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Analysis of plant-microbe interactions on the Styrian oil pumpkin as basis for an advanced breeding strategy

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Kurzfassung

Mikrobielle Gemeinschaften spielen während des gesamten Lebenszyklus einer Pflanze eine zentrale Rolle. Hinsichtlich des Einflusses der Züchtung auf das Mikrobiom und die Wirkung biologischer Kontrollstämme (BCAs) ist jedoch wenig bekannt. Dem steirischen Ölkürbis kommt in Österreich eine große kulturelle Bedeutung zu. Diese Kulturart ist jedoch geprägt durch ihre starke Krankheitsanfälligkeit und die instabilen Reaktionen auf BCAs im Feld. Aufgrund der potentiellen Wirkungen des assoziierten Mikrobioms auf die Pathogenanfälligkeit und die Biokontrolle, wurde im Rahmen dieser Masterarbeit eine umfassende Mikrobiomanalyse durchgeführt. Die Ergebnisse geben erste Einblicke, die für künftige Züchtungs- und Biokontrollstrategien genutzt werden können. Die Mikrobiomanalyse von 14 Ölkürbis-Sorten zeigte einen starken Einfluss des *Cucurbita pepo*-Genotyps auf die Zusammensetzung des Samenmikrobioms, nicht jedoch auf das Rhizosphärenmikrobiom. Die Pflanzenhabitate zeichneten sich durch eine hohe, aber sehr gegensätzliche Diversität und eine stark unterschiedliche taxonomische Zusammensetzung aus. Die Diversität des Samenmikrobioms war geringer und wurde durch einen hohen Anteil an Enterobacteriaceae definiert, die potentielle Phytopathogene wie *Erwinia* und *Pectobacterium* umfassen. Aber auch nutzbringende Bakterien wie *Lysobacter*, *Paenibacillus* und *Lactococcus* trugen zu den mikrobiellen Gemeinschaften bei. Basierend auf diesen vielversprechenden Ergebnissen soll eine Analyse des Mycobioms verschiedener *C. pepo*-Genotypen durchgeführt werden. Dazu wurde eine Peptidnucleinsäuresonde, ein spezifischer PCR-Blocker zur Unterdrückung der Amplifikation von *C. pepo*-Sequenzen, konstruiert und validiert. Das gewonnene Wissen über die Zusammensetzung des *C. pepo*-assoziierten Mikrobioms führte zur Annahme, dass die Genotypspezifität auch für die Behandlung mit BCAs gilt. Daher wurden die Wechselwirkungen von drei Genotypen mit verschiedenen *Serratia plymuthica*-Stämmen mikroskopisch und unter gnotobiotischen Bedingungen im Gewächshaus analysiert. Diese Experimente zeigten, dass Genotypen spezifisch die Besiedelung mit *S. plymuthica*-Stämmen auf Pflanzenoberflächen beeinflussen. Diese Effekte könnten sogar mit der genetischen Variation und dem Inzuchtgrad zusammenhängen. Das Wissen über genotypspezifische Pflanzen-Mikroben Interaktionen eröffnet neue Möglichkeiten für moderne Zucht- und Saatgutbehandlungsstrategien die indigenen mikrobiellen Gemeinschaften und BCAs gezielt zu nutzen.

Abstract

The plant-associated microbiome is crucial for plant physiology, health and fitness, but less is known concerning the relationship of breeding activities with microbiome composition and biological control strategies by means of biological control agents (BCAs). The Styrian oil pumpkin is of cultural importance in Austria, but shows a severe susceptibility to various microbial pathogens and unstable responses to BCAs in the field. As the plant-associated microbiome has potential effects on pathogen susceptibility and biocontrol treatments, a wide-ranging microbiome analysis was performed in this Master´s thesis. The results give insights that might be used for future breeding and biocontrol strategies. The analysis of 14 oil pumpkin cultivars´ plant-associated bacterial microbiomes revealed a strong impact of the *Cucurbita pepo* genotype on the composition of the seed microbiome, but not on the rhizosphere microbiome. The habitats were characterised by a high, but very contrasting diversity and a substantially different taxonomic composition. The diversity of seed microbiomes was lower and the community defined by a high abundance of Enterobacteriaceae family members, which comprise potential phytopathogens like *Erwinia* and *Pectobacterium*. But also potential plant-beneficial bacteria like *Lysobacter*, *Paenibacillus* and *Lactococcus* contributed to the communities. Based on these encouraging results an analysis of the mycobiome of different *C. pepo* genotypes is intended to be performed. For this a peptide nucleic acid probe, a specific PCR blocker for suppression of amplification of *C. pepo* sequences, was designed and successfully validated. The gained knowledge on the *C. pepo*-associated microbiome constitution raised the assumption that plant genotype-specificity also applies for the treatment with BCAs. Therefore, the interactions of three *C. pepo* genotypes with different *Serratia plymuthica* strains, which are acting as beneficial or detrimental plant associates, were analysed microscopically and under gnotobiotic conditions in the greenhouse. These experiments revealed that genotypes specifically influence colonisation with *S. plymuthica* strains on plant surfaces that could even be related to the genetic variation and the degree of inbreeding. The knowledge of genotype-specificity opens up new horizons in next-generation breeding strategies and in seed treatment approaches that are more capable of exploiting beneficial indigenous microbial communities and that take into account genotype-specific interactions with BCAs.

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1 Introduction

By means of this Master´s thesis, plant-microbe interactions on the Styrian oil pumpkin are analysed and discussed in order to establish a basis for integrating this knowledge in an advanced breeding strategy. In the following introductory subchapters the Styrian oil pumpkin as an agronomically important crop in Styria (Austria) and recent findings of cultivar-specific plant-microbe interactions are described.

1.1 The Styrian oil pumpkin

The Styrian oil pumpkin *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb., sometimes referred to as var. *oleifera*, belongs to the family of Cucurbitaceae and has tradition in cultivation and use in Austria and neighbouring south-eastern countries since three centuries with international importance today. Due to health-promoting constituents of the seeds, the crop is of commercial and medical relevance. The plant is monoecious, cross-pollinating and produces seed-carrying berries (Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft 2015, Bürstmayr et al. 2014, Grube et al. 2011, Teppner et al. 2000). Young plants and ripe fruits are illustrated in Figure 1.

Figure 1: The Styrian oil pumpkin: Young plants (A) and ripe fruits (B).

The morphological structure of *C. pepo* seeds comprises an embryo which is made up of a radicle, hypocotyl, plumule as well as two distinct photosynthetic cotyledons. Further components are a thin endosperm, remains of the nucellus and a testa (seed coat) consisting of five layers (Figure 2). A specific characteristic of the Styrian oil pumpkin is the missing

lignification of the seed coat. The loss of the lignification of the thick and leathery testa is the result of a natural mutation, which is believed to have occurred about 140 years ago in western Styria. The protochlorophyll content of the chlorenchyma, the fifth testa layer, is responsible for the characteristic olive-green seed colour (Figure 3) (Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft 2015, Bürstmayr et al. 2014, Zraidi 2005, Teppner 2000, Tschermak-Seysenegg 1934, Saatzucht Gleisdorf GmbH).

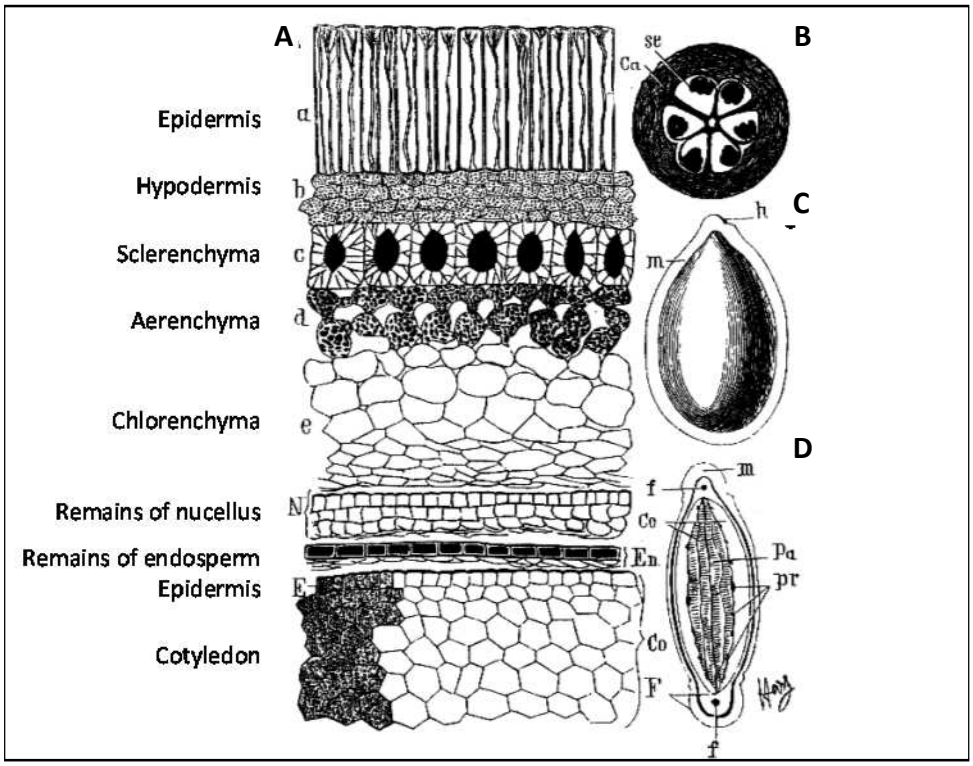

Figure 2: Transverse section of *C. pepo* seed coat (A), cross-section of ripe fruit (B), hulled seed (C) and cross-section of hulled seed (D). Ca: pericarp, se: seeds, h: hilum, m: margin callosity, F: seed coat, f: vascular bundle, Co: cotyledon, Pa: palisade layer, pr: procambium strands (Teppner 2000, drawing from Harz 1885).

Figure 3: Seeds of different *C. pepo* genotypes with (A) and without lignification (B) of the seed coat.

The Styrian oil pumpkins´ seeds are characterised by their high oil content (Bürstmayr et al. 2014). The seed oil has a characteristic dark-green colour and its aroma and nutty taste are primarily influenced by the cultivar and the particular processing methods. The specific soil and climatic conditions of cultivation areas can also affect the quality of the oil, e.g. fatty acid composition and vitamin E content (Murkovic et al. 1996). The Styrian pumpkin seed oil is produced according to the specification as Protected Geographical Indication (PGI) pursuant to EC Regulation 1263/96 ECJ L 163/19 ff/96 (Bundesministerium für Land- und Forstwirtschaft, Umwelt und Wasserwirtschaft 2015). In 2016, the acreage of the Styrian oil pumpkin reached a peak level with 39,450 ha in Austria, and the market demand for this high-value crop continues to rise.

Because of the lack of lignification of the seed coat the seeds are highly susceptible to abiotic and biotic stress factors during germination (Heinisch and Ruthenberg 1950). Thus, commercially available Styrian oil pumpkin seeds are treated with chemical strippers, mainly with synthetic fungicides or copper-based products. Sowing of untreated seeds generally results in a drastic reduction of germination rates or germination fails totally, if weather or soil conditions are unfavourable after sowing. A disease responsible for high yield losses is the fruit rot caused by the consortium of *Didymella bryoniae* and *Pectobacterium carotovorum* (former *Erwinia carotovora*) (Grube et al. 2011) or *Erwinia atrosepticum*. Leaves of adult plants can be infested by fungi such as *Didymella bryoniae* or *Phyllosticta cucurbitacearum* (Bedlan 2012) and by bacterial pathogens like *Xanthomonas campestris* pv. *cucurbitae*, *Pseudomonas syringae* or *P. viridiflava* resulting in water-soaked (Grube et al. 2011) and necrotic lesions of foliage (Huss 2009-2012). Further important pathogens affecting adult plants of the Styrian oil pumpkin are viruses like Zucchini Yellow Mosaic Virus (ZYMV), Watermelon Mosaic Virus (WMV) and Cucumber Mosaic Virus (CMV) (Pachner 2016, Winkler 2000, Saatzucht Gleisdorf GmbH). A severe virus infestation can cause substantial yield losses (Huss 2009-2012).

A strong cultivar-specificity was shown in evaluations of registered oil pumpkin cultivars regarding the susceptibility to fruit rot (AGES 2016, Winkler et al. 2008). A high genotype-specificity has been widely shown for the interactions of plants with pathogens (Neupane et al. 2015, Bruns et al. 2012, Rubiales and Niks 1996) and thus breeding for resistances against pathogens is common practice (Ashkani et al. 2015, Pachner et al. 2015, Niks et al. 2011,

Roane 1973). Conversely, genotype-specific beneficial plant-microbe associations have not been considered in breeding strategies thus far.

Current breeding programs aim to develop new cultivars with improved quality traits (e.g. oil content), resistances against pathogens (e.g. ZYMV resistance genes) and agronomic characteristics (e.g. enhanced yield) besides adaptation to present climatic conditions and the current agronomic practices (Bürstmayr et al. 2014, Saatzucht Gleisdorf GmbH).

1.2 Cultivar-specific plant-microbe interactions

The plant-associated microbiome is crucial for plant physiology, health and fitness (Vandenkoornhuyse et al. 2015, Berendsen et al. 2012) and recent studies support a close symbiotic relationship between plants and their associated microorganisms, as reviewed by Berg et al. (2016).

Plants are recognised as meta-organisms, which harbour countless microbial inhabitants. The conditions in the rhizosphere, defined as the soil zone closely surrounding the root, are influenced by root exudates, which are of central importance in plant-microbe interactions (Hiltner 1904). The major reservoir for the establishment of functional interactions is the plant surrounding soil. Various studies characterised the diversity and the related function of the rhizosphere microbiome and the impact of the plant genotype and the surrounding bulk soil. Besides the rhizosphere, there are other densely colonised compartments of the plant, like the phyllosphere, endosphere and spermosphere of seeds, which are colonised by particular microbial communities (Fonseca-García et al. 2016, Erlacher et al. 2014, Berg et al. 2013, Lopez-Velasco et al. 2013, Bakker et al. 2012, Lundberg et al. 2012, Berg and Smalla 2009, Smalla et al. 2001, Hiltner 1904). The spermosphere is known as a microbial dynamic zone of soil which is surrounding a germinating seed (Nelson 2004).

The hologenome theory postulates that the holobiont comprising the plant host and its associated microbiome co-evolve as one heritable unit of selection (Zilber-Rosenberg and Rosenberg 2008). Gopal and Gupta (2016) reviewed that the microbiome regulates the holobiome fitness and Terrazas et al. (2016) stated that from an ecological point of view the holobiont and not the plant itself responds to biotic and abiotic stresses. Microorganisms

work in interlocked networks (van der Heijden and Hartmann 2016) and keystone species essentially influence plant-microbiome interactions and stress responses (Agler et al. 2016, Wei et al. 2015).

Plant-associated microorganisms, which are referred to as plant microbiome, can help plants to fend off diseases, promote stress resistance, stimulate growth and occupy space that would otherwise be taken up by pathogens. They influence crop yield and quality as well (Berg et al. 2013) and contribute to disease suppressiveness of soils. Specific microbes may influence the plant health, but their effectiveness is strongly influenced by the whole community (Berendsen et al. 2012). Microbial richness and evenness of the microbiome members (relative abundance) in the rhizosphere are of particular importance for the maintenance of plant health, as reviewed by Bakker et al. (2012).

Berendsen et al. (2012) reported that different plant genotypes are able to establish specific microbial communities in the rhizosphere and that genotypes can selectively affect the associated microbes in order to help to fend off diseases, i.e. by the recruitment of specific beneficial microorganisms. In regard to this, a promising new strategy of breeding activities might be the development of cultivars which are able to selectively influence or exploit functions of the soil microbiome. The composition of root exudates is different among plant species and cultivars as well. The targeted manipulation of exudates might be an interesting agricultural approach in order to enhance yield, disease suppressiveness or other traits (Bakker et al. 2012). Germida and Siciliana (2001) reported that the microbial diversity, which is associated with old wheat cultivars, differs from the diversity related to modern cultivars. The old wheat cultivars were associated with a high diversity of rhizobacteria, whereas the modern cultivars were dominated by fast-growing Proteobacteria. The plant species and the soil type have a great impact on structure and function of rhizosphere microbial communities. Even the phylogenetic position of a plant species influences bacterial community composition (Berg and Smalla 2009). Gopal and Gupta (2016) summarised that the plant-associated microbiome offering genetic variability to plants is a tool which can be selected in congruence with the plant genome as holobiont system to produce next-generation crops by new breeding strategies.

Recent studies showed that breeding activities influence the seed- and root-associated bacterial communities (Perez-Jaramillo et al. 2016, Cardinale et al. 2015, Bouffaud et al.

2014, Aleklett and Hart 2013, Peiffer and Ley 2013). Especially the influence of the whole seed microbiome on plant health has gained more interest (Barret et al. 2015, Aleklett and Hart 2013). Seeds are of particular concern as microbial carriers because they are involved in the transmission of both beneficial and pathogenic microorganisms from one generation to another (Johnston-Monje et al. 2016).

Plants which are able to selectively use and recruit beneficial functions of the microbiome and enhancing this function could increase agricultural sustainability (Bakker et al. 2012). Breeding plants for beneficial plant-microbe interactions is an emerging field mainly focusing on below ground interactions in the rhizosphere (Bakker et al. 2012, reviewed in Wissuwa et al. 2009), but the influence of the seed microbiome is still unknown.

1.3 Biological control

As reviewed by Berg (2009) the use of microorganisms as biological control agents (BCAs) by exploiting the beneficial plant-microbe interactions is an encouraging and sustainable approach for supporting both organic and conventional agriculture concepts. Biocontrol is defined as the suppression or control of pathogens by means of bioactive microorganisms. This can be achieved by various mechanisms comprising a changing constitution of microbial communities, competition for a certain ecological niche or for nutrients, active lysis or antibiosis. But the severeness of diseases can also be reduced by promotion of germination capacity, plant development and growth and also by enhancing the stress tolerance, e.g. by supporting nutrient acquisition and hormonal stimulation. A lot of bacterial genera are known for plant growth promotion, like *Bacillus*, *Pseudomonas* and *Serratia*, but also fungi like *Trichoderma*. Based on these organisms biofertilisers, phytostimulators and biopesticides are developed and used in agriculture (Berg 2009). HRO-C48 for examples is the basic component of the commercially available biocontrol product RhizoStar® and it is known for its growth promotion effect besides its antagonistic activity (Berg et al. 1999, Kalbe et al. 1996).

Since the genus of *Serratia*, which belongs to the Enterobacteriaceae family, includes potential biocontrol agents for the cultivation of the Styrian oil pumpkin, different

Serratia plymuthica strains were used for targeted greenhouse experiments in this Master´s thesis. *S. plymuthica* 3Rp8 and S13 have positive effects in *C. pepo* cultivation. The 3Rp8 strain is known for its plant growth promotion (Adam 2015) and S13 for its germination rate enhancement (Fürnkranz et al. 2012). For *S. plymuthica* 3Re4-18 and HRO-C48 no explicit positive effect in *C. pepo* cultivation is known. They were chosen because of their positive effects in biocontrol of *Rhizoctonia solani* in lettuce cultivation in the case of *S. plymuthica* 3Re4-18 (Grosch et al. 2012, Scherwinski et al. 2008) and because of the enhancement of plant growth and disease suppression (*Verticillium* wilt and *Phytophthora* root rot) in cultivation of strawberries (Kurze et al. 2001) and oilseed rape (*Rhizoctonia solani*) (Kai et al. 2007) in the case of *S. plymuthica* HRO-C48. *S. plymuthica* 4Rx13 is known for its antagonistic activity (Berg et al. 2002) and its production of high amounts of sodorifen, a newly discovered and unusual volatile organic compound (Weise et al. 2014). An application on *C. pepo* seeds inhibits germination strongly (Adam 2015). This effect was already published for *Arabidopsis thaliana* (Weise et al. 2013, Verspermann et al. 2007).

1.4 Aims of the Master´s thesis

The high importance of oil pumpkin cultivation in Styria under challenging climatic conditions, the high susceptibility of this crop to various microbial pathogens and the differing and unstable responses to applied biological control agents led to the high interest in the constitution of the crop-associated microbiome, especially regarding cultivar-specific patterns. This knowledge could be (re-)integrated into sustainable next-generation breeding strategies exploiting native plant-associated microbes as well as applied biological control agents with targeted effects on plant development and performance.

The main objective of this thesis was a wide-ranging seed and rhizosphere microbiome analysis of 14 *Cucurbita pepo* genotypes based on a 16S rRNA gene sequencing approach targeting the bacterial diversity. Those cultigens comprised an open-pollinated cultivar, three hybrids and their pedigree components (four inbred lines) and five segregating lines of the Styrian oil pumpkin as well as one zucchini hybrid. These microbiome analyses are a first step towards gaining a deeper understanding of genotype-dependent differences in plant-microbe interactions.

The results of the plant-associated microbiome analysis of different genotypes raised the assumption that genotype-specificity of plants on cultivar level with regard to interactions with the microbiome also applies for the treatment of crops with biological control agents. Therefore, the interactions between young *C. pepo* plants of different cultivars with different beneficial as well as detrimental *S. plymuthica* strains were investigated by means of greenhouse experiments and confocal laser scanning microscopy in order to determine if the *C. pepo* cultigen affects the effect of biological control agents.

Based on the encouraging results of the bacterial microbiome, which is associated with *C. pepo* genotypes, an analysis of the mycobiome is intended to be performed. Part of this Master´s thesis was to design a peptide nucleic acid probe, which is a specific PCR blocker, for suppression of amplification of *C. pepo* sequences, which are similar to the ITS-1 amplicon target site in order to allow a detailed analysis of the fungal plant-associated community.

2 Materials and Methods

2.1 Characterisation of *Cucurbita pepo* genotypes

Upon selection of the *C. pepo* genotypes for analysis, the focus was toward coverage of cultivars with a high market share (three-way cross hybrids GL Opal and GL Rustikal) including their pedigree components (inbred Line A, B, C and D as well as the single cross hybrid Gleisdorfer Diamant (Gl. Diamant)). An open-pollinated cultivar frequently used in organic agricultural systems, GL Classic, and six other cultigens bred in countries other than Austria were included to broaden the spectrum of genotypes. Characteristics of the selected cultivars are summarised in Table 1. The geographic origin records the country in which a genotype was selected or bred. With the exception of the single cross zucchini hybrid Naxos, the seeds used for amplicon sequencing approaches were harvested from plants grown on three different field sites near Gleisdorf (province of Styria, Austria). Post-harvest processing of those seeds was performed according to standard procedures of the Saatzucht Gleisdorf GmbH breeding station. Figure 4 illustrates seeds of the different genotypes. The special attribute of this thesis was the access to protected breeding material.

A hybrid variety is established by crossing two parental inbred lines, which are derived from different populations or genepools. A breeding line is considered as inbred line after six to seven selfing generations, which cause inbreeding depression effects. A cross between two unrelated homozygous lines leads to a heterosis effect, which is defined as the increased efficiency of the progeny in yield or other agronomic traits compared to the parental component´s mean. Single cross hybrids are the result of crossing two inbred lines. Three-way cross hybrids are the product of a single cross hybrid and an inbred line, which possibly increases the yield even further. If a cultivar is characterised by heterozygote genes and is normally multiplicated by open pollination, then it is called a population variety. Segregating lines are not yet homozygous lines (e.g. F3, F4 generations), with still segregating genes and consequently segregating phenotypes as well (Miedaner 2010).

Table 1: Characteristics of *Cucurbita pepo* genotypes selected for microbiome analysis

Genotype name	Field No.	Category	Pedigree	Botanical classification	Geographic origin	Harvest year	Field origin	GPS data of field origin	Vining type	Disease suscept.	ZYMV tolerance	Origin of seeds
Line A	1	Inbred line (nl)	-	*C. pepo* var. *styriaca*	Austria	2014	Pfarrhoffeld	47°06'48.4"N 15°42'06.9"E	Short vines	Leaf-edge necrosis	Tolerant	Mixed fruits
Line B	2	Inbred line (nl)	-	*C. pepo* var. *styriaca*	Austria	2014	Pfarrhoffeld	47°06'48.4"N 15°42'06.9"E	Long vines	Unknown	Tolerant	Mixed fruits
Line C	3	Inbred line (nl)	-	*C. pepo* var. *styriaca*	Austria	2013	Teichacker	47°07'01.8"N 15°42'24.8"E	Short vines	Large leaf necrosis	Tolerant	Single fruit
Line D	4	Inbred line (nl)	-	*C. pepo* var. *styriaca*	Austria	2014	Pfarrhoffeld	47°06'48.4"N 15°42'06.9"E	Short vines	Unknown	Tolerant	Mixed fruits
Gl. Diamant	6	Single cross hybrid (nl)	Line A x Line B	*C. pepo* var. *styriaca*	Austria	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Short vines	Unknown	Tolerant	Mixed fruits
GL Opal	7	Three-way cross hybrid (nl)	Gl. Diamant x Line C	*C. pepo* var. *styriaca*	Austria	2014	Unknown	47°08'04.9"N 15°40'58.4"E	Short vines	Unknown	Tolerant	Mixed fruits
GL Rustikal	8	Three-way cross hybrid (nl)	Gl. Diamant x Line D	*C. pepo* var. *styriaca*	Austria	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Short vines	Unknown	Tolerant	Mixed fruits
GL Classic	9	Open-pollinated cultivar (nl)	-	*C. pepo* var. *styriaca*	Austria	2014	Pfarrhoffeld	47°06'48.4"N 15°42'06.9"E	Long vines	Unknown	Suscept-ible	Mixed fruits
Naxos	10	Single cross hybrid (nl)	Unknown	*C. pepo* var. *pepo*	The Netherlands	Unknown	Unknown	Unknown	Bush	Unknown	Tolerant	Mixed fruits
Line E	11	Segregating line (nl)	-	*C. pepo*	Germany	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Bush	Unknown	Unknown	Single fruit
Line F	12	Segregating line (l)	-	*C. pepo*	Slovenia	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Long vines	Unknown	Unknown	Single fruit
Line G	13	Segregating line (nl)	-	*C. pepo*	Slovenia	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Long vines	Unknown	Unknown	Single fruit
Line H	14	Segregating line (l)	-	*C. pepo*	China	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Short vines	Unknown	Unknown	Single fruit
Line I	15	Segregating line (l)	-	*C. pepo*	China	2014	Teichacker	47°07'01.8"N 15°42'24.8"E	Long vines	Unknown	Unknown	Single fruit

nl no lignification of seed coat, *l* lignification of seed coat

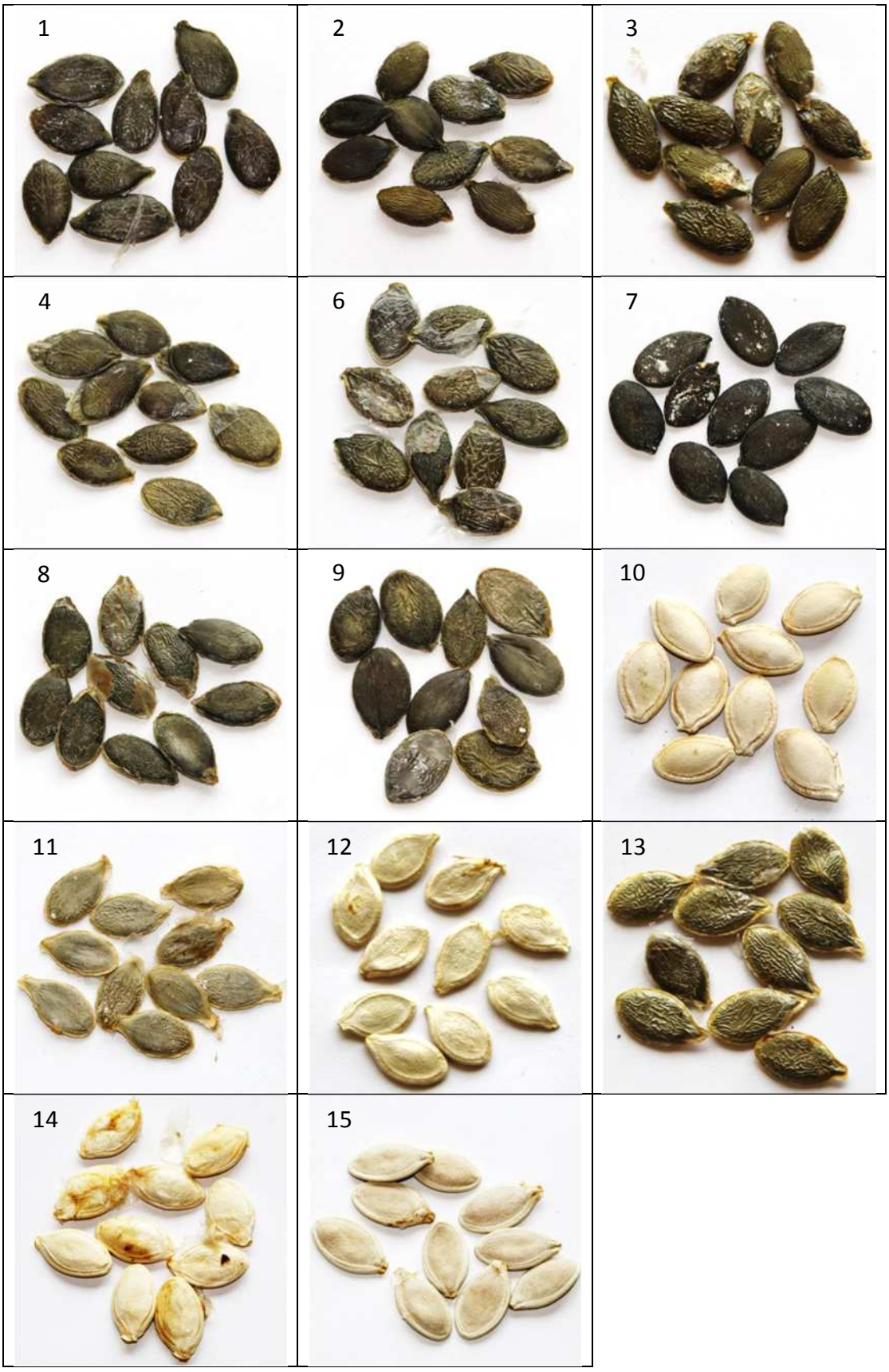

Figure 4: *C. pepo* seeds of four inbred lines (1-4), four hybrids (5-7, 9), one population cultivar (8) and five segregating lines (10-14). The numeration is congruent with the field number listed in Table 1.

2.2 Analysis of plant-microbe interactions

2.2.1 Sampling of seeds, rhizosphere and soil

For the seed microbiome analysis, 40 seeds of each genotype were washed five times for one minute with 50 ml sterile deionised water and soaked in 25 ml sterile deionised water for four hours on a rotary shaker at 100 rpm. Subsequently, the seeds were divided into four replicates of ten seeds each and were ground with a pestle in 10 ml 0.85% NaCl in sterile bags (Nasco Whirl-Pak), as illustrated in Figure 5. For each replicate, a 3 ml suspension was pelleted by 20 min centrifugation at 4°C and 13,500 g in a 2 ml reaction tube. The pellets were stored at -70°C until DNA isolation.

