earlier (22). The qPCR results were transformed to numbers of cells per gram by calculating the mean copy number of insertion sequences per cell (25 copies of IS*1245* in *M. avium* subsp. *hominissuis*, 15 copies of IS*901* in *M. avium* subsp. *avium*, and 15 copies of IS*900* in *M. avium* subsp. *paratuberculosis*).

RESULTS

***In vitro* experiment.** The qPCR analyses showed that mycobacteria were present in all of the leaf and stem samples from the plants grown on artificially contaminated medium. The quantity of mycobacteria was 3 orders of magnitude smaller than the quantity present in the substrate medium. The negative control gave no signal.

The culture results are presented in Table 1. The decontamination method clearly resulted in much lower yields in culture than

TABLE 1 qPCR and culture examinations of stem and leaf samples from *in vitro*-grown bean plants[a]

Plant part and no. or parameter	No. of cells/g[b]	No. of CFU/g after cultivation: Without decontamination: MB + PANTA[c]	MB[d]	HEYM[e]	Leslie[f]	With decontamination: MB + PANTA	MB	HEYM	Leslie
Stem									
1	6.31×10^6	>1,000	>1,000	100	0	150	0	0	0
2	1.54×10^7	100	0	0	200	20	0	0	0
3	7.74×10^6	1,000	>200	>1,000	200	10	10	0	0
Mean (SD)	9.81×10^6 (4.88×10^6)	3,700 (5,474)	3,400 (5,716)	366 (550.7)	133.33 (115.5)	60 (78.1)	3.33 (5.7)	0 (0)	0 (0)
Leaf									
1	6.70×10^6	200	>200	1,000	0	0	100	100	10
2	1.74×10^6	>200	>200	500	100	0	0	0	0
3	3.49×10^6	>100	>200	>1,000	0	0	100	10	100
Mean (SD)	3.97×10^6 (2.51×10^6)	166 (57.73)	200 (0)	3,833 (5,346)	33.33 (57.7)	0 (0)	66.67 (57.7)	36.67 (55)	36.67 (55)
Negative control	0	0	0	0	0	0	0	0	0

[a] The input concentration of *M. avium* subsp. *hominissuis* culture added to the agar was 10^9 cells/ml.
[b] Determined by qPCR assay for IS*1245*.
[c] MB + PANTA, Middlebrook M7H11 plus PANTA.
[d] MB, Middlebrook M7H11 without antibiotics.
[e] HEYM, Herrold egg yolk medium.
[f] Leslie, Leslie medium.

did sample processing without the decontamination step. The comparison of four different culture media also shows that the best recovery was obtained with Middlebrook M7H11 agar. The addition of PANTA antibiotics had no adverse effect on mycobacterial growth. Using electron microscopy, we observed structures similar to bacterial cells in the plant tissues (Fig. 1). Using Ziehl-Neelsen microscopy of plant tissue sections 15 to 20 nm thick, we were able to observe acid-fast rods. We made similar observations by fluorescence microscopy (Fig. 1). Mycobacteria were observed by microscopy only in the bean plants grown *in vitro*, where the number of cells per gram exceeded 10^6.

Field experiment. In the field experiment with the tomato plants, the results from qPCR are shown in Fig. 2 and 3. *M. avium* subsp. *paratuberculosis* DNA was present in the entire stem and leaf samples from the plants in group 1 at the three sampling times. The fruit samples from this group were positive only at the

