Hidden microorganisms in horticultural plants and their interaction with the host

Endophytes are defined as organisms that colonize plant tissue for a considerable period of their life cycle without causing any symptoms in the host plant. Their interaction with the host is intriguingly complex, ranging from mutualism to commensalism and parasitism [1,2]. Many publications demonstrate that endophytes can influence the biosynthetic pathways of the host and even contribute to synthesis of secondary metabolites or plant hormones, possibly indicating mutual adaptation during the evolution process [2]. Nevertheless, many aspects of the plant - endophyte interaction remain unresolved: it is not clear what genetic and biochemical features are responsible for colonization and for the type of interaction. It is not even known whether plants would be fit enough to survive in their natural environment without their microbial endophytes [3,4].

Answering these questions is also of great practical importance, since mutualistic endophytic microorganisms can stimulate plant growth, enhance tolerance to abiotic stressors such as drought, and may confer resistance against pathogens or herbivores [5]. Moreover, it is now widely recognized that their use as a biological control agent could open new approaches in crop management. Biological control is especially desirable for those horticultural plants that are continuously harvested and are consumed unprocessed. Bell pepper (*Capsicum annuum* L.) belongs in this category of vegetables. It is an economically important cash crop grown in greenhouses or open fields worldwide.

The aim of our project was to identify endophytic bacteria and fungi in pepper and open up the way to analyze their possible beneficial effects on physiological processes and stress tolerance of the host, as well as the potential risks originating from their presence in agricultural practice and in the food chain. A detailed analysis of species composition of endophytic bacteria and fungi was carried out in parallel from plants grown in hydroponic culture in greenhouse or in sandy soil under field conditions. Different organs of the cultivars ‘Hó F1’ and ‘Kárpia F1’ were analyzed and compared. Analyses of endophytic bacteria was carried out by the Department of Microbiology and Biotechnology, while fungi and viruses were investigated by the Department of Plant Physiology and Plant Biochemistry. Virological studies were restricted to the *Partitiviridae* and *Endornaviridae* families, but in addition to the *Capsicum* genus partitiviruses from the *Beta* genus were also included. In case of the latter viruses cloned sequences and adequate plant material were already available from earlier research of our group, and such questions concerning virus-host interaction could be better targeted. Partiti- and endornaviruses are known for life-long association with their hosts without inducing symptoms.

1. Analysis of endophytc bacteria

1.1. Isolation, typing and identification of putative endophytic bacteria of sweet pepper

Putative bacterial endophytes were isolated from the planting seeds, roots, stems, leaves and fruits of the ‘Hó’ and ‘Kárpia’ cultivars of sweet pepper *Capsicum annuum L.* var. *grossum* grown in hydroponics or soil. Altogether 288 bacterial colonies were isolated, which belonged to 234 phenotypic groups and formed 200 different genotypic (RAPD-PCR) clusters.

Molecular identification of the representative isolates selected from the RAPD clusters (altogether 200 strains) was done by sequencing the PCR products of the 16S rRNA gene, while for strains belonging to the *Pseudomonas* genus the *rpoB* gene was also amplified and sequenced.

Identification of the 200 endophytic strains at species or genus level indicated high level of biodiversity and abundance of the genera of the *Pseudomonas, Bacillus, Pantoa, Rhizobium, Enterobacter* and *Microbacterium*, which represented ca. 60% of the isolates.

Distribution of the endophytic strains according to the combinations of cultivars ('Hó' and 'Kárpia') and growth conditions (hydroponics and soil) showed that *Pseudomonas, Bacillus, Rhizobium* and *Microbacterium* were present in all the four combinations, while *Enterobacter, Delftia, Pantoea* and *Staphylococcus* strains were isolated in relatively high ratio at least in tree cases of the four combinations.

