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STSM-Report Elsayed

COST Action: FA1103
TRAINEE: Tarek Elsayed

STSM TITLE:

The colonization behavior of tomato plants by endophytic bacteria with *in vitro* antagonistic activities against *R. solanacearum*

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MOTIVATION FOR THE STSM:

Ralstonia solanacearum (biovar 2/race3), the causal agent of bacterial wilt in potato, tomato and many other plant species, is a quarantine phytopathogen. This pathogen can survive in the soil for several years and can spread very fast via water streams and latent infections are particularly problematic. Endophytic biocontrol agents might be a solution as they occupy the same ecological niches colonized by *R. solanacearum.*

A total of approximately 2,016 bacterial isolates were checked for their ability to inhibit the growth of *R. solanacearum* in a dual-culture assay. BOX-PCR fingerprints were used to investigate the diversity of antagonists and to detect similar genotypes in the different plant spheres and soil types. Distinct fingerprints were formed and similar fingerprints were grouped together. One representative isolate from each BOX cluster was identified based on the 16S rRNA gene sequencing. The presence of several biocontrol metabolites was evaluated as well as some plant growth promoting activities and biological control related functional genes such as pyrrolnitrin (*prnD*), 2,4-diacetylphloroglucinol (phl), phenazine-1-carboxylic acid (phz) and hydrogen cyanide (*hcnBC*) were also estimated.

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Moneymaker) were treated with the *gfp*-tagged antagonistic bacterial culture suspensions OD_{600} = 1, seeds were germinated in a potting soil and transferred to pots filled with 300 g field soils (4 replicates each and 4 seedlings per pot). A post inoculation drenching was applied 8 days post sowing with 10 ml bacterial suspension OD = 1.00 of each isolate , after 24 days drenched with 5 ml of additional two

antagonistic isolates tagged with the *cfp* and *yfp* genes in order to investigate the co-colonization by different antagonistic isolates. The infection was done at 28 days post sowing with 10 ml of *R. solanacearum* B3B. Samples were taken 15 and 28 days after sowing. Samples were divided into rhizosphere samples (n=4) with tightly attached soil, endorhiza samples where the same roots were surface sterilized and shoots samples. One plant per pot was used as one replicate and the other seedling growing in the same pot were used for the microscopic study. The antagonists CFU counts were estimated using different antibiotic combinations, 1) King's agar B supplemented with ampicillin, chloramphenicol, cycloheximide and rifampicin for the total numbers of antagonistic bacteria, 2) King's agar B supplemented with ampicillin, chloramphenicol, cycloheximide, streptomycin and gentamicin for counting *gfp*-tagged antagonistic bacteria and 3) King's agar B supplemented with ampicillin, chloramphenicol, cycloheximide and kanamycin to count the *cfp* and *yfp* tagged antagonistic bacteria. Total Community DNA (TC-DNA) was extracted. Plant parameters were also estimated.

GOALS OF THE STSM:

The aims of the stay in the laboratory of Dr. Stephane Compant (head Prof. Dr. Angela Sessitsch) were:

1- To localize the colonization pattern of single antagonistic isolates and consortia in different plant microenvironments using confocal microscope using either *gfp* strains or Dope-FISH.
2- To verify whether the co-existing of both endophytic isolates and *R. solanacearum* will leads to control the pathogen or create more entrance sites for the pathogen to attack the root system.

DESCRIPTION OF THE WORK CARRIED OUT:

Work carried out before the STSM.

Tomato seeds (*Lycopersicon esculentum*Mill. cv. Moneymaker) were treated with the *gfp*-tagged antagonistic bacterial culture suspensions OD = 1, seeds were germinated in a potting soil and transferred to pots filled with 300 g field soils (4 replicates each and 4 seedlings per pot). A post inoculation drenching was applied 8 days post sowing with 10 ml bacterial suspension OD_{600} = 1.00 of each isolate, after 24 days drenched with 5 ml of additional two antagonistic isolates tagged with the *cfp* and *yfp* genes. The infection was done at 28 days post sowing with 10 ml of *R. solanacearum* B3B. Samples were taken 15 and 28 days after sowing. Samples were divided into rhizosphere samples (n=4) with tightly attached soil, endorhiza samples where the same roots were surface sterilized and shoots samples. One plant per pot was used as one replicate and the other seedling growing in the same pot were used for the

microscopic study. Plant materials were fixed for the Fluorescence and confocal laser scanning microscopy examination.

