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Internalisation of mycobacteria in plants confirmed by microscopy, culture and quantitative real time PCR examination

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Abstract

The environment is a reservoir of non-tuberculous mycobacteria and is considered as 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 internalisation into plant tissue through intact as well as damaged root systems in different types of plants grown *in vitro* and under field conditions. The substrate in which plants were seeded was previously contaminated with different strains of *Mycobacterium avium* (10^8 to 10^{10} cells/gram of soil), and faeces from animals infected with paratuberculosis. We detected *M. avium* subsp. *avium*, *hominissuis* and *paratuberculosis* in the stems and leaves of the plants using both culture and quantitative real time PCR (qPCR). The presence of mycobacteria in the plant tissues was confirmed by microscopy. The number of mycobacteria found inside plant tissue was several orders of magnitude lower (up to 10^4 cells/gram of tissue) than the initial amount of mycobacteria present in the culture media or substrate. These findings lead us to the hypothesis that plants may play a role in the spread and transmission of mycobacteria to other organisms in the environment.

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40 Introduction

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42 Non-tuberculous mycobacteria cause a wide range of diseases in animals and
43 immunocompromised individuals. Mycobacterial infection is acquired mainly through
44 the respiratory and the gastrointestinal tract. Mycobacteria are ubiquitously
45 distributed and some members are present in high numbers in natural and man-
46 made environments; thus, they pose a constant risk to susceptible species of animals
47 and immunocompromised humans. The diversity of mycobacteria in the environment
48 was studied using combination of molecular biology methods (1). This enabled
49 qualitative and quantitative analysis and detection of sequences of pathogenic
50 mycobacteria in all types of tested soil. Non-tuberculous mycobacteria have been
51 described as causal agents in different types of diseases, most often pulmonary, skin
52 and soft tissue infections (2). Although members of the *Mycobacterium avium*
53 complex are usually associated with pulmonary disease, colonization and infection of
54 the gastrointestinal tract in AIDS patients has also been described (3). However, the
55 route of transmission is usually unclear. Water has been proposed as a main
56 reservoir (4), while infections through aerosol from soil have also been described (5).
57 Little information is available regarding the possibility of plants or food of vegetable
58 origin being contaminated with mycobacteria. Likewise, only a small number of
59 studies were concerned with food as a source of infection in humans.A study of the
60 food as a source of exposure of mycobacteria to HIV positive patients detected
61 mycobacteria in seven out of 121 samples examined (6). A later study compared the
62 genotypes of *M. avium* isolates from patients and foods using PCR- Restriction
63 fragment lenth polymorphism and demonstrated a link between them (7). Non
64 tuberculous mycobacteria were isolated from from salads, leak, lettuce, mushrooms,

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and other vegetables as well as apple juice. Twenty nine isolates were obtained from 46 samples, with the predominant isolated species being *M. avium* (8).

Studies investigating the contamination of vegetables with mycobacteria have not proven whether mycobacteria can be present inside the plant tissue. In a few studies mycobacteria were identified in or on the surface 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 from the genus *Mycobacterium* were found on leaves as well as soil samples. The sequence obtained from conventionally grown lettuce was most similar to *M. alvei*. Also, *M. avium* subsp*. paratuberculosis* (*M. a. paratuberculosis*) was detected in grass samples using qPCR (12).

Plants in aquatic environment are also known to harbour mycobacteria. *M. avium* was detected in a red beed samples from a constructed wetland, and plants were also selected as possible reservoirs of *M. ulcerans* in the environment (9;13).

The penetration of bacteria, namely *Salmonella* and *E. coli* into plant tissues has been studied previously (14; 15; 16; 17). It was shown that motile bacteria can enter the plant through roots or even hydathode on the leaves of tomato plants (18). The persistence of bacteria inside the plant tissue depends most probably on the conditions inside the plant. The survival of *Salmonella* in basil was limited to a few days (17). To the authors' knowledge, there is no record of internalisation 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 media, or in faeces from infected cattle, can penetrate into the intact or damaged

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plant tissue of two different plant species through their root system. To this end, we first analysed the presence of mycobacteria into *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 which are not in contact with contaminated soil. Moreover, both plants can be routinely cultured in laboratory conditions.