1.2. Phylogenetic diversity of the endophytic bacterial isolates

Phylogenetic trees based on the partial 16S rDNA sequences were constructed presenting the filogenetic relations of the 200 putative endophytic strains. In the course of phylogenetic analyses 16S rDNA sequences of the strains were compared and strains harbouring identical sequences were separated into operational taxonomic units (OTUs).

Phylogenetic analysis of the whole, pepper associated culturable endophytic population indicated an extended diversity, because the 200 investigated strains constituted as high as 100 OTUs in the tree. All but two of the OTUs were assigned to three big phyla, namely Proteobacteria, Firmicutes and Actinobacteria. The two OTUs, which represented distinct phyla belonged to *Bacteroidetes* (Class Flavobacteria) and *Deinococcus – Thermus* (class Deinococci), respectively.

1.3. Repartition of the endophytic bacterial biota in the different plant organs

Comparing the ratio of the strains isolated from the different plant organs it could be concluded that the proportion of bacterial strains derived from the roots and green vegetative organs of seedlings was much higher (46%), than those isolated from that of mature plants (23%). The proportion of strains originated from the fruits was unexpectedly high (29.5%), while only three strains (1.5%) were isolated from the planting seeds. More detailed analysis showed that the types of cultivation systems (hydroponic or soil) did not have any significant influence on the proportion of the endophytic strains comparing the seedlings and mature plants. But almost three-times more strains were derived from fruits harvested from soil-cultivated plants than from those of hydroponic cultivated ones. Comparing the number and ratio of the strains originated from the roots and the green organs of the hydroponic and soil cultivated pepper it could be concluded that on one hand, the roots of the hydroponic plants were a much more rich reservoir of endophytes than that of the soil grown plants and on the other hand almost five-times more endophytic strains were originated from the roots than the green organs in the case of hydroponic plants, while this ratio was only two in case of the soil cultivated plants.

Detailed analysis of the endophytic bacterial populations, phylogenetic trees and repartition of the endophytic isolates in the different plant organs can be found in Füstös et al., 2017b [7].

1.4. Effect of the isolated putative endophytic bacterial strains on seed germination

Endophytic bacteria may either support seed germination via their growth promoting activity or be neutral in this respect. We therefore, used the quantitative seed germination test as a screening method for evaluating the potential endophytic nature of the putative endophytic bacterial isolates.

The results of the seed germination test showed that the involved 100 putative endophytic bacterial strains could be separated into three main groups; one group (19 % of strains) had

germination inhibiting activity in the range of 43-88 % inhibition, while the majority of the strains (65%) belonged to the neutral group. Strains belonging to the third group (16%) had germination stimulating ability. For further work aiming to investigate the internalization capability of selected endophytes via seed bacterization only those strains were taken into consideration, which had a stimulatory effect on pepper seed germination.

1.5. Internalization of seed inoculated endophytic bacteria in pepper plants

In order to investigate the true endophytic nature of the selected two putative endophytic strains (*Chryseobacterium sp.* FPBSKK1 and *Pseudomonas* sp. HPBBIK3), we inoculated sweet pepper seeds with these bacteria separately. Inoculated seeds were germinated and seedlings were grown under aseptic conditions.

Internalization of both putative endophytic strains has been proven by culture based as well as by culture independent techniques. Inoculated bacteria were isolated on selective culture media, and were detected by PCR amplification of DNA extracted directly from the plant tissues. Visualization of the inoculated bacterial cells in the internal pepper tissues was performed using the FISH-CLSM technique. Our results indicated that both of the inoculated putative endophytic bacteria were present in the root and stem tissues of the examined samples, therefore they could be considered real endophytes of sweet pepper.

Detailed analysis of the internalization of the seed inoculated bacteria in sweet pepper can be fund in Füstös et al., 2017c [8].

