Thesis of PhD dissertation

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BIODIVERSITY OF CULTURABLE ENDOPHYTIC BACTERIA FROM SWEET PEPPER (*CAPSICUM ANNUUM* L. VAR. *GROSSUM*) AND MODELING THEIR COLONIZATION

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1. INTRODUCTION AND OBJECTIVES

Sweet pepper is a worldwide known and popular vegetable which is consumed with predilection by the consumers as a raw or boiled/fried food, because it has favourable flavour, organoleptic and nutritional-physiological properties as well.

This crop is often consumed raw, thus it is important to investigate from food safety and microbiological aspects too, because illnesses associated with raw plant-based foods have increasing tendencies worldwide and. Most of these illnesses are connected to bacteria originated from animals, but illnesses caused by opportunistic pathogens from the surface/inside of crops are also well known.

In order to understand more clearly the plant – bacteria relationship and through this we can reduce the food safety risk of the crop it is necessary to analyse bacteria living inside the plant (bacterial endophytes) in terms of quality and quantity. Since diversity of bacterial endophytes is highly influenced by climate, cultivation conditions, plant species and the cultivar, during my research work I have investigated two sweet pepper cultivars grown in two different cultivation conditions. However, identification of bacteria living inside the plant has important role not only in revealing their biodiversity, but also in confirmation of presence of opportunistic and human pathogenic bacteria as well.

Based on denomination, endophytic bacteria can be divided into two groups. The first is a cluster for putative (potential) endophytic bacteria, which are microorganisms detected from asymptomatic and surface disinfected plants independently from the detection techniques (traditional cultivation based and molecular techniques), while the second group is formed by real (or "true") endophytic bacteria. In order to award this later denomination, in addition to the above mentioned criteria, microscopic evidence of their residence inside the plant tissues is necessary as well. The plant - bacteria relationship is generally considered of mutualistic nature, where the bacteria are using nutrients provided by the plant while also enjoying the protective role of the internal plant environment against adverse environmental factors. In contrast, the plants receive stress tolerance increment and plant growth stimulation from these beneficial endophytic bacteria. On the one hand, the bacterial impact on plants could happen in a direct way through production of phytohormone-like molecules and by regulation of ethylene level. On the other hand, it could happen in an indirect way through their inhibitory effects against phytopathogens and by degradation of the plant toxins. However, there also exists bacterial endophytes which have antagonistic effects against human pathogens and bacteria with such characteristics may be used as biocontrol agents. Here especially culturable endophytes must be underlined, which can also be used in practice for such purposes.

During my PhD work I have aimed the isolation, identification, determination of biodiversity and analysis of effect on pepper seed germination of culturable endophytic bacteria from two sweet pepper cultivars grown in two, nowadays widespread cultivation conditions. Furthermore, my aim was to select mutualistic strains and prove their real endophytic nature. Finally, I wanted to investigate the internalisation of human pathogenic and human pathogen modelling strains in pepper plants through artificial infection of pepper seeds, as well as their inhibition under the impact of selected true endophytes.

Realization of the objectives had the following steps:

1. Isolation of putative bacterial endophytes from Hó and Kárpia sweet pepper cultivars grown in traditional (soil system) and new (hydroponic) conditions.
2. Organising of the putative endophytes into similarity groups based on phenotyping and RAPD-PCR (**R**andom **A**mplification of **P**olymorphic **D**NA-**P**olymerase **C**hain **R**eaction) genotyping, thereafter molecular identification of the selected strains representing these genotypic groups,based on rRNA and *rpoB* gene sequences.
3. Investigation of biodiversity of the strains and their relationships through phylogenetic analyses: construction of phylogenetic trees based on 16S rRNA gene sequences, thereafter selection of one representative strain from each taxonomical unit of the trees in order to prove their real endophytic nature.
4. Investigation of the effects for the putative endophytic strains on germination of pepper seed.
5. Method development in order to detect putative endophytic and human pathogenic or pathogen modelling strains with PCR and FISH-CLSM (**F**luorescence ***I****n* ***S****itu* **H**ybridisation- **C**onfocal **L**aser **S**canning **M**icroscopy).
6. Investigation of internalisation for putative endophytic and human pathogenic or pathogen modelling strains and colonisation by artificial infection of pepper seeds.
7. Investigation of the effects of true endophytic strains on human pathogenic and human pathogen modelling strains by *in vitro* contact inhibition and agar diffusion analyses.