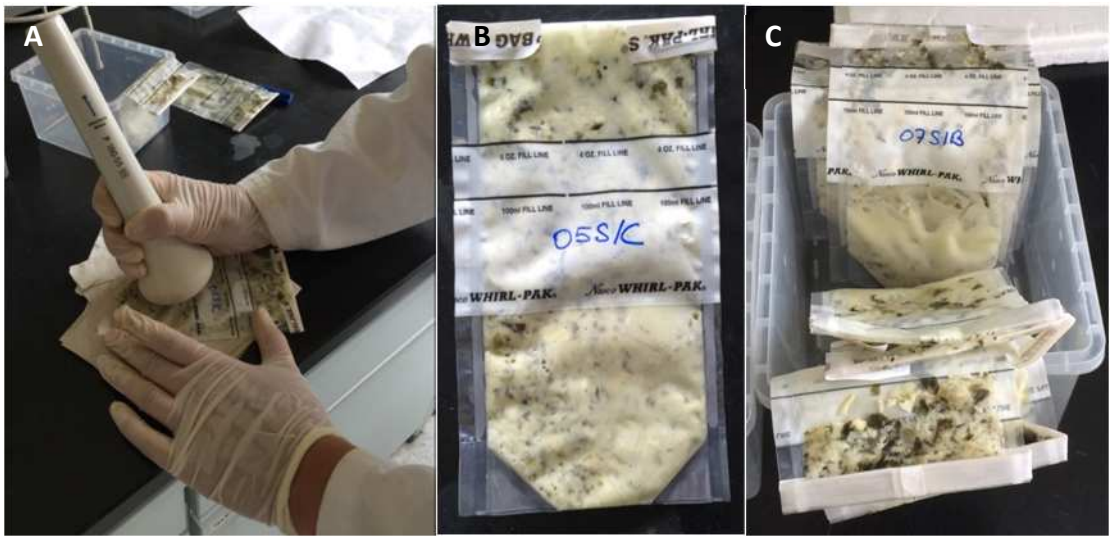

Figure 5: Preparation of *C. pepo* seed samples for DNA isolation: Grinding of seeds with NaCl in sterile bags (A) and homogenised seed suspension (B, C).

For the rhizosphere microbiome analysis, 40 seeds per genotype of the same seed lots used for seed microbiome analysis were coated with 0.3 g of the fungicide Maxim® XL (Syngenta) and split into four replicates. Seeds were sown at a field site at the breeding station of Saatzucht Gleisdorf GmbH (47°06'57.3"N, 15°42'31.3"E) in randomised plots. The trial layout is depicted in Figure 6. The soil of the field site is described as gleyed loose brown soil, loamy silt and cover loams on a quaternary terrace deficient in lime, with a pH-value of 6.5. During the growing season no fertiliser, herbicides, fungicides or pesticides were applied. When required the plant vines were put back into their respective plots and weeding was done.

1	5	9	14
2	11	12	15
3	8	6	16
4	1	7	13
5	2	10	12
6	3	15	11
7	16	14	9
8	4	5	10
9	13	16	7
10	14	3	8
11	15	2	6
12	9	1	5
13	7	11	4
14	6	8	1
15	12	13	2
16	10	4	3
1. Rep	2. Rep	3. Rep	4. Rep

Figure 6: Randomised trial layout of 16 *C. pepo* genotypes. The plot numbers are congruent to the field numbers listed in Table 1. 40 plants of each genotype were grown in four replicates. The distance among plants in rows was 30 cm, between rows 1 m. Sowing depth was 3.5 cm.

In parallel, 40 seeds per genotype were sown without fungicide coating on the same field site. As only 2.7% of those plants emerged, the rhizosphere samples had to be taken from plants grown from fungicide treated seeds (93.1% germination rate). One month after sowing, rhizosphere material from four randomly chosen plants per plot was sampled and pooled under semi-sterile conditions. For sampling, the soil around the root body of the plant was loosened. Then the roots were carefully exposed and dragged out of the soil (Figure 7).

Figure 7: Sampling of rhizosphere material of *C. pepo* plants in the field.

The root morphology of a *C. pepo* plant is exemplarily shown in Figure 8. The plants exhibited a dumose and branched root system with numberless subtle hairy roots.

Figure 8: Morphology of sampled root and rhizosphere material of a *C. pepo* plant. One square of the raster corresponds to 0.25 cm².

Additionally, four bulk soil samples were taken from random places at the field site. The rhizosphere and soil material was cooled until subsequent sample preparation. Thereafter, 5 to 7 g of each rhizosphere and soil replicate were suspended in 50 ml 0.85% NaCl and homogenised by a 3 min bag mixer (stomacher) treatment. Root material close to and distant to the main root was used as well as fine roots. Then 4 ml of the homogenised solution were pelleted as described above (Figure 9). The pellets were stored at -70°C until DNA isolation.

Figure 9: Preparation of the rhizosphere samples of *C. pepo* plants for DNA isolation: Weighing of rhizosphere material (A, B), bag mixing (stomaching) and pelleting of the rhizosphere-suspension (C, D).

2.2.2 DNA extraction

The DNA extraction was performed using a modified protocol of the FastDNA™ SPIN Kit for Soil (MP Biomedicals). For this purpose, an appropriate amount of sodium phosphate buffer (987 µl/sample) was mixed with MT buffer (soil lysis buffer) (122 µl/sample). First, the pelleted sample was resuspended in 600 µl of the buffer mixture, transferred to a Lysing Matrix E tube and filled up with the buffer mixture until the start of the vertical line on the tube. The suspension was homogenised in the FastPrep® instrument for 40 seconds at a speed setting of 6.0 and centrifuged at 12,000 x g for 15 minutes to pellet debris. Subsequently, the supernatant was transferred to a 2 ml reaction tube containing 250 µl PPS (protein precipitation solution) and mixed by inverting the tube ten times. The pellet was precipitated via centrifugation at 12,000 x g for 5 minutes. Afterwards the supernatant was added to 1.0 ml resuspended BM (binding matrix) suspension in a 2 ml reaction tube and mixed briefly. In order to allow binding of the DNA, the reaction tubes were inverted by hand for 2 minutes. To allow settling of the matrix, the tubes were stored for 5 minutes on

the bench. After this step, at least 500 µl of the limpid supernatant were discarded and the binding matrix resuspended in the remaining amount of supernatant. At least 600 µl of the mixture were transferred to a SPIN™ filter and centrifuged at 12,000 x g for 1 minute. Afterwards the catch tube was emptied, 500 µl SEWS-M solution (nucleic acid wash solution) containing ethanol added and the pellet gently resuspended using the force of the liquid flow from pipetting. The filter was centrifuged at 12,000 x g for 1 minute, the catch tube emptied and replaced subsequently. Without addition of any solution, the filter was centrifuged again at 12,000 x g for 2 minutes to dry the matrix of the residual wash solution. Then the catch tube was replaced with a new, clean catch tube and the SPIN™ filter was air dried for 5 minutes at room temperature. Next, the binding matrix was gently resuspended in 55 µl of nuclease-free water (ROTH) that was prewarmed to 55°C. Filter and resuspended binding matrix were incubated for 5 minutes at 55°C in a heat block. Finally, the filter was centrifuged at 12,000 x g for 1 minute to bring the eluted DNA into the new clean reaction tube.

The isolated DNA samples were checked using UV-VIS spectrophotometric technology (NanoDrop™ 2000c Spectrophotometer, Fisher Scientific).

To validate the results of the spectrophotometric measurements, certain samples with low and high DNA concentrations were checked by agarose gel electrophoresis. The electrophoreses were performed using 1 x TAE (TRIS-Acetate-EDTA) buffer with 0.8% agarose and the small gel chamber of genX®press (MultiSUB) at 100 Volt for 50 minutes. Afterwards, the gels were stained in a 0.0001% ethidium bromide solution for 20 minutes and bleached for 10 minutes in deionised water. The graphical analyses were performed with the gel imaging instrument BIO-RAD GelDoc 2000 and the software Quantity One® 4.6.9 (Basic). On each gel 2 µl GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific) were loaded next to the slots with 3 µl DNA isolation product mixed with 1.5 µl loading dye.

Test PCRs and subsequent agarose gel electrophoresis were performed with selected samples in order to determine if a purification of samples is necessary prior to PCR amplification for sequencing.

Until further downstream applications the samples were stored at -20°C and -70°C.

2.2.3 Amplicon sequencing

2.2.3.1 16S rRNA gene amplification

For sequencing, to determine the bacterial diversity in the rhizosphere and seed samples, the variable region 4 of the 16S rRNA gene was amplified with 515f and 806r primers extended by individual barcodes (Caporaso et al. 2011, Turner et al. 1999). The 16S rRNA gene comprises conserved regions, which are identical among all bacteria, and variable regions that encompass specific sites which are unique to individual bacterial taxa, as illustrated in Figure 10 (Alimetrics Ltd. 2016).

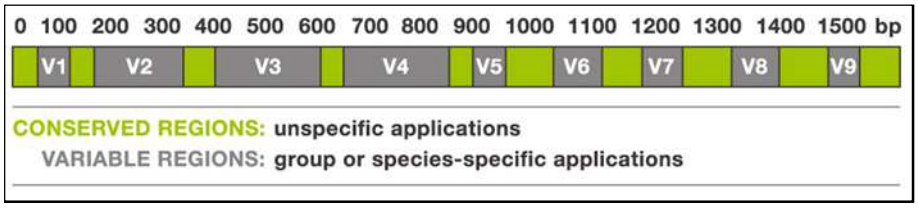

Figure 10: Conserved (green) and variable (grey) regions of 16S rRNA gene of bacteria (Alimetrics Ltd. 2016).

The sequences of the primers 515f-BC1 and 806r-BC1 are exemplified below. The barcode sequence is underlined:

Forward Primer: 5´-ACAGATCGTGCCAGCMGCCGCGGTAA-3´

Reverse Primer: 5´-ACAGATCGGACTACHVGGGTWTCTAAT-3´

For PCR amplification, a modified protocol of Lundberg et al. (2013) including synthetic peptide nucleic acid PCR clamps (PNAs) for blocking the amplification of mitochondrial and plastid 16S rRNA gene sequences of plants, which can account for more than 80% of the resulting sequences, was applied. The DNA fragments were amplified with the Thermocycler (Biometra®) upon the subsequent program:

96°C	5 min	initial denaturation	
96°C	1 min	denaturation	30 cycles
78°C	5 sec	PNA annealing	
54°C	1 min	primer annealing	
74°C	1 min	elongation	
74°C	10 min	final elongation	
10°C		finish state	

The PCR batch used is listed below:

6.00 µl	Taq-&Go™ DNA Polymerase (Mastermix 5xC; MP Biomedicals)
0.45 µl	100 µM PNA Mix (1 : 1 mix 100 µM mPNA and 100 µM pPNA; PNA Bio)
20.15 µl	Nuclease-free H_2O (ROTH)
1.20 µl	515f-BC primer (5 µM; Microsynth)
1.20 µl	806r-BC primer (5 µM; Microsynth)
1.00 µl	Template DNA (DNA isolation product)
30.00 µl	Total volume

The resulting PCR product was examined by performing a gel electrophoresis using a gel made of 0.5 x TBE (Tris/Borate/EDTA) buffer with 1% agarose. Usually the medium and small gel chambers of genX®press (MultiSUB) were used. For small gels 100 Volt for 50 minutes and for medium gels 100 Volt for 30 minutes followed by 120 Volt for 25 minutes were applied. The gels were stained in a 0.0001% ethidium bromide solution for 20 minutes and bleached for 5 minutes in deionised water. The graphical analyses were performed with the gel imaging instrument BIO-RAD GelDoc 2000 and the software Quantity One® 4.6.9 (Basic). On each row of a gel one slot was loaded with 2 µl GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific) and 3 µl PCR product mixed with 2.5 µl loading dye were applied in separate slots.

Three independent PCR amplifications were performed per replicate sample. The template dilution of PCR batch was adjusted based on the electrophoresis results. The dilutions of extracted DNA ranged from undiluted to a dilution factor of 200.

The triplicate amplification products were pooled and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) protocol for centrifugation. First, an equal volume (81 µl) of Membrane Binding Solution was added to each PCR amplification product. Then SV Minicolumns were placed in Collection Tubes and the PCR amplification products transferred into the corresponding columns. The columns containing the amplification products were incubated for 1 minute at room temperature. After this step, the SV Minicolumns were centrifuged for 1 minute at 14,000 rpm and the liquid in the Collection Tubes discarded. Washing was performed by adding 700 µl of Membrane Wash Solution (diluted with 95% ethanol) to the SV Minicolumns. The columns were centrifuged for 1 minute at 14,000 rpm

afterwards. Then the Collection Tubes were emptied and the washing step repeated using 500 µl Membrane Wash Solution and centrifuging for 5 minutes at 14,000 rpm. The Collection Tubes were emptied and centrifuged again for 1 minute with lids open to allow evaporation of ethanol residues. Next the columns were transferred to clean 1.5 ml reaction tubes and 25 µl of Nuclease-Free Water were added to the centres of the columns. The columns were incubated at room temperature for 1 minute and subsequently centrifuged for 1 minute at 14,000 rpm. Finally the SV Minicolumns were removed and the samples checked performing a gel electrophoresis based on the above mentioned conditions and by UV-VIS spectroscopy whether the purification was successful.

The PCR products were adjusted volumetrically to reach equimolarity of each sample in one common pool for 16S rDNA sequencing. Amplicon sequencing was performed with the Illumina MiSeq V2 sequencing platform (2 × 150 bp paired-end) by GATC Biotech (Germany).

2.2.3.2 ITS amplification

To determine the fungal diversity in two rhizosphere and two seed samples the ITS-1 region of the fungal ribosomal RNA transcribed unit, flanked by a 18S and a 5.8S partial fragment, was amplified with the corresponding barcoded primer set ITS1-F and ITS2 extended by individual barcodes (Gardes and Bruns 1993, White et al. 1990). Similar to the 16S rRNA gene of bacteria, the rRNA gene of fungi is composed of highly conserved structural rDNA regions and highly variable regions, the internal transcribed spacer (ITS) regions, which allow classification of fungi (Underhill and Iliev 2014). The fungal ribosomal RNA transcribed unit including ITS-1 and ITS-2 is depicted in Figure 11.

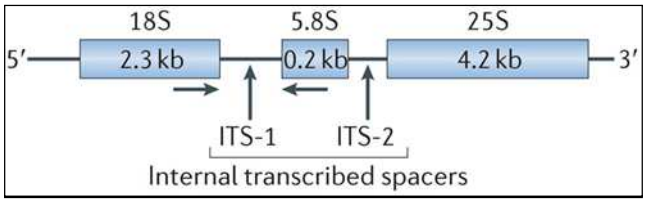

Figure 11: Fungal ribosomal RNA transcribed unit including ITS-1 and ITS-2 (modified according to Underhill and Iliev 2014).

Below the sequences of the primers ITS1-F-BC1 and ITS2-BC1 are exemplified. The barcode sequence is underlined:

Forward Primer: 5´-AGTCACACTTGGTCATTTAGAGGAAGTAA -3´

Reverse Primer: 5´-AGTCACAGCTGCGTTCTTCATCGATGC-3´

The DNA fragments were amplified with the Thermocycler (Techne TC-PLUS, Bibby Scientific) upon the subsequent program:

95°C	5 min	initial denaturation	
95°C	30 sec	denaturation	30 cycles
58°C	35 sec	annealing	
72°C	40 sec	elongation	
72°C	10 min	final elongation	

For ITS sequencing the corresponding DNA fragment was amplified using the following PCR batch:

6.00 µl	Taq-&Go™ DNA Polymerase (Mastermix 5xC; MP Biomedicals)
19.10 µl	Nuclease-free H_2O (ROTH®)
0.90 µl	$MgCl_2$ (25 mM; New England BioLabs GmbH)
1.50 µl	ITS1-F-BC primer (5 µM; Microsynth)
1.50 µl	ITS2-BC primer (5 µM; Microsynth)
1.00 µl	Template DNA (DNA isolation product)
30.00 µl	Total volume

The resulting PCR product was examined by electrophoresis, as described above in subchapter 2.2.3.1. Three independent PCR amplifications were performed per sample. The template dilution for PCR batch was adjusted based on electrophoresis results. The dilutions of extracted DNA ranged from undiluted to a dilution factor of 10. The triplicate amplification products were pooled and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) protocol for centrifugation (see chapter 2.2.3.1). The quality of purification was checked by electrophoresis based on the above mentioned conditions and by UV-VIS spectroscopy.

Finally, the PCR products were adjusted volumetrically to reach equimolarity of each sample in one common pool for ITS rDNA sequencing. Amplicon sequencing was performed with the Illumina MiSeq V2 sequencing platform (2 × 150 bp paired-end) by GATC Biotech (Germany).

2.2.4 Bioinformatic and statistical analyses

2.2.4.1 16S rDNA amplicons

Following de-multiplexing of raw reads and clipping of sequencing adapters, read pairs were joined and sorted according to sample-specific barcodes. Joint reads were further analysed with the open-source bioinformatics pipeline QIIME 1.9.1 (Caporaso et al. 2010a). First, reads were quality (Phred score ≥20) and length (290-300 bp) filtered, and primer and barcodes flanking the reads were clipped. Chimeric sequences were removed by means of the de novo UCHIME method (Edgar et al. 2011, Edgar 2010). Remaining sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity level using the de novo UCLUST clustering method with default parameters (Edgar 2010). The most abundant representative read per OTU was picked and taxonomically assigned using RDP Classifier 2.2. (Wang et al. 2007) based on the reference database Greengenes release gg_13_8 (DeSantis et al. 2006). The alignment of reads was performed using PyNAST (Caporaso et al. 2010b). Additionally, an approximately-maximum-likelihood phylogenetic tree using FastTree 2.1.3 (Price et al. 2010) was generated.

Each replicate was comprised of 11,245 to 276,132 sequences after initial data processing. Prior to statistical analyses, the mitochondrial (ranging from 0.6% to 32.9% per replicate) and plastid (0.3% to 6.8%) sequences with plant origin and unassigned OTUs were excluded by filtering the OTU table. Additionally, out of four replicates per sample, the replicate with the lowest read number was discarded. As the remaining replicates comprised of 3758 to 256,248 sequences, the subsequent analyses were performed after normalising the sequence number per replicate to 3758.

The species richness by means of alpha-diversity was determined by calculating the Shannon H´ (Shannon 1997) and the Chao1 (Chao and Bunge 2002, Chao 1984) indices as well as by rarefaction analysis (Gotelli and Colwell 2001, Heck et al. 1975). For illustration of taxonomic

composition, the open-source software Circos (Krzywinski et al. 2009) was applied among others. The evenness in the *C. pepo* samples was recorded by means of the Heip evenness measure (Heip 1974), which is based on the Shannon diversity index. Statistical analyses to calculate significance of differences in diversity indices were performed using the nonparametric Kruskal-Wallis Rank Sum Test (Hollander and Wolfe 1973) and the Pairwise Test for Multiple Comparisons of Mean Rank Sums (Nemenyi-Test) (Sachs 1997) implemented in the open source data analysis software RStudio (RStudio Team 2015). Nonparametric analyses of similarities (ANOSIM) were calculated according to Fierer et al. (2010) and Clarke (1993) and beta-diversity analysed by means of an Unweighted UniFrac-based principal component analysis using Emperor (Vazquez-Baeza et al. 2013, Lozupone and Knight 2005). Network analysis was performed with the open source bioinformatics software Cytoscape (Shannon et al. 2003). A non-metric multidimensional scaling (NMDS) analysis was done using the open source data analysis software RStudio and the function metaMDS {vegan} (Faith et al. 1987, Minchin 1987) with calculation of the distance matrix based on a Bray-Curtis algorithm. For illustration heatmaps based on OTU abundances were created. Data processing was also performed in MS Excel.

2.2.4.2 ITS amplicons

The basis bioinformatic processing of ITS amplicon sequencing data from de-multiplexing of raw-reads to generate a phylogenetic tree was performed as already described for the 16S amplicon sequencing data (chapter 2.2.4.1). The analysed samples, which correspond to a replicate of the *C. pepo* rhizosphere or seed samples, comprised 16,896 to 221,425 sequences after initial data processing. Since the data were only used as a first insight into fungal diversity, no further processing was carried out. Data were not normalised for visualisation.

For characterisation of unidentified Plantae taxa, which constituted a considerable part of the fungal community associated with *C. pepo*, the OTU table gained by initial data processing was filtered. Single- and doubletons were removed and unidentified Plantae selected. Single- and doubletons are informative for taxonomic analyses, but were disregarded here for the sake of simplicity. For subsequent analyses the representative

sequences of the OTUs were gained by filtering the FASTA file. Further data processing was carried out in MS Excel. Multiple sequence alignments were done with Clustal Omega (McWilliam et al. 2013). Blast analyses of representative sequences were performed against the NCBI nucleotide database for highly similar sequences (megablast) with default settings (Morgulis et al. 2008, Zhang et al. 2000).

2.3 Design of a peptide nucleic acid DNA clamp

Because of the large proportion of unidentified Plantae sequences in the seed-associated communities, their sequence similarity and their presumable origin from pumpkins a peptide nucleic acid (PNA) DNA clamp was designed to block this amplification in subsequent ITS amplifications of *C. pepo* samples.

The PNA is an artificially synthesised polymer similar to RNA and DNA, but the bases are linked to the backbone by methylene carbonyl bonds like in peptides. The bondage between PNA and DNA is stronger than between DNA and DNA because of the absence of electrostatic repulsion due to the absence of charged phosphate groups. PNA probes are very stable, *in vitro* and *in vivo*. Because of their strong binding affinity and specificity to their target DNA and the fact that they are not recognised by DNA polymerases as primer, the PNAs can be used as sequence-specific clamps which block PCR (PNA Bio 2016). PCR reaction can be blocked by means of elongation arrest of polymerase (Figure 12) or competitive binding between primers and PNA probe (Wintzingerode et al. 2000).

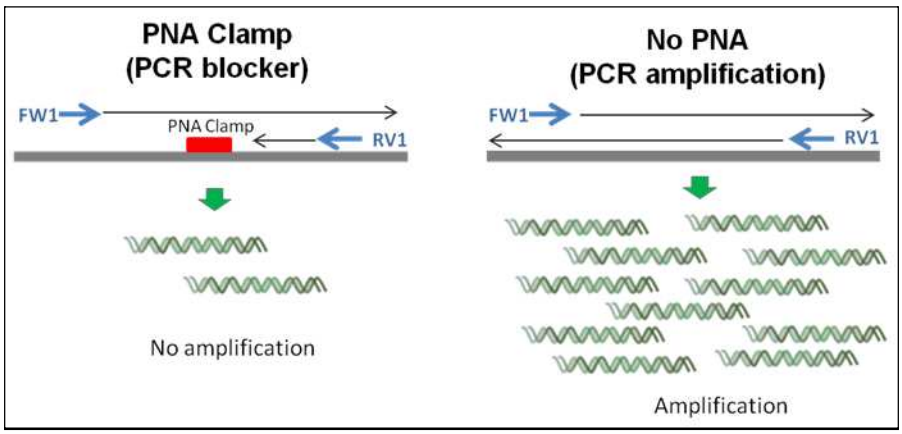

Figure 12: Principle of a peptide nucleic acid (PNA) PCR blocker (PNA Bio 2016).

2.3.1 pepoPNA construction

The PNA acting by arresting the polymerase in the elongation process was designed based on criteria of PNA Bio (2016) and scientific publications as described below.

According to Lundberg et al. (2013) the optimal length of a PNA oligomer for elongation arrest is between 13 bp and 17 bp with an annealing temperature above the temperature of the primers used in the PCR reaction to be blocked. As long as the melting temperature (T_m) of the PNA is higher than that of primers, the PNA probe anneals to the DNA template before the primers do. The PNA melting temperature should be above the temperature of the extension cycle (Terahara et al. 2011). As Terahara et al. (2011) reported, one mismatch between template DNA and PNA probe do not affect PCR blocking, in contrast to two or more mismatches. PNA Bio (2016) provided following guidelines for PNA design. The anti-parallel orientation for duplex formation is preferred by PNA probes. For blocking either the sense or reverse complement sequence can be used. If it targets genomic DNA, it does not matter since it is double stranded. The melting temperature of the PNA/DNA-duplex is roughly 1°C/bp higher than that of the corresponding DNA/DNA-duplex. And the recommended size is between 12 and 21 bases. Because of the high binding affinity the design of a long PNA is usually not needed. Self-complementary sequences should be avoided, because PNA/PNA interaction is stronger than PNA/DNA interaction as well as Purine-rich sequences (>60%), because they tend to aggregate due to low solubility in aqueous solution. Purine stretches over six residues, especially four or more Guanine residues, are not good either. Additionally, a miniPEG gamma modified PNA resulting in a stereogenic centre at the γ-carbon atom can convey advantages such as an increased T_m (5-8°C/substitution) providing higher affinity, improved solubility, less self-aggregation and more stable PNA-DNA duplexes.

As a first step of PNA design, a multiple sequence alignment of unidentified Plantae sequences was checked for appropriate sites, preferably a site with total base identity of all representative sequences. Those sequence parts were checked regarding melting temperature as well as purine and GC contents among other criteria by the help of the PNA Tool provided by PNA Bio (2016), Blast analyses against the NCBI nucleotide database for highly similar sequences (megablast) with default settings (Morgulis et al. 2008, Zhang et al.

2000) and searching the sequences in the FASTA file with total representative sequences and the file with unidentified Plantae sequences. Subsequently, it was checked, if the apparently suitable candidate is contained in the UNITE database version 6 (reference database for ITS amplicons used by QIIME). For the pepoPNA design the PCR protocol already used for the ITS amplicons, as described in chapter 2.2.3.2, was taken as starting point where the PNA should be fitted in.

The designed pepoPNA oligomer was finally ordered at PNA Bio Inc. (USA) and named pepoPNA.

2.3.2 Validation of pepoPNA functionality

After *in silico* analyses, the pepoPNA functionality was validated *in vitro* by single strand conformation polymorphism (SSCP) subsequently to PCR amplification using different pepoPNA concentrations and by sequencing.

For these analyses the samples used for ITS amplicon sequencing (two seed samples (02sc, 08sa) and two rhizosphere samples (02ra, 08ra)) as well as two additional seed samples were used (10sd, 13sa). The aim was to cover not only samples of the Styrian oil pumpkin (inbred Line B (02) and three-way cross hybrid GL Rustikal (08)), but also samples of genetically more distinct genotypes (Dutch zucchini hybrid Naxos (10) and segregating Line G with geographic origin in Slovenia (13)) in order to determine if the pepoPNA functionality is sufficient for all genotypes investigated.

2.3.2.1 PCR amplification

Based on the 16S amplicon PCR protocol, which included mPNA and pPNA and has worked well, and also upon consideration of Lundberg et al. (2013), the ITS amplicon protocol was extended with an annealing step for pepoPNA for blocking *C. pepo* sequences:

95°C	5 min	initial denaturation	
95°C	30 sec	denaturation	30 cycles
78°C	5 sec	pepoPNA annealing	
58°C	35 sec	primer annealing	
72°C	40 sec	elongation	
72°C	10 min	final elongation	

According to the PNA Tool of PNA Bio (2016) the predicted T_m of pepoPNA including the miniPEG gamma modification is 77.6-80.6°C. In order to ensure high specificity of binding, an annealing temperature for pepoPNA at 78°C was chosen. Also the primers do not yet bind at this temperature, as their recommended annealing temperature according to T_m calculator of New England Biolab is at 44°C without, or 53°C including the barcodes. Also the established 16S amplicon protocol is similar: an annealing temperature of 78°C was used for mPNA and pPNA with melting temperatures at 79.6°C and 82°C respectively.

The composition of the PCR batch was also retained, but three pepoPNA concentrations were tested: 0.5 µM (PNA1), 0.77 µM (PNA2) and 1 µM (PNA3) final concentration in PCR batch. 0.77 µM was the final concentration of mPNA and pPNA in the 16S amplicon PCR protocol, which worked sufficiently. A PCR batch for ITS amplicons including pepoPNA comprised:

6.0 µl	Taq-&Go™ DNA Polymerase (Mastermix 5xC; MP Biomedicals)
1.5 µl	ITS1-F-BC primer (5 µM; Microsynth; Gardes and Bruns 1993)
1.5 µl	ITS2-BC primer (5 µM; Microsynth; White et al. 1990)
1.0 µl	Template DNA (DNA isolation product, diluted appropriately)
0.9 µl	$MgCl_2$ (25 mM; New England BioLabs)
0.15-0.30 µl	pepoPNA (100 µM; PNA Bio)
18.95-18.8 µl	Nuclease-free H_2O (ROTH)
30.0 µl	Total volume

Details of primer sequences are illustrated in chapter 2.2.3.2. The delivered lyophilised pepoPNA was dissolved in distilled and nuclease-free water to a stock concentration of 100 µM. The pepoPNA stock was long-term stored at -20°C and during working progress at 4°C.

The resulting PCR product was examined by performing a gel electrophoresis using an agarose gel made of 0.5 x TBE (Tris/Borate/EDTA) buffer with 1% agarose. Usually the medium and small gel chambers of genX®press (MultiSUB) were used. For the small gels 100 Volt for 50 minutes and for the medium gels 100 Volt for 30 minutes followed by 120 Volt for 25 minutes were applied. The gels were stained in a 0.0001% ethidium bromide solution for 20 minutes and bleached for 5 minutes in deionised water. The graphical analyses were performed with the gel imaging instrument BIO-RAD GelDoc 2000 and the software Quantity One® 4.6.9 (Basic). On each row of a gel one slot was loaded with 2 µl GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific 2015) and 3 µl PCR product mixed with 2.5 µl loading dye were applied in separate slots.

The concentration of template DNA was adjusted by appropriate dilutions of the DNA isolation product. This was judged by gel electrophoresis. A distinct band was the objective, two in most cases of ITS amplicons. The standard protocol of the respective sample was adapted accordingly, and the same template concentration was then used for the pepoPNA variants. The dilutions were in a range of undiluted, or even concentrated, to a dilution factor of ten.

The same protocols were used for seed and rhizosphere samples.

Subsequently, the PCR amplification products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) protocol for centrifugation (described in chapter 2.2.3.1). The purified product was eluted with 25 µl nuclease-free water (Promega). The purity and nucleic acid concentration in the samples were determined by UV-VIS spectrophotometric technology (NanoDrop™ 2000c Spectrophotometer, Fisher Scientific).

2.3.2.2 Single strand conformation polymorphism

For analysis of pepoPNA functionality by means of single strand conformation polymorphism (SSCP) the purified PCR products (chapter 2.3.2.1) were used.

Prior to SSCP the double-stranded nucleic acids were processed into single-stranded nucleic acids by digestion of the 5´ phosphorylated strands with the Lambda exonuclease (New England BioLabs). 20 µl of purified PCR product were mixed on ice with 4 µl of reaction solution consisting of 1.6 µl Lambda exonuclease (8 U) and 2.4 µl 10 x Lambda exonuclease reaction buffer (New England BioLabs). The mixture was incubated for 60 minutes at 37°C. Then 20 µl loading buffer were added to each sample for a 2 minute denaturation process at 95°C. 1000 µl of loading buffer contained 950 µl deionised formamide (95%), 4 µl 2.5 M NaOH solution, 5 µl 5% bromphenol blue solution and 41 µl deionised water. The folding was achieved on ice for at least five minutes. Until SSCP the samples were stored at -20°C. After storage, just before application, the samples were denatured and folded again.