Materials and Methods

In vitro experiment

Mycobacterium avium subsp. *hominissuis* (*M. a. hominissuis*, field isolate obtained from infected swine) was grown under laboratory conditions on Middlebrook broth (M7H9) with enrichment (OADC, Beckton and Dickinson) with constant shaking for two weeks. *M*. a. hominissuis was chosen due to its rapid availability and fast growth *in vitro*. The concentration of IS*1245* was quantified using qPCR (19). To 20 ml of Murashige and Skoog agar (Duchefa, Haarlem, The Netherlands) at a temperature of 50º C 10^9/ml of the suspension were added. Cultivation was performed in 250 ml glass cylindrical vessels. Surface sterilisation of the seeds of beans (Pinto beans; *Phaseolus vulgaris*) was performed by submerging them in 3% Sodium hypochlorite for three minutes, followed by three subsequent washes in sterile water (each 5 minutes). Sterilised seeds were planted on the agar and grown under laboratory conditions for two weeks. Samples for microscopy, mycobacterial culture and qPCR were collected from leaves and stems, using sterile forceps and scissors.

Field experiment

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For the field experiment, we used *Mycobacterium avium* subsp. *paratuberculosis* (*M. a. paratuberculosis,* reference strain CAPM 6381) and *Mycobacterium avium* subsp. *avium* (*M. a. avium,* reference strain CAPM 5889). These subspecies were chosen due to their possible presence in the soils after fertilization with faeces from infected animals. Both strains were grown on Middlebrook broth (M7H9) with enrichment (OADC, Beckton and Dickinson) and mycobactin J (Allied Monitor) with constant shaking for one month.

Tomato seeds (*Solanum lycopersicum*) were planted in pots containing one litre of a commercially available substrate of potting soil. The substrate was tested using qPCR and it was negative for the presence of *M. a. avium* and *M. a. paratuberculosis* prior to inoculation. Twenty four plants were tested in total; nine of them were grown in soil contaminated with *M. a. avium*, twelve were grown in soil contaminated with *M. a. paratuberculosis* and three served as negative controls. Three groups of plants were grown in soil contaminated with *M. a. avium* and four groups were grown in soil contaminated with *M. a. paratuberculosis*. Each group contained three plants. In the first group, the substrate was contaminated with *M. a. avium* (10^8 cells /L of potting soil) or *M. a. paratuberculosis* (10^{10} cells /L of potting soil) suspension immediately before the seeding. Ten millilitres of the suspension was added to the soil and the soil was mixed using glass spatula. In the second and third groups, the suspensions were added two weeks after seeding, in the proximity of the main roots, with the difference that the roots of the third group were mechanically damaged a syringe needle. The fourth group consisted of plants seeded in the substrate mixed with faeces from a cow clinically ill with paratuberculosis. The amount of cells in the

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substrate after the addition of faeces was 10^6 cells/ L of potting soil (quantified using qPCR).

The plants were grown in a phytotron, where day temperature was adjusted to 22 ºC, humidity at 29% and CO_2 concentration at 630ppm. There were three collection points. Samples from leaves, stems, and fruits (where available), were collected two, four, and eight weeks after seeding (for group 1) and after contamination (for groups 2 and 3).

Additionally, we performed distribution analysis on two selected plants, one grown on *M. a. avium* contaminated soil and the other on *M. a. paratuberculosis* contaminated soil. After eight weeks, we analysed stems and leaves from every 5 cm height of the plant, as well as roots, fruit and pollen samples (the pollen sample was collected from several plants during flowering phase).

Sample examination

Light and fluorescence microscopy

Microscopy was performed on histological cuts of stem (15-20 µm using a cryostat), root and leaf tissue, which were stained according to the Ziehl Neelsen method. Slides were analysed under 1000x magnification on an Olympus microscope BX41. Slides for fluorescent microscopy were prepared using a primary polyclonal rabbit anti-*Mycobacterium* antibody, and a secondary Cy3-labelled anti-rabbit 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 (12-16 hours) at 4 ºC. After three washes in PBS, the secondary antibody was applied for one hour at room

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temperature. After the final wash in PBS, slides were mounted with mounting medium and analysed under 1000x magnification on an Olympus BX41 microscope (Olympus, Japan). For the samples stained with fluorescent antibody the 510-550 nm filter was used.