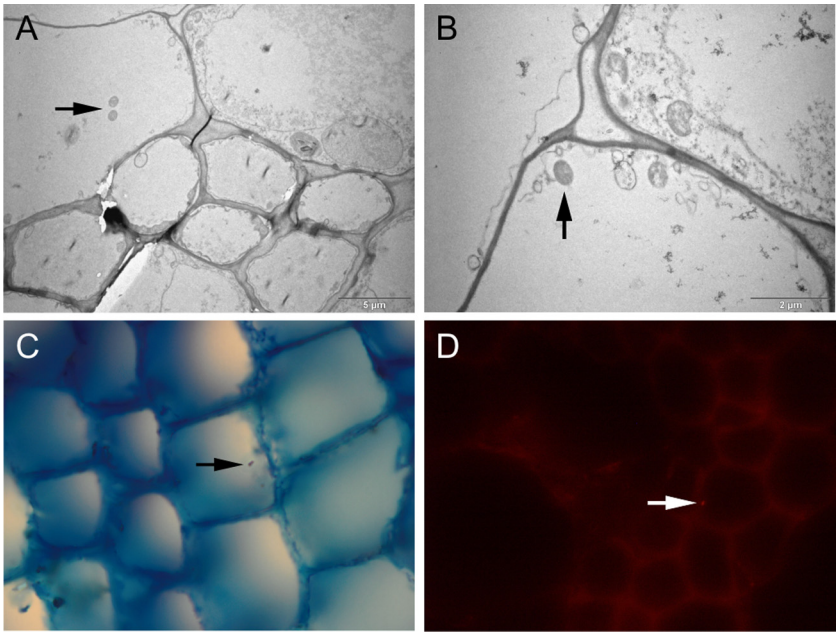

FIG 1 Microscopy of bean plant stems sections containing *M. avium* subsp. *avium*. (A, B) Transmission electron microscopy. Arrows indicate structures similar to bacterial cells. (C) Ziehl-Neelsen staining of bean plant stem tissue. A mycobacterium is stained red against a blue background. (D) Specific-antibody-labeled *M. avium* subsp. *avium* inside bean plant stem tissue. Fluorescent rods were observed inside plant transport cells.

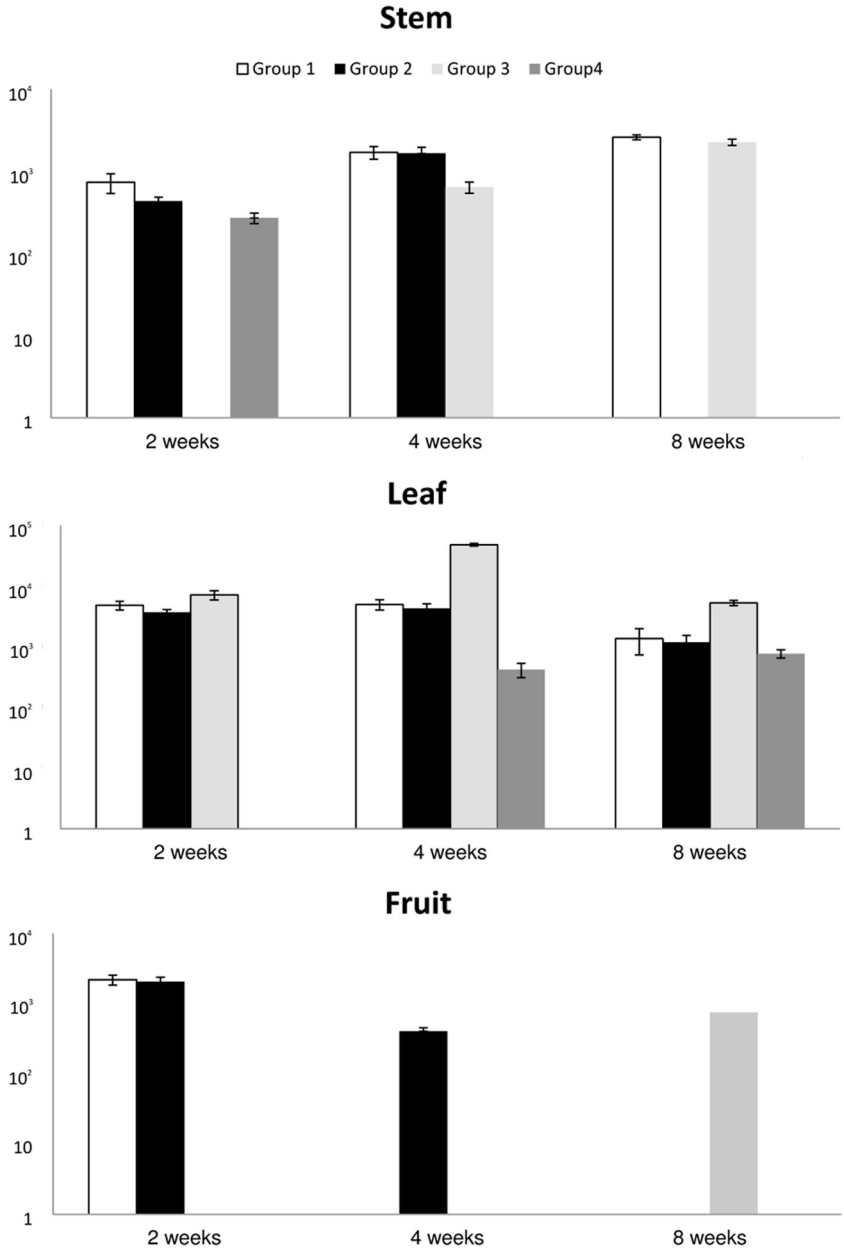

FIG 2 Detection of *M. avium* subsp. *paratuberculosis* DNA inside tomato plants.