For the SSCP 52 ml of the gel combined:

19.73 ml	Ultrapure water (Barnstead)
10.4 ml	5 x TBE (1000 ml constituted 54 g Tris HCl, 27.5 g boric acid, 20 ml 0.5 M EDTA, filled up with ultrapure water)
21.57 ml	2 x MDE gel solution (Biozym Scientific)
275 µl	10% APS
27.5 µl	TEMED

The gel filled in the gel mould was overlaid with ethanol during polymerisation for approximately three hours. Afterwards the ethanol was rinsed off with deionised water and the polymerised polyacrylamide gel embedded into the TGGE MAXI System (Biometra). The buffer chambers were filled with 1 x TBE. Then 8 µl of samples and 2 µl of GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific 2016) for monitoring purposes were applied into the respective slots of the gel. Electrophoresis was performed at 400 V, 50 mA and 26°C for 17 hours.

Following the electrophoresis for visualisation the gel was fixed in 300 ml 10% acetic acid solution for 30 minutes. After a washing step with deionised water for five minutes, which

was repeated three times, the gel was incubated for 30 minutes in 300 ml of a 0.1% silver nitrate solution with 2 ml formaldehyde (37% wt/vol). Then the gel was rinsed with deionised water for 10 seconds. The development was performed by incubation in 500 ml 3% NaOH solution with 2 ml formaldehyde (37% wt/vol) in the dark until bands became visible. The reaction was stopped in 300 ml of a 10% acetic acid solution for 30 minutes and the conservation done in 300 ml 10% ethanol including 13% glycerol for 30 minutes. Subsequently the gel was fixed physically and dried at room temperature for two days. The developed gel with the visible bands was then scanned.

2.3.2.3 Sequencing of SSCP-derived bands

Deviating bands in the lanes of the standard PCR variants on the SSCP gel were excised and sequenced in order to verify that the blocked sequences refer to the targeted *C. pepo* sequences.

13 bands of the standard PCR variant which differed from pepoPNA variants (Figure 44) were excised with a scalpel and the gel slices treated with 150 µl crush and soak buffer according to Sambrook et al. (1993) for five days. The crush and soak buffer consisted of 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA (pH 8.0) and 0.1% SDS. Subsequently the supernatant was transferred into a new reaction tube and centrifuged at 13,000 rpm and 4°C for 10 minutes. The supernatant was again transferred to a new reaction tube and 570 µl 70% ethanol were added. The tubes were stored at -20°C for six hours for precipitation and then centrifuged at 13,000 rpm and 4°C for 20 minutes. Afterwards the supernatant was discarded and the remaining pellets in reaction tubes were dried for 0.5 hours. Finally the precipitated nucleic acid was resuspended in 50 µl of 10 mM Tris HCl (pH 8.0). The samples were stored at -20°C till further usage.

The PCR amplification of excised bands was performed with the standard protocol for ITS amplicons excluding the pepoPNA step, as shown in chapter 2.2.3.2. Due to the low DNA concentration after isolation from the polyacrylamide gel, the maximum possible template quantity (20.1 µl) was used for weak bands and 15 µl template DNA for thicker bands. The resulting PCR product was examined by performing an agarose gel electrophoresis (details in chapter 2.2.2).

The PCR amplification products selected for sequencing were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) protocol for centrifugation. The purified product was eluted with 25 µl nuclease-free water (Promega), as describe in chapter 2.2.3.1. The purity and nucleic acid concentration in the samples were determined by UV-VIS spectrophotometric technology (NanoDrop™ 2000c Spectrophotometer, Fisher Scientific).

For sequencing 100 ng of template DNA were united with 4 µl of reverse primer (27 bp; 5 mM) and nuclease-free water to a total volume of 14 µl. The sequencing was performed by LGC Genomics (Germany) with Sanger sequencing method.

2.3.3 Establishment of a PCR protocol including pepoPNA

Based on the knowledge gained so far, some aspects and possible optimisations of the PCR protocol including the pepoPNA probe to block *C. pepo* sequences were summarised.

2.4 Analysis of genotype-specific responses to BCAs in the greenhouse

For greenhouse experiments seeds of the *C. pepo* genotypes GL Rustikal, Gl. Diamant and Line D were primed with *Serratia plymuthica* strains and were grown under gnotobiotic conditions in germination pouches and in sterile soil. The effect on tissue colonisation, hypocotyl length and germination capacity as well as on fresh and dry weight dependent on the genotype and the *Serratia* strains used for priming was investigated.

2.4.1 *Serratia plymuthica* strains

For the greenhouse experiments and CLSM analyses of different *C. pepo* genotypes five *Serratia plymuthica* strains were used: *S. plymuthica* 3Rp8, 3Re4-18, S13 and HRO-C48, which were presumed as beneficial acting strains, and 4Rx13, which was considered to be

harmful to *C. pepo* plants, according to related publications as described in chapter 1.3. The bacterial isolates and the respective references are listed in Table 2.

Table 2: Abbreviation of strain denomination used in this Master´s thesis, strain denomination, NCBI accession number and references of the *S. plymuthica* strains used for experiments and CLSM analyses of different *C. pepo* genotypes

Abbreviation	Bacterial isolate	NCBI accession number	References
3Rp8	*Serratia plymuthica* 3Rp8	CP012096	Adam et al. 2016a Berg et al. 2002
3Re4-18	*Serratia plymuthica* 3Re4-18	CP012097	Adam et al. 2016a Berg et al. 2005
S13	*Serratia plymuthica* S13	CP006566.1 (NC_021659.1)	Fürnkranz et al. 2012 Müller et al. 2013
4Rx13	*Serratia plymuthica* 4Rx13	CP006250.1 (NC_021591.1)	Berg et al. 2002 Weise et al. 2014
HRO-C48	*Serratia plymuthica* HRO-C48	NZ_LTDN00000000 (PRJNA224116)	Kurze et al. 2001

In order to be able to visualise the bacteria on different tissues of *C. pepo* genotypes, transformed strains were used, which harbour the pIN69 vector as carrier for the respective fluorescence proteins. The pIN69 vector was derived from pBBR1MCS plasmids (Vergunst et al. 2010). It is rhizosphere stable and cannot be mobilised (Δmob) in order to prevent a plasmid transfer between bacteria during experiments. The pIN69 plasmid contains a trimethoprim resistance (TpR) to be used as selection marker. The antibiotic trimethoprim is a dihydrofolate reductase inhibitor of prokaryotes. The different cloned genes of fluorescence proteins are controlled by a tac promoter. The pIN69 vectors carrying genes for GFP, DsRed2, mNeptune and eBFP2 fluorescence proteins and *S. plymuthica* 3Rp8 tagged with the pIN69 vector with DsRed2 were derived from the Master´s thesis of Murgu (2014). The transformed *S. plymuthica* strains S13-GFP, 3Re4-18-mNeptune and 4Rx13-eBFP2 were provided by the Master´s thesis of Adam (2015).

In the subsequent chapters, only the abbreviated strain denomination or strain denomination followed by the fluorochrome name are used for the not modified and modified *Serratia plymuthica* strains respectively.

2.4.2 Washing and priming of seeds

All seeds used for the greenhouse experiments were washed before seed priming. First the rough dirt like remainings of the fruit flesh or soil was removed by a quick sweeping off with water. Then the seeds were shaken in a 1% NaOCl solution for 2 minutes. Afterwards they were washed six times for 30 seconds and once for two minutes. Finally, they were dried in sterile petri dishes with the lid open in the clean bench for four hours at room temperature (Figure 14).

The day before the seed priming was performed, the appropriate overnight cultures were prepared. For each strain 20 ml of LB medium (Luria/Miller, Carl Roth; (25 g/l)) were inoculated and incubated at 30°C and 100 rpm. The ONCs were checked macroscopically if they were correspondingly turbid and by plating on LB agar plates (Luria/Miller, Carl Roth; (25 g/l, 2% agar) in order to determine the CFU number by means of the dripping method, as illustrated in Figure 13. The media were prepared with or without trimethoprim, depending on the bacterial strains used (with/without pIN69 vector harbouring trimethoprim resistance gene). By means of this method two drops (10 µl each) of the dilutions (10^{-4} to 10^{-6}) were dripped on agar plates. Then the plates were tilted in two ways so that the drops dispensed close to the edges of the plate. The plates were incubated at room temperature or at 30°C for one day.

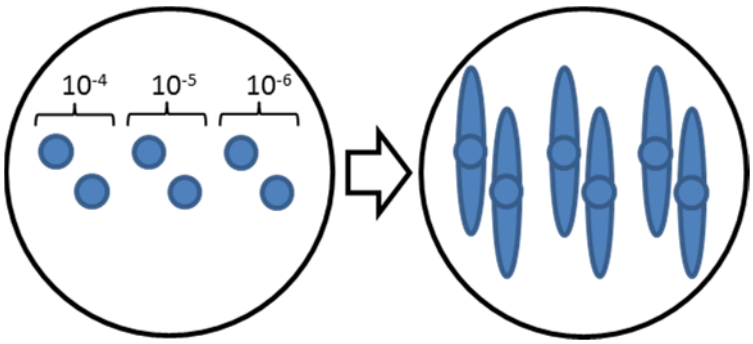

Figure 13: Dripping method for determination of CFU/ml of a suspension. The circles correspond to 10 µl droplets of a certain dilution (10^{-4} to 10^{-6}) of a suspension, which are dispensed in two directions by tilting the agar plate.

For seed priming five to ten pumpkin seeds and 36 lettuce seeds, depending on the experiment, were put in a sterile petri dish and 2 ml of the respective ONC as well as 18 ml of 0.85% NaCl were added. In the case of a mixture of different strains, the 2 ml bacterial suspension was divided accordingly. The seeds of the negative control (NegC) were treated with 20 ml of 0.85% NaCl. Afterwards the petri dishes were closed and sealed with parafilm

and the seeds incubated for four hours at 100 rpm and room temperature (Figure 14). After incubation, the bacterial suspension was removed and the seeds dried roughly for two hours in the clean bench.

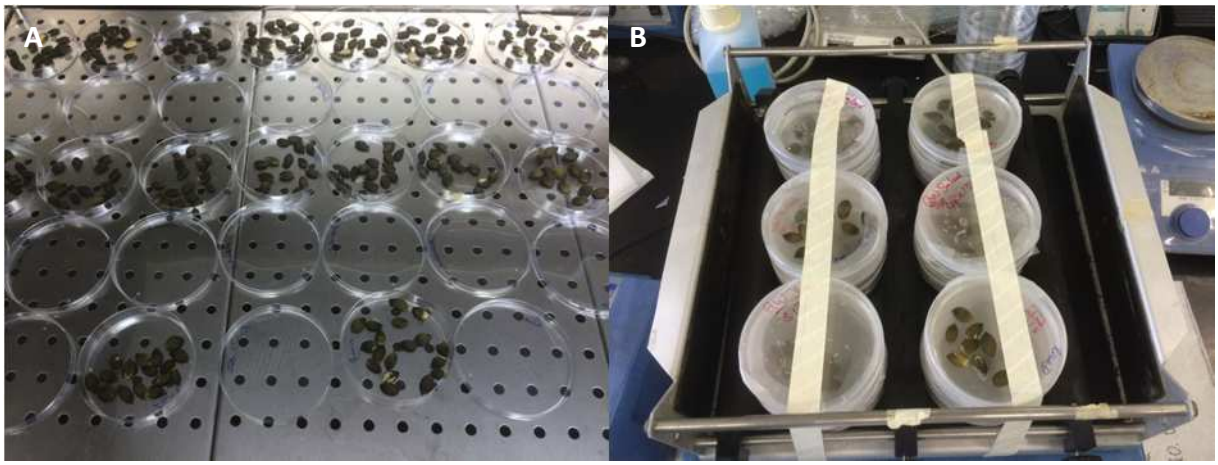

Figure 14: Drying of washed pumpkin seeds in the clean bench (A) and incubation of inoculated seeds at 100 rpm and room temperature (B).

2.4.3 Germination test with *S. plymuthica* 4Rx13

In order to determine if the *S. plymuthica* strain 4Rx13 inhibits germination of different cultivars in an extent which was documented by Adam (2015) for the cultivar GL Rustikal, five *C. pepo* genotypes (GL Rustikal, Gl. Diamant, Line A, Line B and Line D) were tested for germination capacity after 4Rx13 seed priming.

For the examination five washed and primed seeds (see chapter 2.4.2) of each genotype were put in a petri dish with two filter papers and 5 ml deionised water were added. The petri dishes were incubated at 20°C in the dark and the germination process monitored and the radicle size recorded twice.

2.4.4 Determination of tissue colonisation, hypocotyl length and fresh weight

2.4.4.1 Analysis of *C. pepo* plants grown in germination pouches

For the analysis of plant-microbe interactions in germination pouches (cyg™ seed germination pouch, Mega International, USA) three *C. pepo* cultivars GL Rustikal, Gl. Diamant and Line D were used. Five seeds of each genotype were primed with one transformed *S. plymuthica* strain (3Rp8-DsRed2, S13-GFP, 3Re4-18-mNeptune or 4Rx13-eBFP2), the seeds of the mixed treatment with a mixture of 3Rp8-DsRed2, S13-GFP and 3Re4-18-mNeptune, and the seeds of the negative control (NegC) solely with 0.85% NaCl, as described in chapter 2.4.2 and illustrated in Table 3.

Table 3: Experimental design of *C. pepo* plants primed with different transformed *S. plymuthica* strains and grown in germination pouches. The Mix corresponds to an inoculation mixture of 3Rp8-DsRed2, S13-GFP and 3Re4-18-mNeptune. Five seeds of each genotype with the same inoculum strain were sown in one pouch

***S. plymuthica* strain** ***C. pepo* genotype**	3Rp8-DsRed2	S13-GFP	3Re4-18-mNeptune	4Rx13-eBFP2	Mix	NegC
GL Rustikal	5	5	5	5	5	5
Gl. Diamant	5	5	5	5	5	5
Line D	5	5	5	5	5	5

Five seeds of the same genotype and inoculation variant were sown in one autoclaved germination pouch. The seed germination pouches are built up by a paper wick forming a trough, which is surrounded by a plastic pouch. The seeds were placed in the trough. At sowing 20 ml deionised, autoclaved water were added (Figure 15 A, B). Three pouches of the different genotypes with the same inoculum were placed in an upright position in plastic boxes. The pouches were arranged in a way which protected the developing roots from light. The boxes were closed and sealed with parafilm, in order to keep the moisture in the boxes, and incubated at 20°C in the dark.

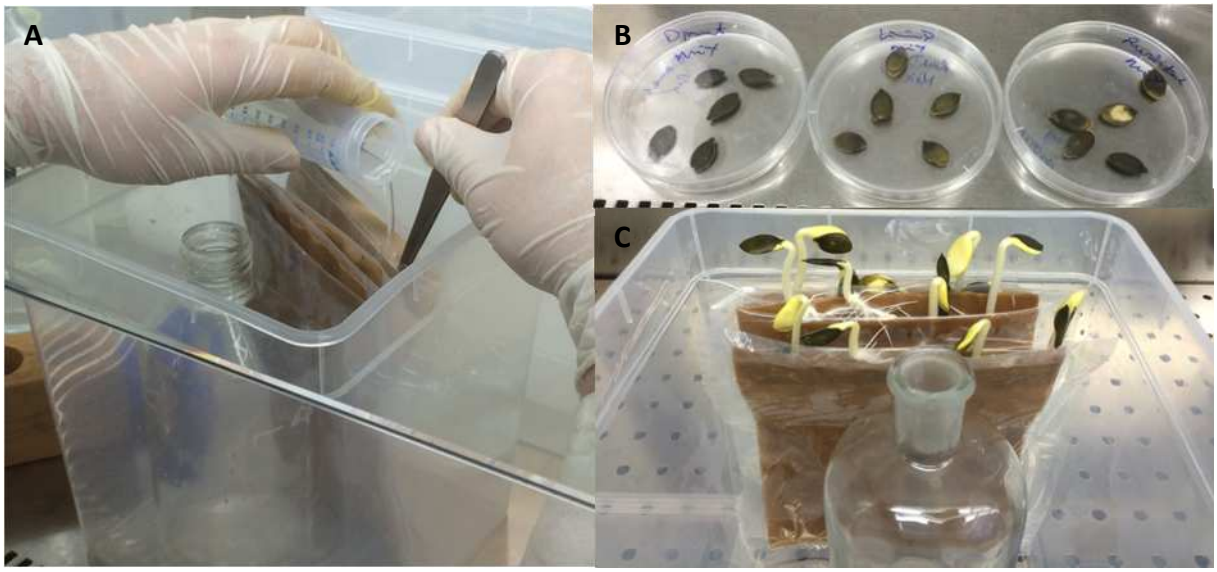

Figure 15: Growing *C. pepo* plants in germination pouches. A: Sowing and watering of seeds. B: Inoculated seeds ready for sowing. C: Seedlings six days after sowing.

Germination and plant development were monitored regularly. After six days, when they showed a sufficient germination level (Figure 15 B), the box lid was removed and replaced by a box put the upside down onto the box with the pouches in order to give the plants more space to grow. The gap between the boxes was sealed with parafilm and tape to preserve the moisture inside the box. At this time the pouches were rewatered with 10 ml each. Afterwards, the boxes were incubated at 23°C with 13 hours light in the greenhouse for additional seven days. During plant development the number of germinating plants and in the first experiment the hypocotyl length were recorded. Plant material was used for CLSM and reisolation analyses 12 days and 13 days after sowing respectively.

Sowing and all other steps until harvesting were performed under aseptic conditions.

The whole experiment including reisolation was repeated with the same experimental design (Table 3), but three repetitions. The boxes were put into the greenhouse after seven days. Rewatering with 20 ml deionised water was done seven days after sowing. Harvest and reisolation were carried out after 15 days of growing.

2.4.4.2 Reisolation of inoculated bacteria

The reisolation of inoculated strains from *C. pepo* plants grown in germination pouches was done 13 days and 15 days post inoculation in the first and second experiment respectively, when the first true leaf was in development (Figure 16).

Figure 16: *C. pepo* plants grown in germination pouches in a plastic box (A). Plants of the cultivars Line D, Gl. Diamant and GL Rustikal (left to right) in germination pouches 12 days after sowing (B).

For reisolation 0.7 to 1.9 g roots per pouch (equal amounts of all root parts) and all above ground plant parts from hypocotyl to leaves of two young plants (referred to as leaf in subsequent chapters) per pouch (1.4 to 3.6 g) were weighed in sterile whirl-paks (Nasco) (Figure 17), 5 ml of 0.85% NaCl added and ground until homogenisation (Figure 18).

In the second experiment all of the roots of the three repetitions were pooled (11.2 g to 19.0 g), meaning the roots of 15 plants. Stems and leaves of two plants of one repetition (7.8 g to 15.0 g) were used, meaning six plants were pooled in total per cultivar-treatment-combination. The average fresh weight per plant was evaluated as well. 10 ml of 0.85% NaCl were used for the homogenisation step.

In the case of seeds inoculated with 4Rx13-eBFP2, one third of the not germinated seeds (in total ~0.2 g in the first and ~0.5 g in the second experiment) was sampled (Figure 17 A).

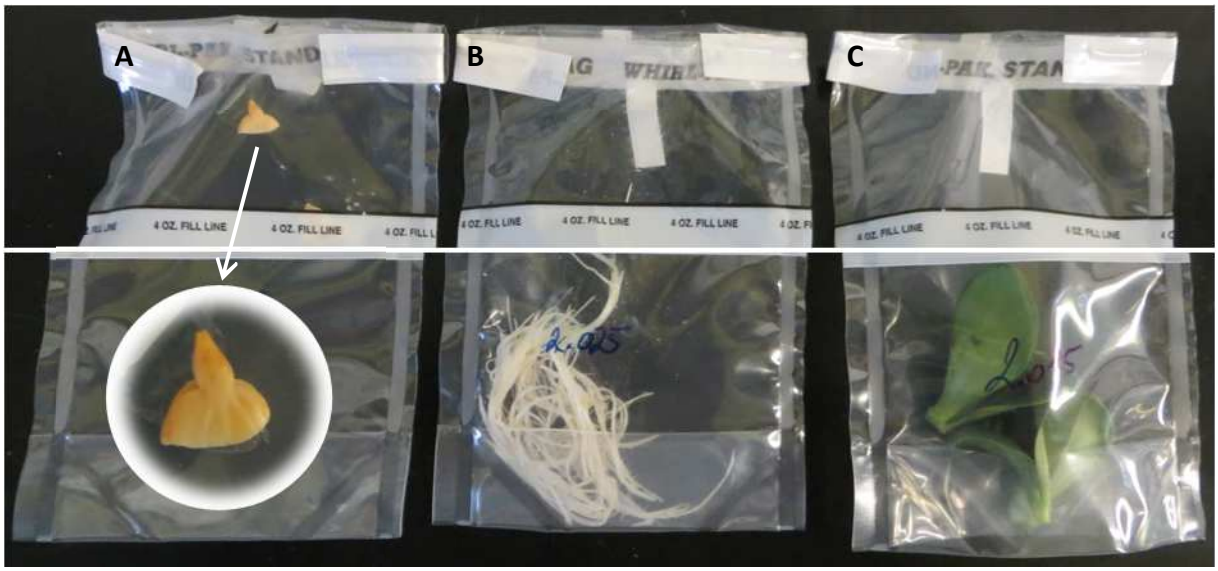

Figure 17: Seed (A), root (B) and leaf (C) samples for reisolation of inoculated transformed *S. plymuthica* strains from *C. pepo* tissues.

Figure 18: Macerated leaf and root samples of *C. pepo* for reisolation of transformed *S. plymuthica* strains from *C. pepo* tissues.

Subsequently, depending on plant amount used, dilution series of all samples to a dilution factor of 10^{-6} were established. 10 µl of three dilutions of each sample were plated on LB agar plates (Luria/Miller, Carl Roth; 25 g/l, 2% agar) containing 50 µg/ml trimethoprim (Sigma Aldrich, 290.32 g/mol; >99% crystallised) by means of the dripping method, as described in chapter 2.4.2 and illustrated in Figure 13. The plates were incubated at 30°C for one day.

The CFU of both repetitions of the most appropriate dilution were counted (Figure 50) and the mean CFU/g FW (fresh weight) calculated based on this formula:

$$\frac{CFU}{g\ FW} = \frac{CFU\ count * F * V_{whirl-pak}}{V_{droplet} * W_{tissue}(g)}$$

CFU … colony forming units
FW … fresh weight
F … dilution factor
V … volume
W … weight

2.4.5 Determination of fresh and dry weight

2.4.5.1 Analysis of *C. pepo* and *L. sativa* plants grown in sterile soil

In order to determine the fresh and dry weight in dependence of plant-microbe interactions, pumpkin and lettuce plants were grown in sterile soil in plastic boxes. For this experiment, three *C. pepo* cultivars GL Rustikal, Gl. Diamant and Line D and the *Lactuca sativa* cultivar Gelber Winter were used. Five (in two repetitions) and nine seeds (in four repetitions) of each *C. pepo* and *L. sativa* genotype respectively were primed with one *S. plymuthica* strain (3Rp8, S13, 4Rx13, 3Re4-18, HRO-C48). The seeds of the negative control (NegC) were incubated with 0.85% NaCl. The experimental design is illustrated in Table 4.

Table 4: Experimental design of *C. pepo* and *L. sativa* plants primed with different *S. plymuthica* strains grown in sterile soil. Five or nine seeds of one pumpkin genotype or lettuce respectively and with the same inoculum strain were grown in one plastic box. Two replicates of experiments with *C. pepo* and four replicates with *L. sativa* were performed

***S. plymuthica* strain** / ***C. pepo* genotype**	3Rp8	S13	3Re4-18	4Rx13	HRO-C48	NegC
GL Rustikal	2 x 5	2 x 5	2 x 5	2 x 5	2 x 5	2 x 5
Gl. Diamant	2 x 5	2 x 5	2 x 5	2 x 5	2 x 5	2 x 5
Line D	2 x 5	2 x 5	2 x 5	2 x 5	2 x 5	2 x 5
***L. sativa* cultivar**						
Gelber Winter	4 x 9	4 x 9	4 x 9	4 x 9	4 x 9	4 x 9

In advance, approximately 7 cm soil (Gramoflor, Germany) was filled in big plastic boxes (21 cm/21 cm/22 cm; 475 g soil) for pumpkin and small boxes (11 cm/9 cm/9 cm; 180 g soil) for lettuce seedlings. Gramoflor substrate is a standard soil peat, moderately to highly decomposed raised bog peat and weakly fertilised. The substrate contains primary and micronutrients which are necessary for plant growth. Available nutrients are in the range of 50-300 mg/l N, 80-300 mg/l P_2O_5 and 80-400 mg/l K_2O. Salt content is below 1.5 g/l and pH

($CaCl_2$) in the range of 5.0 to 6.5. The boxes including the soil were autoclaved twice. The sterility of the soil was checked by plating soil samples on PDA agar (26.5 g/l potato extract glucose broth (ROTH), 2% agar).

After washing and priming, five seeds of the same pumpkin genotype and inoculation treatment were placed in the soil in a depth of 2 cm in one autoclaved plastic box. In the case of lettuce, nine seeds were placed separately in a depth of 0.5 cm per box. The moisture of the soil was between 61% and 74% in the big boxes and between 68% and 71% in the small boxes, determined by the MB 35 Moisture Analyser (Ohaus Corporation, USA). At sowing 50 ml and 20 ml deionised water were added respectively. The boxes were closed with a lid and sealed with parafilm, in order to preserve the moisture in the boxes, and incubated in the greenhouse for 12 days and 15 days at 23°C with 13 hours light. The germination and plant development were monitored and the number of viable plants noted.

The sowing and all other steps until harvesting were performed under aseptic conditions.

2.4.5.2 Determination of fresh and dry weight

The determination of fresh and dry weight of *C. pepo* and *L. sativa* plants grown in sterile soil in plastic boxes was done after 12 days and 15 days after seeding respectively, when the first true leaf was in development in the case of pumpkin seedlings and when lettuce plants had developed around five true leaves.

For weight determination all plants of a variant were cut at the bottom of the hypocotyl by being careful to avoid any contamination with soil (Figure 19 A-C, Figure 20 A). The replicates were assessed separately. The plants were transferred into paper bags, and the fresh weight determined accordingly (Figure 19 D). The pumpkin plants were dried in a drying chamber at 105°C for three days, the lettuce plants for four days and the dry weight measured subsequently (Figure 19 E, Figure 20 B). The progress of the drying process was monitored with the MB 35 Moisture Analyser (Ohaus Corporation, USA). The remaining water content in dry leaf material was below 1%. In the time between drying process and weighing the bags with dry plant material they were placed in an exsiccator. The fresh and dry weights were determined including tiny and weak plants.

Figure 19: *C. pepo* seedlings (A and B) and their roots (C) primed with a *S. plymuthica* strain, and harvested plant tissue for determination of fresh (D) and dry weight (E).

Figure 20: *L. sativa* seedlings primed with a *S. plymuthica* strain (A) and harvested plant tissue for determination of fresh and dry weight (B).

2.5 Visualisation of bacterial colonisation patterns

2.5.1 Analysis of fluorescence signals with Bio-Rad ChemiDoc™ XRS System

The Bio-Rad ChemiDoc™ XRS System (Bio-Rad Laboratories) detects fluorescence and colorimetric data. The system combines an Universal Hood III, a light-tight enclosure, illumination of UV and white light and a charge coupled device (CCD) camera to capture images in real time. Available lighting settings are UV Trans, Red Epi, Green Epi, Blue Epi, White Epi, Blue Trans and 'no light source'. Possible filter settings are 'standard filter', 695/55, 605/50, 530/28 filter and 'no filter'. The combination of light source and filter settings enables detection of an expected signal. For documentation of fluorescence signals of isolated bacterial strains and plant tissues the Image Lab Software Version 4.0.1 was used. The settings for visualisation of fluorescent proteins GFP, DsRed2 and mNeptune expressed by *S. plymuthica* strains harbouring the pIN69 plasmid are listed in Table 5.

Table 5: Properties of fluorescence proteins expressed by pIN69 plasmid (trimethoprim resistance, 50 µg/ml Tp in medium) in transformed bacterial strains and settings for visualisation by BIO-RAD ChemiDoc™ XRS System (Bio-Rad Laboratories)

Plasmid pIN69			
Fluorescence protein	**GFP**	**DsRed2**	**mNeptune**
Excitation wavelength (nm)	475	563	600
Emission maximum (nm)	505	583	650
Bio-Rad ChemiDoc™ XRS System			
Excitation light	Blue Epi	Green Epi	Red Epi
Emission filter	Filter 4: 530/28	Filter 3: 605/50	Filter 2: 695/55

2.5.2 Visualisation of bacterial colonisation patterns by CLSM

2.5.2.1 Confocal Laser Scanning Microscopy

For visualisation of colonisation patterns of certain transformed *S. plymuthica* strains (see chapter 2.4.1) on Styrian oil pumpkin tissue samples, the TCS SP Confocal Laser Scanning Microscope DM5500Q (Leica Microsystems, Wetzlar, Germany) was used. For the excitation of the samples four lasers of class 3B were available: 635 nm, 488 nm and 532 nm in the visible light range as well as 405 nm in the UV range. The emission filters can be adjusted manually. Eight channels for fluorescence and one for transmitted light microscopy are available. The confocal images were gained with the LeicaACS APO 40x oil CS objective lens (NA, 1.15). Thus images with a 400-fold magnification were generated.

During scanning procedure the light emitted by the laser at a certain excitation wavelength is directed to the sample, which consequently emits light with a higher wave length. This emitted light is detected and the signal is transferred to the computer. The software converts the signals into pixels of a digital image. The generation and processing of images was performed with the software Leica LAS AF version 3.0 (Leica Microsystems CMS).

2.5.2.2 Sample selection and preparation

From greenhouse experiments, i.e. from germination pouches, samples of roots and above ground plant tissues were prepared for CLSM analyses. Also different sections of primed,

germinated seeds, i.e. testa layers and embryo constituents (Figure 21), were examined two and three days post seeding.

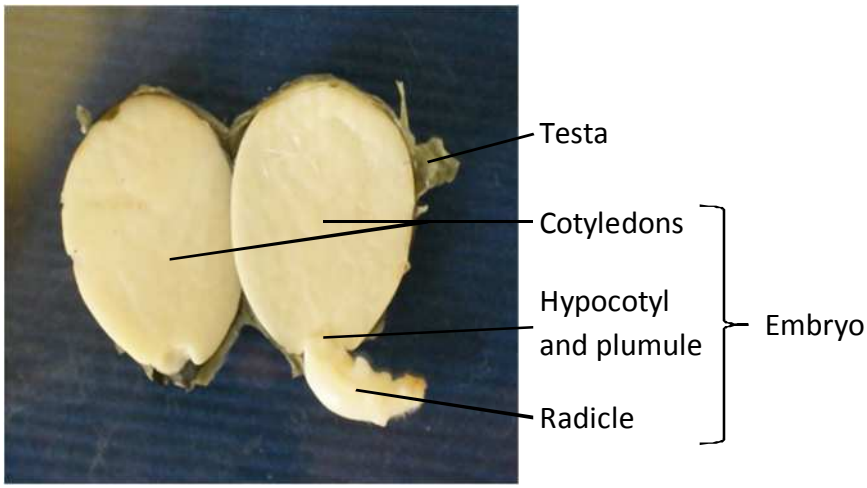

Figure 21: Structure of germinating *C. pepo* seeds.