2. MATERIALS AND METHODS

The **isolation of endophytic bacteria** was effectuated by traditional microbiological culturing methods from Kárpia and Hó cultivars of soil and hydroponic cultivated sweet peppers (*Capsicum annuum* var. *grossum*) as well as from seeds used for cultivation. The pepper plants were grown at the Experimental and Research Farm of Faculty of Horticultural Sciences, Szent István University, Soroksár in 2012, and seeds were also originated from there.

Based on their phenotypic characteristics (macro- and micro-morphological) the **isolates were sorted into phenotypic groups**, then from each phenotypic group one representative isolate was selected for further analyses.

Subsequently, genomic DNAs of the representative **isolates were typed by RAPD-PCR method**, and from each RAPD type one representative strain was chosen for further work.

In the next step, there **were detected strains from the *Pseudomonas* genus** by applying a genus specific PCR reaction. Their *rpoB* gene was amplified by using a specific PCR reaction. In addition, 16S rRNA coding rDNA genes of all representative strains were amplified as well.

In case of *rpoB* gene **amplicons were sequenced** from one direction by using the reverse primer. Likewise, the reverse primer was used for nucleotide order determination in case of 16S rRNA genes, however in some cases almost the complete 16S rRNA was sequenced by applying primers directed outward from inside. Determined sequences were compared with those of the type strains deposited in database by using the program BLAST in case of *rpoB*, respectively server EzTaxon in case of 16S rRNA genes.

For **phylogenetic analysis** 16S rRNA sequences were used, from which identical ones were classified into OTUs (**O**perational **T**axonomic **U**nits) and from each OTU one representative strain was selected for further investigations. Thereafter, phylogenetic tree was constructed by applying the most suitable substitution model and the Maximum Likelihood statistic method (Felsenstein, 1981).

In order to analyse the **effects of the representative bacterial strains on germination of pepper seeds**, fifty surface disinfected pepper seeds were used in case of each strain. Seeds were incubated for six hours in an approximately 10^8 CFU/ml concentrated bacterial suspension, then they were placed onto germination papers and germinated for 10 days. The number of germinated seeds was counted and evaluated in comparison to the control (ISTA, 2003; Niranjan Raj *et al.*, 2003; Chandrashekhara *et al.*, 2007).

Internalisation of bacteria in plant tissues was simulated by infection of pepper seeds, where internalisation of five different bacterial strains (*Chryseobacterium hispalense* FPBSKK1 and *Pseudomonas* sp. HPBBIK3 as putative endophytes, *Listeria innocua* 1010, *Escherichia coli* ATCC 8739, *Listeria monocytogenes* CCM 4699 as pathogen modelling and pathogenic strains) was analysed separately. Furthermore two controls (sterile and non-sterile) were used as well. Seedlings grown from seeds were cultivated for a 35-40-day-growth stage, thereafter they were processed.

Bacteria from **plants inoculated by *C. hispalense* FPBSKK1 and *P.* sp. HPBBIK3 as well as form control plants** were isolated by traditional cultivation method, followed by grouping based on their colony morphology. In the next steps only one representative isolate from each group was used. DNAs of the representative isolates and the putative bacterial endophytes used for inoculation (as positive controls) were analysed with specific primer pairs designed in this work. In case of those isolates which produced the proper amplicons, the 16S rRNA genes were amplified and sequenced. Thereafter, the determined nucleotide sequences were compared with 16S rRNA gene sequences of strains used for plant inoculation. Finally, the genomic DNAs originated from isolates and strains used for inoculation were typed by RAPD-PCR as well.

***E. coli* ATCC 8739 inoculated** seedling tissues were analysed on Chromocult® coliform selective agar plates, while ***L. innocua* 1010 and *L. monocytogenes* CCM 4699 inoculated** ones were tested on ALOA *Listeria* selective chromogen agars.