first sampling point 2 weeks postinoculation. The samples from group 2 gave similar results, although leaf stem samples were negative 8 weeks after inoculation, but DNA was detected in fruit samples at 4 weeks postcontamination. Stem samples from group 3 (with damaged roots) were positive 4 and 8 weeks after contamination, but leaf samples were positive at all three time points. Fruit samples were positive 8 weeks after contamination with mycobacteria. The quantities were similar in all of the samples and ranged from 10^2 to 10^4 cells/g of tissue. Leaf and stem tissue samples from group 4 were positive, but fruit samples were not. The quantity of IS*900* reached up to 10^3/g of tissue (Fig. 2). The samples from tomato plants grown in soil contaminated with *M. avium* subsp. *avium* gave similar results. Stem samples from group 1 were positive at 4 and 8 weeks after seeding, but leaf samples were positive at all sampling points. All of the stem and leaf samples from group 2 were positive, but none of the fruit samples were. Leaf samples from group 3 were positive at all of the time points tested, and fruit samples were positive at 2 weeks after contamination. The quantity of *M. avium* subsp. *avium*-specific DNA ranged from 10^1 to 10^5 IS*901* copies/g of tissue (Fig. 3).

We also obtained three isolates from cultivation. Two of the isolates (*M. avium* subsp. *avium*) from stems of plants in groups 2 and 3 were obtained at the 2-week sampling time, and one isolate (*M. avium* subsp. *paratuberculosis*) from group 2 was obtained 1 month after seeding. The samples from the control group were negative. The qPCR results of the distribution of mycobacteria inside the plant are shown in Fig. 4. Mycobacteria were concentrated mostly in the root samples, and their quantity decreased

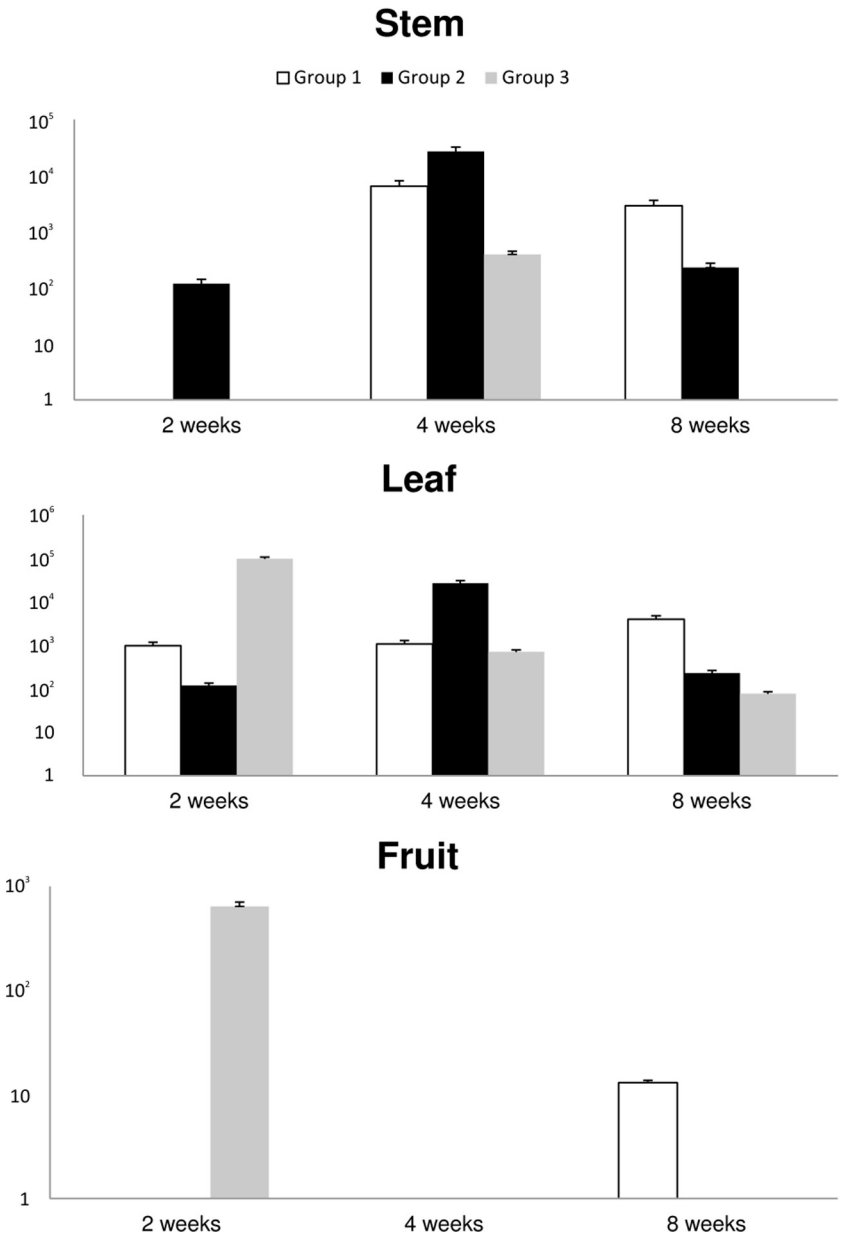

FIG 3 Detection of *M. avium* subsp. *avium* DNA inside tomato plants.

through the fruit, although we also detected the target sequence in pollen samples.