1.6. Colonization ability of *Escherichia coli* and *Listeria monocytogenes* in the endosphere of sweet pepper

Fruits and vegetables can be transmission vehicles of human opportunistic and obligate pathogenic bacteria, persisting in inner tissues for shorter or longer periods or colonizing the plants as facultative endophytes. We investigated the ability of commensal *E. coli* and pathogenic *L. monocytogenes* strains to internalize sweet pepper seedlings via seed bacterization, as germinating seeds and roots are important infiltration sites for entry of enteric bacteria. By combining cultivation dependent and independent (PCR and FISH-CLSM) techniques we could not demonstrate any stable colonization of the inoculated strains in the inner tissues, not even a transient persistence could be suspected by PCR amplification of DNA being present in dead or VBNC cells. These results suggest that there is a low risk associated with internalized enteric or human pathogenic bacteria via germinating seeds in sweet pepper (Füstös et al., 2017a, [6]).

2. Analysis of endophytic fungi

2.1. Effect of cultivar and agrotechnology on colonization rate by cultivable endophytic fungi (Halász et al., 2016, [15])

Samples from root, leaf, stem and fruit were taken at the same 4 time points as for identification of endophytic bacteria. To assess the effect of open field versus greenhouse we only included the isolates from August and October, since the seedlings for both sites had been reared together under greenhouse conditions. For these two timepoints we started with a total of 1607 samples, i.e. with nearly 200 samples per combination (site, cultivar, sampling time), and the number of outgrowing colonies was 436 and 218 from field and greenhouse samples, respectively. Colonization rates were lower in plants grown in hydroponic culture in greenhouse. Statistical evaluation verified a significant difference between production sites at $\alpha<0.1\%$ and also between the two cultivars under field conditions, but not in the greenhouse. Colonization was consistently higher in cv. ‘Hó’ than in ‘Kárpia’; such differences were not observed in colonization by bacteria.

We also investigated the temporal progression of the overall colonization rate. All samples (2260) were included in this analysis; in April and in May they were collected from roots, stems and leaves, in August and in October fruits were also included. At the seedling stage, i.e. in April and May, we did not observe any difference in fungal colonization rates, but this pattern significantly changed in August and October. In these months we observed a continuous increase in colonization – in remarkable contrast to endophytic bacteria.

To our knowledge fungal colonization of *C. annuum* at different developmental stages (seedling, flowering and fruiting stage) has only been described by Paul and co-workers [9] to date, who observed an even more enhanced colonization rate in all organs at the fruiting stage than we did.

Of all investigated organs roots seem to be the most highly colonized at all sampling times. At the fruiting stage old leaves and pedicles represent the most strongly colonized organs. High colonization of pedicles is possibly interconnected to its porous tissue structure that may accommodate hyphal growth better. Colonization rates of young and old pericarp tissue were relatively low, although a strong enhancement was observed in pericarp of fruits nearing biological ripeness in October. Fungal infection of pepper pods, especially that of pericarps has been reported in *Capsicum* and the cancerogenic aflatoxins produced by *Aspergillus* species are considered a major health risk in chili powder [10–11]. In Hungary *Alternaria* sp. was most frequently isolated from mouldy pepper pods [12].

2.2. Identification of endophytic fungi

Isolated fungi were first divided in morphotypes on the basis of colony morphology. DNA was isolated from selected strains and ITS sequences were amplified by using ITS1 and ITS4 primers. Putative endophytes were identified on the basis of their ITS sequences. Identification was mainly possible at the genus level only. Our isolates arise from at least 19 genera: *Alternaria, Cladosporium, Penicillum, Acremonium, Chaetomium, Fusarium, Lewia, Arthirinium, Aspergillus, Cercospora, Colletotrichum, Galactomyces, Myrothecium, Paecilomyces, Plectosphaerella, Pyrenochaeta, Rhizopycnis, Verticillium and Xylaria*. By far the most frequently occurring genus was *Alternaria*, at least 10 *Alternaria* strains were found, partly in morphologically different groups [13].