Analysis of DNA isolated from the plants was effectuated with primers designed in this work and already published ones. *L. innocua* 1010 and *L. monocytogenes* CCM 4699 inoculated seedling tissues were analysed with 27f/ Lis659R, while *E. coli* ATCC 8739 inoculated ones with 27f/ Ec/Sh473R primer pairs. Specific amplicons generated by Chr22F/ Chr818R primer pair (specific for *C. hispalense* FPBSKK1) were sequenced, thereafter compared to the 16S rRNA gene sequences of *C. hispalense* FPBSKK1 strain and to sequences available in the server EzTaxon as well. Samples from which specific amplicons could be generated by Pse393F/ Pse618R primer pair (specific for *P.* sp. HPBBIK3) were amplified with 1070F/ 1492R primer pair specific for bacterial rDNA, then the smaller (448 bp) amplicon was cleaned and sequenced. The determined sequences were compared to the 16S rRNA gene sequences of *P.* sp. HPBBIK3 strain and to sequences available in EzTaxon server as well.

During **FISH-CLSM analysis** all bacterial cells present in the samples were detected by EUB mix probe, while bacterial strains used for plant inoculation were detected with the following specific probes (*- designed during in this work): *C. hispalense* FPBSKK1 – **Chryseo_643***; *P.* sp. HPBBIK3 – **Pseudo_828***; *L. innocua* 1010 or *L. monocytogenes* CCM 4699 – **Lis-637** and

Lis-comp*; *E. coli* ATCC 8739 – **Ec/sh_453**; Bacterial cells in sterile and non-sterile control plants – with all specific probes separately. Microscopic investigations were performed with a Zeiss LSM 710 type (Carl Zeiss AG, Germany) CLSM and the image formatting with the ZEISS ZEN 2 lite (Carl Zeiss AG, Germany) program.

Effects of the selected endophytic bacteria (*C. hispalense* FPBSKK1 and *P.* sp. HPBBIK3) **on pathogenic and pathogen modelling** (*L. monocytogenes* CCM 4699, *E. coli* ATCC 8739, *L. innocua* 1010) **bacteria** were analysed with contact inhibition and agar diffusion methods. PGYS agar plates were inoculated with a standardized suspension of the pathogenic and pathogen modelling strains, then three drops from the suspensions of bacterial endophytes were pipetted onto the surfaces of the agar plates and incubated at different temperatures.

3. RESULTS AND DISCUSSIONS

3.1. Isolation of putative endophytic bacteria

Altogether 170 putative endophytic bacteria were isolated. A few isolates were derived from seeds, while in contrast 142 isolates were cultured from the seedlings. From the matured plants a few bacteria (23) were also isolated. In terms of pepper cultivars almost half as much isolates (60) were originated from Hó as Kárpia cultivar (110). Taking into consideration the cultivation conditions it can be concluded that more isolates were cultured from hydroponic (98) than from soil (67) grown plants.

3.2. Phenotypic characterisation of putative endophytic isolates

Because of the high amount of isolates and for easy handling separate phenograms for Gram-positive and Gram-negative isolates were constructed. In this division the 69 Gram-positive isolates formed 56, while the 101 Gram-negative formed 82 groups. Overall, the 170 isolates could be grouped into 138 clusters. Isolates belonging to the same group probably belong to the same strains, therefore during the succeeding investigation only one representative isolate was selected from each group in order to reduce the number of isolates. Screening of the identical isolates is also important in order not to distort the quantitative diversity results of the whole microbial population.

3.3. Genotyping of the isolates by RAPD-PCR method

The 56 Gram-positive representative isolates formed 45, while the 82 Gram-negative bacteria were grouped into 55 clusters, thus the total of 138 isolates separated based on their phenotypic characteristics could be sorted into 100 RAPD-PCR genotypes. The number of Gram-positive, unique phenotypic isolates was decreased by more than 19%, while Gram-negative ones even greater, by more than 32% due to genotyping.

As in the previous chapter, from all clusters only one representative isolate was chosen which could be considered as a strain. Thus, altogether 100 strains were used in the identification session.

3.4. Molecular identification of the isolated strains based on *rpoB* and 16S rRNA gene sequencing

Amplification of 16S rRNA gene was successfully done in case of all 100 strains. As a first step the sequencing was effectuated by using the reverse primer, then the whole gene was sequenced

in such cases, when the species level identification was not possible due to insufficient information.