DISCUSSION

The food safety of vegetables has been of increasing concern since recent outbreaks of *Salmonella* and *E. coli* were traced back to vegetables and sprouts. Much research has focused on these two pathogens and their potential for penetration versus surface contamination of vegetables (23, 24). Solomon et al. (25) described the migration of *E. coli* O157:H7 from contaminated soil into the tissue of lettuce. There have also been studies on the internalization of *Salmonella* into tomato plants through roots or even hydathodes (18). Although mycobacteria were detected in vegetables previously, the present study is the first to confirm their internalization inside plant tissue. The large numbers of *M. avium* cells used in our experiment may have biased the results; however, a recent study showed that the concentration of mycobacteria in soil, as well as their diversity, is high (1).

Plants or vegetables have been suspected as possible sources of food-borne mycobacterial diseases (6, 8). Typing of *M. avium* isolates from food and patients showed the same DNA patterns (8). Our results demonstrate that mycobacteria can be taken up by the root systems of plants, even plants with intact roots. The persistence of mycobacteria in soil, manure, and different parts of the environment has been demonstrated previously (12, 26). *M. avium* subsp. *paratuberculosis* remained viable in manure after 55 weeks (26). Manure from domestic animals is used as fertilizer in fields where crops or vegetables are grown. Therefore, it is plausi-

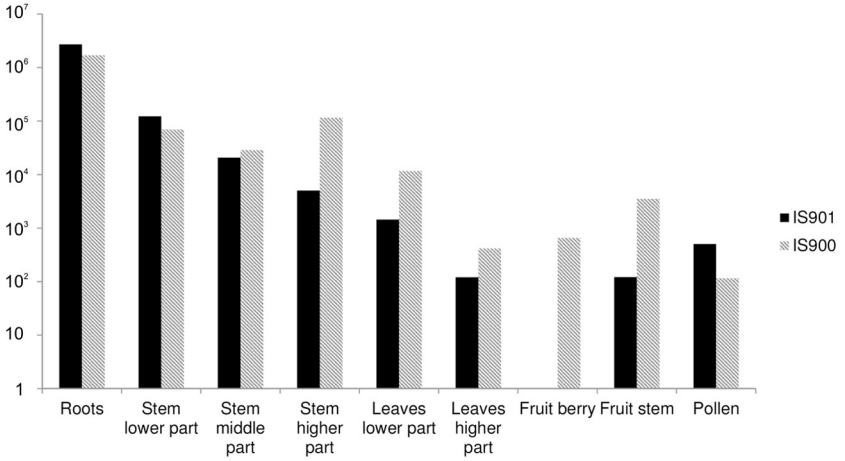

FIG 4 Distribution of *M. avium* subsp. *avium* and *paratuberculosis* DNA inside tomato plants.

ble that because of its presence inside plants, there might be a risk of infection of grazing animals or of humans. Although in the present study we have proven the presence of mycobacteria inside plant tissue, we have not performed any experiments regarding its pathogenicity. Future research should test the pathogenicity of mycobacteria after their internalization in plants. The next step would be to feed animals such plants to see whether this route of transmission is plausible.

In our study, mycobacteria were present inside plant tissue for at least 2 months after the contamination of potting soil. This may be due to the properties of mycobacteria, as well as the environment inside the plant tissue. A study of the survival of *Salmonella* in basil showed a decline after only 3 days (17), although other studies have detected *Salmonella* in tomato fruit samples 49 days after inoculation (27).

Regarding the distribution of mycobacteria inside the plants, *M. avium* subsp. *avium* and *paratuberculosis* DNA quantities were highest in the roots and gradually decreased along the height of the plant. The presence of mycobacterial DNA in the fruit and pollen samples is noteworthy regarding food safety and further spread of mycobacteria. However, we did not obtain an isolate from these samples by culture.

In conclusion, we have demonstrated the internalization of mycobacteria into different types of plants; furthermore, their distribution within the plants was found to be even. However, the concentration of mycobacteria found inside plant tissue was several orders of magnitude smaller than the initial concentration of mycobacteria present in the culture medium or substrate. Mycobacteria are probably passively taken inside the roots rather than actively penetrate the root epidermis. This passive intake could be facilitated by the relatively small size of mycobacterial cells. Although mycobacteria inside plant tissue pose a possible risk of transmission, we suspect that the subsequent handling of vegetables and secondary surface contamination with mycobacteria might play a bigger role in the transmission of the infectious agent.

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