Thin cuts of the tissues were gained by cutting the seeds with an aseptic scalpel. Afterwards the fragments were transferred to a carefully cleaned microscope slide with a 1.2 mm to 1.5 mm indentation (Thermo Fisher Scientific), 50 µl to 100 µl of 0.85% NaCl were added, the slide covered with a cover glass, air bubbles removed and sealed with nail polish.

For fluorescence microscopy it is important to ensure that certain plant parts, i.e. roots, were grown in the dark. Also during the sample preparation handling they need to be protected from light in order to avoid the formation of autofluorescent substances and to conserve the fluorescence activity.

2.5.2.3 Visualisation of labelled bacterial strains

The transformed bacterial strains on tissues of the Styrian oil pumpkin harboured the pIN69 plasmid and therefore expressed fluorescence proteins which are excitated by the lasers of the microscope and emit light in a certain wavelength spectrum. The excitation and emission wavelengths of the respective proteins are summarised in Table 6.

Table 6: Fluorescence properties of fluorescence proteins expressed by pIN69 plasmid (trimethoprim resistance, 50 µg/ml Tp in medium) in transformed bacterial strains and settings for visualisation by TCS SP Confocal Laser Scanning Microscope DM5500Q (Leica Microsystems, Wetzlar, Germany)

Plasmid pIN69				
Fluorescence protein	**eBFP2**	**GFP**	**DsRed2**	**mNeptune**
Excitation wavelength (nm)	383	475	563	600
Emission maximum (nm)	448	505	583	650
Leica DM5500Q non-confocal				
Filter cube	GFP	GFP	N2.1	N2.1
Leica DM5500Q confocal				
Excitation laser (nm)	405	488	532	532 (635)
Emission filter (nm)	495-546	495-550	580-640	640-685

Visualisation of plant tissue structures was enabled by autofluorescence. Autofluorescence was achieved with the 405 nm excitation laser beam, causing emission of light caught by the filter with a range of 495 to 546 nm.

For illumination of autofluorescent plant structures, S13-GFP and 3Rp8-Dsred2/3Re4-18-mNeptune the optimal offset (processing of background signal) was adjusted in a range of -3 to 0, -15 to -1 and -9 to -1 respectively. The gain (signal multiplication by photomultiplier) settings ranged from 880 to 1250, 703 to 980 and 800 to 1107. Excitation lasers of wavelengths 405 nm, 488 nm and 532 nm were used with an intensity of 29.2% to 56.6%, 36.3% to 50.9% and 25.5% to 84.4%, respectively.

Z-stacks comprising 10 to 104 confocal planes were acquired within Z-steps of 0.7 to 1 µm. Maximum Z-projection images of confocal stacks were established for gaining maximal possible information.

The fluorescence protein mNeptune was also visible when using the settings for the visualisation of DsRed2. Consequently, 3Rp8-DsRed2 and 3Re4-18-mNeptune were not distinguishable on tissue surfaces of *C. pepo* plants inoculated with a mixture of those strains. Visualisation of 4Rx13-eBFP2 did not work either since autofluorescence of plant tissues was surveyed with the same wavelength settings.

3 Results

3.1 Analysis of plant-microbe interactions

In the subsequent sections of this chapter preparatory results of microbial community analysis by means of an amplicon sequencing strategy – ranging from DNA extraction to sequencing – are summarised.

3.1.1 DNA extraction

The isolated DNA of seed and rhizosphere samples was checked using UV-VIS spectrophotometric technology. The quality of extracted DNA was sufficient. Most of the 260/280 ratios were between 1.8 and 2.0. Deviating ratios are due to contaminants like proteins and phenols. The concentrations ranged from 27.0 ng/µl to 344.1 ng/µl. But due to interfering agents, the concentrations are quantitative not reliable and only quality measures.

To validate the results of the spectrophotometric measurements certain samples with low (09rb, 02rd, 04rd) and high (03rd, 07rd, 05ra, 05sa, 01sb) DNA concentrations were checked by agarose gel electrophoresis (Figure 22). The agarose gel showed an acceptable isolation result in all cases, meaning quite intense smears along the lanes caused by isolated DNA. Apparently, the isolated DNA of rhizosphere samples was different from that of seed samples, as the smears differed in the respective lines.

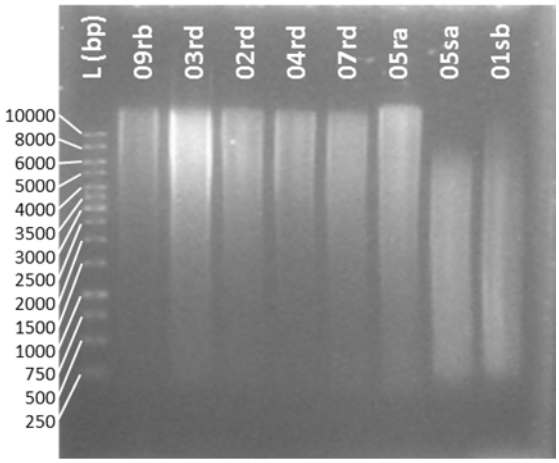

Figure 22: Agarose gel (0.8% agarose in 1 x TAE buffer, stained with ethidium bromide) to check the quality of the DNA isolation products of rhizosphere (09rb, 03rd, 02rd, 04rd, 07rd, 05ra) and seed samples (05sa, 01sb) of *C. pepo*. 2 µl of GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific 2016) and 3 µl DNA isolation product mixed with 1.5 µl loading dye were applied. The electrophoresis was performed at 100 Volt for 50 minutes.

Test PCRs with selected samples and subsequent agarose gel electrophoresis showed quite accurate bands and no smears. Therefore purification was not necessary after DNA isolation in advance to PCR amplification for sequencing.

3.1.1 Amplicon sequencing

3.1.1.1 16S rRNA gene amplification

The resulting PCR products of 16S rRNA gene amplification for sequencing were examined by electrophoresis. An exemplary agarose control gel is depicted in Figure 23. The expected size of the band of the amplification product was in the range of 300 bp. The bands of appropriate template dilutions were quite precise without considerable smears or primer amplifications. A smear along the lane could be caused by a too high template amount or by interfering agents (samples 01rc and 02rd). If the used amount of template DNA was too low, no band was visible, but unspecific primer amplification products appeared (sample 02ra).

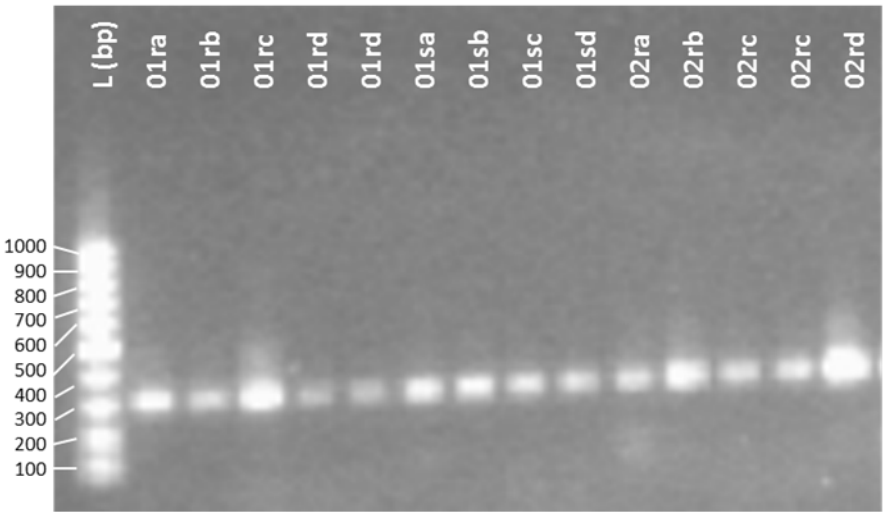

Figure 23: Agarose gel (1% agarose in 0.5 x TBE buffer, stained with ethidium bromide) to check the quality of the 16S rRNA gene amplification products of rhizosphere and seed samples of *C. pepo*. 2 µl of GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific 2015) and 3 µl PCR product mixed with 2.5 µl loading dye were applied. The electrophoresis was performed at 100 Volt for 30 minutes followed by 120 Volt for 25 minutes.

The purified amplicons were checked by UV-VIS spectrophotometry. The 260/280 ratio was between 1.82 and 1.90. The nucleic acid concentration was between 72.5 and 154.4 ng/µl. Both, the quality and quantity of the amplicon samples were appropriate for subsequent sequencing.

3.1.1.2 ITS amplification

PCR amplification products of the fungal ITS-1 region were checked by electrophoresis and UV-VIS spectrophotometry. The quality and quantity of purified PCR amplicons were appropriate for subsequent sequencing. The 260/280 ratio was between 1.87 and 1.89 and the nucleic acid concentration between 73.2 and 110.8 ng/µl.

3.2 Analysis of plant-bacteria interactions

As described in the introductory chapter 1, the agronomic importance of the Styrian oil pumpkin in Austria, the high susceptibility to a range of pathogens and the unstable and yet not fully clear responses to biological control agents forced a wide-ranging microbiome analysis. These analyses revealed a high diversity in different plant-associated habitats and also cultigen-specific interactions of plants and bacteria. The results are described and depicted in the subsequent sections.

3.2.1 Bacterial communities associated with seeds, rhizosphere and soil

The analysed seed, rhizosphere and bulk soil microbiomes were characterised by a diverse, but very different taxonomic composition. The proportional structure of the plant-associated bacterial communities at phylum level is depicted in Figure 24. Proteobacteria predominated seed (83%), rhizosphere (41%) and soil (24%) microbiomes and considerable proportions of the phyla Firmicutes (11%, 8% and 6%) and Actinobacteria (2%, 17% and 15%) were found in all habitats. Thaumarchaeota belonging to Archaea as well as Acidobacteria, Bacteroidetes, Chloroflexi, Nitrospirae, Gemmatimonadetes, Planctomycetes and Verrucomicrobia contributed to the microbiomes of the rhizosphere and soil and only to minor degree to the seed microbiome.

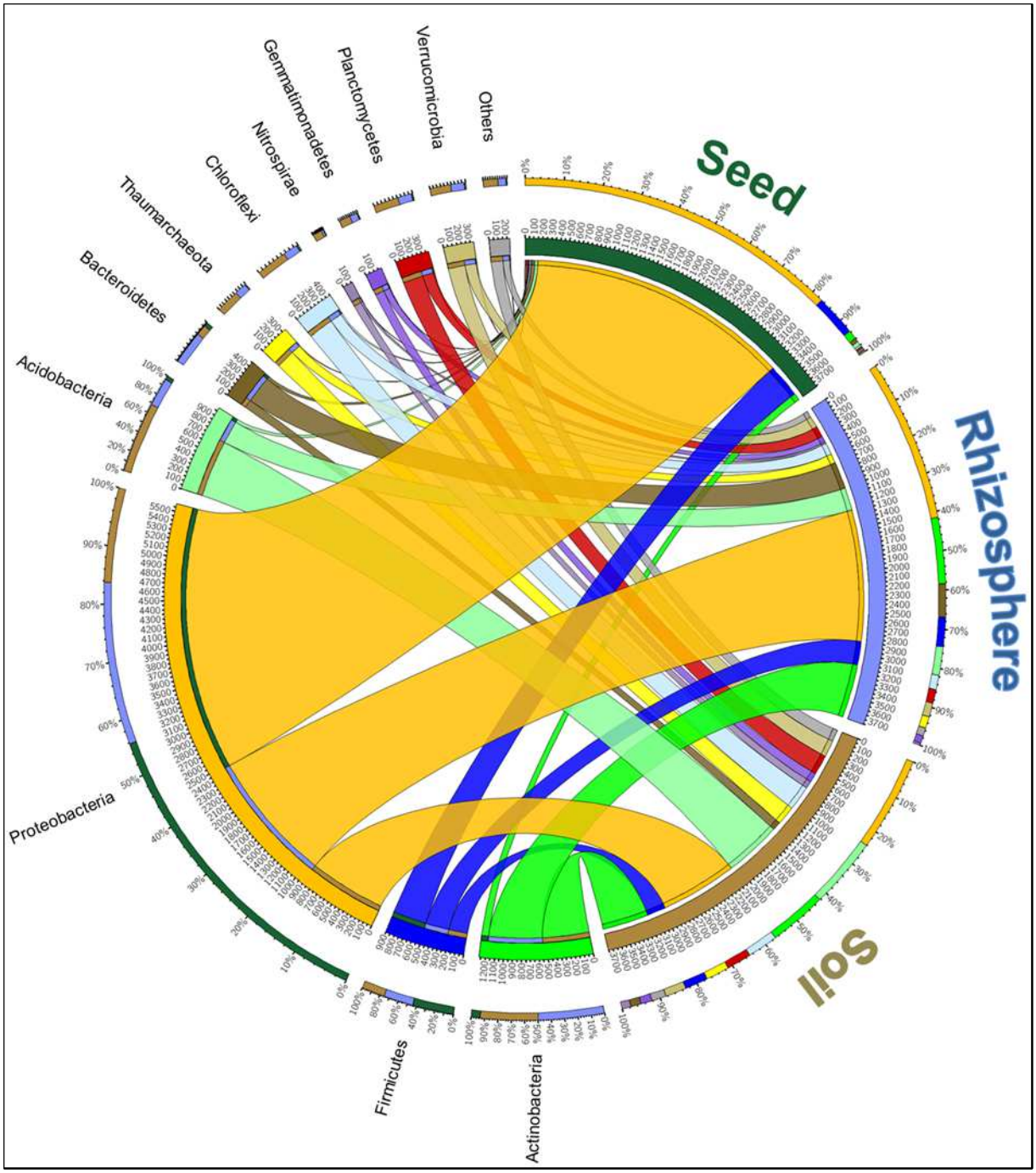

Figure 24: Proportional structure of bacterial communities at phylum level associated with rhizosphere and seeds of *C. pepo* genotypes as well as soil (outer circle). Taxa with a proportion lower than 1.5% in all three habitats are summarised as 'Others'. Values within the inner circle indicate the number of reads of a phylum within the normalised dataset (Adam et al. 2016b).

OTUs representing the core microbiome were calculated separately for each of the habitats seed, rhizosphere and soil and then summarised (Figure 25). Differences in the relative abundances of those OTUs between the core microbiomes exist for several Nitrososphaeria, Acidobacteria, Chloracidobacteria and Chloroflexi OTUs, which occurred in a higher proportion in the soil microbiome compared to the rhizosphere and seed microbiomes, whereas several Bacilli, Actinobacteria, Saprospirae, Alpha-, Beta- and parts of the Gammaproteobacteria OTUs occurred to a greater extent in the rhizosphere microbiome in comparison to the soil and seed microbiomes. The seed core microbiome was dominated by high abundances of eight Gammaproteobacteria (seven Enterobacteriaceae and one Pseudomonadaceae) and two Bacilli (one *Lactococcus* and one *Exiguobacterium*) OTUs.

Since the genus of *Serratia*, which belongs to the Enterobacteriaceae family, includes potential biocontrol agents (BCAs) for the cultivation of the Styrian oil pumpkin, these abundances were analysed in detail. This genus was detected in extremely minor abundances in 16 out of 56 seed samples ($7.14*10^{-5}$%) and in 6 out of 56 rhizosphere ($1.96*10^{-5}$%) samples. In contrast, in bulk soil samples the genus of *Serratia* was not detected.

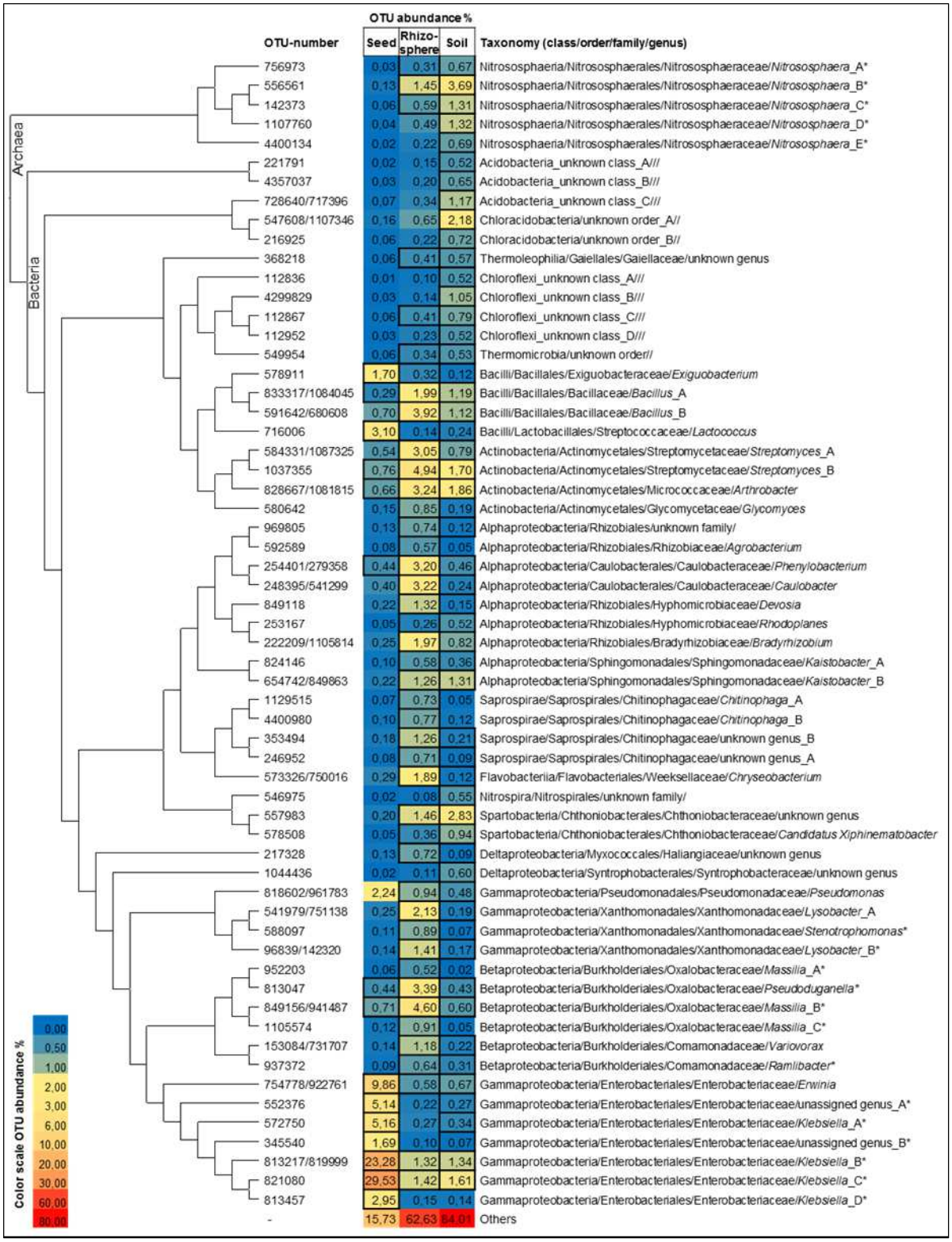

Figure 25: Core microbial communities represented by 16S rRNA gene sequences in the seeds and rhizosphere of *C. pepo* genotypes as well as in the bulk soil. Relative abundance values of taxa belonging to the core of a habitat are framed, while other abundance values are not belonging to the core in the respective habitat. OTUs with abundance lower than 0.5% in all core microbiomes are summarised as 'Others'. Taxa marked with asterisks were complemented with additional taxonomic information from NCBI database, while the other denominations were derived from Greengenes database. The phylogenetic tree using the representative sequences of the OTUs was calculated with the NCBI tree method fast minimum evolution (maximum sequence difference 0.75) and illustrated in MEGA7 (Adam et al. 2016b).

3.2.2 OTU distribution and diversity analyses

The comparison of the seed and rhizosphere core microbiomes with the bulk soil microbiome shows that seeds and rhizosphere shared only 10.5% of the total OTUs including OTUs from soil, whereas the rhizosphere and the bulk soil shared 32.6% of OTUs (Figure 26 A). Within the rhizosphere OTUs 16.8% were conserved in seeds and soil as well, 4.5% were derived solely from seeds and 49.6% solely from soil. Apparently, the seed microbiome has a smaller influence on the rhizosphere communities than the soil microbiome. 29.0% of the rhizosphere OTUs were unique. Some of those OTUs could have been derived from the seed testa, but were removed due to the washing and soaking procedure for seed microbiome analysis. This probably released a number of microorganisms to the washing suspension. Another source of inoculum could have been rare soil bacteria that were below the detection level in the soil microbiome analysis but have been enriched in the rhizosphere due to the rhizosphere effect. A detailed analysis of the contribution of Enterobacteriaceae to the communities revealed that major proportions of those OTUs observed were unique for seeds, whereas in rhizosphere and soil no unique Enterobacteriaceae were detected (Figure 26 B). The same trends were observed when the analyses were calculated on *C. pepo* genotype level: the family of Enterobacteriaceae was strongly associated with the seed as habitat.

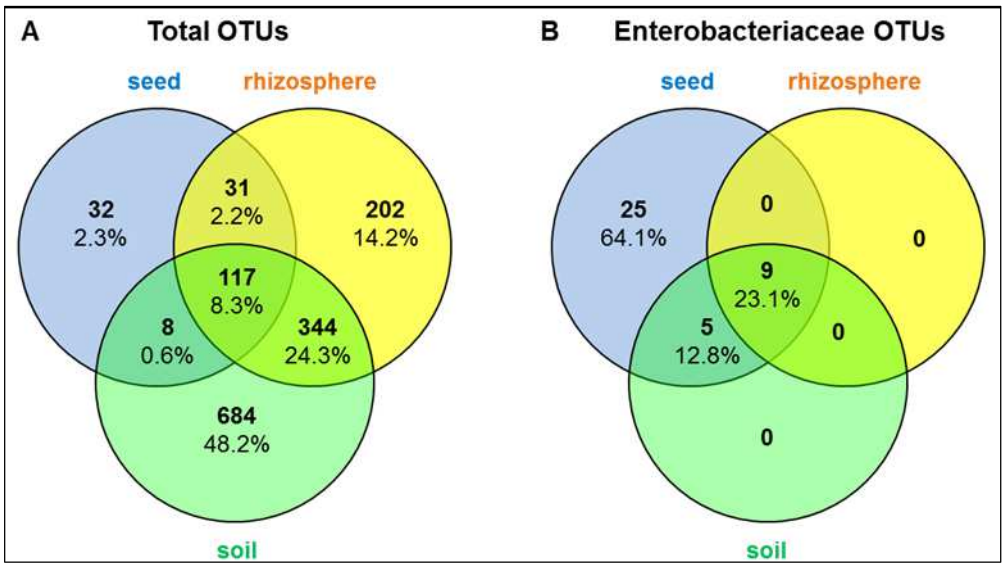

Figure 26: OTU distribution within the habitats: Total OTUs (A) and Enterobacteriaceae OTUs (B) in seed and rhizosphere of *C. pepo* genotypes as well as in bulk soil. Fraction of samples that OTU was observed in to be considered as 'core': 50% (Adam et al. 2016b).

A considerable fraction of bacterial diversity was covered in the analysed microhabitats according to rarefaction analysis (graph of diversity vs. sequencing depth) of sample origin with observed OTUs as rarefaction measure (Figure 27). The rarefaction curve of seed samples showed a flat slope, no saturation yet, but indicated that greater sequencing depth wouldn´t result in the detection of considerable more OTUs. The slope of the rarefaction curve of rhizosphere samples was steeper, but still sufficient. The curve of the soil samples was even steeper, indicating that more OTUs could have been observed upon deeper sequencing.

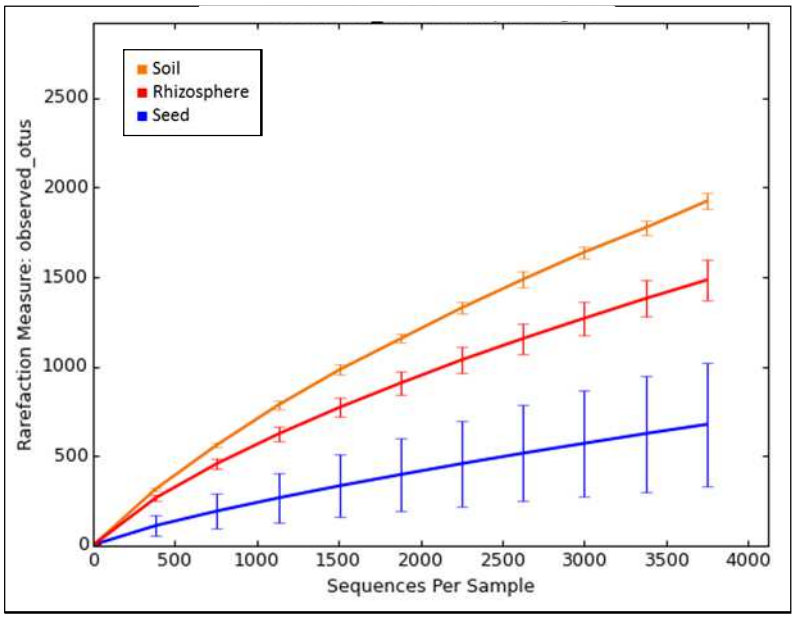

Figure 27: Alpha-diversity analysis of rhizosphere, seed and soil samples of *C. pepo* cultivars: Rarefaction curves of observed OTUs.

Alpha diversity measures Chao1 and Shannon indices revealed that the species richness in the rhizosphere was significantly higher than in the seeds (calculated with the QIIME script alpha_diversity.py; paired t-test, significance level $\alpha = 0.01$, p-values = 0.003), whereas the richness in the soil was significantly higher than in the rhizosphere. The Heip index (calculated with the QIIME script alpha_diversity.py) indicated that the evenness in the seeds was considerably lower than in the rhizosphere and soil, meaning that the relative abundance of taxa was not evenly distributed in seeds. The seed microbiomes of the genotypes Line E, Gl. Diamant and Line G showed a higher alpha diversity (Shannon diversity index H´ of 8.6, 7.9 and 7.1, respectively and Heip evenness index E´ of 0.29, 0.17 and 0.16, respectively) than the other investigated genotypes. In contrast to the rhizosphere,

differences in alpha diversity among the seeds of several genotypes were significant (Table 7).

Table 7: Bacterial species richness, evenness and coverage in rhizosphere (R) and seeds (S) of different *C. pepo* genotypes and of soil at an even sequence depth of 3758 (Adam et al. 2016b)

Denomination	Shannon diversity index (H´)[1]		Heip evenness index (E´1:0)[2]		Chao1 diversity index (OTU no.)[3]		Observed OTUs		Coverage[4]	
	R	S	R	S	R	S	R	S	R	S
Line A	9.6	3.7[a]	0.48	0.04[a,b]	4,634	1,326[a]	1,592	334[a]	0.34	0.25[a]
Line B	9.3	4.1[a,b]	0.44	0.03[a]	3,971	2,081[a,b]	1,472	468[a,b]	0.37	0.22[a]
Line C	9.5	4.6[a,b]	0.47	0.04[a,b]	4,168	2,650[a,b]	1,578	565[a,b]	0.38	0.21[a]
Line D	9.4	4.9[a,b]	0.45	0.05[a,b]	4,369	2,936[a,b]	1,534	613[a,b]	0.35	0.21[a]
Gl. Diamant	9.5	7.9[b]	0.46	0.17[a,b]	4,819	5,651[b]	1,596	1,399[b]	0.33	0.25[a]
GL Opal	9.3	5.1[a,b]	0.42	0.06[a,b]	4,772	2,017[a,b]	1,499	549[a,b]	0.31	0.27[a]
GL Rustikal	9.5	4.4[a,b]	0.46	0.05[a,b]	4,642	1,646[a,b]	1,543	412[a,b]	0.33	0.25[a]
GL Classic	9.0	4.4[a,b]	0.39	0.05[a,b]	3,823	1,602[a,b]	1,362	431[a,b]	0.36	0.27[a]
Naxos	9.3	4.5[a,b]	0.43	0.04[a,b]	4,105	3,144[a,b]	1,444	561[a,b]	0.35	0.18[a]
Line E	9.1	8.6[b]	0.41	0.29[b]	3,704	4,019[a,b]	1,395	1,324[b]	0.38	0.33[a]
Line F	9.3	4.7[a,b]	0.44	0.05[a,b]	3,833	1,985[a,b]	1,448	481[a,b]	0.38	0.24[a]
Line G	9.4	7.1[a,b]	0.46	0.16[a,b]	4,229	3,821[a,b]	1,509	1,124[b]	0.36	0.29[a]
Line H	9.1	4.7[a,b]	0.40	0.05[a,b]	3,889	1,858[a,b]	1,366	498[a,b]	0.35	0.27[a]
Line I	9.3	5.9[a,b]	0.43	0.08[a,b]	4,015	2,573[a,b]	1,450	739[a,b]	0.36	0.29[a]
Average	9.3	5.3[a,b]	0.44	0.08[a,b]	4,212	2,665[a,b]	1,485	678[a,b]	0.35	0.25[a]
Soil	10.2		0.61		5,116		1,900		0.37	

1 estimation of species diversity (a higher number indicates a higher diversity); 2 distribution of individuals over OTUs (tends to 0 as the evenness decreases in species-poor communities, tends to 1 as the individuals are increasingly distributed equally in communities); 3 nonparametric richness estimator; 4 ratio of observed OTU number to estimated OTU number; a, b different alphabetic characters indicate statistical significant differences

3.2.3 Genotype-specific colonisation patterns of seeds

The microbial seed communities of all *C. pepo* genotypes were dominated by Proteobacteria with 83% on average, whereof 64% belonged to the family of Enterobacteriaceae. Genotype-specific colonisation patterns of the seeds were evident in the open-pollinated cultivar GL Classic and in the inbred Line D (Figure 28). In those genotypes, the genus *Erwinia* was part of the microbiome with a relative abundance of 38% and 33% respectively. A detailed analysis of the sequences clustered in *Erwinia* genus revealed that they contained sequences of the important pathogen *Pectobacterium carotovorum* (syn. *Erwinia carotovora*). The genus *Pseudomonas* was present in all genotypes but was enriched in four out of six cultivars bred in other countries than Austria. Firmicutes were enriched in the two three-way cross

hybrids GL Opal (19%) and GL Rustikal (36%), as well as in the two inbred Lines B and D and the segregating Line F. Within the Firmicutes, the genus *Lactococcus* was more abundant in four out of eight cultivars bred in the province of Styria (Austria) and only low abundant in cultivars bred in other countries. The genus Acinetobacter was more prevalent in the segregating lines, especially in Line H with 11%. Actinobacteria were observed to a higher extent in Line E, Line G and Gl. Diamant. These three genotypes had similar communities which were more diverse than those of the other genotypes, congruent with the results of the calculated diversity indices.