Work carried out in the host instate

Fixed plant materials were examined using the confocal laser scanning microscopy (Olympus Fluoview FV1000 with multi-line laser FV5-LAMAR-2 HeNe(G)laser FV10-LAHEG230-2) directly for the samples fixed for the GFP examination. Pictures were taken at 405, 488, 633 nm wavelengths and then merged (RGB). The images were edited and analyzed with the softwares ImageJ and Imaris. Fluorescence *in situ* hybridization was carried out using probes EUB mix (equivalent mixture of EUB338, EUB338II, EUB338III) to target all the bacteria and a specific probe for a target taxon (alpha, gamma-proteobacteria and actinobacteria, pictures were also aken as mentioned before.

Results

Control tomato plants

Control tomato plants with no inoculation with the antagonistic isolates or infected with *Ralstonia solanacearum* can be seen in (Fig.1).

Fig.1.control tomato plants showing no colonization with any fluorescent tagged bacteria in the root surface or in the root endophytic compartments.

Tomato plants inoculated with *Pseudomonas koreensis* (299)

Tomato plants inoculated with *P. koreensis* 299, which showed in the greenhouse experiment a good biological control activity against the plant pathogen *Ralstonia solanacearum*, were heavily colonized in the root tips as well as the elongation parts of the roots, most of the tested root tips were colonized by the *gfp*-tagged *P. koreensis* 299 (Fig.2).