The *rpoB* gene amplification was used in case of strains of *Pseudomonas*, because in itself the 16S rRNA gene does not provide enough information for the identification to the members of *Pseudomonas* genus at species level. However, in many cases it is not sufficient to analyse only the *rpoB* gene, because there is also an increasing need for other housekeeping (like *gyrB*, *rpoD*, etc.) and specific gene sequence information for the accurate identification of *Pseudomonas* genus members at species level, according to the findings of Bennasar *et al.* (2010) and Mulet *et al.* (2010).

Summarising the results of molecular identification it can be concluded, that more than 50% (53 strains) out of 100 strains were successfully identified at species level. The remaining part was identified only at genus level. However, in case of members of *Pseudomonas* this ratio decreased to 29%. Consequently, for *Pseudomonas* genus members and other additional isolates with increased species diversity or even for potential novel strains the identification was possible primarily at genus level.

3.5. Results of phylogenetic analysis

The 16S rRNA gene sequences of the 100 strains were compared in order to identify identical ones, thus it was possible to separate them into 64 different OTUs. Biodiversity and relationships of strains based on phylogenetic tree constructed by using strains representing the 64 OTUs were analysed. Based on these results high level of biodiversity of the strains could be concluded.

γ-Proteobacteria was the most populated class with 21 OTUs (33%), which was followed by the Gram-positive Bacilli class (17 OTUs, 27%) with low GC content in DNA and Gram-positive Actinobacteria class (14 OTUs, 22%) with high GC content. Furthermore, 7 OTUs (11%) were originated from α-Proteobacteria, while β-Proteobacteria was represented by 3 OTUs (5%). Flavobacteria class was represented only with *C. hispalense* FPBSKK1, while Deinococci class with *Deinococcus* sp. SEFSRH1 strain.

Based on the repartition of the OTUs on the phylogenetic tree, it can be established that *Pseudomonas* OTUs were the most dominant (12 OTUs, 19%) not only in γ-Proteobacteria class but also among all classes.

The following strains should be underlined which are sufficiently far from the other strains in point of their relationship: the *Pseudomonas* sp. HPBBIK3, *Cupriavidus campinensis* SEPRH20, *Deinococcus* sp. SEFSRH1, *Brevundimonas* sp. FPBTIH1, *Paenibacillus* sp. SESRK1, *Brevibacillus centrosporus* SESRK14, *Leucobacter tardus* HPBSKH1 and *C. hispalense*

FBBSKK1. Based on these strains it was possible to most likely select those ones, for which it was possible to design specific oligonucleotide probes in order to execute FISH analysis.

3.6. Repartition of the putative endophytic strains in pepper plants

In terms of cultivation conditions it can be assessed that in total more strains (54) were derived from hydroponic grown plants, than from soil grown (43) ones, which is truer if it is zoomed into seedlings (48 from hydroponic and 31 from soil grown seedlings). In contrast to the former assessments this ratio was reversed and even more pronounced in case of the fruits. Summing up the strains originated from plant organs, most of them - as it was expected - were originated from the roots, while from green organs and fruits less than one third, 18-18 strains were isolated, and the fewest strains (3) were derived from the seeds. In terms of the fruit, which is the most important from food safety aspects, it was possible to isolate strains belonging to *Enterobacter cancerogenus* and *Pantoea brenneri* species, described as opportunistic human pathogens. Analysing the cultivars it can be assessed, that in total near twice as many (66) strains were originated from Kárpia, than from Hó (34) cultivar. The same phenomenon was observed after detailed assessments, however isolates from fruits of the hydroponic grown plants were exceptions.

In case of pepper plants grown in hydroponic system the *Pseudomonas* and *Bacillus*, while the plants grown in soil system the *Rhizobium* and *Microbacterium* genera were presented in the largest number, however, all four genera were present in both cultivation conditions. Strains occurred in fewer extents from *Micrococcus, Staphylococcus, Paenibacillus* and *Enterobacter* genera, in peppers grown under both cultivation conditions. Several genera were represented exclusively in hydroponics grown plants like *Cupriavidus, Delftia, Acidovorax, Stenotrophomonas, Leucobacter, Kocuria, Cohnella, Brevibacillus* and *Lysinibacillus*, while members of the *Pantoea, Leclercia, Deinococcus, Brevundimonas, Ochrobactrum, Curtobacterium, Clavibacter* and *Leifsonia* genera could be isolated only from soil grown plants.