Electron microscopy

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

Cultivation of mycobacteria

Samples from stems and leaves were washed in 3 % Sodium hypochlorite for 3 min and sterile water (3 times for 5 minutes) prior to culture in order to avoid possible surface contamination. Samples from tomatoes were cut aseptically and the inside part of the fruit was cultured. Cultivation of 1g of each sample (homogenized in 2 ml of phosphate buffered saline) was performed without any decontamination, and from an additional 1 g with decontamination according to Fischer et al. (20). Briefly, the homogenised sample was treated with 1 M HCl for 20 minutes, and subsequently was neutralised using 2M NaOH. The material was inoculated onto four different culturing media (Table 1): Herrold egg yolk medium, Leslie medium, Middlebrook MH711 with antibiotic mixture PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) and without antibiotics. The Middlebrook MH711 was

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obtained from BD Diagnostics (Denmark). The rest of the media were prepared in our laboratory, as described (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 their efficiency. Due to 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 0.25g of tissue were achieved using the commercially available PowerFood Microbial DNA isolation kit (MoBio, USA) with certain modifications to the original protocol. Initial homogenization of the samples was done in a MagnaLyser (Roche, Germany) at 6400 rpm/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 the lysis buffer (700 µl) was used and an additional step of heating at 65º C for 10 min with shaking at 1400 rpm was included. The remaining steps were performed according to the manufacturer's recommendations. DNA was eluted in 100 µl of pre-heated TE buffer (Amresco, USA) and used subsequently in the qPCR reaction. Triplex qPCR for simultaneous detection of IS*1245* and IS*901* was performed for every sample in duplicate for the plants contaminated with *M. a. avium* and *M. a. hominissuis*, according to Slana et al. (19). For the tomato plants contaminated with *M. a. paratuberculosis*, qPCR for detection of IS*900* was performed as described earlier (22). The qPCR results were transformed to cells/g by calculating the mean copy number of insertion sequences per cell (25 copies of IS*1245* in *M. a. hominissuis*, 15 copies of IS*901* in *M. a. avium* and 15 copies of IS*900* in *M. a. paratuberculosis*).

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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 media. The quantity of mycobacteria was three order of magnitude lower than the quantity present in the substrate media. The negative control gave no signal.

The results from culture are presented in Table 1. The decontamination method clearly resulted in much lower yields in culture than samples processed without the decontamination step. The comparison of four different culturing media also shows that the highest recovery was obtained with the use of Middlebrook M7H11 agar. The addition of PANTA antibiotics had no adverse effect on the mycobacterial growth.

Using electron microscopy, we observed structures similar to bacterial cells in the plant tissues (Figure 1). Using Ziehl Neelsen microscopy on plant tissue sections of 15-20nm thickness we were able to observe acid-fast rods. We made similar observations using fluorescence microscopy (Figure 1). Mycobacteria were observed under microscopy only in the bean plants grown *in vitro*, were 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 Figure 2 and Figure 3. *M. a. paratuberculosis* DNA was present in the entire stem and leaf samples at the three sampling times in the plants from group 1. The fruit sample from this group was positive only at the first sampling point two weeks post inoculation. The samples from group 2 gave similar results, although leaf stem

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samples were negative 8 weeks after inoculation, but DNA was detected in fruits at four weeks post contamination. Stem samples from group 3 (with damaged roots) were positive four and eight weeks after contamination, but leaf samples were positive in all three time points. Fruits were positive eight weeks after contamination with mycobacteria. The quantity was similar in all of the samples and ranged between 10^2 to 10^4 cells per gram of tissue. Samples from group 4 were positive in leaf and stem tissue but not in fruits. The quantity of IS*900* reached up to 10^3/g of tissue (Figure 2). The samples from tomatoes grown in soil contaminated with *M. a. avium* gave similar results. Stem samples were positive in group 1 at four and eight weeks after seeding, but leaf samples were positive at all sampling points. In samples from group 2 all of the stem and leaf samples tested positive, but none of the fruit samples. Leaf samples from group 3 were positive in all time points, fruit sample was positive at two weeks after contamination. The quantity of *M. a. avium* specific DNA ranged between 10^1 to 10^5 IS*901* copies per gram of tissue (Figure 3).