1- Rhizosphere

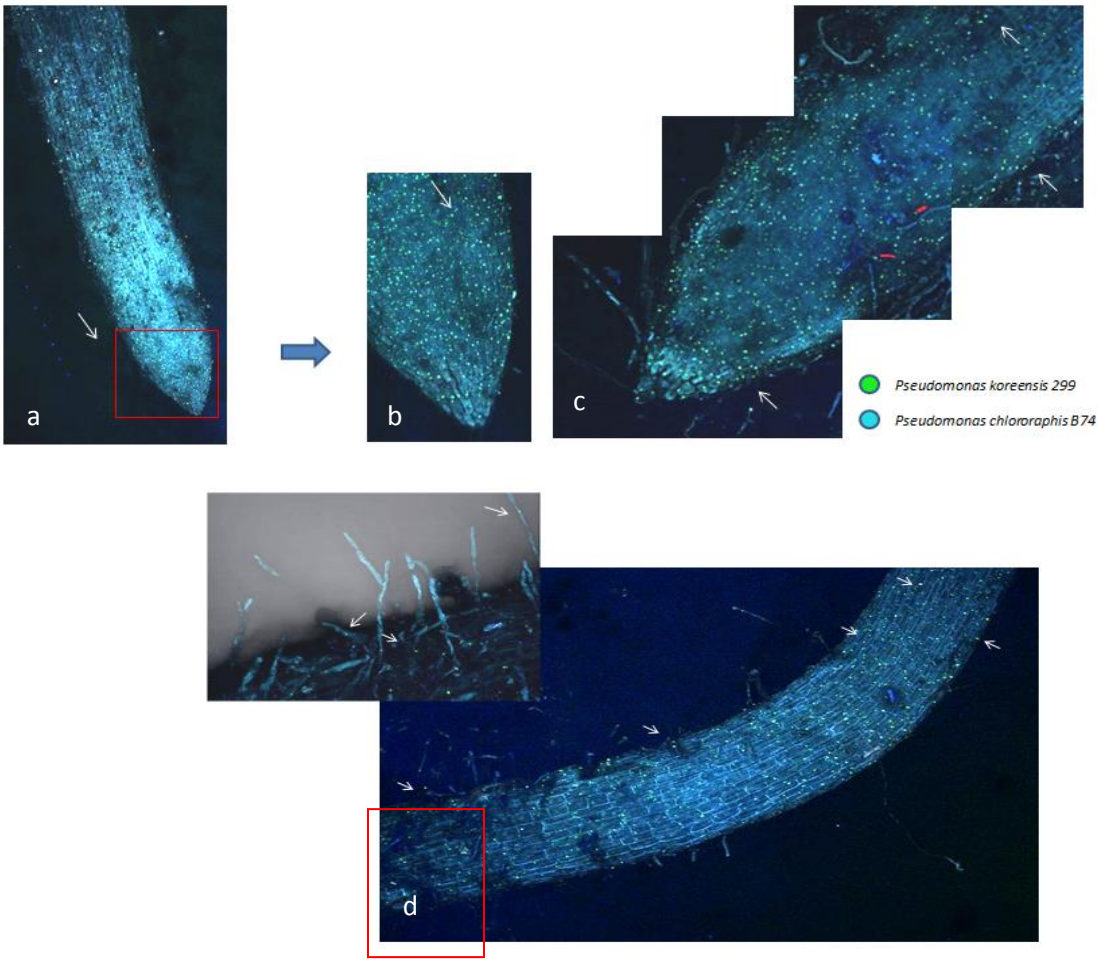

Fig.2. The rhizosphere of tomato plants colonized *by P. koreensis* 299, (a) tomato secondary root colonization (b) root tip magnified covered with the green fluorescent tagged bacteria (c) another root tip colonized by the *gfp* tagged *P. koreensis* 299, (d) secondary root colonized with the *gfp*-tagged bacteria with a magnification on the root hairs

2- Root endophytic compartments

Tomato root endophytic compartments were investigated by examining root sections, antagonistic bacteria tagged with the green fluorescent protein were only found deep in the epidermis, while the *cfp*-tagged antagonistic bacteria *Pseudomonas chlororaphis*B74 could be found also in the endodermis of tomato roots (Fig.3).

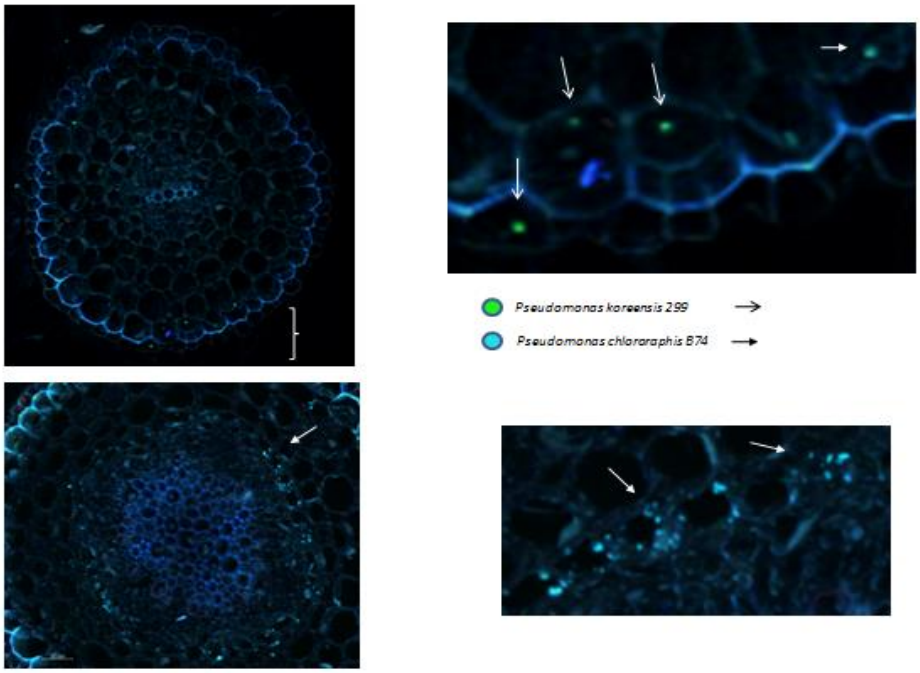

Fig.3. tomato plants root sections showing the colonization of epidermis by the *gfp*-tagged bacteria *P. koreensis* 299 and the endodermis by the *cfp*-tagged bacteria *P. chlororaphis* B74

Tomato plants inoculated with *Pseudomonas umsongensis* (215)

Tomato plants inoculated with *P. umsongensis* 215, which also showed in the greenhouse experiment a good biological control activity against the plant pathogen *Ralstonia solanacearum*, were also colonized in the root tips as well as the elongation parts of the roots (Fig.4).