Strains originated from Hó and Kárpia cultivars were derived in large numbers from *Pseudomonas* and *Rhizobium* genera, furthermore strains occurred also in large numbers from *Microbacterium* in case of Hó, while from *Bacillus* genus in case of Kárpia cultivars. Though in lower extent, it was possible to isolate from both cultivars the following genera: *Enterobacter, Leclercia, Stenotrophomonas, Paenibacillus, Curtobacterium*. In terms of the uniquely occurring genera, it was possible to isolate fewer strains from Hó (*Pantoea, Cupriavidus, Acidovorax, Deinococcus, Leucobacter*) cultivar, than from Kárpia (*Delftia, Brevundimonas, Ochrobactrum,*

Cohnella, Lysinibacillus, Brevibacillus, Rothia, Kocuria, Micrococcus, Clavibacter, Leifsonia, Staphylococcus).

3.7. Effects of the putative endophytic strains from different OTUs on pepper seed germination

In order to distinguish the real endophytes from the putative endophytes, it is necessary to prove visually their presence (colonisation) in the internal plant tissues, which would have been difficult to fulfil for all the 64 OTU-representing strains. Therefore, a new method was used in order to screen for non-endophytes and also for revealing those mutualist endophytic traits, which directly stimulate the seed germination.

In the followings only those strains were considered as putative endophytes, which induced a minimal 90% pepper seed germination rate compared to the control. 10.9% of the strains showed strong, while 9.4% of them showed slight inhibitory effects on seed germination. Most of the strains (near 64.1%) were taking part of the neutral category and just a small part of them, 15.6% were able for significant stimulation. Probably it is worth to searching for real endophytes among this 79.7%.

From the deviation of measurements it can be concluded that this method has a minor uncertainty. A stimulatory limit (min. 110% germination promotion) was imposed due to the large number of strains with neutral effects, and in the followings only those strains were used which corresponded to this criterion. Through this it was possible to minimize the number of strains used for further investigations. Half of these strains (10) are members of *Pseudomonas*, and a rest of them were derived from *Leclercia, Curtobacterium, Pantoea, Chryseobacterium* and *Rhizobium* genera. Unfortunately, *Pseudomonas* sp. SEPRK23 strain with the highest stimulatory effect on germination could not be identified at species level.

Comparing the 10 strains with stimulatory effects to those 8 strains looking suitable for FISH probe design based on phylogenetic analysis, the number of strains was reduced to two. One of them was the *P.* sp. HPBBIK3, which was not identified at species level, but many *Pseudomonas* species were described in the scientific literature as endophyte (Prieto és Mercado-Blanco, 2008; Andreote *et al.*, 2009; Pandey *et al.*, 2012), therefore it was considered as potential candidate. The other strain, marked as FPBSKK1, was identified as *Chryseobacterium hispalense*, from which species one strain was described as **p**lant **g**rowth **p**romitng **b**acterium (PGPB) (Montero-Calasanz *et al.*, 2013a), thus this strain has good chance to be a true endophyte (considering its life strategy, probably it is a facultative endophyte, as most of the PGPB (Gaiero *et al.*, 2013)).

3.8. Results of the analysis of bacteriobiota derived from seedlings developed from pepper seeds inoculated with putative bacterial endophytes

Bacterial strains were successfully cultured from internal plant tissues by traditional microbiological methods. Followed the colony morphological grouping, identity of the strains used for inoculation and cultured from plants was determined by specific PCR and 16S rRNA gene sequencing. As a further confirmation similarity of their molecular fingerprints was established by RAPD-PCR.

Based on specific PCR and 16S rRNA gene sequence analysis the identity of strains was also confirmed by using the total plant DNA as template.