We obtained also three isolates from cultivation. Two of the isolates (*M. a. avium*) were from the stems in group 2 and 3, at the two week sampling time, and one isolate (*M. a. paratuberculosis*) from group 2 was obtained one 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 Figure 4. Mycobacteria were mostly concentrated in the root samples and their quantity decreased through the fruit, although we detected the target sequence also in the pollen sample.

Discussion

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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 internalisation of *Salmonella* into tomato plants through roots or even hydathodes (18). Although mycobacteria were detected in vegetables previously, the present study is the first one to confirm their internalisation inside the plant tissue. The large numbers of *M. avium* cells used in our experiment may have biased the results; however, recent study showed that the quantity of mycobacteria in soil as well as their diversity is high (1).

Plants or vegetables have been suspected as possible routes for foodborne mycobacterial diseases (6, 8). Typing of *M. avium* isolates from food and patients showed same DNA patterns (8). Our results demonstrate that mycobacteria can be taken up by the root systems of plants, even in plants with intact roots. The persistence of mycobacteria in soil, manure and different parts of the environment has been demonstrated previously (12, 26). *M. a. paratuberculosis* remained viable in manure after 55 weeks (26). Manure from domestic animals is used as fertilizer on fields where crops or vegetables are grown. Therefore, it is plausible that due to its presence inside plants, there might be a risk of infection to grazing animals or to humans. Although in the present study we have proven the presence of mycobacteria inside the plant tissue, we have not performed any experiments regarding its pathogenicity. Future research should be focused on the testing of the pathogenicity of mycobacteria after the internalisation in plants. Next step would be

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feeding animals with such plants in order to see whether this route of transmission is plausible.

In our study, mycobacteria were present inside the plant tissue for at least two months after contamination of the potting soil. This may be due to the properties of mycobacteria as well as the environment inside the plant tissue. A study on the survival of *Salmonella* in basil showed a decline after only three days (17), although other studies have detected *Salmonella* in tomato fruits 49 days after inoculation (27). Regarding the distribution of mycobacteria inside the plants, *M. a. avium* and *M. a. paratuberculosis* DNA quantity was 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 using culture.

In conclusion, we have demonstrated the internalisation of mycobacteria into different types of plants; further, the distribution within the plant was found to be even. However, the number of mycobacteria found inside plant tissue was several orders of magnitude lower than the initial amount of mycobacteria present in the culture media or substrate. Mycobacteria are probably passively taken inside the roots rather than actively penetrate root epidermis. The passive intake could be also 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 transmission of the infectious agent.

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Table 1. qPCR and culture examination of stem and leaf samples in *in vitro* grown beans; Input concentration of *Mycobacterium avium* subsp. *hominissuis* culture added to the agar was 10^9 cells/ ml.

Part of beans	Plant No.	qPCR IS*1245* (cells/g)	Cultivation without decontamination (CFU)				Cultivation with decontamination (CFU)			
			MB+PANTA	MB	HEYM	Leslie	MB+PANTA	MB	HEYM	Leslie
Stem	1	6.31×10^6	>1000	>1000	100	0	150	0	0	0
	2	1.54×10^7	100	0	0	200	20	0	0	0
	3	7.74×10^6	1000	>200	>1000	200	10	10	0	0
Mean		9.81×10^6	3700	3400	366	133,33	60	3,33	0	0
Standard deviation		4.88×10^6	5474	5716	550.7	115.5	78.1	5.7	0	0
Leaf	1	6.70×10^6	200	>200	1000	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	>1000	0	0	100	10	100
Mean		3.97×10^6	166	200	3833	33.33	0	66.67	36.67	36.67
Standard deviation		2.51×10^6	57.73	0	5346	57.7	0	57.7	55	55
Negative control		0	0	0	0	0	0	0	0	0