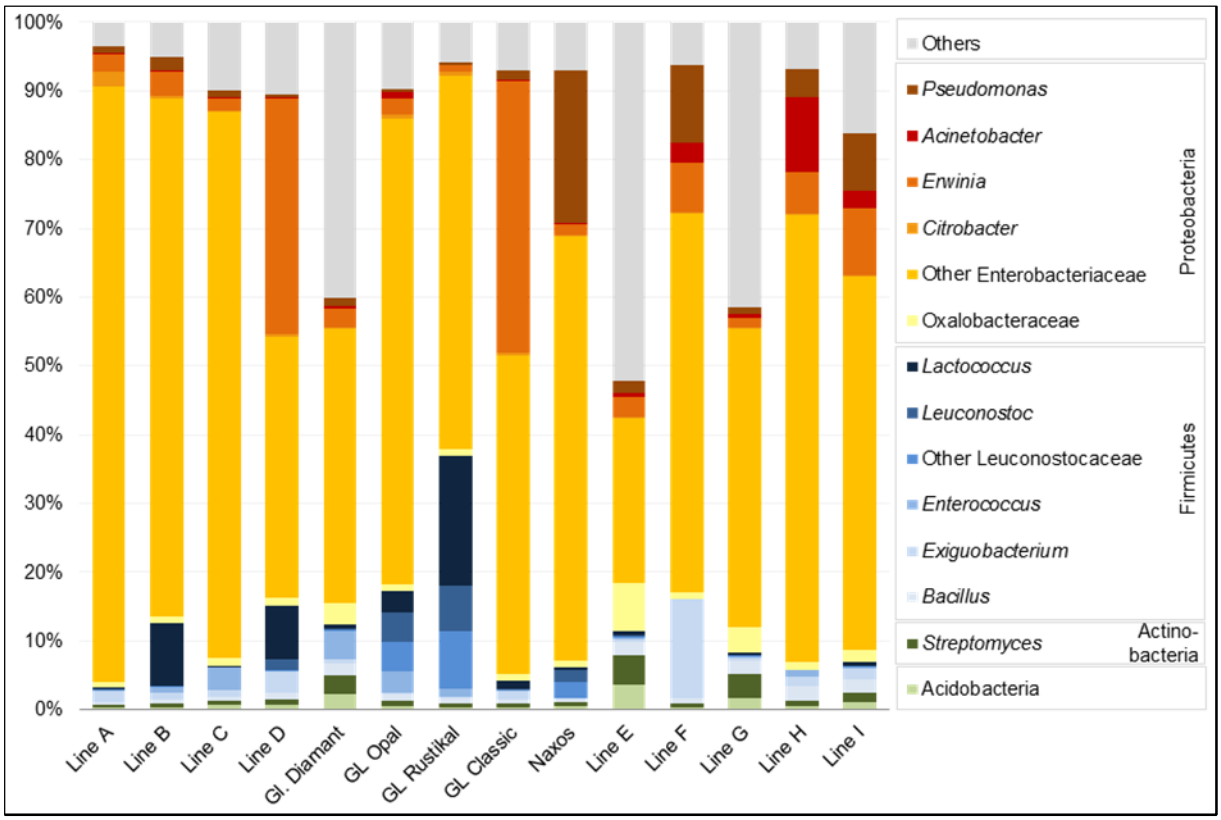

Figure 28: Structure of bacterial taxa within seeds of *C. pepo* genotypes with a proportion higher than 2% in at least one genotype. Taxa with lower proportions are summarised as 'Others' (Adam et al. 2016b).

No specific patterns concerning the field site origin of studied seeds were observed. According to the nonparametric analysis of similarities (ANOSIM) the genotype (R = 0.527, p-value = 0.001) has greater influence on the bacterial community composition than the field origin of the seeds (R = 0.181, p-value = 0.008). The principal coordinate analysis also showed that seed samples are clustering more due to their respective genotypes, than because of field site origin of seeds, as illustrated in Figure 29.

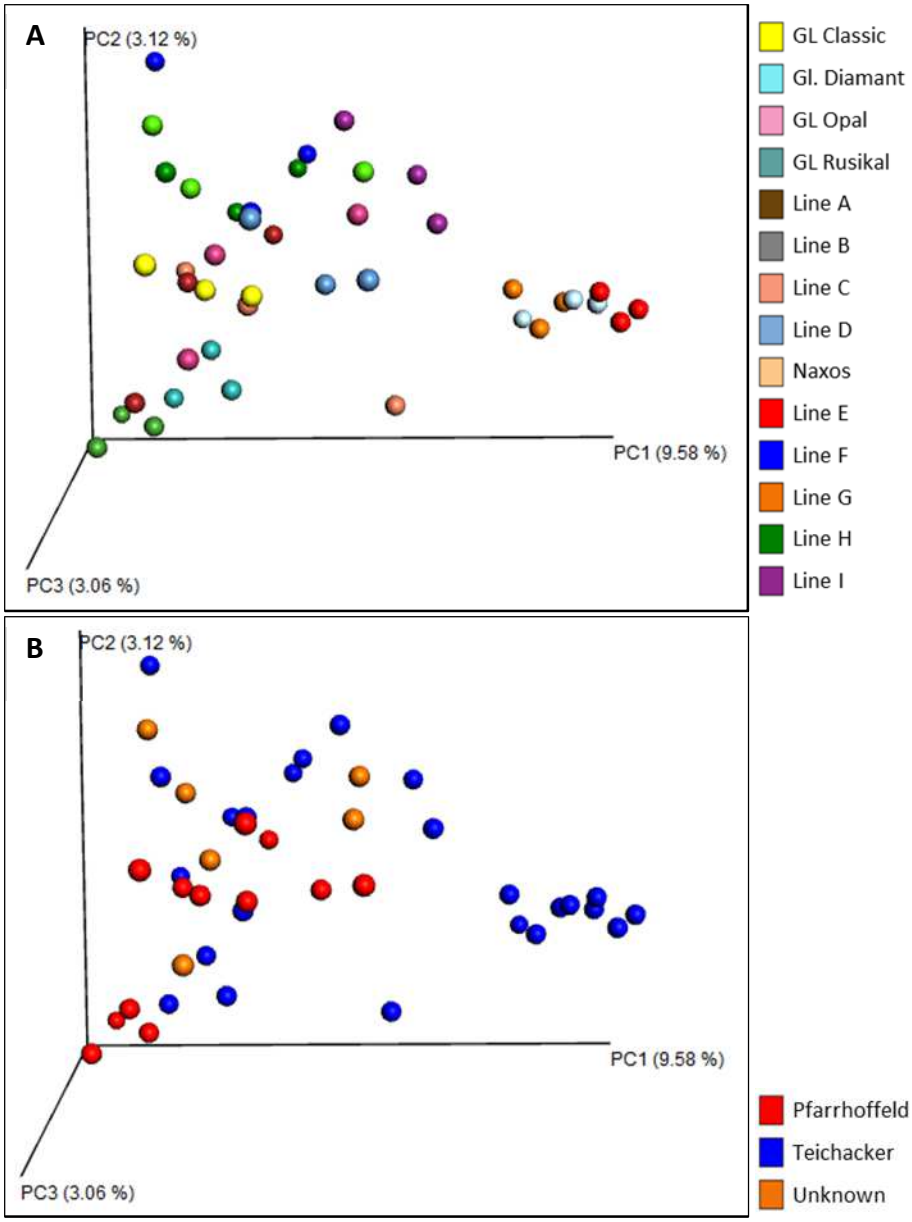

Figure 29: Unweighted UniFrac-based 3D PCoA plot of bacterial OTUs detected in seed microbiomes of *C. pepo* genotypes, coloured according to *C. pepo* genotype (A) and field origin of seeds (B). Axes are not drawn to scale.

While only 21 OTUs showed significantly different abundances among the rhizospheres (Kruskal-Wallis test, $\alpha = 0.01$), 121 OTUs differed significantly among the seed microbiomes of the 14 genotypes. Six OTUs showed significantly different degrees of abundance in both habitats. These OTUs were assigned to the taxa of *Exiguobacterium*, Chthoniobacteraceae, Nitrospirales, Xanthomonadaceae and *Bacillus*, according to the Greengenes database. In order to visualise the beta diversity and the relationships of seed-associated bacterial taxa with significant different abundances among the genotypes, a non-metric multidimensional scaling (NMDS) analysis of the 14 *C. pepo* genotypes was performed based on a community-by-species matrix comprising of 121 significantly different OTUs (Figure 30).

53 of the 121 different abundant seed-associated OTUs referred to the phylum of Proteobacteria, wherein twelve belonged to the family of Enterobacteriaceae, of which three were further assigned to the genus *Erwinia*. Higher abundances of *Pseudomonas viridiflava* were observed in the seed microbiomes of Line F and Line I as well as in the GL Classic. Furthermore, the abundances of *Lysobacter* and *Paenibacillus* were significantly different among the seed microbiomes of the genotypes investigated. Within this analysis, the phylum Firmicutes comprised of 23 OTUs, wherein eleven OTUs were assigned to the family of Leuconostocaceae and four to Paenibacillaceae. A large proportion of Leuconostocaceae is located at similar coordinates as GL Rustikal and GL Opal within the NMDS plot. A weak clustering is distinguishable for the Lines A, B, C, D, Gl. Diamant, GL Classic, GL Opal and GL Rustikal with the geographic origin in Austria. The three cultivars with a highly diverse seed microbiome, Gl. Diamant, Line E and Line G, together with Line I, are located at similar coordinates as the group of 'Other' bacterial taxa with significantly different abundances. According to the Shannon diversity index shown in Table 7, Line I is the fourth diverse *C. pepo* cultivar.

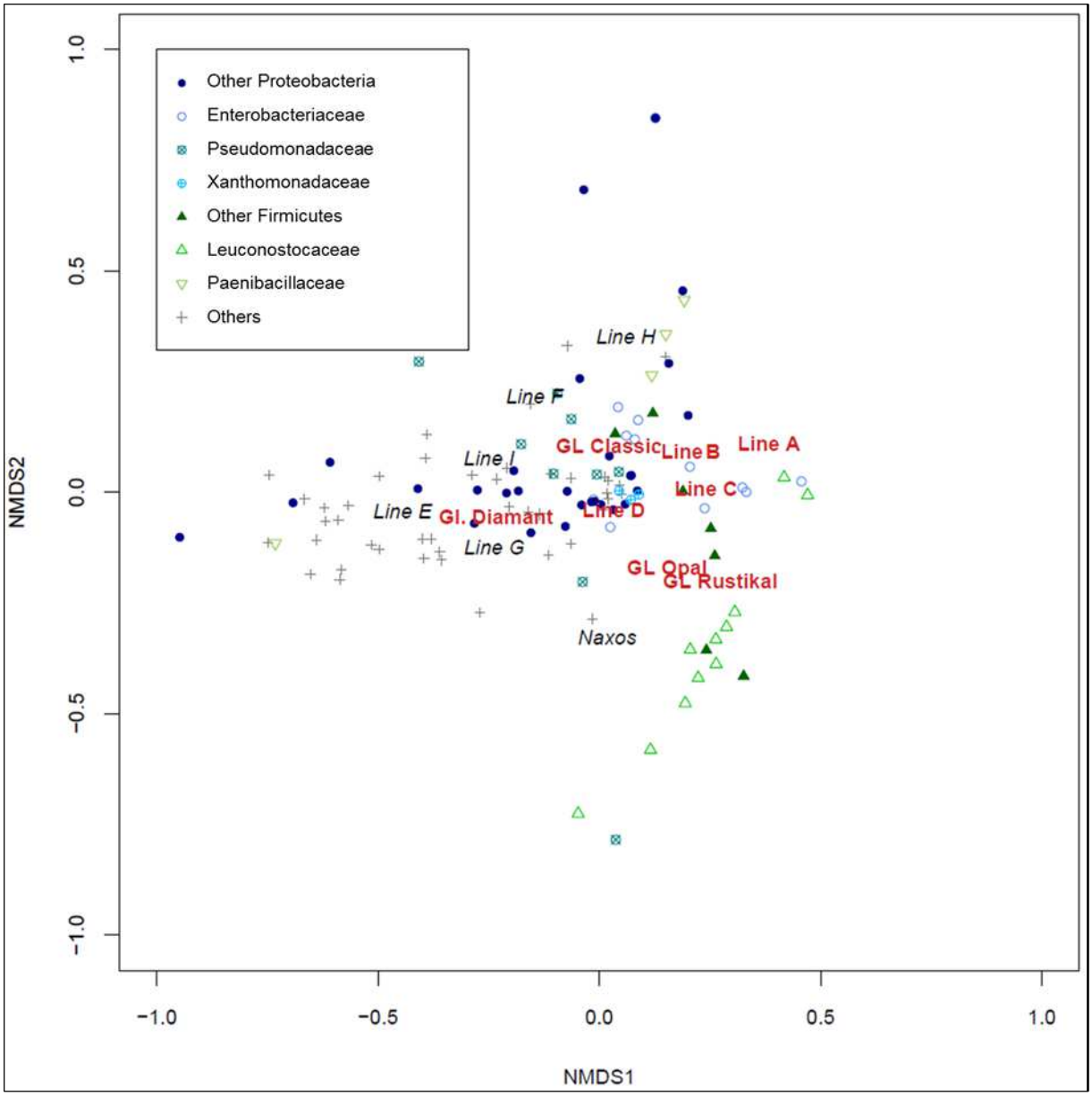

Figure 30: Non-metric multidimensional scaling of 121 significantly different bacterial OTUs in seed microbiomes of 14 *C. pepo* genotypes. Genotypes bred in Austria are written in bold, genotypes bred in the Netherlands (Naxos), Germany (Line E), Slovenia (Line F and G) or China (Line H and I) in italics. The stress (i.e. the discrepancy between 2D configuration and predicted values from regression) was 0.159 (Adam et al. 2016b).

3.2.4 Seed microbiomes of agronomically important cultivars and of GL Rustikal pedigree

When comparing the three agronomically most important cultivars GL Classic, GL Rustikal and GL Opal with the most important component in the hybrid seed production, Gl. Diamant, 14% of the OTUs comprised the core microbiome (Figure 31 A). Gl. Diamant seeds were colonised with 90 unique OTUs compared to the other three genotypes

investigated. Concerning the Enterobacteriaceae (Figure 31 B), 13 of the observed OTUs were shared within all genotypes investigated, 13 OTUs were unique to GL Opal, three to GL Rustikal and five to GL Classic. Gl. Diamant seeds did not harbour unique Enterobacteriaceae OTUs. Three *Erwinia* OTUs were common among all four genotypes, one and three were unique in GL Opal and GL Classic and none were exclusively observed in GL Rustikal and Gl. Diamant.

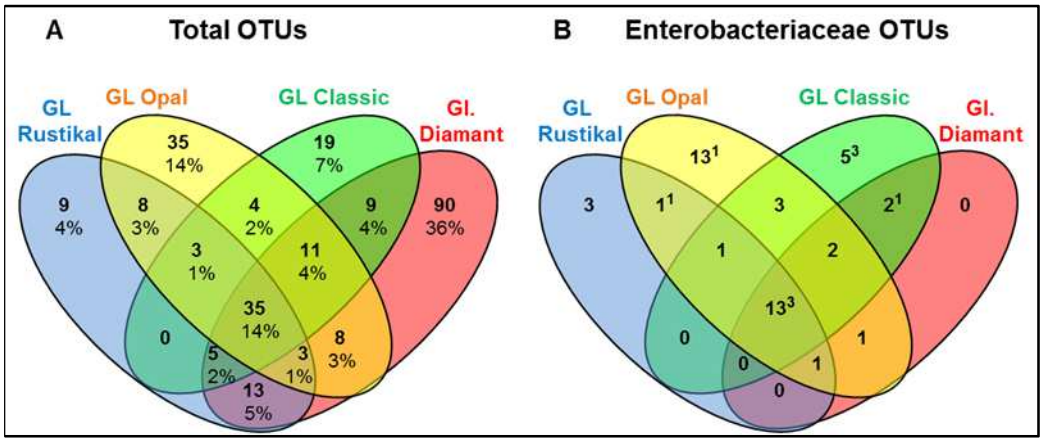

Figure 31: OTU distribution within the seed microbiomes of total OTUs (A) and Enterobacteriaceae OTUs (B) of the three-way cross hybrids GL Rustikal and GL Opal, the single cross hybrid Gl. Diamant and the open-pollinated cultivar GL Classic. OTU numbers of the genus *Erwinia* are in superscript. Fraction of samples that OTU was observed in to be considered as 'core': 100% (Adam et al. 2016b).

To visualise the relationships of seed-associated bacterial taxa among the different genotypes a taxonomic interaction network (illustrated in Figure 32 A) of the three-way cross hybrid GL Rustikal and its pedigree components (relationships shown in Figure 32 B) was created. The seed microbiome of the highly diverse Gl. Diamant was comprised of 117 taxa of which 65 were unique, whereas the seed microbiomes of the other genotypes showed no (Line A, Line D, GL Rustikal) or just one unique taxa (Line B). The family of Enterobacteriaceae dominated the seed associated communities of GL Rustikal pedigree.

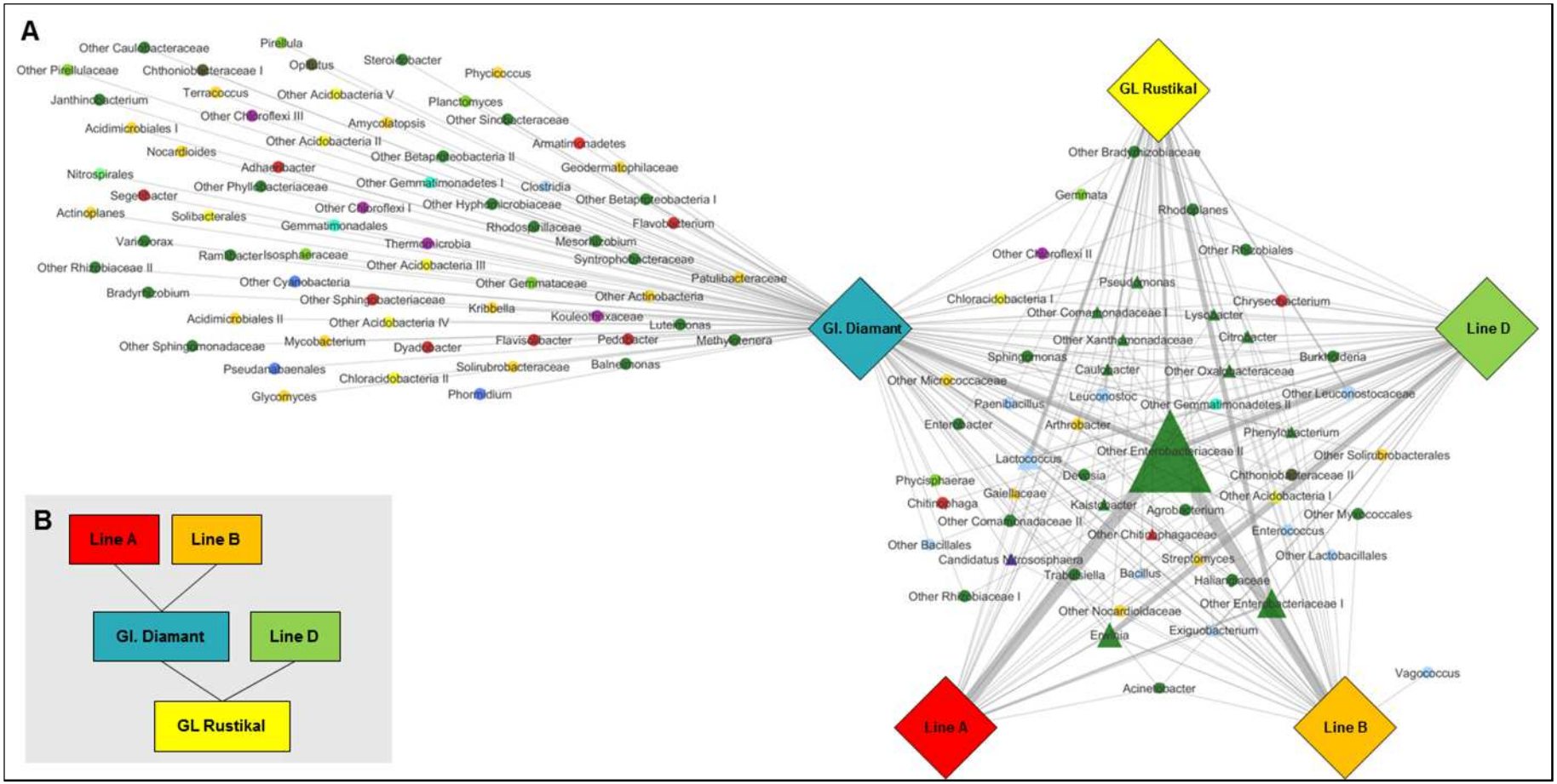

Figure 32: Taxonomic interaction network at genus level: Seed associated bacterial communities of GL Rustikal pedigree (A) and the pedigree component relationships (B). The outer squares in the network illustrate the *C. pepo* genotypes. Seed-associated bacterial taxa with a relative abundance of at least 0.1% are connected with the corresponding genotype by a grey line. The line width correlates with the relative abundance of each taxon connected with the respective genotype. The size of the shape next to the bacterial taxa corresponds to the mean of the relative abundance of all 118 bacterial taxa analysed in this network. The 20 taxa belonging to the core microbiome are shown as triangles. Taxa that are shared by four, three or two genotypes, or that are unique in a genotype are illustrated as circles. Taxa of the same phylum are depicted in the same shape colour. The proportions are not drawn to scale (Adam et al. 2016b).

The analysis of OTU distribution within the GL Rustikal pedigree components (Figure 33) revealed that Gl. Diamant and Line D as parental components shared 22% and 20% of OTUs with GL Rustikal, whereas the genetically more distant components Gl. Diamant and Line D shared 29%. The core microbiome of all five genotypes investigated was comprised of 14% of the OTUs. 14 (30%) of the observed Enterobacteriaceae OTUs, including three *Erwinia* OTUs, were shared within all five genotypes, which could indicate an essential function or that the inheritance of the microbiome is focused on certain taxa. There may be a connection of the higher bacterial diversity in Gl. Diamant with the displacement and competition of Enterobacteriaceae in its seeds.

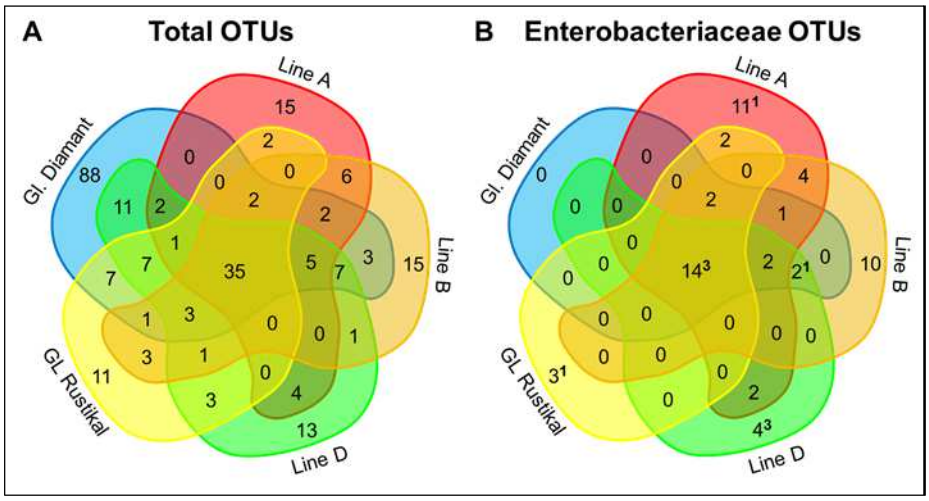

Figure 33: Distribution of total OTUs (A) within the seed microbiomes of the three-way cross hybrid GL Rustikal and its pedigree components and of Enterobacteriaceae OTUs (B). OTU numbers of the genus *Erwinia* are in superscript. Fraction of samples that OTU was observed in to be considered 'core': 100% (Adam et al. 2016b).

The heatmap illustrated in Figure 34 indicates that OTUs of the Enterobacteriaceae family mainly occur in seeds and less in rhizosphere and soil samples. A Blast analysis against the NCBI nucleotide database for Enterobacteriaceae OTUs with an observation count of more than 10 sequences per OTU of the GL Rustikal pedigree component's seed and rhizosphere samples revealed that the 16S rRNA gene sequences of most of the 54 Enterobacteriaceae OTUs had the highest similarity to *Klebsiella* sp. Other OTUs were assigned to *Pantoea* sp., *Salmonella* sp., *Enterobacter* sp., *Trabulsiella* sp., *Yersinia* sp., *Erwinia* sp., *Kluyvera* sp. and *Cedecea* sp. *Pectobacterium carotovorum* was part of the seed microbiome of all genotypes, except GL Rustikal, but was not detected in rhizosphere and bulk soil. This fact could be an indication for seed transmission of this pathogenic bacterium.

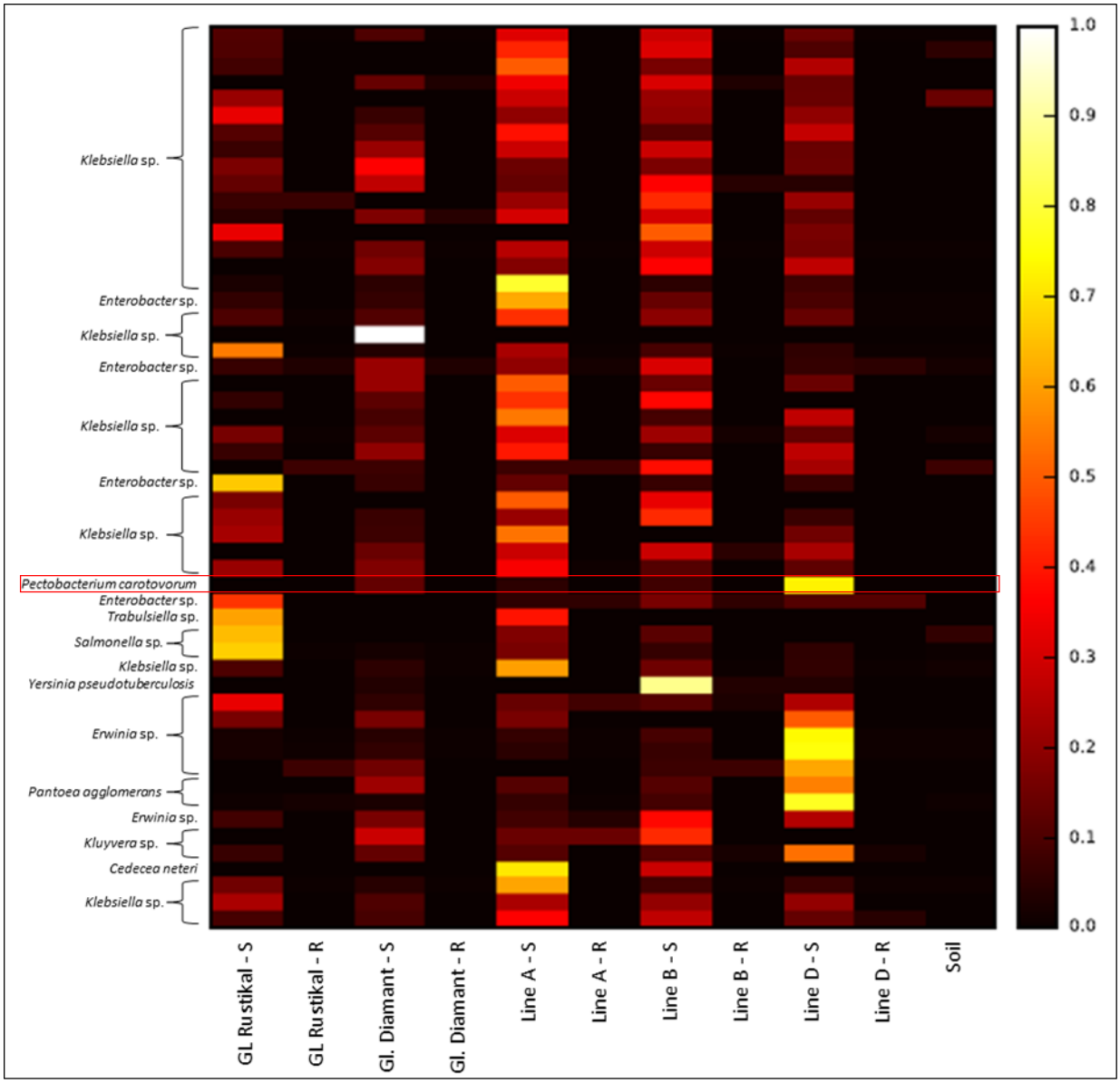

Figure 34: Heatmap of GL Rustikal pedigree components´ seeds (S) and rhizosphere (R) as well as soil Enterobacteriaceae OTUs. OTUs on species level with an observation count ≥10 sequences were analysed. Representative sequences of OTUs were assigned to taxonomy based on a Blast analysis against the NCBI nucleotide database.

3.3 Analysis of plant-fungi interactions

Based on the encouraging results of the bacterial microbiome analysis, which revealed cultivar-specific bacterial communities in seeds and the dominance of Proteobacteria, and therein especially members of Enterobacteriaceae family, which comprises also potential pathogens like *Erwinia* sp., a wide-ranging analysis of the *C. pepo*-associated mycobiome is also intended to be performed. Also fungal pathogens like *Didymella* sp. are potential causal agents of severe yield losses. Prior to this comprehensive analysis of a multitude of different cultigens, the fungal microbiome of only two rhizosphere and two seed samples were analysed in order to get a first insight into community constitution and to decide subsequently if it is worth to analyse the fungal microbiome of all samples. Samples of Line B and GL Rustikal were selected, because the amplification of 16S rRNA gene worked well. Next the fungal diversity of analysed samples is characterised.

3.3.1 Fungal diversity in rhizosphere and seeds of *C. pepo* genotypes

The fungal diversity on phylum level of two rhizosphere and two seed samples of GL Rustikal and Line B determined upon ITS gene amplicon sequencing is depicted in Figure 35. The rhizosphere and seed samples showed a similar pattern of colonisation among them. The rhizosphere-associated fungal community was dominated by Ascomycota (56.5% on average), followed by Zygomycota (16.6%), Chytridiomycota (8.6%) and Basidiomycota (7.9%). Sequences of so called unidentified Plantae were of minor importance (1.3%). The seed microbiome was quite different. Unidentified Plantae dominated both samples; 93.6% in the case of Line B and 91.0% of GL Rustikal. The proportion of fungal sequences was minor, but Ascomycota (3.1% on average) dominated as well.