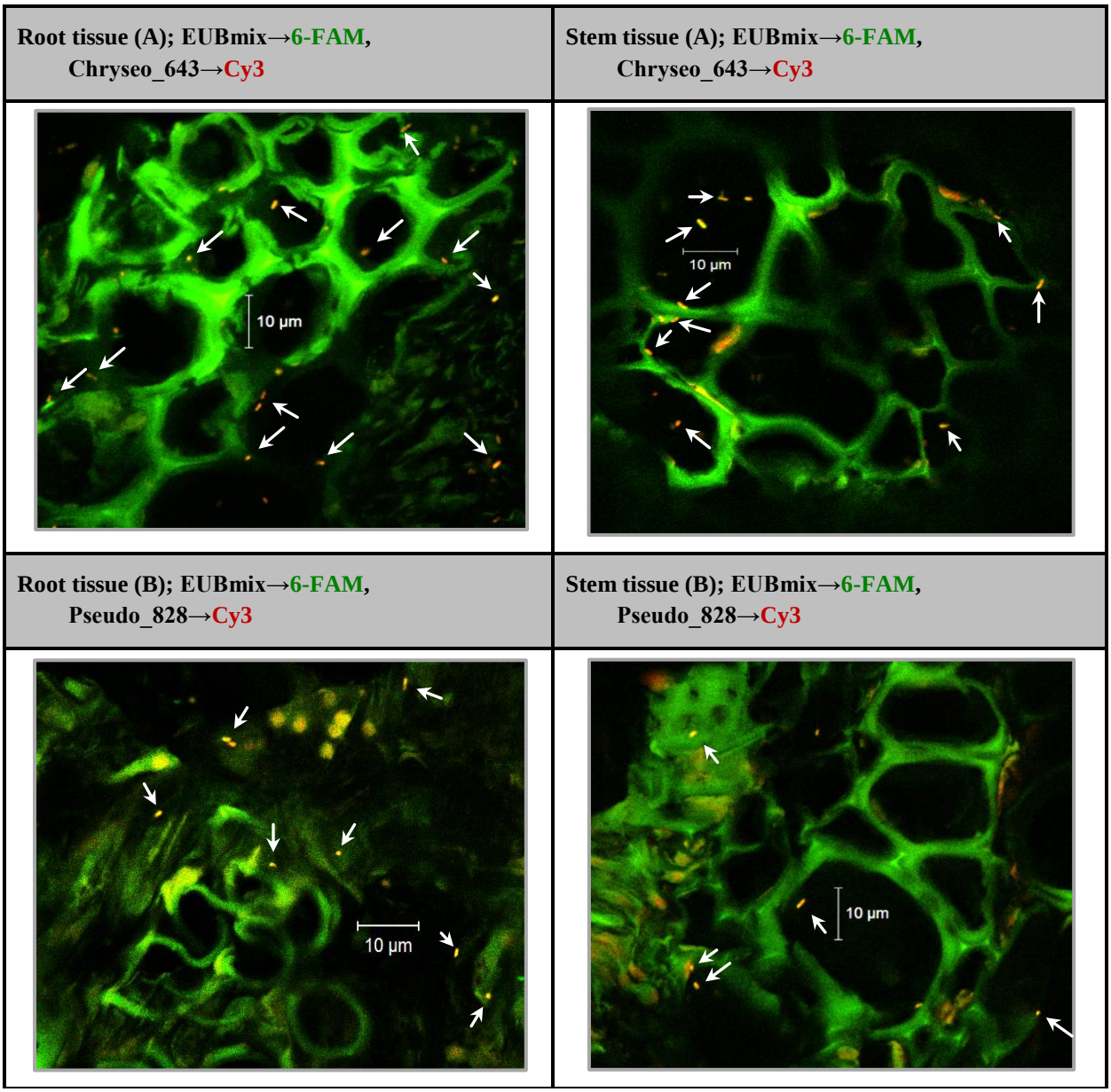

Figure 1. Representative FISH-CLSM images of different plant parts, prepared from plants inoculated with *C. hispalense* FPBSKK1 (A) and *P.* sp. HPBBIK3 (B) strains. Applied probes legend: Probe name → labelling.

Figure 1 represents images made from plant root and stem sections inoculated with *C. hispalense* FPBSKK1 and *P.* sp. HPBBIK3 strains, where the bacterial cells are indicated with arrows. The cells can be seen in yellow, because the fluorophores of general probes (6-FAM) emitted green signal, while the fluorophores of specific probes (Cy3) emitted oranges-red signal, and these two colours together resulted in yellow. In these images there are visible large numbers of *C. hispalense* FPBSKK1 cells in root and stem tissues. This is confirms the internalisation of *C. hispalense* FPBSKK1 cells used for inoculation into seeds and thereafter to other internal plant tissues. Similar phenomenon can be observed in cases of sections made from plants inoculated with *P.* sp. HPBBIK3 strain, except that the number of detected cells was much lower.