1. Middlebrook M7H11+PANTA
2. Middlebrook M7H11 without antibiotics
3. Herrold egg yolk medium
4. Leslie medium

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426 Figure 1. Microscopy of bean stems sections containing *M. a. avium*. A and B: Transmission electron microscopy. C: Ziehl-Neelsen
427 staining of bean stem tissue, a mycobacteria is stained in red against blue background. D: Specific-antibody labelled *M. a. avium*
428 inside bean stem tissue. Fluorescent rods were observed inside the transport plant cells.
429 Figure 2. Detection of *M. a. paratuberculosis* DNA inside tomato plants.
430 Figure 3. Detection of *M. a. avium* DNA inside tomato plants.
431 Figure 4. Distribution of *M. a. avium* and *M. a. paratuberculosis* DNA inside tomato plants.
432

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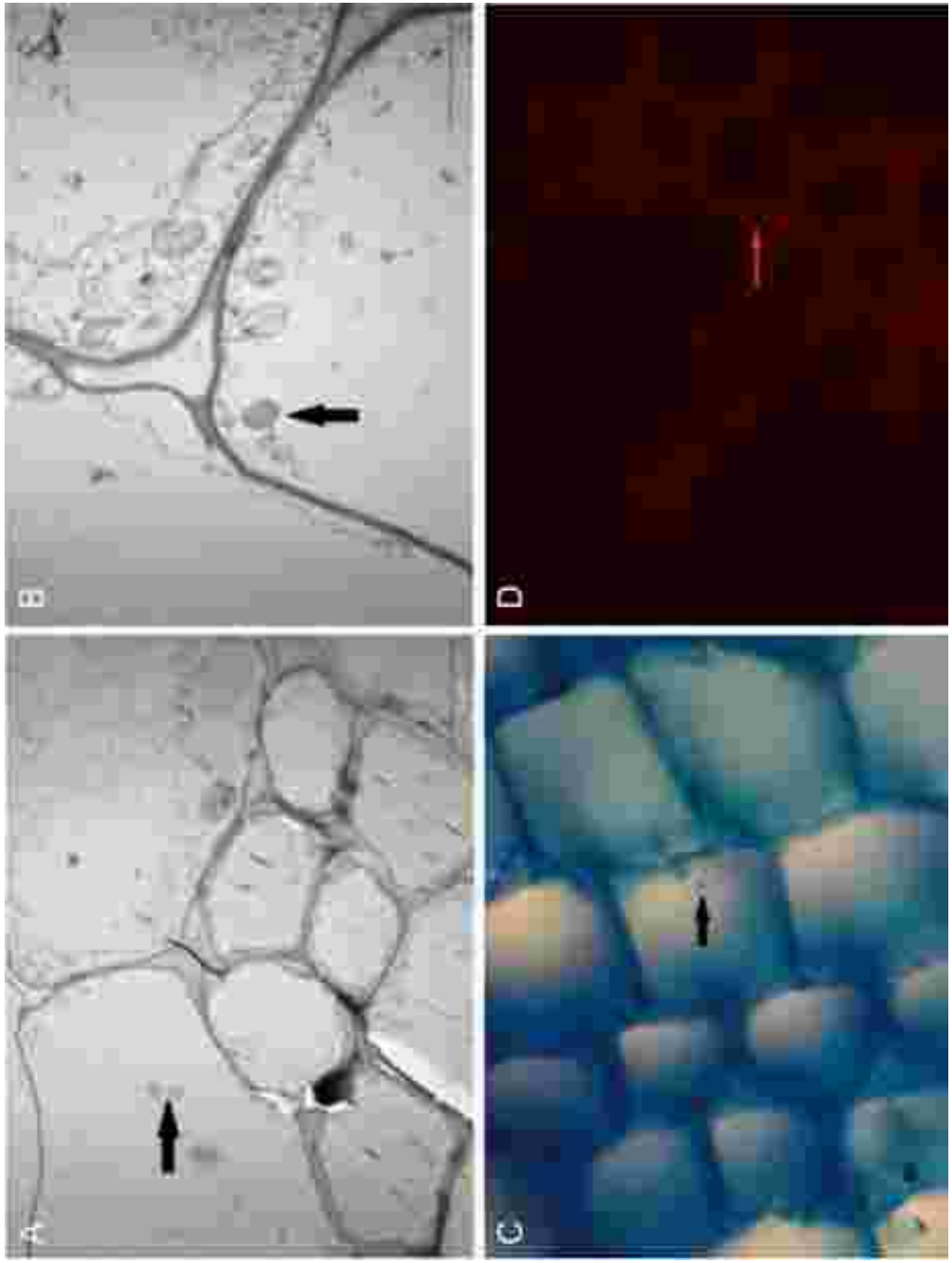