1-Rhizosphere

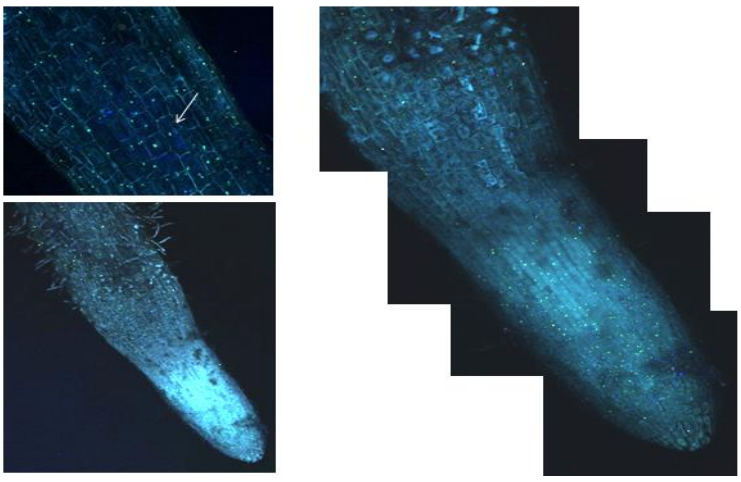

Fig.4. tomato plants inoculation with the *gfp*-tagged antagonistic isolate *P. umsongensis* 215

2- Root endophytic compartments

Antagonistic bacteria tagged with the green fluorescent protein were only found deep in the epidermis, while no *cfp*-tagged antagonistic bacteria could be found in the root endophytic compartmenting (Fig.5)

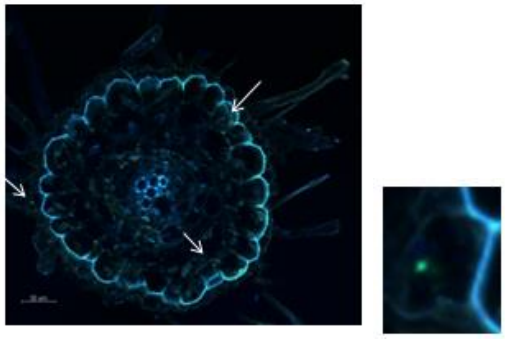

Fig.5. root section of tomato plants inoculated with *P. umsongensis* 215 showing the colonization of epidermis by the *gfp*-tagged bacteria

Tomato plants inoculated with *Pseudomonas rhizosphaerae* 142

Tomato plants inoculated with *P. rhizosphaerae* 142, which showed in the greenhouse experiment a good colonization, while increased the severity of the infection with *R. solanacearum*, were also colonized in the root tips as well as the elongation parts of the roots (Fig.6).

1- Rhizosphere

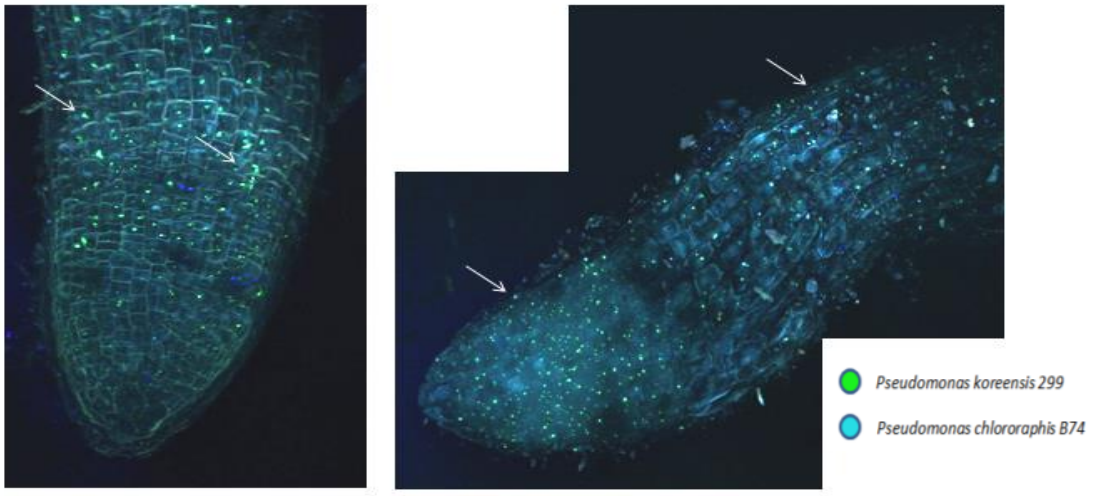

Fig.6. tomato plants inoculated with the *gfp* tagged antagonistic isolate *P.rhizosphaerae*142

2- Root endophytic compartments

Antagonistic bacteria tagged with the green fluorescent protein were only found deep in the epidermis, while no *cfp*-tagged antagonistic bacteria could be found in the root endophytic compartmenting (Fig.7)