Using FISH technique, newly designed probes and confocal laser scanning microscopy it was possible to confirm, visually as well, the presence of the two putative endophytes in the plants (see Figure 1.), thus they can be considered as real endophytes.

Summarising the results we can state that both endophytic strains (*C. hispalense* FPBSKK1 and *P.* sp. HPBBIK3) were successfully detected by the applied methods.

3.9. Results of the analyses of bacteriobiota derived from seedlings grown from pepper seeds inoculated with pathogenic and pathogen modelling bacteria

After incubation of plant tissues on selective media (Chromocult®, ALOA) growth of bacteria which colonies are identical with those of used for plant inoculation was not detected.

During PCR analysis of the DNA isolated from plants, neither the 27f/ Lis659R (applicable for detection of *L. innocua* 1010 and *L. monocytogenes* CCM 4699) nor the 27f/ Ec/Sh473R (applicable for detection of *E. coli* ATCC 8739) primer pairs resulted PCR products.

It was not possible to detect the target cells by FISH technique, but it confirmed the presence of non-target cells in three cases (root and stem tissues inoculated with *E. coli*, and root tissues of sterile control plants).

Based on the summarized results neither the pathogenic (*L. monocytogenes* CCM 4699) or opportunistic pathogen (*E. coli* ATCC 8739), nor the pathogen modelling bacteria (*L. innocua* 1010) were detected by the applied methods, which suggests that the analysed bacteria did not internalize the sweet pepper plant through the seeds.

3.10. Effects of the two endophytic strains on pathogenic and pathogen modelling strains

During seedling cultivation experiments the effects of the two endophytic strains (*C. hispalense* FPBSKK1 and *P.* sp. HPBBIK3) used for plant inoculation were analysed separately against

pathogenic (*L. monocytogenes* CCM 4699), opportunistic pathogen (*E. coli* ATCC 8739) and pathogen modelling (*L. innocua* 1010) strains. Since most of the bacteria are inhibiting other bacteria through production of secondary metabolites, therefore during these experiments the tested endophytic strains were used in their late exponential or early stationer growth stage.

Summarizing the effects of two endophytic strains on pathogenic and pathogen modelling bacteria it can be said, that based on the contact inhibition and agar diffusion analysis the analysed endophytic strains did not show inhibitory effect on the model bacteria, and the stimulatory effect was not significant as well under the tested conditions.

3.11. Novel scientific results

1. I have firstly isolated putative endophytic bacteria from two cultivars (Hó and Kárpia) of the sweet pepper grown in soil and hydroponic systems with culturing method.
2. By adapting and further developing of an internationally accepted germination test I have elaborated and applied a new screening method in order to recognise those strains, which have negative effects on seed germination.
3. In case of the two endophytic strains (*Chryseobacterium hispalense* FPBSKK1 and *Pseudomonas* sp. HPBBIK3) selected on results of pepper seed germination and phylogenetic analysis, I have analysed their internalization in plant by artificial pepper seed infection. I have demonstrated their colonization in different plant organs by designing specific oligonucleotide probes and using the FISH-CLSM technique. I have also supported this by molecular analysis of the plant and the bacterial genomic DNA isolated from inoculated plants.
4. I have analysed the entrance and colonization of pathogenic and pathogen modelling bacteria in plant by artificial infection of the pepper seeds. Using bacterial culturing, specific PCR with new primer pairs, and FISH-CLSM technique I could not detect any of them in the plant, which suggests that the analysed bacteria do not internalize the sweet pepper plant through the seeds.

4. CONCLUSIONS AND SUGGESTIONS

Since nowadays plant based foods consumed raw are increasingly associated with human diseases caused by bacteria, but even more because endophytic bacteria with antagonistic effects on human pathogens were isolated in several cases, it is worth to know more about bacteria living inside the consumable plants.

Large numbers of putative endophytic bacteria were isolated from two sweet pepper cultivars grow in two different cultivation conditions, and after phenotypic grouping and genotyping it was possible to significantly reduce the numbers of absolutely necessary strains (from 170 to 100). As reduction of numbers of representative isolates from phenotypic groups was possible using RAPD-PCR, it indicates that phenotypic grouping is not inefficient enough, therefore its application together with genotyping is advised.