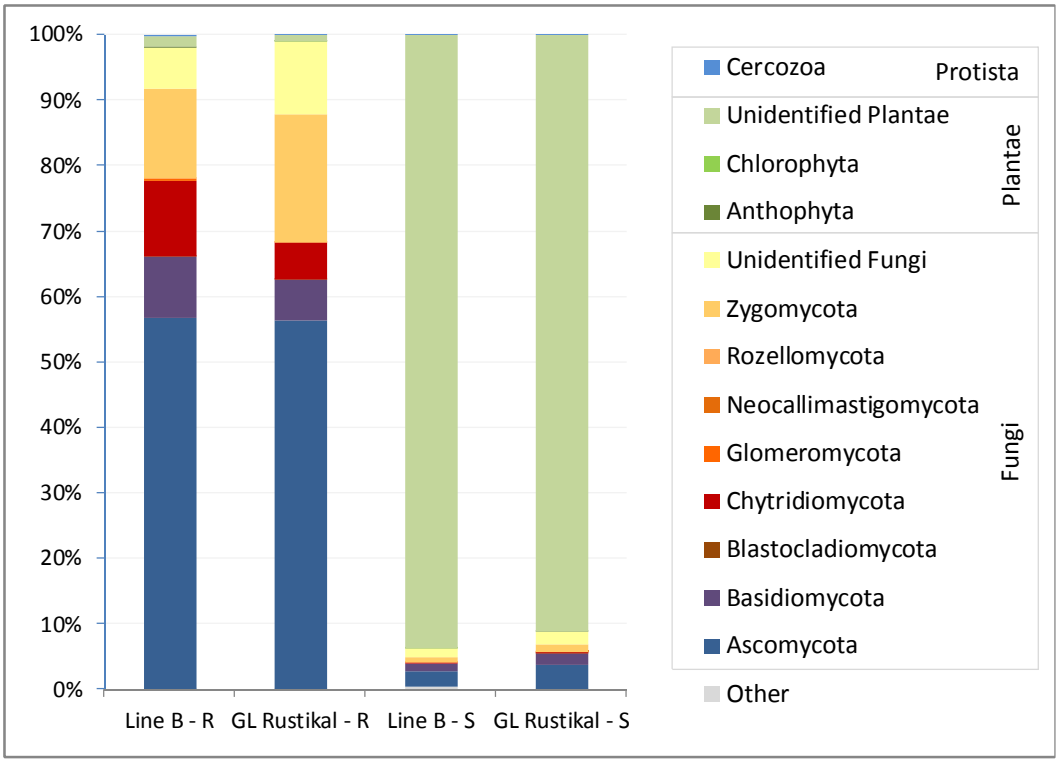

Figure 35: Structure of fungal, plant and Protista phyla in rhizosphere (R) and seed (S) microbiomes of *C. pepo* genotypes. Taxa with no taxonomic assignment are summarised as 'Other'. The results correspond to one sample replicate.

3.3.2 Assignment of fungal ITS amplicon sequences classified as unidentified Plantae

Filtering of OTU table after initial data processing resulted in a list comprising 570 so called denovo OTU numbers in all four samples which were classified as unidentified Plantae on genus level. For a better handling of data, OTUs counting less than 50 sequences in at least one sample were negatively filtered, entailing 41 OTUs. This resulted in a reasonable proportion of sequences which was covered (91-96% of unidentified Plantae). Details of sequence counts and proportions are listed in Table 8.

Table 8: ITS amplicon sequence counts in *C. pepo* genotypes Line B and GL Rustikal rhizosphere (R) and seed (S) samples

Sequence counts	Line B – R	GL Rustikal – R	Line B – S	GL Rustikal – S
Total	137,738	221,425	16,896	115,324
Unidentified Plantae	2215	1903	15,540	103,934
Proportion of total	1.6%	0.9%	92.0%	90.1%
>50 unid. Plantae sequences/OTU in at least one sample	2023	1781	14,547	100,117
Proportion of total unid. Plantae	91.3%	93.6%	93.6%	96.3%
>50 unid. Plantae sequences/OTU in at least one sample (without *Aeonium*)	1913	1552	14,504	99,745
Proportion of total unid. Plantae	86.4%	81.6%	93.3%	96.0%

The representative sequences of the 41 OTUs were assigned to a phylogenetic taxon by means of a Blast analysis against the NCBI nucleotide database. The analysis of 40 sequences resulted in a more or less equal result. The best hit was a partial sequence of the uncultured eukaryote clone CMH469 18S ribosomal RNA gene (Accession KF800560.1). The sequence was published by Rittenour et al. (2014). They characterised fungal communities collected in air and dust samples in homes of asthmatic children. Nearly all other Blast results comprised sequences of genera of Cucurbitaceae family like *Cucurbita*, *Schizocarpum*, *Cionosicys*, *Cionosicyos*, *Cayaponia*, *Selysia, Trichosanthes, Bryonia*, *Gymnopetalum* and *Melothria*. Among the further results were also partial 18S rRNA gene sequences of uncultured fungus clones, for example the Accession FN666025.1. These hits were referenced by medical and pharmaceutical scientific work and prior isolated from cystic fibrosis sputum. A multiple sequence alignment of the 40 representative sequences assigned to the uncultured eukaryote clone revealed that the sequences are very similar with a lot of conserved regions, as already presumed due to the Blast results. Upon sorting the sequences two groups were identified which were even more similar among themselves. The sequences of one group were identified as coding strand and the second as non-coding strand. Subsequently, the reverse complement of the sequences of one group was created and the multiple sequence alignment was established again. Then all 40 representative sequences showed a very high similarity. For illustration, an alignment of two sequences is shown in Figure 36.

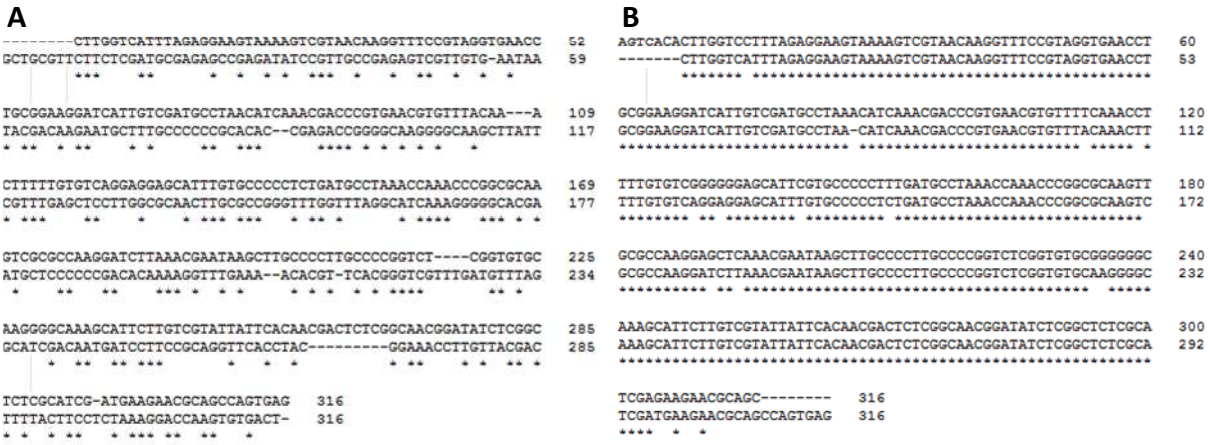

Figure 36: Alignment of unidentified Plantae sequences of groups one and two in the original orientation (A) and the same sequences aligned after creation of the reverse complement of one sequence (B). The * correspond to base identity.

One out of the 41 OTUs was assigned to Crassulaceae family. The best hit of Blast analysis was a partial sequence of *Aeonium spathulatum* internal transcribed spacer 1 (Accession AY082149.1). Further results comprised a lot of *Aeonium* species and other representative genera of Crassulaceae family, i.e. *Aichryson*, *Monanthes* and *Sedum*. But also a partial sequence of an uncultured *Cortinarius* isolate SSCP gel band iCb08 internal transcribed spacer 1 (Accession KF429530.1) was among the Blast results. The isolation source was the plant rhizosphere and the host *Aeonium urbicum*. The sequence did not show so much similarity with the other 40 sequences. Two regions, one at the beginning and one at the end were similar, but the major part of the sequence differed from the others. An alignment of the unidentified Plantae sequence assigned as *Aeonium* with an exemplified unidentified Plantae sequence assigned as uncultured eukaryote clone is depicted in Figure 37. The *Aeonium* OTU constituted 8.5% in average of rhizosphere unidentified Plantae sequences and 0.3% of seed sequences. Consequently the proportion can be neglected.

Figure 37: Alignment of unidentified Plantae sequence assigned as *Aeonium* (bottom) with unidentified Plantae sequence assigned as uncultured eukaryote clone (top). The * correspond to base identity.

Finally, the 40 sequences assigned as uncultured eukaryote clone were assumed as sequences originating from oil pumpkin seeds, at which the ITS amplicon primer sequences fitted randomly. An alignment of an unidentified Plantae sequence with the sequence of a *C. pepo* ITS sequence (Accession EF595858.1) is depicted in Figure 38. It shows a high similarity of the exemplary unidentified Plantae sequence to the pumpkin sequence.

Figure 38: Alignment of *C. pepo* (Accession EF595858.1) (bottom) and unidentified Plantae sequences (top). The * correspond to base identity.

3.4 Design of a peptide nucleic acid DNA clamp

Because of the large proportion of unidentified Plantae in the seed-associated fungal communities, their sequence similarity and their presumable origin from pumpkins a peptide nucleic acid (PNA) DNA clamp was designed to block this amplification in subsequent ITS amplifications of *C. pepo* samples.

3.4.1 pepoPNA construction

As a first step the multiple sequence alignment was checked for appropriate sites. The 15 bp long sequence marked in Figure 39 was chosen as the best pepoPNA candidate. Other sites than the chosen may have been suitable as well with slight restrictions (one mismatch, situated close to the conserved 18S region, different oligomer length) which could have been compensated. The position is located approximately in the middle of the ITS-1 region and characterised by a total sequence identity for the 40 unidentified Plantae sequences assigned as uncultured eukaryote clone. The oligomer does not bind to the unidentified Plantae sequence assigned as *Aeonium*. The position at *Aeonium* sequence differs in 7 bp. Therefore the pepoPNA does not block amplification of this sequence.

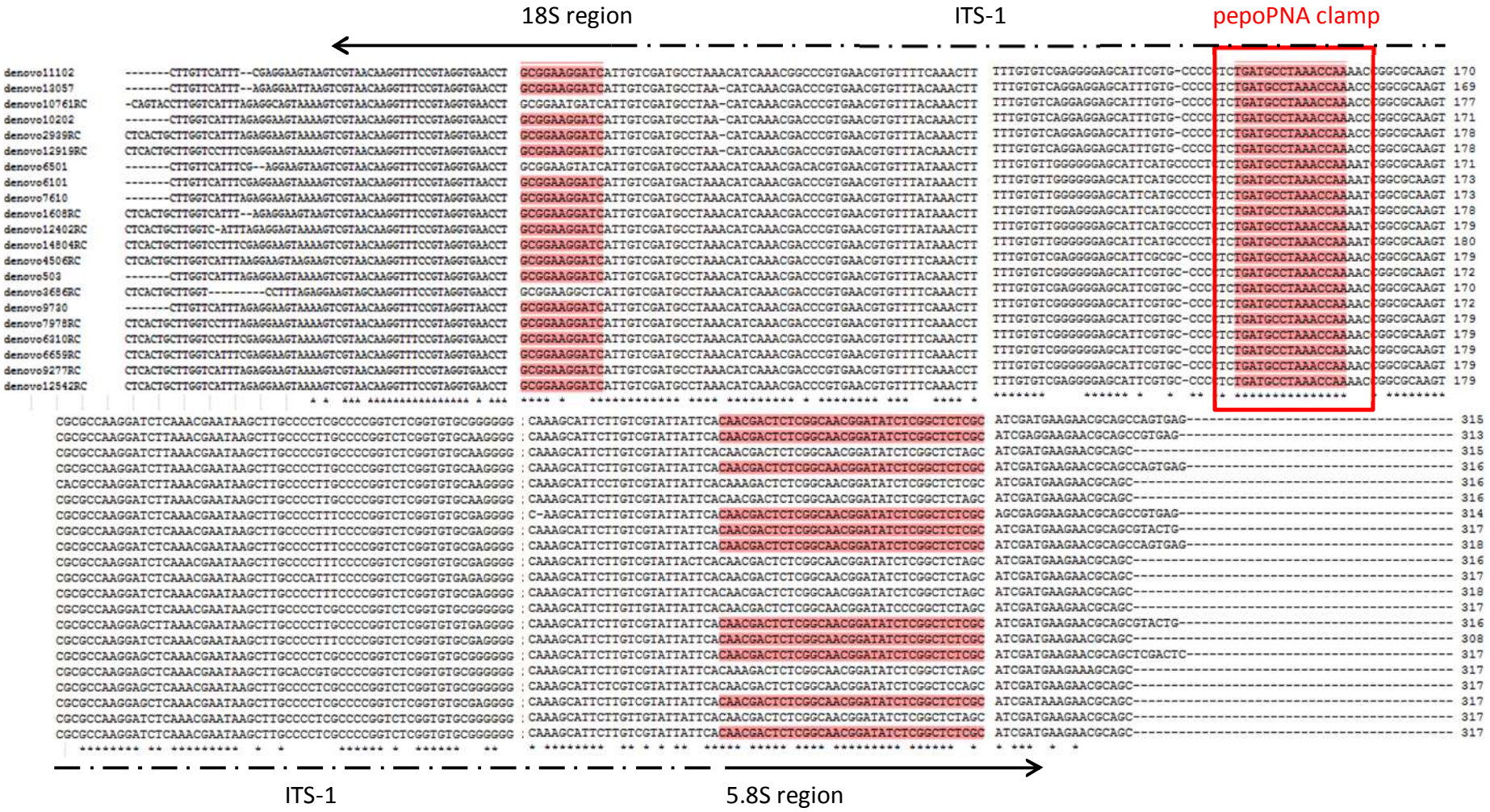

Figure 39: pepoPNA clamp located in multiple sequence alignment of unidentified Plantae sequences. The beginnings of 18S and 5.8S regions are highlighted, as well as the pepoPNA clamp. The * correspond to base identity.

In the case of the pepoPNA sense sequence orientation the number of hits in the FASTA file of representative sequences of unidentified Plantae was congruent to the number of hits in the file containing the whole set of representative sequences (each 239 sequences). Also in the case of the reverse complement sequence the numbers were nearly the same (250 hits and 251 hits respectively).

A Blast analysis of the pepoPNA sequence (sense and reverse complement sequence orientations) against the NCBI nucleotide database returned a list with 100 equal results. Among them were two hits of a partial sequence of *C. pepo* ITS-1 sequences (Accessions KT347508.1 and KT347507.1). An alignment of one of these sequences with an unidentified Plantae denovo sequence revealed a high sequence similarity, as illustrated in Figure 40.

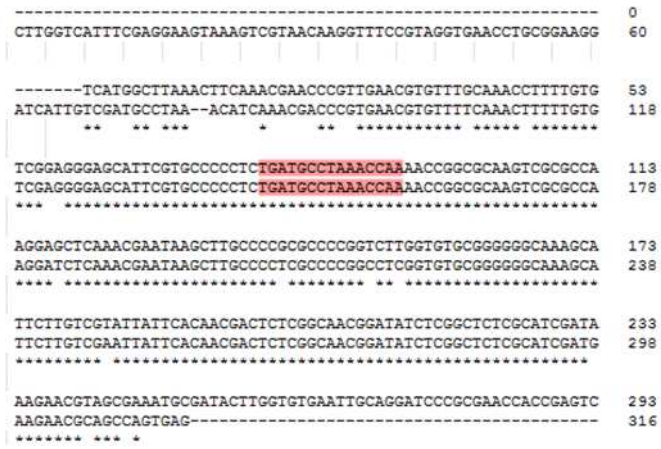

Figure 40: Alignment of *C. pepo* (Accession KT347508.1) (top) and unidentified Plantae sequence (bottom). The * correspond to base identity. The sense pepoPNA sequence oligomer is highlighted.

Among the other results were also four fungal sequences, partial mRNA sequences of a glucanohydrolase and a glucosidase of *Ogataea polymorpha* (Accession XM_018357919.1 and XM_014077075.1) and chromosome sequences of *Komagataella* sp. (Accessions CP014586.1 and CP014710.1). However, since these were sequences outside the rRNA genes, this result can be neglected. Further hits included among others prokaryotic sequences like Metazoa (animals) and Viridiplantae (green plants) as well as Archaea.

It was also checked, if the apparently suitable pepoPNA oligomer is contained in the UNITE database, the reference database for ITS amplicons used by QIIME. The search yielded one hit, an uncultured fungus according to UNITE taxonomy. A Blast analysis revealed the already known uncultured eukaryote clone (Accession KF800560.1). In contrast to the sense sequence, the reverse complement sequence was not found in the sequence collection.

Because of the fact that PNA probes also bind even if there is one mismatch in the formed heteroduplex, also different one-mismatch variants were checked. The Blast analysis provided a list with many hits of ITS sequences of the fungal genus *Rhizoplaca* (e.g. Accessions EF095277.1, HM577236.1 and AY509790.1). In Figure 41 two alignments of the *Rhizoplaca* Accession AY509790.1 with unidentified Plantae denovo sequences in sense and in reverse complement orientation are shown. The sequences showed only minor similarity in both cases. The reverse complement pepoPNA oligomer is identical to the sense sequence with three mismatches, and is located at a different position as the pepoPNA probe itself is dedicated to bind. Since clear matches were missing, the one-mismatch variants were neglected and the chosen pepoPNA sequence was further pursued.

Figure 41: Alignments of *Rhizoplaca chrysoleuca* (Accession HM577236.1) (top) and unidentified Plantae sequences in sense (A) and reverse complement (B) orientation (bottom). The * correspond to base identity. The sense and reverse complement pepoPNA sequence oligomers are highlighted, as well as the pepoPNA reverse complement sequence with three mismatches in the sense sequence (rectangle).

Also Blast analyses of the sense and the reverse complement sequences with a random number of surrounding bases delivered the uncultured fungus and various Cucurbitaceae as results.

Furthermore, the representative sequences excluding unidentified Plantae were checked, if one-mismatch variants of the sense and reverse compliment pepoPNA oligomer match with any sequence. There were three hits. A Blast analysis of these sequences revealed the already known uncultured eukaryote clone (Accession KF800560.1), an uncultured *Rhizoplaca* clone (Accession EF095277.1) as well as an uncultured fungus clone (Accession JX376757.1). But these results could be disregarded.

The evaluation of the sense and reverse complementary sequence of the pepoPNA oligomer by means of the PNA Tool (PNA Bio 2016) is shown in Table 9.

Table 9: Evaluation of the sense and reverse complementary sequence of pepoPNA oligomer by means of the PNA Tool (PNA Bio 2016)

	Sense sequence	**Reverse complementary sequence**
Complete sequence	tga tgc cta aac caa	ttg gtt tag gca tca
T_m at 4 µM	72.6°C	70.8°C
Base count	15	15
Base composition	A = 6 (40.0%) T = 3 (20.0%) G = 2 (13.3%) C = 4 (26.7%)	A = 3 (20.0%) T = 6 (40.0%) G = 4 (26.7%) C = 2 (13.3%)
Purines (%)	53.3%	46.7%
GC content	40.0%	40.0%
Purine stretch	3 (AAA)	3 (AGG)
pepoPNA chemical formula	$C_{161}H_{201}N_{89}O_{42}$	$C_{163}H_{204}N_{84}O_{48}$
Molecular weight	4054.9	4107.9
Extinction coefficient	158.4 ml/(µmole*cm)	153.9 ml/(µmole*cm)
1 ODU	6.3 nmole	6.5 nmole
pepoPNA sense sequence (5' to 3')	TGATGCCTAAACCAA	TTGGTTTAGGCATCA
Reverse complementary sequence (5' to 3')	TTGGTTTAGGCATCA	TGATGCCTAAACCAA
Comment	Redesign recommended Purine content > 50%	Good

After consultation with an employee of PNA Bio the sense sequence was chosen and some recommended modifications were made. The reverse complement sequence has less purine in general, but contains a number of guanins, which is worse than adenins. Consequently the sense orientation is more suitable. Also two lysines were added at 3´-end for clamping purpose, because it is important to have a good solubility.

For the pepoPNA design the PCR protocol already used for the ITS amplicons, as described in chapter 2.2.3.2, was taken into consideration. The melting and annealing temperatures of primers of PCR protocol for ITS amplicon sequencing were proven by the help of the T_m calculator of New England BioLabs. According to this tool, the recommended annealing temperature for PCR reaction was 44°C and the T_ms without barcodes at 49°C and 58°C.

Including the barcodes the suggested annealing temperature was 53°C, the T_ms at 58°C and 64°C.

As recommended by Lundberg et al. (2013), the T_m of the designed pepoPNA probe (72.6°C, illustrated in Table 9) is higher than that of the primers and slightly higher than that of extension cycle (72°C). Based on Lundberg et al. (2013), who have established a very similar PCR protocol, in which the PNA with a T_m of 80°C works well, the T_m of pepoPNA was increased additionally. Another aspect which supported an increase in T_m was the initial similarity in temperature of extension cycle. 72.6°C as T_m is basically acceptable, but one gamma modification was added to increase the melting temperature for 5~8°C. This temperature is also congruent to the PCR protocol including mPNA and pPNA used for 16S amplicon sequencing (chapter 2.2.3.1).

The finally ordered pepoPNA oligomer was constituted as shown in Figure 42. The complete pepoPNA order is attached in the appendix.

```
            N-term                  C-term
Sequence : tga tgc ct*a aac caa-KK
            (*: miniPEG gamma modification)
```

Figure 42: pepoPNA oligomer for PCR blocking in ITS amplicon sequencing including two lysines for solubility and a miniPEG gamma modification to increase T_m.

3.4.2 Validation of pepoPNA functionality

After *in silico* construction and analyses, the pepoPNA functionality was validated *in vitro* by single strand conformation polymorphism (SSCP) following a PCR amplification using different pepoPNA concentrations and by sequencing.

3.4.2.1 PCR amplification

The PCR amplification of rhizosphere and seed samples with different pepoPNA concentrations already provided a first indication for pepoPNA functionality. In contrast to the standard PCR variant without pepoPNA, no amplification product was observed with pepoPNA variants concerning the seed samples (Figure 43). Remembering, unidentified

Plantae sequences to be blocked constituted more than 90% of seed samples (described in chapter 0).

Consequently also the amount of amplification product was not sufficient. Therefore the PCR reactions of seed samples were repeated with an increased template amount.

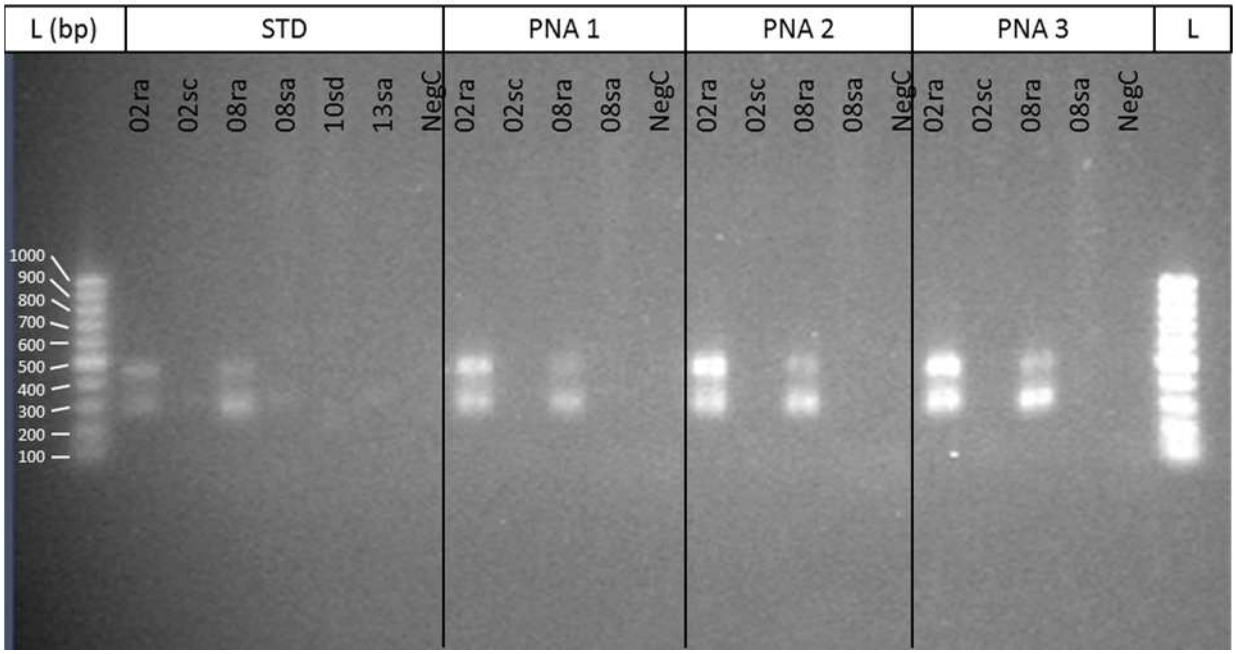

Figure 43: Agarose gel (1% agarose in 0.5 x TBE buffer, stained with ethidium bromide) to check the quality of the ITS amplification products of rhizosphere and seed samples of *C. pepo* using a PCR protocol including pepoPNA. STD: Standard PCR batch without pepoPNA. PNA1/2/3: 0.50/0.77/1.00 µM pepoPNA concentration in PCR batch. L: 2 µl of GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific Inc. 2015) and 3 µl PCR product mixed with 2.5 µl loading dye were applied. NegC: Negative control, i.e. no template. The electrophoresis was performed at 100 Volt for 30 minutes followed by 120 Volt for 25 minutes.

The purity and nucleic acid concentration in the samples were determined by UV-VIS spectrophotometric technology (NanoDrop™ 2000c Spectrophotometer, Fisher Scientific). The ratio of 260/280 was in the range of 1.83 to 1.92, which indicates a sufficient purity of all samples. The nucleic acid concentration was in the range of 63.6 ng/µl to 94.0 ng/µl.

3.4.2.1 Single strand conformation polymorphism

The SSCP electrophoresis provided the confirmation of pepoPNA functionality (Figure 44), as described below.

Regarding the rhizosphere samples (02ra, 08ra), there was no apparent difference between the standard and the pepoPNA variants. The *C. pepo* sequences to be blocked only comprised 1.3% on average (Figure 35 in chapter 3.3.1).

In the case of seed samples (02sc, 08sa, 13sa) blocking of *C. pepo* sequences could be validated. In all three seed samples, a similar result was obtained. Three really dominant bands and a thinner band in the standard variant were significantly weaker or missing in the pepoPNA variants. These bands are indicated by points and lines. The sequence to be blocked comprised more than 90% of fungal sequences in seed samples (Figure 35 in chapter 3.3.1). The different pepoPNA variants yielded a similar result. Only a slight tendency was observed depending on pepoPNA concentration.

The SSCP analysis also gives insight into the fungal communities of rhizosphere and seed samples of *C. pepo*. The result clearly shows the high diversity in the rhizosphere samples, and the lower diversity and evenness in the seed samples, which has already been observed with regard to bacterial diversity.

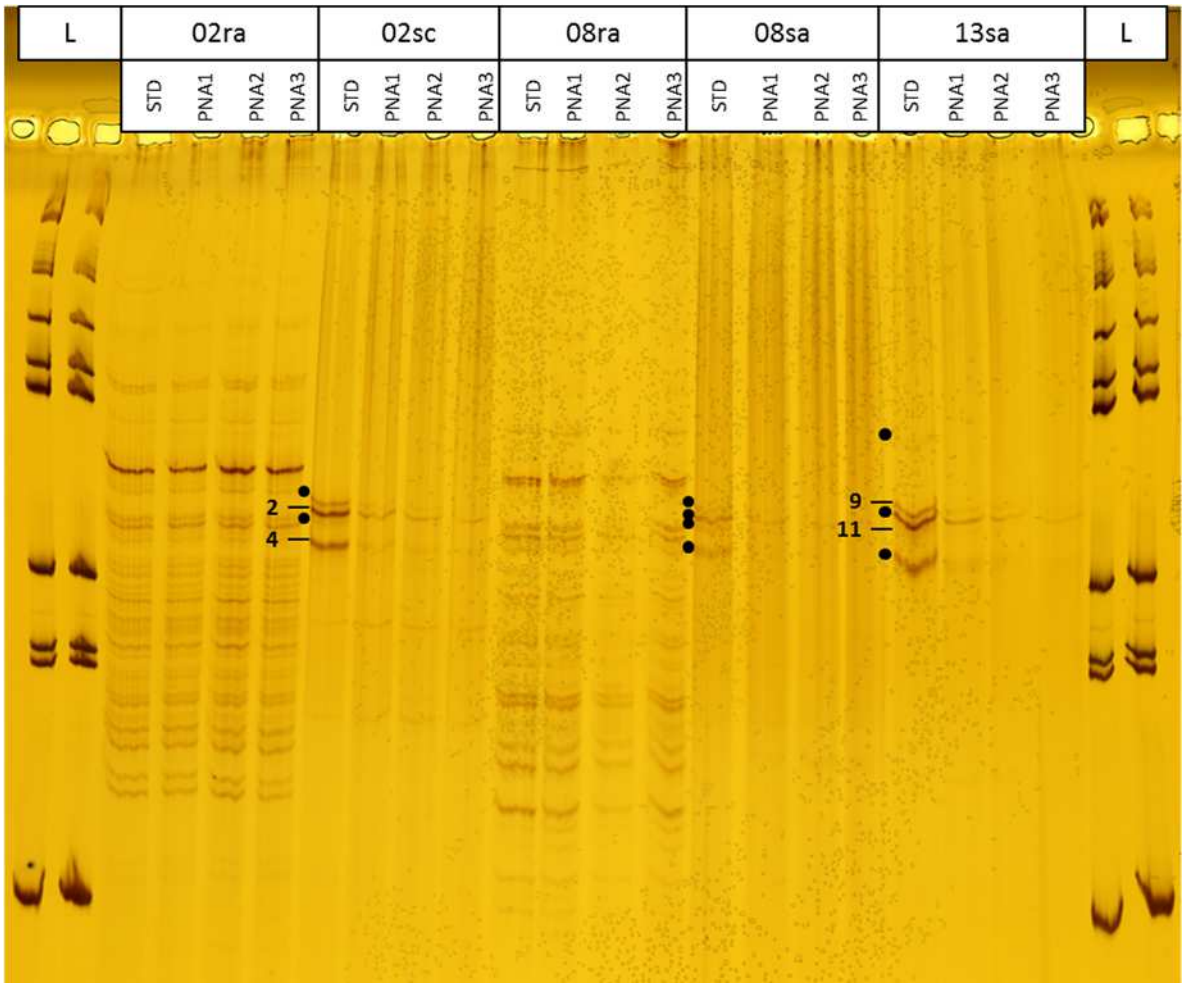

Figure 44: SSCP (8% polyacrylamide gel in 1 x TBE buffer, stained with silver nitrate) to validate pepoPNA functionality in blocking *C. pepo* sequences in ITS amplification protocols. STD: Standard PCR batch without pepoPNA. PNA1/2/3: 0.50/0.77/1.00 µM pepoPNA concentration in PCR batch. L: 2 µl of GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific Inc. 2016) and 8 µl PCR product were applied. NegC: Negative control, i.e. no template. Points and lines indicate prevalent bands in STD protocols in contrast to pepoPNA protocols. The electrophoresis was performed at 400 Volt, 50 mA and 26°C for 17 hours.

3.4.2.2 Sequencing of SSCP-derived bands

For validation of SSCP results of pepoPNA functionality respective bands of the SSCP gel, i.e. sequences which had been blocked, were excised and sequenced.

The DNA isolated from SSCP polyacrylamide gel was amplified with standard PCR protocol for ITS amplicon sequencing without pepoPNA. The control agarose gel is depicted in Figure 45. The smears in some samples might be due to high template amounts, but this should not be a problem for subsequent steps. Consequently, one band of each lane position of the SSCP gel with a sufficient amplification product was chosen for sequencing, i.e. 02sc2, 02sc4, 13sa9 and 13sa11 (numeration 2, 4, 9 and 11 in Figure 44). Due to 260/280 ratio, which was

between 1.84 and 1.86, the purity of PCR product was sufficient. The nucleic acid concentration of the four selected products was between 63.5 and 75.6 ng/µl.