Fungi often harbor dsRNA viruses which may alter their morphology as well as their virulence. We selected 28 strains to extract total RNA and detect high molecular weight dsRNA on immunoblots by using dsRNA-specific monoclonal antibody. The presence of HMW dsRNA can be taken as indication for the presence of fungal viruses. DsRNA was detected in 6 isolates belonging to *Alternaria, Xylaria, Acremonium* and *Sarocladium* genera [14].

Until now only one publication [9] described endophytic fungi in bell pepper, grown in Korea. 10 of the genera are identical to those identified in our experiments, the others (11) were different.

3. Partiti- and endornaviruses in the *Capsicum* genus

In Hungary only *C. annum* is cultivated, but in other regions of the world other members of the genus, *C. chinense* and *C. frutestens* are also being produced and several further *Capsicum* species are used by breeders. *Capsicum* species are known to harbor dsRNA viruses belonging to the *Partitiviridae* or *Endornaviridae* family. Plant viruses from these families have no known vector and are transferred probably only by pollen and seed. Similar to endophytic fungi, these viruses are usually not causing any symptoms and stay associated with their hosts life-long. Although it was suggested by several authors that these viruses may have an effect on host's physiology, definite proofs are still lacking.

3.1. Identification of new dsRNA viruses in *C. chinense*

In earlier experiments we systematically analyzed the occurrence of HMW dsRNA in a large panel of Capsicum species and strains. The dsRNA pattern observed led us to the hypothesis that in *C. chinense* probably more than one partitiviruses occur which may to be different from those described in *C. annuum*. We also detected large dsRNA molecules indicating the possible presence of one or more endornavirus. Interestingly, in *C. baccatum* we never found any partitivirus, while putative endornavirus sequences were always detected. Since these conclusions were drawn on the basis of the

pattern and length of dsRNA species, we decided to provide sequence-based evidence as well and started to clone putative partitiviral sequences from *C. chinense*. As starting material a plant containing only one pair of dsRNA at 1.4 and 1.5 bp and a large dsRNA with a size characteristic for endornaviruses was chosen.

Cloning and sequencing most of the RdRp (RNA-dependent RNA-polymerase) segment has shown that in *C. chinense* a new *Deltapartitivirus* is present, which significantly differs from PCV1 and PCV2 partitiviruses described earlier in *C. annuum*. We made several attempts to clone the coat protein (CP) segment of the virus as well but did not succeed, because always RdRp-related sequences were delivered after cDNA synthesis. The key step of the synthesis is efficient denaturing of genomic dsRNA segments. We believe that since usually the RdRp encoding segment is much easier to denature, slight contaminations from this segment repressed synthesis of CP-specific cDNA. We observed this phenomenon even after separate elution of genomic segments from PAA gels, because of the close proximity of the corresponding bands in the gel [16].

Having a collection of different plants from *C. annuum*, *C. chinense*, *C. frutestens* and *C. chacoense* we investigated by RT-PCR whether the new virus may be present in other species. First we established that the expected PCR-products were synthesized from all *C. chinense* samples containing the 1.4-1.5 kbp pair of dsRNA. DsRNA bands of this size were also observed in some *C. frutestens* plants which also gave a positive reaction in RT-PCR with our primers. Similar results were obtained in *C. chacoense* (3 lines investigated), but no RT-PCR products were seen in *C. annuum* samples. To verify the data PCR products were sequenced. The sequence of the 720 bp long PCR product was completely identical in all investigated samples, except for *C. frutestens* where 2 nucleotide exchanges were found. Our results show that the same deltapartitivirus may be present in all three related species. It is tempting to speculate that the new virus that we tentatively call *Pepper cryptic virus 3* (PCV3) might have arouse before the separation of these 3 *Capsicum* species during evolution.

We also cloned part of the ≥14 kbp dsRNA. Sequence information allowed unequivocal identification of the genomic dsRNA of a putative endornavirus belonging to the *Betaendornavirus* genus. Its nucleotide sequence identity to Hot pepper endornavirus (NC 027920.1) and to Bell pepper endornavirus (NC 015781.2) is well below 75%, therefore it may be considered as a new virus.