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Figure 2. Detection of *M. a. paratuberculosis* DNA in tomato plants

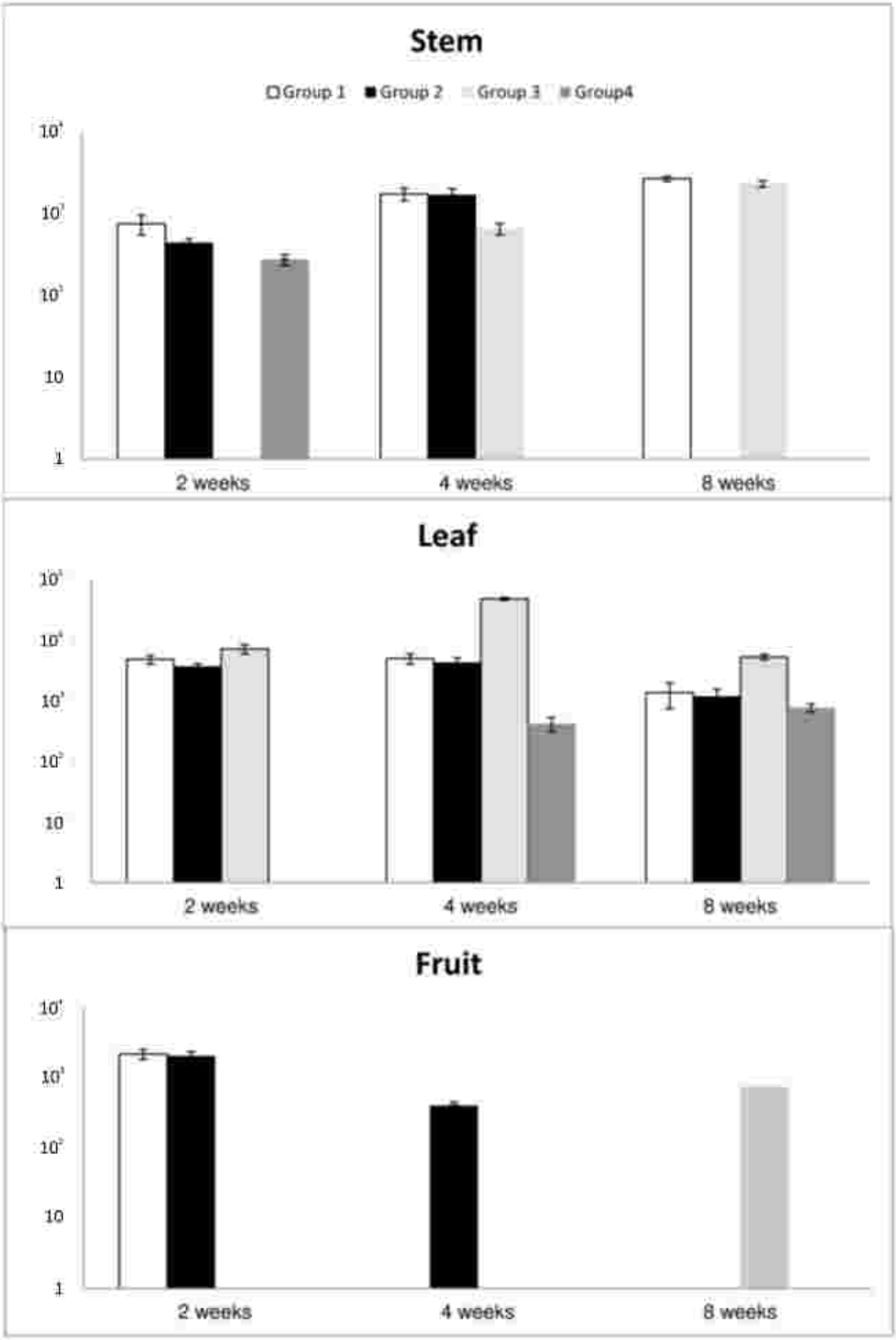

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Figure 3. Detection of *M. a. avium* DNA in tomato plants