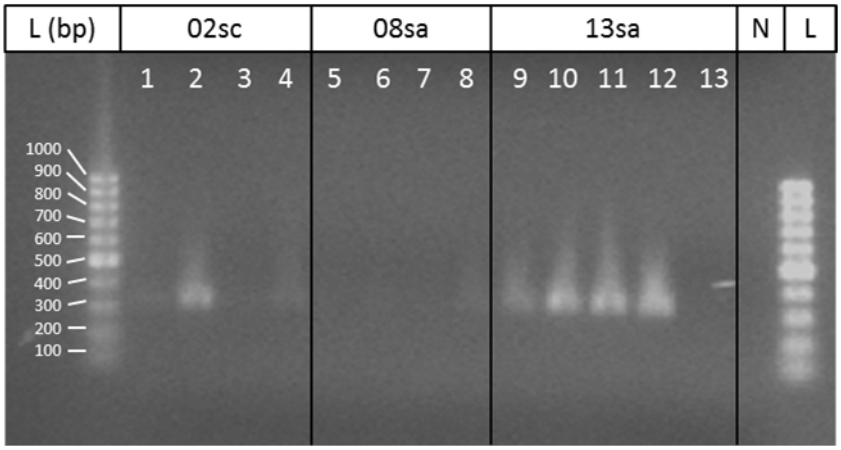

Figure 45: Agarose gel (1% agarose in 0.5 x TBE buffer, stained with ethidium bromide) to check amplification of DNA excised from SSCP for validation of pepoPNA functionality in seed samples of *C. pepo*. The numbers of 1 to 13 correspond to the respective bands, marked in Figure 44. L: 2 µl of GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific Inc. 2015) and 3 µl PCR product mixed with 2.5 µl loading dye were applied. N: Negative control, i.e. no template. The gel was run at 100 Volt for 30 minutes followed by 120 Volt for 25 minutes.

For validation of SSCP results the four chosen bands were sequenced. The multiple sequence alignment of sequenced samples revealed a high similarity, as depicted in Figure 46. The pepoPNA sequence was also part of all sequences. Thus, the functionality of the pepoPNA could be confirmed. The different positions of the bands in the SSCP lanes were due to different sequence lengths.

```
02sc4       --------------ACGACAAGAATGCTTTGCCCCTCGCACACCGAGACCGGGGCAAGGG
13sa9       ---GTTGTGATAATACGACAAGAATGCTTTGCCCCTCGCACACCGAGACCGGGGAAAGGG
02sc2       GTCGTTGTGATAATACGACAAGAATGCTTTGCCCCCCCGCACACCGAGACCGGGGCAAGGG
13sa11      ---GTTGTGATAATACGACAAGAATGCTTTGCCCCCCCGCACACCGAGACCGGGGCAAGGG
                          ********************* ****************** *****

02sc4       GCAAGCTTATTCGTTTGAGATCCTTGGCGCGACTTGCGCCGGTTTTGGTTTAGGCATCAG
13sa9       GCAAGCTTATTCGTTTGAGATCCTTGGCGCGACTTGCGCCGGTTTTGGTTTAGGCATC--
02sc2       GCAAGCTTATTCGTTTGAGCTCCTTGGCGCGACTTGCGCCGGTTTTGGTTTAGGCATCAA
13sa11      GCAAGCTTATTCGTTTGAGATCCTTGGCGCGACTTGCGCCGGTTTTGGTTTAGGCATCAA
            ******************* ************************************

02sc4       AGGGGGCACAAATGCTCCCCCCGACACAAAAAGTTTGAAAACACGTTCACGGGTCGTTTG
13sa9       -------------------------------------------------------------
02sc2       AGGGGGCACGAATGCTCCCCCCGACACAAAAAGTTTGAAAACACGTTCACGGGTCGTTTG
13sa11      AGGGGGCACGAATGCTCCCCCCGACACAAAAAGTTTGAAAACACGTGCACGGGTCGTTTG

02sc4       ATGTT-------
13sa9       ------------
02sc2       ATGTTTAGGCAT
13sa11      ATGTTTAGGCAT
```

Figure 46: Multiple sequence alignment of sequenced SSCP bands to confirm pepoPNA functionality. The pepoPNA probe sequence is highlighted. The * correspond to base identity.

A Blast analysis in the NCBI nucleotide database of the four sequenced excised SSCP-bands showed the desired result, the same hits as identified by pepoPNA design. Again the uncultured eukaryote clone with the same accession number (Accession KF800560.1)

showed the highest similarity, as well as *Cucurbita* sequences and uncultured fungi sequences. Since the fungi hits belonged to an uncultured fungus, which does not necessarily have to be a fungus, the results were accepted. The sequences of this study (Accessions FN666051.1, FN666025.1 and FN666070.1) could be due to pollen contaminations which were inhaled into cystic fibrosis sputum for example as well.

3.4.3 Establishment of a PCR protocol including pepoPNA

Based on the knowledge gained so far some aspects and possible optimisations of the PCR protocol including the pepoPNA probe to block *C. pepo* sequences upon ITS amplification are summarised in the following paragraphs.

The PCR amplicon protocol including the pepoPNA is only intended to be used for seed samples and not for rhizosphere samples since the unidentified Plantae sequences have made up only a minor percentage in the rhizosphere in contrast to seeds.

Basically, the PCR protocol including the pepoPNA worked well with all three pepoPNA concentrations, as described in chapter 2.3.2.1. Consequently the PNA2 variant, i.e. a final concentration of 0.77 µM pepoPNA in PCR batch, could be a good compromise of pepoPNA amount and sufficient functionality.

If blocking of desired sequences is not sufficient, it can be improved by lowering of annealing temperature of pepoPNA and by increasing the pepoPNA concentration in the PCR batch according to Ørum (2000).

In contrast, in the case of problems with yielded PCR product, the pepoPNA concentration should be lowered. At lower temperatures, as it is the case in the primer annealing step with 58°C, the pepoPNA could bind unspecifically.

In general, there is no upper limit for template DNA amount. Very few fungal sequences in template, requiring high template amounts, may cause smears in lanes of control gel. A solution to this would be a nested PCR strategy. In a nested PCR the pepoPNA would have to be used in both PCRs in order to prevent unwanted amplification. In this case a lower pepoPNA concentration would be sufficient enough. For a nested PCR strategy, the number

of cycles in the PCR protocol could be reduced, but the cycles are generally adapted to template amount and yielded PCR product.

3.5 Analysis of genotype-specific responses to BCAs in the greenhouse

The results of the comprehensive analysis of the plant-associated microbiome of different *C. pepo* genotypes raised the assumption that genotype-specificity of plants on cultivar level with regard to the abundance and interactions with the microbiome also applies for the targeted treatment of crops with biological control agents. If so, in next-generation breeding strategies cultivars could be raised, which are better able to exploit promoting effects of native and applied biological control agents. In order to investigate genotype-specific responses to BCAs in greenhouse experiments, seeds of the *C. pepo* genotypes GL Rustikal, Gl. Diamant and Line D were primed with *Serratia plymuthica* strains. The plants were grown under gnotobiotic conditions in germination pouches and in sterile soil, and subsequently the effects on tissue colonisation, hypocotyl length and germination capacity as well as on fresh and dry weight dependent on the genotype-strain combination were investigated.

3.5.1 Germination test with *S. plymuthica* 4Rx13

In order to determine if the *S. plymuthica* strain 4Rx13 inhibits germination of different cultivars in an extent which was documented by Adam (2015) for GL Rustikal, five *C. pepo* genotypes were tested for germination capacity after 4Rx13 seed priming.

First signs of germination, meaning the swelling of the seeds and a slight radicle growth, were monitored two days after test start. The radicle length was documented twice, eight and 14 days post seeding (dps), as illustrated in Figure 47. Four out of five GL Rustikal seeds developed a small radicle, and one to three in the cases of Line A, Line B and Line D. In the case of Gl. Diamant the germination was totally inhibited. From 8 dps to 14 dps a slight increase of the number of small radicles of genotypes GL Rustikal and Line B was observed.

The radicle was not larger than 0.5 cm in all cases. Obviously, 4Rx13 inhibited germination of all five genotypes investigated.

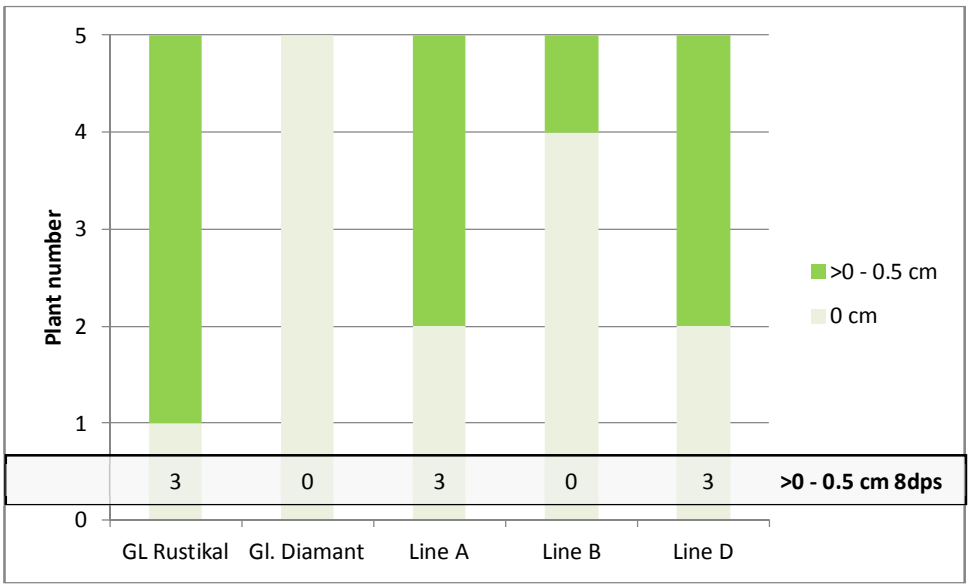

Figure 47: Radicle length of germinated seeds eight (numbers in grey bar at the bottom) and 14 days (bar chart) post seeding in the germination test of five *C. pepo* genotypes with *S. plymuthica* 4Rx13 priming. Five seeds of a variant were grown in one petri dish with watered filter paper in one replicate.

From day to day the colour of the radicle turned from white and slightly yellow to brown and in some cases to lightly black and the seeds started to get mouldy, as illustrated in Figure 48.

Figure 48: *C. pepo* seeds aborting germination in early radicle development stage as a consequence of priming with *S. plymuthica* 4Rx13.

3.5.2 Analysis of primed *C. pepo* plants grown in germination pouches

3.5.2.1 Experiment 1: Hypocotyl length and tissue colonisation

For the analysis of plant-microbe interactions under gnotobiotic conditions in germination pouches the three *C. pepo* cultivars GL Rustikal, Gl. Diamant and Line D were primed with one transformed *S. plymuthica* strain, a mixture of them or solely with 0.85% NaCl as negative control.

The concentrations of the different strains in the ONCs were determined based on the dripping method, as described in chapters 2.4.2 and 2.4.4.2. The concentrations of the bacterial suspensions were in the order of magnitude 10^9 CFU/ml, as listed in Table 10. For seed priming (see chapter 2.4.2) a 1:10 dilution of the ONC was used.

Table 10: CFU concentrations of *S. plymuthica* strains in the ONCs for seed priming in experiment 1

***S. plymuthica* strain**	**CFU/ml**
3Rp8-DsRed2	$1.50*10^9$
S13-GFP	$3.50*10^9$
4Rx13-eBFP2	$2.85*10^9$
3Re4-18-mNeptune	$3.18*10^9$

During the growing phase the hypocotyl length of the seedlings was documented and an index calculated (Figure 49). 4Rx13-eBFP2 suppressed germination of all genotypes completely. Only a small radicle, not bigger than 0.5 cm, was developed by some seeds. With progressing time, there was no further growth and the radicles turned from white and slightly yellow to brown. When considering the plant counts and the index, plants treated with 3Rp8-DsRed2 showed consistently the best results concerning hypocotyl length. Also plants treated with a mixture of 3Rp8-DsRed2, 3Re4-18-mNeptune and S13-GFP were among the better. Gl. Diamant was the genotype with the largest hypocotyls, but also the hypocotyls of the negative control were among the longest. In the case of Line D S13-GFP promoted the hypocotyl length of the plants in the same extent considering the index as 3Rp8-DsRed2 did.

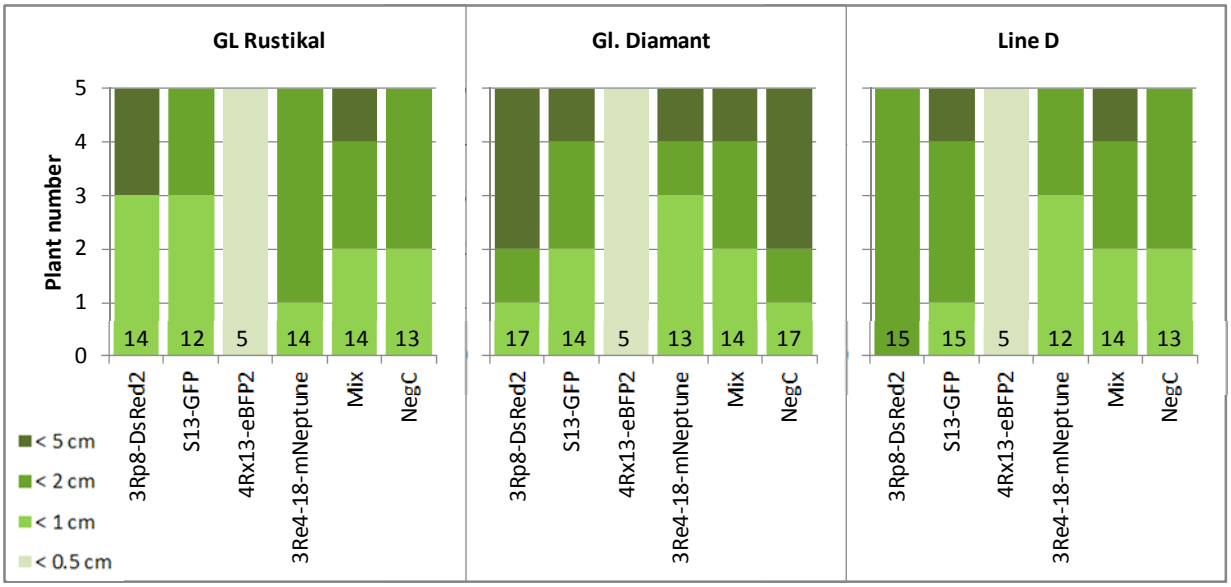

Figure 49: Hypocotyl length of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in germination pouches, five days post seeding. Experiment 1. NegC: Negative control. Five plants of a variant were grown in one pouch in three replicates. Calculation of the index shown in the bottom of the bars: Plant number x 1/2/3/4 depending on hypocotyl length (< 0.5 cm, < 1 cm, < 2 cm, < 5 cm respectively).

13 days after seeding root and leaf material of the plants was harvested, homogenised and aliquots plated in order to determine the CFU/g fresh weight of plant material. Three dilutions of the reisolated mixture of *S. plymuthica* strains (3Rp8-DsRed2, S13-GFP and 3Re4-18-mNeptune) from a *C. pepo* root sample, dropped on LB agar plates containing trimethoprim by means of the dripping method (chapter 2.4.2), are shown in Figure 50 exemplary. The CFU/g FW were calculated based on the formula described in chapter 2.4.4.2.

Figure 50: CFU of different dilutions (10^{-2} to 10^{-4}) of the mixture of *S. plymuthica* strains (3Rp8-DsRed2, S13-GFP and 3Re4-18-mNeptune) reisolated from a *C. pepo* root sample, dropped on LB agar plates containing 50 µg/ml trimethoprim.

The results revealed a high colonisation density of *C. pepo* roots of all genotypes with all *S. plymuthica* strains (Figure 51). The colonisation patterns of the different strains were quite

similar among the genotypes. The above-ground plant parts were less colonised. The numbers of reisolated CFU/g FW were one or two orders of magnitude lower in comparison to roots. Root and leaf colonisation did not correlate. 3Rp8-DsRed2 dominated root and leaf tissues of all cultivars. The CFU/g FW of 4Rx13-eBFP2 is not directly comparable with the others, since the value is based on the seed and radicle quantity and not on root or leaf fresh weight. The high density is presumable due to a remaining high cell density of seed priming. The colonisation of GL Rustikal plants was also high with S13-GFP and 3Re4-18-mNeptune, followed by the mixture of 3Rp8-DsRed2, S13-GFP and 3Re4-18-mNeptune. But the colonisation of all three treatments was one order of magnitude below the 3Rp8-DsRed2 treatment. In the case of Gl. Diamant the root and leaf colonisation with 3Re4-18-mNeptune and the mixture were similar and the colonisation with S13-GFP was slightly lower. Regarding Line D 3Re4-18-mNeptune and the mixed treatment resulted in a higher CFU number upon reisolation than S13-GFP treatment. Since transformed *Serratia* strains were also detected on plants of the negative control, the experiment was repeated.

Details of root and leaf weights, CFU counts of the respective dilutions and the calculated CFU/g FW are listed in Appendix-Table 1.

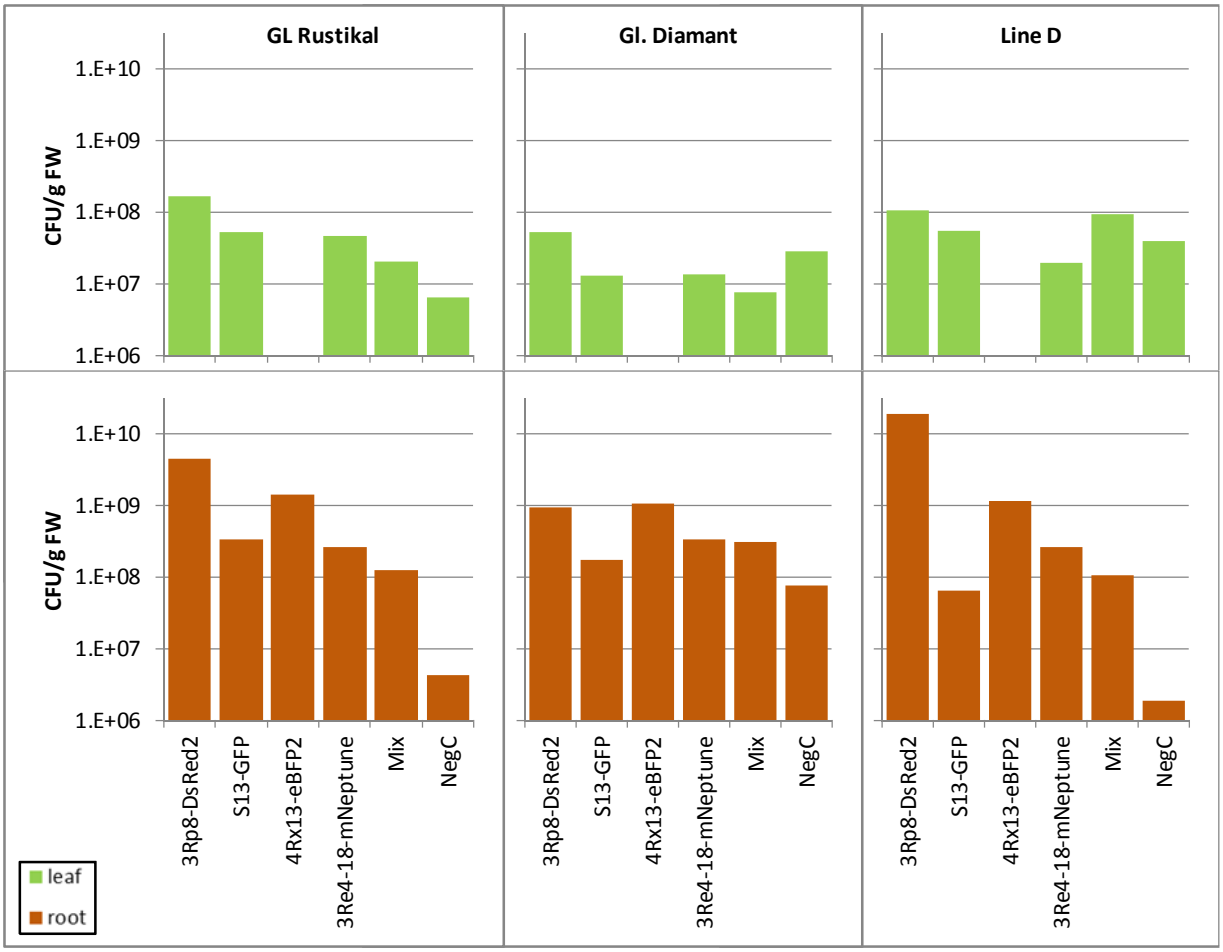

Figure 51: Reisolated CFU/g fresh weight of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in germination pouches, 13 days post seeding. Experiment 1. NegC: Negative control. Five plants of a variant were grown in one pouch in one replicate. The CFU/g FW of 4Rx13-eBFP2 is only given for the sake of completeness, but not directly comparable with the others, since the value is based on the seed and radicle quantity and not on root or leaf fresh weight. The CFU/g FW are drawn in logarithmic scale.

3.5.2.2 Experiment 2: Fresh weight and tissue colonisation

For the analysis of plant-microbe interactions by the means of germination pouches the three *C. pepo* cultivars GL Rustikal, Gl. Diamant and Line D were primed with one transformed *S. plymuthica* strain, a mixture of them or solely with 0.85% NaCl as control. The experiment 2 is a repetition of the experiment 1 with a similar concept.

The concentrations of the bacterial ONC suspensions were in the orders of magnitude from 10^8 to 10^9 CFU/ml, as listed in Table 11. For seed priming (see chapter 2.4.2) a 1:10 dilution of the ONC was used.

Table 11: CFU concentrations of *S. plymuthica* strains in the ONCs for seed priming in experiment 2

***S. plymuthica* strain**	**CFU/ml**
3Rp8-DsRed2	$1.37*10^9$
S13-GFP	$4.35*10^9$
4Rx13-eBFP2	$3.10*10^9$
3Re4-18-mNeptune	$5.80*10^8$

15 days after sowing, root and leaf material of the plants was harvested, weighed, homogenised and aliquots plated in order to determine the fresh weight and the CFU/g fresh weight of plant material.

The fresh weight of root and leaf material per plant is related to the cultivar. The three-way cross hybrid GL Rustikal was ranking first, the single cross hybrid Gl. Diamant second and the inbred Line D third in terms of fresh weight. This corresponds to breeding effects, as agronomic traits like the yield is increased in hybrids due to the strong heterosis effect, which occurs by crossing of inbred lines, which are themselves characterised by inbreeding depression effects. Seed priming with 4Rx13-eBFP2 suppressed germination of all genotypes completely. As observed in experiment 1, the radicle started to grow in some cases, but stopped after a few millimeters and turned brownish. There were hardly any differences and higher weights compared to the untreated controls. Concerning GL Rustikal, the treatment with 3Rp8-DsRed2 resulted in a slight increase in plant weight. Also in the case of Line D the treatment with 3Rp8-DsRed2 and with 3Re4-18-mNeptune as well led to an increase. Regarding seed and root weights on their own, no tendencies were observed.

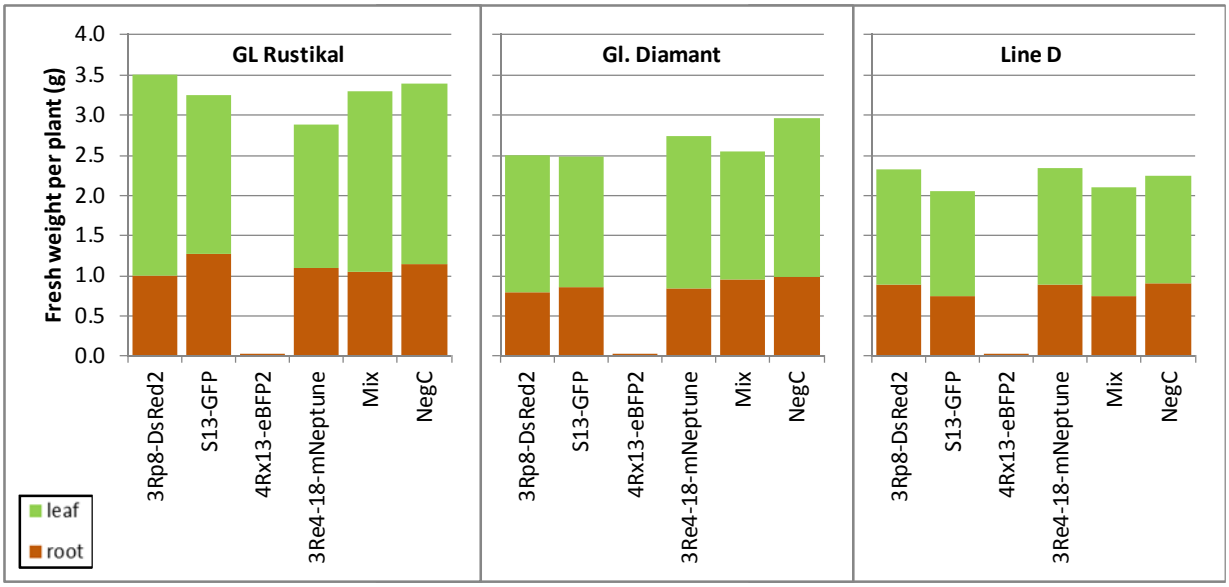

Figure 52: Fresh weight of *C. pepo* cultivars root and leaf material after seed priming with different *S. plymuthica* strains grown in germination pouches, 15 days post seeding. Experiment 2. NegC: Negative control. Five plants of a variant were grown in one pouch in three replicates. The weight of plants treated with 4Rx13-eBFP2 is only given for the sake of completeness, but not directly comparable with the others, since the value is based on the seed and radicle quantity and not on root or leaf fresh weight. The weights of root and leaf material per plant are stacked. The height of the entire bar corresponds to the total fresh weight per plant.

C. pepo roots and leaves grown in germination pouches in the second experiment revealed a high colonisation density of *C. pepo* roots of all genotypes with all *S. plymuthica* strains as well (Figure 53). The colonisation patterns of the different strains were quite similar among the genotypes, but different among the seed treatments. The colonisation of above-ground plant parts was more relevant regarding the strains 3Rp8-DsRed2 and S13-GFP than in experiment 1. This extent of colonisation was not observed with the mixture of 3Rp8-DsRed2, 3Re4-18-Neptune and S13-GFP, although it included both of the strains. Root and leaf colonisation slightly correlated in this experiment. Colonisation of roots was more intense in general. 3Rp8-DsRed2 dominated the plant tissues in all cases, followed by S13-GFP. The CFUs of S13-GFP associated with Line D were noticeable higher than the CFUs reisolated from the other genotypes. The CFU/g fresh weight reisolated from GL Rustikal and Gl. Diamant plants primed with 3Rp8-DsRed2 exceeded the other treatments to a great extent. 4Rx13-eBFP2 was the third in all genotypes, but the CFU/g FW of 4Rx13-eBFP2 is not directly comparable with the others, since the value is based on the seed and radicle quantity and not on root or leaf fresh weight. After treatment with the mixture less CFU/g FW could be reisolated, although the dominant 3Rp8-DsRed2 and S13-GFP strains were part of it. This could be due to a competitional effect of the strains. In all cases, in contrast to experiment 1, considerably smaller CFU counts of 3Re4-18-mNeptune and no

transformed bacteria were reisolated from control plants. The strains 3Rp8-DsRed2 and S13-GFP seem to be more comfortable with *C. pepo* plants than the others.

Details of root and leaf weights, CFU counts of the respective dilutions and the calculated CFU/g FW are listed in Appendix-Table 2.

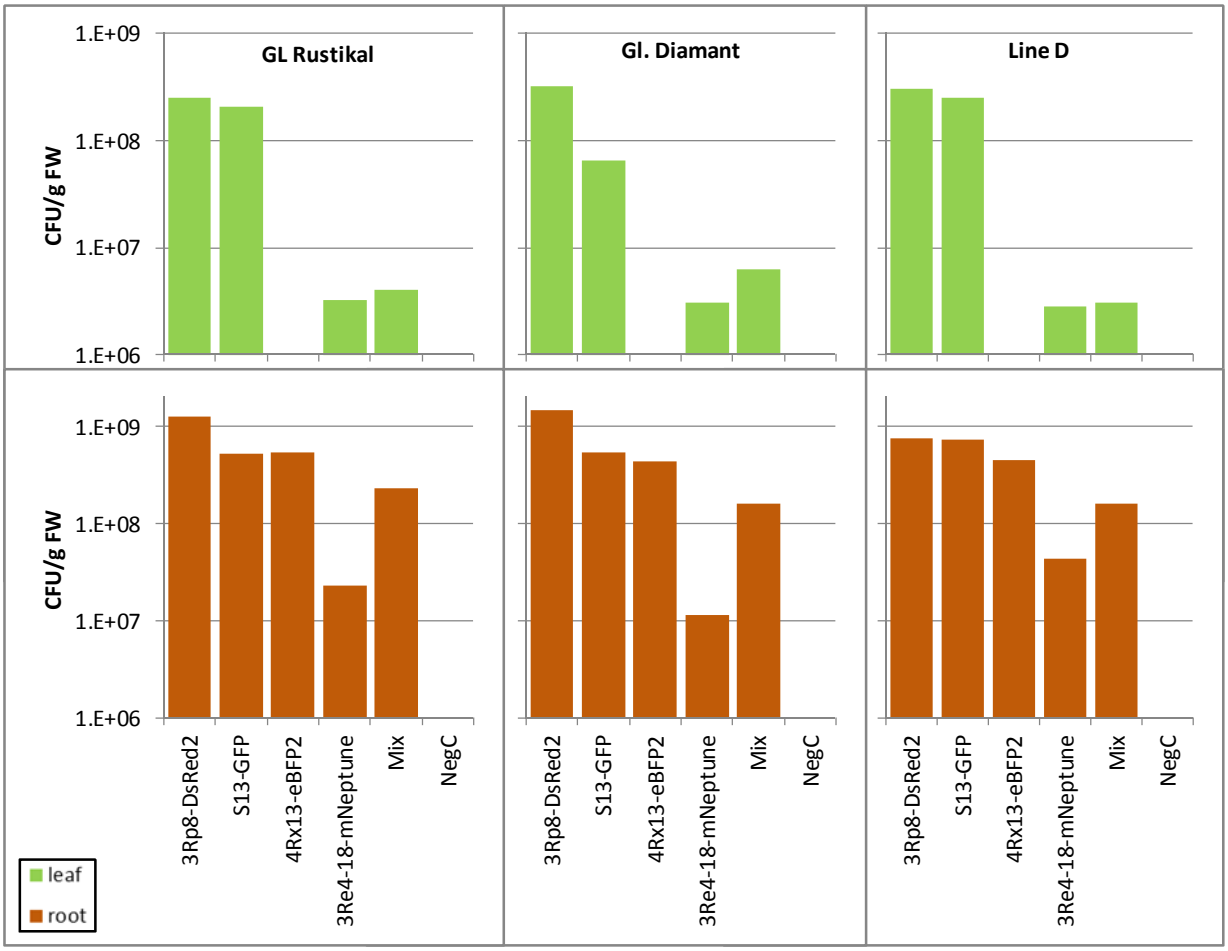

Figure 53: Reisolated CFU/g fresh weight of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in germination pouches, 15 days post seeding. Experiment 2. NegC: Negative control. Five plants of a variant were grown in one pouch in three replicates. The CFU/g FW of 4Rx13-eBFP2 is only given for the sake of completeness, but not directly comparable with the others, since the value is based on the seed and radicle quantity and not on root or leaf fresh weight. The CFU/g FW are drawn in logarithmic scale.