3.2. Transmission of partiti- and endornaviruses through interspecific crosses

Survival of partiti- and endornaviruses completely depends on their continuous and well-regulated interaction with their host. Since they don't have movement proteins, their distribution proceeds only by seed and pollen. Although a striking similarity between some plant and fungal partitiviruses can be observed and horizontal transmission between the two kingdoms is presumed to have a role in their evolution, partitiviruses are usually restricted to their host species and even cultivar specific variations can be observed [17]. Since we observed species-dependent differences in the viral dsRNA-pattern in the *Capsicum* genus, we wanted to find out whether this pattern can be changed by crossing different pepper species, i.e. can the hybrids support viruses any of the parental viruses.

Unfortunately, literature data and breeder's experiences are rather contradictory with respect of the possible outcome of interspecific crosses in *Capsicum*. We tried several parent combinations and generated many seeds, but germination rates were extremely low, and in several crosses none of the seeds germinated. The most successful crosses were those between *C. annuum* 'Kalorez' (containing PCV1) and *C. baccatum* (containing only an endornavirus). These and all other hybrid plants were analyzed, but we were only able to detect the virus coming from the female partner in practically all combinations [18]. Because of the low germination rates we did not continue the project.

4. Approaches to understand peculiarities of partitivirus – host interaction

In order to investigate partitivirus-host interactions we changed our experimental system and carried out our studies using plant hosts from the *Beta* genus and their BCV1 and BCV2 viruses which were cloned

and characterized in our earlier experiments. Aided with these molecular tools and a well characterized plant material we could target questions concerning virus-host interactions directly.

4.1. Production of transgenic tobacco plants to express the coat protein of Beet cryptic virus 1

Partitiviruses coexist lifelong with their hosts and show remarkably little variation in their sequence. The question arises whether this constant association is due simply to an excellent parasitic strategy of the virus or to its mutualistic behavior. Until now only one chance finding delivered direct experimental evidence for the latter. Nakatsukasa-Akune et al. [19] showed than transgenic expression of the coat protein of White clover cryptic virus 1 (WCCV1) inhibited nodulation and conferred a certain degree of protection against infection. WCCV1 is an Alphapartitivirus like Beet cryptic virus1 cloned and characterized in our earlier experiments [17]. Therefore, we planned to express BCV1 CP in tobacco to clarify whether protection can also be detected in other, non-legume systems. First a full-length clone of the CP sequence was constructed and built together with GFP as fluorescent indicator. For expression in tobacco the oestrogene-inducible XVE system, developed by Zuo et al. [20] was used [21]. Unfortunately, only two transgenic plants could be raised to full-sized plants. In these plants integration of the CP and GFP sequence was shown, but detectable protein expression was not achieved.

4.2. Host-specific changes in partitivirus genome – a possible way for evolution?

Beet cryptic virus 1 and -2 occur in several species/subspecies of the *Beta* genus such as *B. maritima,* sugar beet, chard and beetroot. In earlier experiments we determined the sequence of BCV1 and BCV2 from sugar beet, but sequences from other hosts were not investigated. Since these hosts presumably differ more from each other than sugar beet cultivars, we decided to determine the number and site of mutations in comparison to our reference sequence.

The highest number of mutations (89) was found in BCV1 RdRp sequence in chard, in the CP sequence less, only 24 nucleotide exchanges were identified. Most of these mutations, however, did not result in amino acid changes. At amino acid level only one mutation in CP and 6 in RdRp were observed. The same was true for BCV2 in beetroot, here 4-7 point mutations and only 2-3 amino acid exchanges were found. Moreover, even these few exchanges were mainly conservative, indicating that probably a strong selection pressure is acting *in vivo* to preserve the exact protein sequence [22].