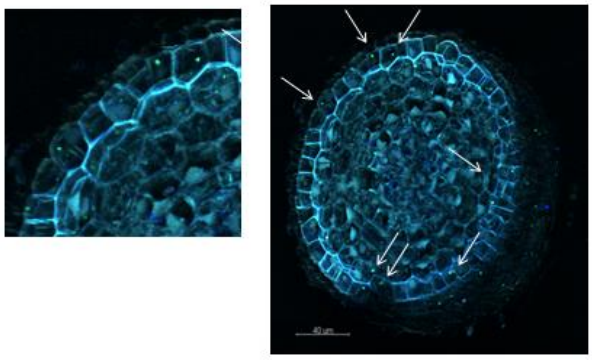

Fig.7. root section of tomato inoculated with *P. rhizosphaerae142* showing the colonization of epidermis by the *gfp*-tagged bacteria

Fluorescence *in situ* hybridization

By using the Fluorescence *in situ* hybridization we could determine the colonization sites in the endophytic compartments, by applying the EUB probes in conjunction with the gammaproteobacteria specific probe we could localize the sites colonized by all the bacteria which marked with the green color while the sites colonized by the members of the gammaproteobacteria (including *Pseudomonas*) marked with the yellow color.

1- Control tomato plant

Examining the control tomato plants (no inoculation and no infection) reveled that tomato endophytic compartments are indigenously colonized (Fig.8) no gammaproteobacteria were observed.

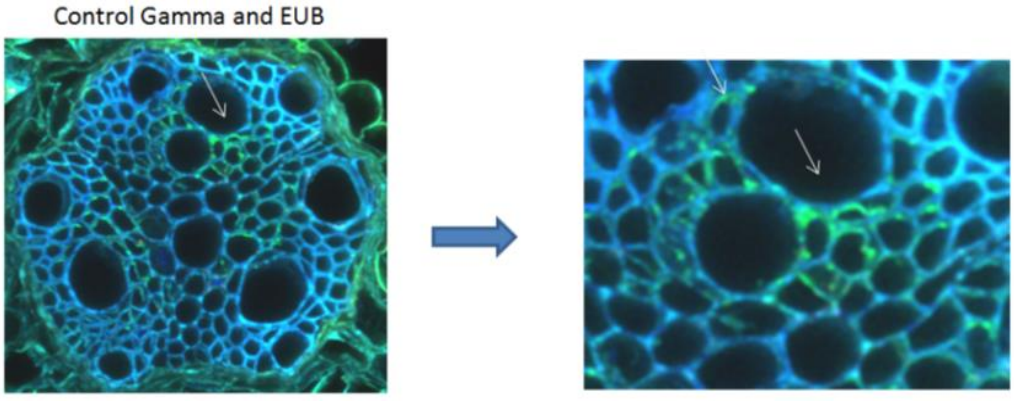

Fig. 8. Non inoculated and non infected tomato plant root sections showing indigenous microbial communities colonizing the root endophytic compartments.

2- Tomato plants infected with *Ralstonia solanacearum*

Tomato plants infected with *Ralstonia solanacearum* were more colonized by bacteria in the root endophytic compartments, which might indicate the presence of *Ralstonia solanacearum* in the xylem vessels of the roots (Fig.9).

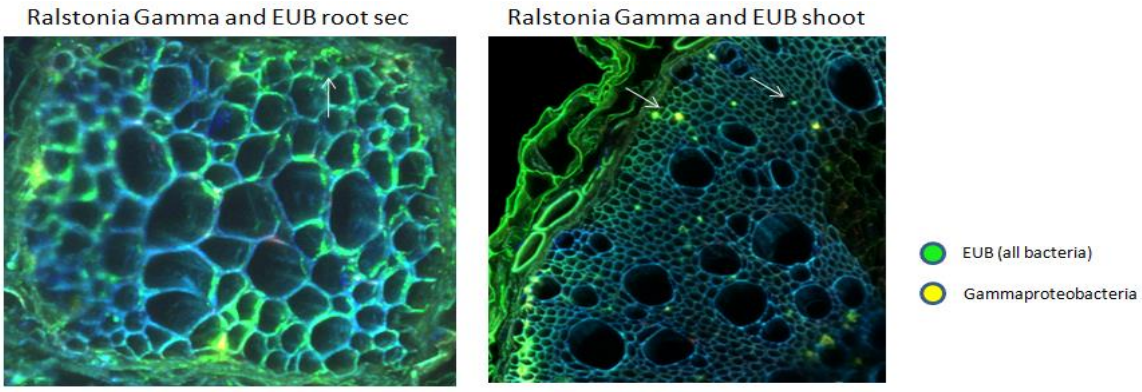

Fig.9. Infected tomato plan root sections showing increase in microbial population colonizing the root endophytic compartment as well as shoot xylem vessels