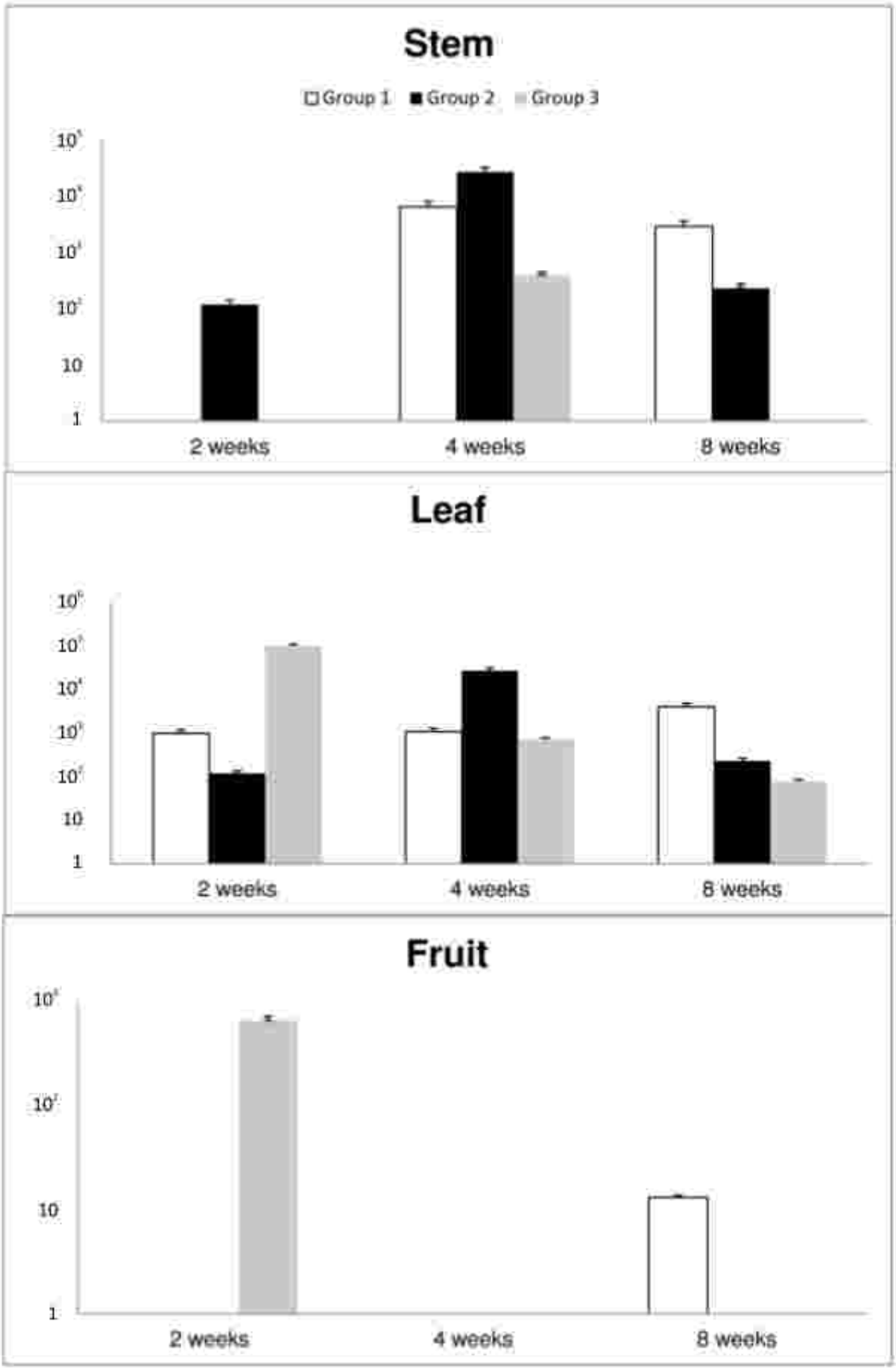

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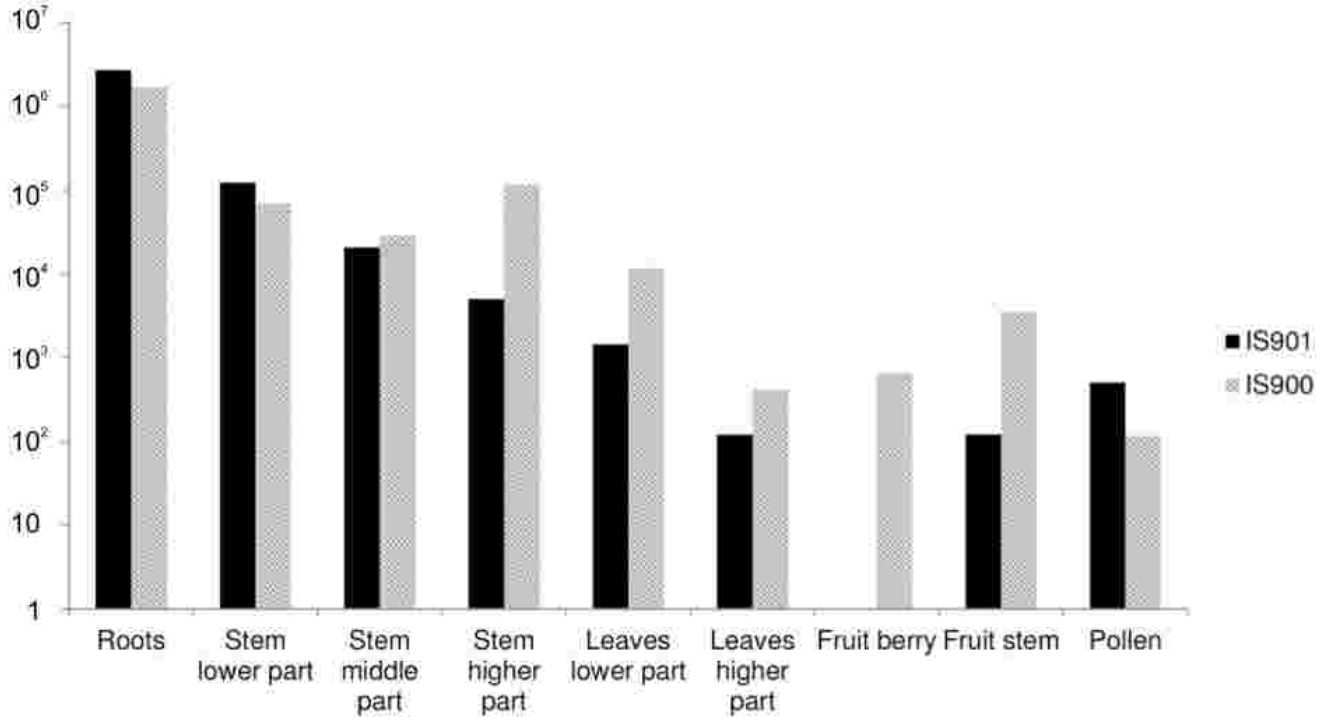
Figure 4. Distribution of M. a. avium and M. a. paratuberculosis DNA inside the tomato plants



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