Lately, rather surprisingly, a partitivirus sequence from *Rheum palmatum* was deposited in GeneBank. This sequence is practically identical to our BCV2 RdRp and CP1 sequence, the presence of a second coat protein was not indicated. By sequence comparison we found 5 point mutations were found in the nucleotide sequence of RdRp, 3 of them resulted in amino acid exchanges as well. The amino acid sequence of CP1 was completely identical with our CP1 sequence. Chines rhubarb, *R. palmatum* (*Amaranthaceae*) and sugar beet, *B. vulgaris subsp. vulgaris var. altissima* (*Polygonaceae*) are only very distantly related. They both belong to ordo *Charyophyllales*, but to very distant families. Therefore, it seems highly improbable that BCV2 was already present in their ancestors before separation of these families occurred. Although the authors did not comment on this interesting finding and as yet no publications are available, the result is exciting and - when verified – would strongly favor partitivirus transmission by a vector. We investigated two *R. palmatum* plants and could not detect any BCV2 related sequences by RT-PCR using a set of different BCV2-specific primers [23]. From these results we conclude that the presence of BCV2 is not a general feature of *R. palmatum* plants. We are waiting for further data supporting the presence of the complete BCV2 sequence in *Rheum.*

4.3. Acquisition of a second coat protein – a possible way to evolution?

As a rule, partitiviruses consist of two genomic segments, encoding the RdRp and the CP, respectively. Although in some cases the presence of an additional third segment which possibly encodes a second CP was reported, such results are treated presently with extreme mistrust by the Taxonomy Commission.

When cloning BCV2 in earlier experiments, we also identified 3 genomic segments, although the virus was described to be bipartite in the literature. We could show that the presence of the

third segment was probably overlooked because two segments comigrate in PAA-TBE or agarose gels. The gene products of the individual CP-encoding segments could be identified in purified virus preparations by mass spectrometry. Unfortunately, there is a long-standing contradiction in the literature with respect to the size of the BCV2 CP. Some authors detected coat proteins in virus preparations with a size more or less corresponding to the coding capacity, while others – like us - found significantly lower molecular masses than expected. This apparent contradiction could be resolved by varying conditions during virus isolation, including protease inhibitors in the solutions and mass spec (MALDI-TOF and MS-MS) analysis of all protein fragments present in virus preparation. Our results indicate that during virus purification proteolytic cleavage of CP-s at more than one preferential site may occur and some cleavage products stay associated with the virions.

To support the tripartite nature of BCV2 we determined the copy number of genomic segments by qPCR in different cultivars. 8 sugar beet cultivars (Pirat 4, Apollo, Sporta, Kawerta, Kazansky, Teri, Kawegiga and Kawemono) and a beetroot cultivar (Bíborgömb) were tested in repeated independent experiments. We found that all 3 genomic segments were present in all samples. Although the ratio of the two CP-encoding segments was variable in different cultivars, their amount was comparable. The largest, about 5-fold difference between the copy number of these segments was found in 'Apollo'. Because of the consistent presence of both CP-encoding segments, their similar concentration in the samples and the common 5'- and 3'-terminal sequences we believe that BCV2 is a tripartite virus having two structurally similar, but clearly distinct coat proteins. The copy number of BCV2 segments varied in different cultivars between 10^6-10^8 copies / 50 ng total RNA. Interestingly, while in case of the bipartite BCV1 the copy number of CP-encoding genomic segment (dsRNA2) was usually larger than that of the RdRp segment (dsRNA1), in BCV2 carrying plants the amount of dsRNA1 segment was larger than or equal to the sum of CP-encoding copies in most cases.

Although both CP1 and CP2 proteins can be detected in purified virions, it is still not clear whether and at what ratio can they occur in individual particles. On the basis of the similarity of the predicted structure of CP1 and CP2 and the coexistence of both proteins in all hosts we expect that they possibly build up the shell of the particles jointly.

Taken together, our studies proved the tripartite nature of BCV2 and we believe that more tripartite partitiviruses will be found in future. Considering the strong counterselection against mutations, acquisition of a third genome segment could open up new possibilities for partitivirus evolution.

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