3-Tomato plants inoculated with *Pseudomonas koreensis* 299

The root sections of tomato plant inoculated with *P. koreensis* 299 reveled also increase in the colonization by the Gammaproteobacteria which might also indicate the colonization by members of Pseudomonas (Fig.10).

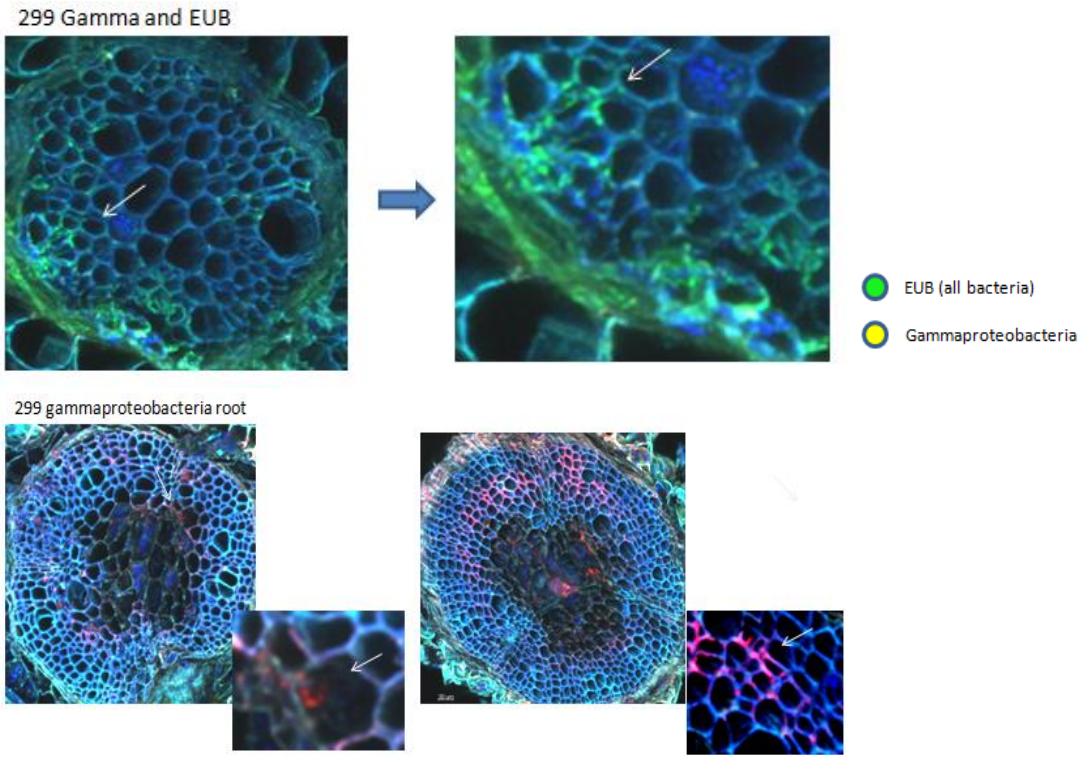

Fig. 10. Tomto plants inoculated with *P. koreensis* 299

Conclusion

The results obtained from the greenhouse experiment showed the successful colonization of tomato plant roots rhizosphere and endophytic compartments by the antagonistic bacteria *P. koreensis* 299 and *P. umsongensis* 215, also confirmed the biologically control of *Ralstonia solanacearum* in plant. The microscopy study also confirmed the colonization of the roots with the tested *gfp*-tagged antagonistic isolates. The co-existing of both endophytic isolates and *R. solanacearum* can lead to control the pathogen but might also with other bacterial isolates create more entrance sites for the pathogen to attack the root system. More analysis is required to investigate the effect of inoculation and infection on total microbial community composition using FISH, and to locate the presence of *Ralstonia solanacearum* in different plant endophytic compartments.