Fifty-three percentage of the 100 strains could be identified at species level, which may suggest the existence of potentially new species among them. In case of isolates from *Pseudomonas* genus this ratio was less than 30%, even if the partial sequencing of *rpoB* was effectuated as well. Therefore, sequencing of further genes (universal and specific) is necessary, and the existence of database contains all gene sequence information for all accepted, identified type strain is also important.

Most of the isolates were originated from roots, because large numbers of bacteria can be found in the rhizosphere and the root surrounding soil. It was possible to isolate many bacteria from above-ground green organs and from fruits as well; from this last organ it was possible to isolate two strains from the *Enterobacter cancerogenus* and *Pantoea brenneri* species described as opportunistic pathogens. The presence of these strains may draw attention to the food safety risk of this fruit in case of immunocompromised individuals. Of course, I have isolated many potential mutualistic strains from the *Pseudomonas, Bacillus, Rhizobium* and *Microbacterium* genera as well. In terms of growth conditions it can be assessed that in total more strains were derived from hydroponic plants, than from soil grown ones. In contrast, this ratio was reversed in case of the fruits, which suggests that, apart from fruits, hydroponic cultivation has higher contribution in the increment of endophytic biodiversity, than soil system. Furthermore, in terms of the cultivated varieties more strains were originated from Kárpia than from Hó cultivar, which is suggesting that the biodiversity of endophytes - apart from many other factors - may depend not only on cultivational conditions, but also on cultivated varieties, respectively on their combination.

Concerning their effects on pepper seed germination, 64.1% of the analysed strains had neutral effect, while 15.6% had stimulatory effect, therefore they counted as putative endophytes. Analysing their effects on pepper seed germination could be useful, because by applying this method it is possible to reveal the presence of surface disinfection surviving, non-endophytic bacteria. Furthermore, by eventual development of this method the endophytes with direct plant growth promotion can be detected.

During the analysis of seedlings infected with different bacterial strains, detection of pathogenic and pathogen modelling strains was not possible with any of the methods and from any of the samples. From these results it can be concluded that they could not penetrate into the plant endosphere, or if they internalized they could not survive the plant cultivation period. In the future it would be worthwhile to infect seeds with the mixture of these human pathogen modelling/human pathogenic bacteria and endophytes (in the same or different ratios) in order to reveal the effects of endophytes on the internalization and survival of human pathogens. This would be essential in disclosing the approach: how do real endophytes affect the pathogen internalization?

What regards the *in vitro* experiments, the two proved endophytic strains certainly did not have either stimulatory or inhibitory effects on pathogenic and pathogen modelling strains. Obviously, this does not mean that none of the 64 representative strains has antagonistic effects against pathogenic bacteria. Therefore, in the future it would be worthwhile to analyse other strains in order to prove their endophytic nature and analyse their effects upon human pathogens.

5. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

Articles in journals

Journals with impact factor

Belák, Á., Héher B., **Füstös, Z.**, Maráz, A. (2014) Endophytic bacteria from *Capsicum annuum* var. *grossum* cultivars and their inhibitory effects on *Listeria monocytogenes*. *Acta Alimentaria*, Vol. 43 (Suppl.), pp. 9-20. (IF/2014: 0,274)

Füstös, Z., Belák, Á., Kovács, M., Maráz, A. (2016) Culturable bacterial endophytic community of *Capsicum annuum* L. var. *grossum*: biodiversity and distribution in the plant (manuscript prepared for publication)

Füstös, Z., Belák, Á., Maráz, A. (2016) Internalisation of endophytic and human pathogenic bacteria in *Capsicum annuum* L. var. *grossum. Acta Microbiologica et Immunologica Hung.* (manuscript prepared for publication)

Conferences

Hungarian conferences (abstracts)

Füstös, Z., Belák, Á., Kovács, M., Maráz, A. (2012): *Isolation, characterization and molecular identification of endophytic bacteria from different Capsicum annuum cultivars*. Annual Congress of the Hungarian Society for Microbiology 2012. Keszthely. Book of abstracts 13. p.

Füstös, Z., Belák, Á., Kovács, M., Héher, B., Maráz, A. (2013): *Különböző Capsicum annuum var. grossum paprikafajták endofita baktériumainak izolálása, jellemzése és molekuláris biológiai vizsgálata*. Hungalimentaria 2013. „Kockázatbecslés, önellenőrzés, élelmiszerbiztonság" scientific conference. Budapest. Program 39. p.

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