The ratio of *S. plymuthica* strains 3Rp8-DsRed2, 3Re4-18-mNeptune and S13-GFP in the bacterial mixture after reisolation was approximated by means of the dripping method as exemplified in Figure 54 and Figure 55, for which the fluorochromes of transformed bacteria were illuminated by Bio-Rad ChemiDoc^{TM} XRS System. Since the agar plates contained trimethoprim, it was expected that the strains which are able to grow on those plates contain the pIN69 plasmid harbouring the trimethoprim resistance gene as well as the respective fluorochrome gene. Despite this, in some cases no clear assignment was possible. Above ground plant parts were colonised almost similar by the different strains. In contrast, root tissues were dominated by 3Rp8-DsRed2 and S13-GFP. 3Re4-18-mNeptune was of minor occurrence. A strong dominance and capability of colonisation of 3Rp8-DsRed2 on roots was observed (see Figure 51 and Figure 53). The colonisation of roots was much stronger than that of leaves, in accordance with the results obtained by both reisolation experiments. Regarding the different genotypes, no specific differences could be recorded beside a lower colonisation of above ground plant parts of Line D. This tends to be consistent with the reisolation results for 3Rp8-DsRed2 and S13-GFP in this experiment, but the abundance of the various *S. plymuthica* strains in the mixture was similar among the three genotypes.

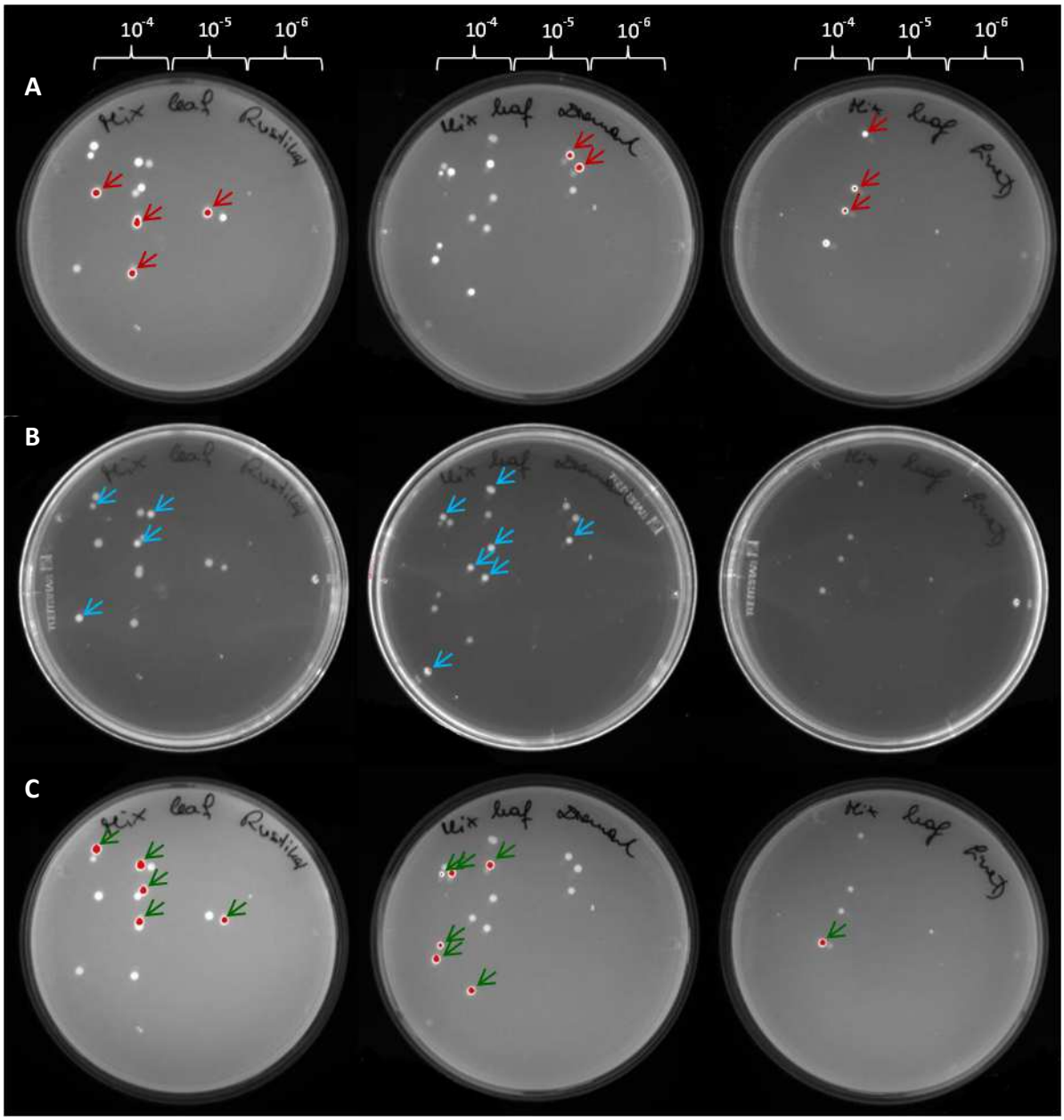

Figure 54: Reisolated CFU from *C. pepo* cultivars above ground plant tissues after seed priming with a mixture of *S. plymuthica* strains 3Rp8-DsRed2, 3Re4-18-mNeptune and S13-GFP and plants grown in germination pouches 15 days post seeding, visualised by Bio-Rad ChemiDoc™ XRS System. Reisolated bacterial suspension was plated by means of the dripping method on LB agar plates containing 50 µg/ml Tp. $10^{-4}/10^{-5}/10^{-6}$ indicate the dilution factors of bacterial suspension. A/B/C: Signal of 3Rp8-DsRed2 (red arrows), 3Re4-18-mNeptune (blue arrows) and S13-GFP colonies (green arrows), respectively. *C. pepo* genotypes GL Rustikal, Gl. Diamant and Line D are shown from left to right. Non-indicated colonies could not be clearly assigned.

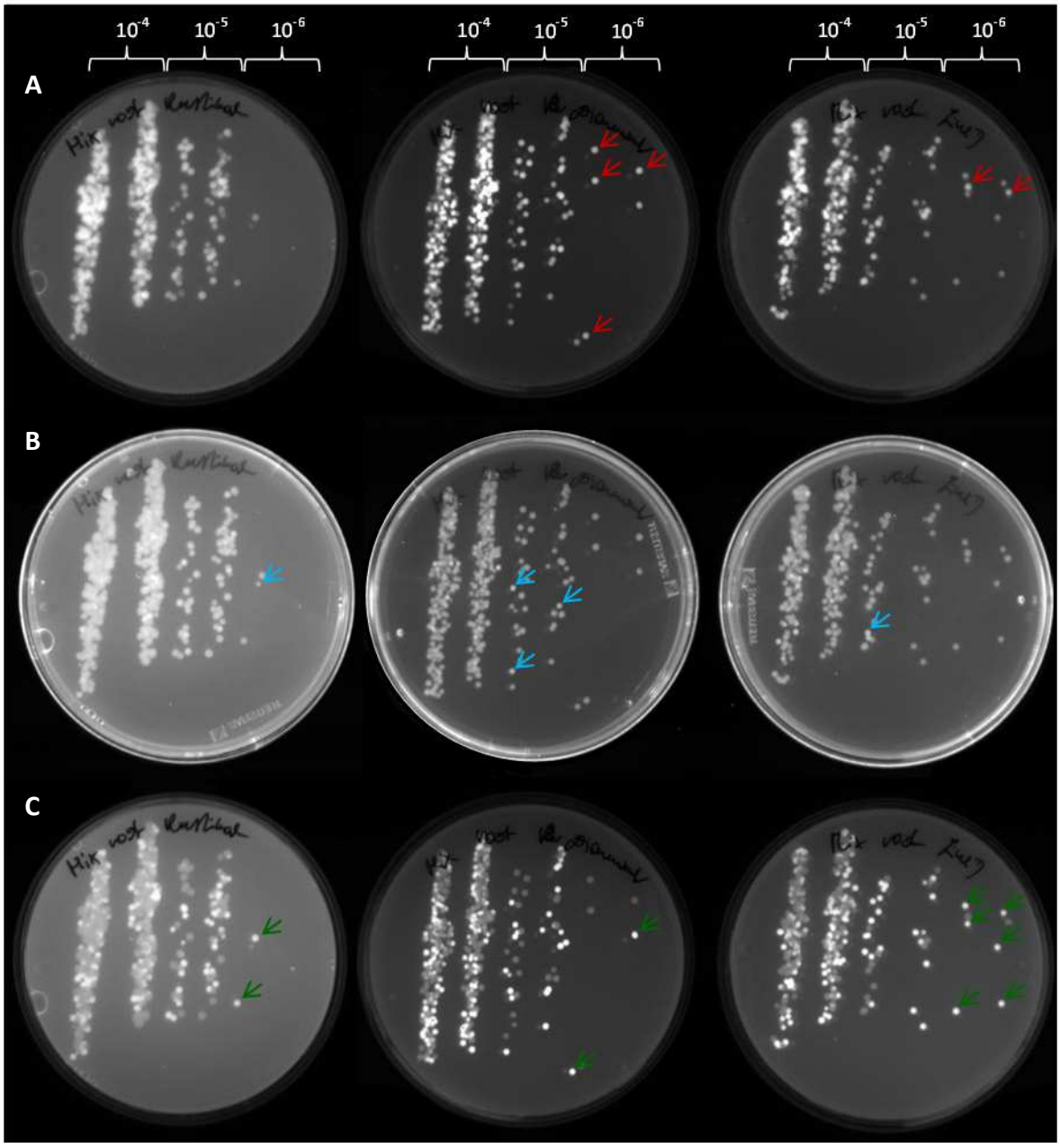

Figure 55: Reisolated CFU of *C. pepo* cultivars´ roots after seed priming with a mixture of *S. plymuthica* strains 3Rp8-DsRed2, 3Re4-18-mNeptune and S13-GFP and plants grown in germination pouches 15 days post seeding, visualised by Bio-Rad ChemiDoc™ XRS System. Reisolated bacterial suspension was plated by means of the dripping method on LB agar plates containing 50 µg/ml Tp. $10^{-4}/10^{-5}/10^{-6}$ indicate the dilution factors of bacterial suspension. A/B/C: Signal of 3Rp8-DsRed2 (red arrows in 10^{-6}), 3Re4-18-mNeptune (blue arrows in 10^{-6} and for purpose of clarification some examples in 10^{-5}) and S13-GFP colonies (green arrows in 10^{-6}), respectively. *C. pepo* genotypes GL Rustikal, Gl. Diamant and Line D are shown from left to right. Non-indicated colonies in 10^{-6} could not be clearly assigned.

3.5.3 Analysis of primed *C. pepo* and *L. sativa* plants grown in sterile soil

3.5.3.1 Experiment 3: Fresh and dry weight of *C. pepo* plants

In order to determine the relationship of plant fresh and dry weight with plant-microbe interactions, the three *C. pepo* genotypes GL Rustikal, Gl. Diamant and Line D were primed with different *S. plymuthica* strains and grown in sterile soil in plastic boxes.

The concentrations of the strains in the ONCs were in the order of magnitude of 10^9 CFU/ml, as listed in Table 12. For seed priming (see chapter 2.4.2) a 1:10 dilution of the ONC was used.

Table 12: CFU concentrations of *S. plymuthica* strains in the ONCs for seed priming in experiments 3 and 4

S. plymuthica strain	CFU/ml
3Rp8	$2.60*10^9$
S13	$4.15*10^9$
4Rx13	$4.15*10^9$
3Re4-18	$4.95*10^9$
HRO-C48	$2.95*10^9$

Six days after sowing the number of germinated seeds was evaluated (Figure 56). All seeds of Gl. Diamant sprouted. Regarding GL Rustikal all seeds with the exception of those treated with 4Rx13 emerged completely. Seeds of Line D showed a worse germination capacity. Only seeds of the negative control and of the 3Re4-18 treatment grew completely. The 4Rx13 and HRO-C48 treatments showed the worst plant number. As observed upon the reisolation experiments, 4Rx13 treatment of seeds could be the reason for a lower germination capacity, but the effect was apparently not as strong as in germination pouches, in which the germination was supressed totally independent of genotypes. The growth suppression of this genotype was maybe due to a poorer germination capacity or due to inbreeding depression effects of the inbred line. The high germination and growth capacity of all genotypes despite a treatment with 4Rx13 grown in sterile soil in comparison to germination pouches, in which the germination was suppressed totally, could be related to the soil environment and to nutrient supply in sterile soil. In contrast, the seedlings in the

germination pouches were exposed to air, only watered and not supplied with any additional nutrients.

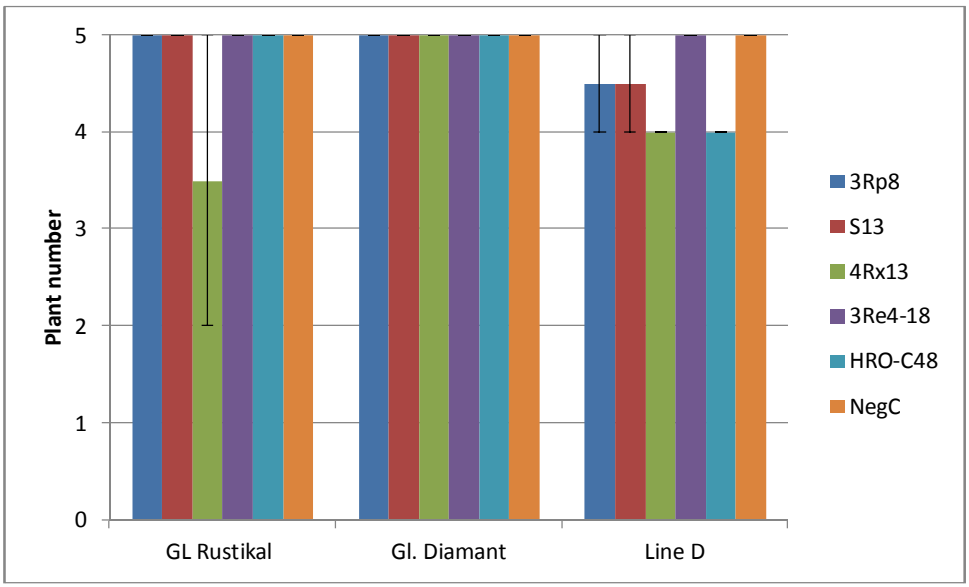

Figure 56: Average number of germinated seeds of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in sterile soil, six days post seeding. NegC: Negative control. Five plants of a cultivar-strain combination were grown in one box in two replicates.

With regard to the fresh weight per plant, a similar cultivar dependent tendency as in experiment 2 (chapter 3.5.2.2) was observed in this experiment (Figure 57). The plants were harvested twelve days after sowing. The fresh weight of above-ground plant parts per plant was greatest for the three-way cross hybrid GL Rustikal, followed by the single cross hybrid Gl. Diamant and the inbred Line D as the third. For GL Rustikal and Gl. Diamant, the strain 3Rp8 was most beneficial for the fresh weight per plant compared to the control, followed by 3Re4-18. For GL Rustikal the effect was bigger in both cases. For GL Rustikal, the fresh weights of plants which were treated with the strains S13, 4Rx13 and HRO-C48 were somewhat lower, but the effects were similar and the weights bigger than the weights of plants of the control, even upon a treatment with 4Rx13. In the case of Gl. Diamant the plant fresh weight after seed priming with those strains was lower than the weight of control plants. For Line D the treatment with S13 increased the fresh weight compared to control, but all of the other strains did not show positive effects. As in experiment 2, the positive effect of S13 on Line D is also noticeable here. The higher weight could be related to good colonisation, as observed in experiment 2 (chapter 3.5.2.2). Also the dominance of 3Rp8 in GL Rustikal and Gl. Diamant correlates with the colonisation pattern in experiment 2. The hypocotyl length of experiment 1 also correlates with this result (chapter 3.5.2.1).

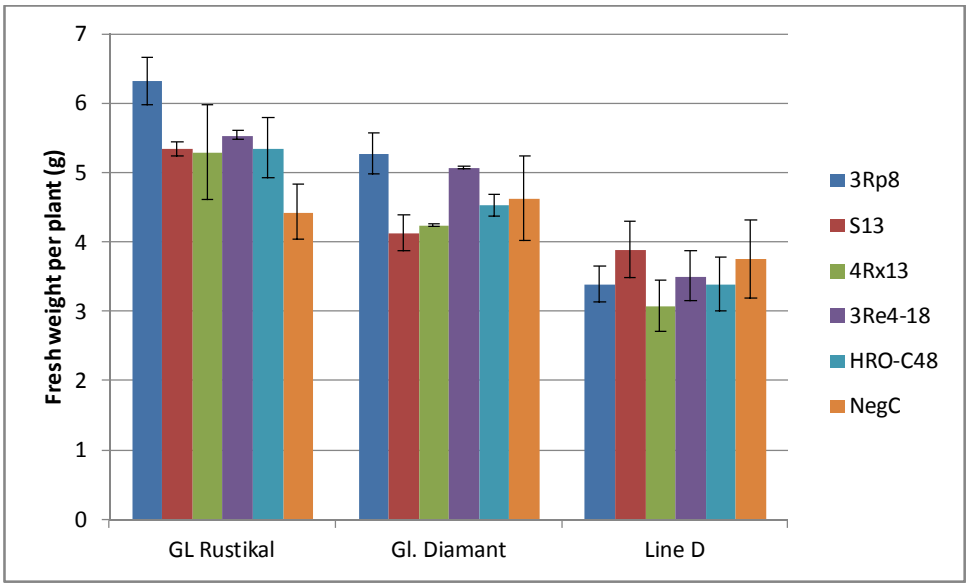

Figure 57: Average fresh weight per plant of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in sterile soil, twelve days post seeding. NegC: Negative control. Five plants of a cultivar-strain combination were grown in one box in two replicates.

Regarding the dry weight per plant a similar cultivar dependent tendency as by fresh weight was observed (Figure 58). Also the effects of the seed treatments were similar. For GL Rustikal 3Rp8 and 3Re4-18 showed noticeable effects on dry weight, but surprisingly 4Rx13 as well. The plant dry weight of cultivars treated with S13 and HRO-C48 was lower, but still higher than the control. Concerning Gl. Diamant the weight of plants treated with 3Rp8 and 3Re4-18 was as high as the weight of the control. The plant weight of the S13, 4Rx13 and HRO-C48 treatments was lower than the control. For Line D S13 showed the best effect on dry weight compared to the control. The weights of the other variants were similar and below the control. In this result, too, 3Rp8 and S13 dominated again dependent on genotype.

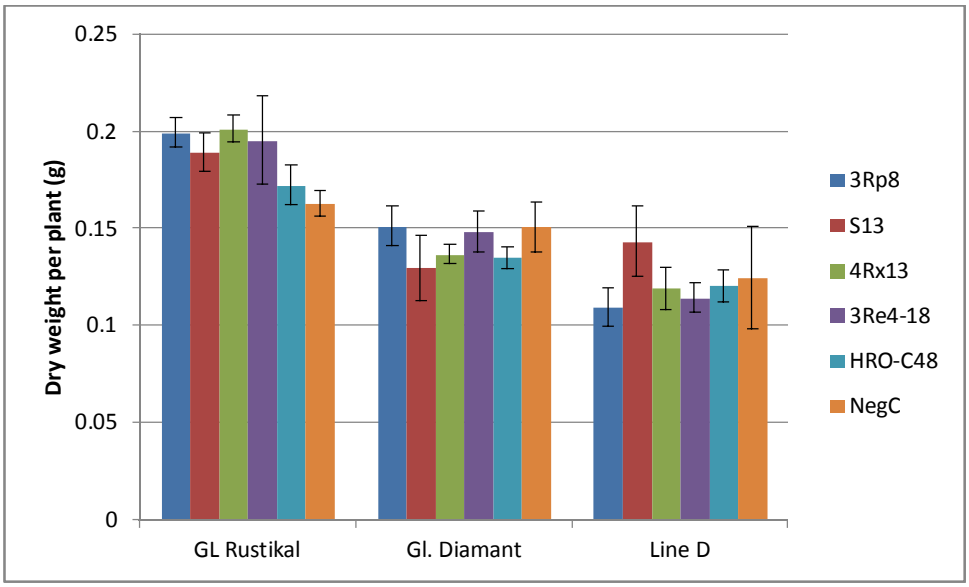

Figure 58: Average dry weight per plant of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in sterile soil, twelve days post seeding. NegC: Negative control. Five plants of a cultivar-strain combination were grown in one box in two replicates.

3.5.3.2 Experiment 4: Fresh and dry weight of *L. sativa* plants

The relationship of fresh and dry weight with plant-microbe interactions was also investigated with the *L. sativa* cultivar Gelber Winter, which was primed with different *S. plymuthica* strains and grown in sterile soil in plastic boxes.

The concentrations of the strains in the ONCs which were used for seed priming are listed in Table 12 (chapter 3.5.3.1).

Six days after sowing, the number of germinated seeds was evaluated (Figure 59). The germination rate of lettuce seedlings was only 4.5 to 7.5 plants in average of 9 plants. The highest plant number was gained upon a seed treatment with HRO-C48. The numbers of plants of all other treatments were below the control. This is an opposite effect compared to the pumpkin results, in which HRO-C48 showed no effect.

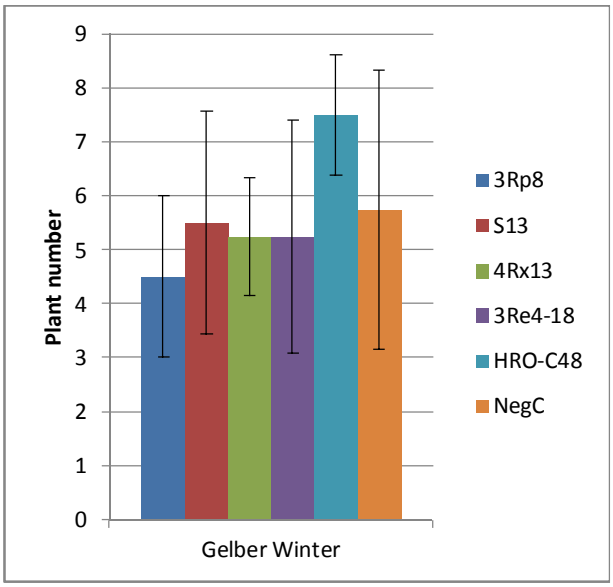

Figure 59: Average number of germinated seeds of *L. sativa* cultivar Gelber Winter after seed priming with different *S. plymuthica* strains grown in sterile soil, six days post seeding. NegC: Negative control. Nine plants of a treatment were grown in one box and in four replicate boxes.

15 days after sowing the plants were harvested. Regarding the fresh weight per lettuce plant, an opposite tendency to the plant number was observed. 3Rp8 and S13 promoted the weight most, followed by 4Rx13 and 3Re4-18. The fresh weight of plants treated with HRO-C48 was similar to the control (Figure 60).

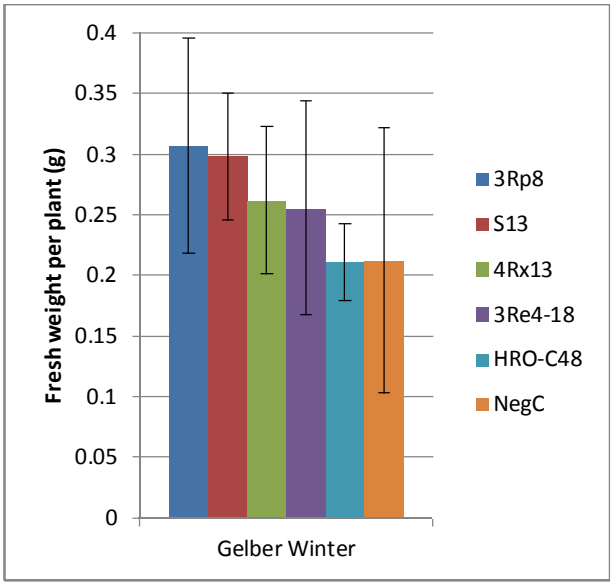

Figure 60: Average fresh weight per plant of *L. sativa* cultivar Gelber Winter after seed priming with different *S. plymuthica* strains grown in sterile soil, 15 days post seeding. NegC: Negative control. Nine plants of a variant were grown in one box and in four replicate boxes.

The results in terms of dry weight showed a promotion by S13, followed by 3Rp8. The treatments with the other strains did not show a positive effect compared to the control (Figure 61).

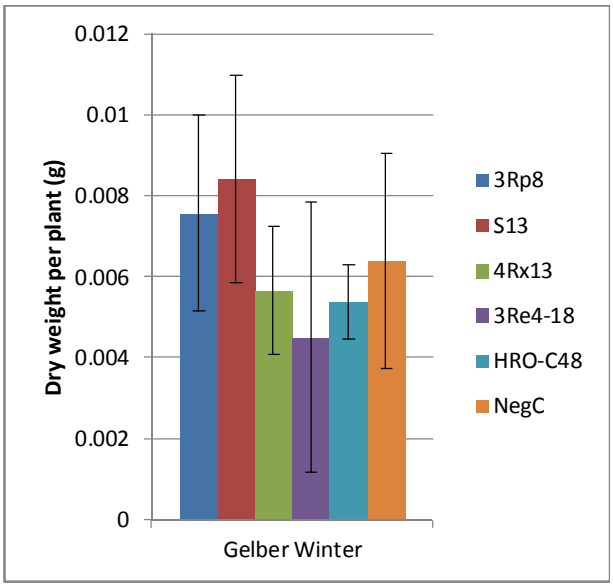

Figure 61: Average dry weight per plant of *L. sativa* cultivar Gelber Winter after seed priming with different *S. plymuthica* strains grown in sterile soil, 15 days post seeding. NegC: Negative control. Nine plants of a variant were grown in one box and in four replicate boxes.

3.6 Visualisation of bacterial colonisation patterns

Due to the specific seed physiology of the Styrian oil pumpkin (Figure 2), microorganisms are able to penetrate easily into inner compartments of the seeds, i.e. into layers of the seed coat and close to the embryo. But, microorganisms are also transmitted from seeds to developing plant parts, i.e. to the hypocotyl, cotyledons and roots. The colonisation patterns of certain transformed *S. plymuthica* strains on Styrian oil pumpkin roots, cotyledons and seeds upon seed priming were visualised by means of confocal laser scanning microscopy, as illustrated in the following subchapters.

Germinating seedlings of GL Rustikal, Gl. Diamant and Line D are illustrated in Figure 62. A strong, branched root system tightly covered with hairy roots of GL Rustikal is visible. In contrast, the root systems of plants of other genotypes were weaker. Differences in plant vigour of genotypes are due to heterosis of hybrid breeding.

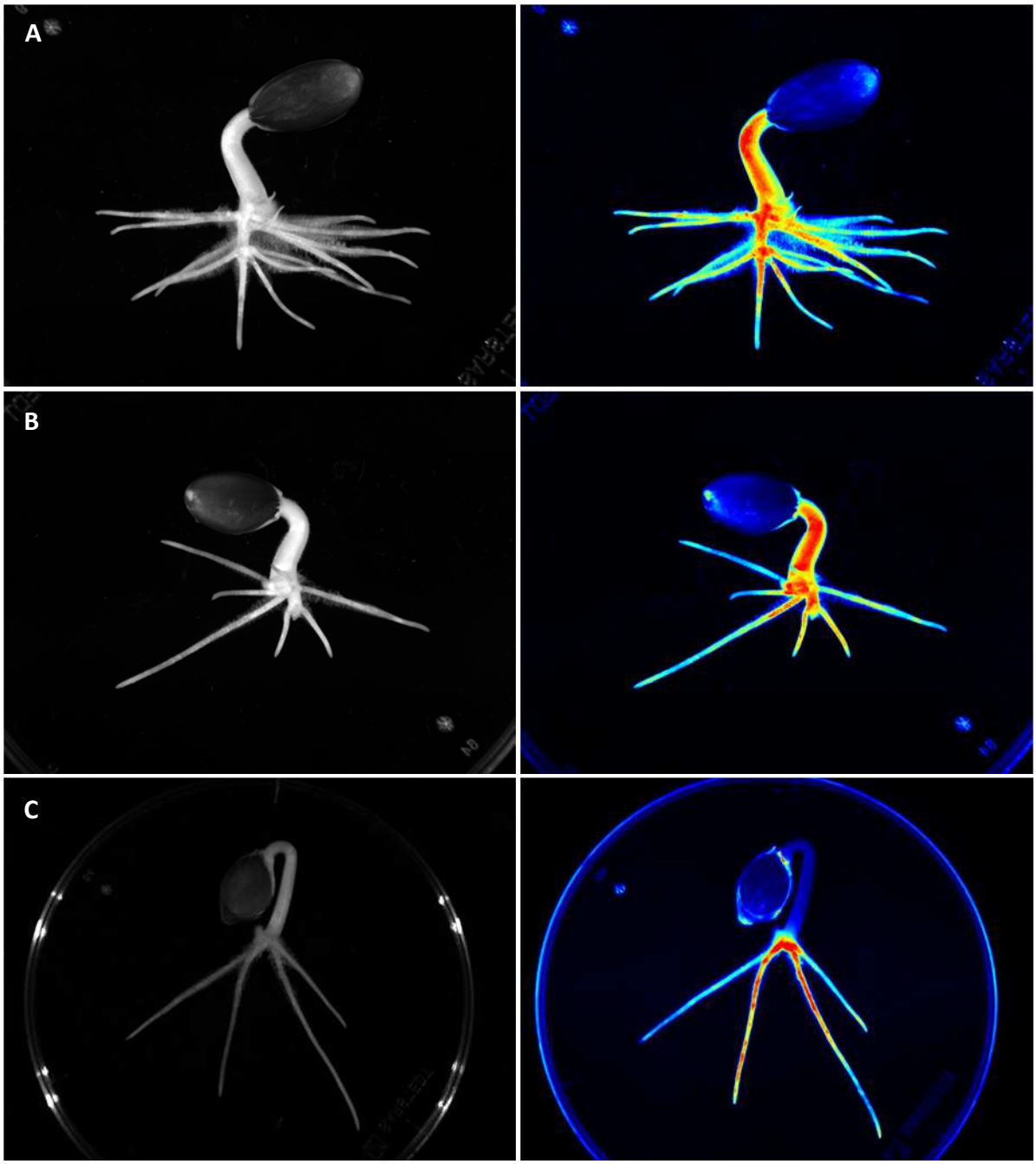

Figure 62: GL Rustikal (A), Gl. Diamant (B) and Line D (C) seedlings grown in germination pouches visualised by Bio-Rad ChemiDoc™ XRS System (false colouring based on autofluorescent plant tissues) five days post seeding. Seeds of GL Rustikal and Gl. Diamant were primed with a mixture of *S. plymuthica* 3Rp8-DsRed2, S13-GFP and 3Re4-18-mNeptune, seeds of Line D solely with S13-GFP.

3.6.1 Colonisation of roots

Hairy roots of *C. pepo* seedlings were densely colonised with *S. plymuthica* 3Rp8-DsRed2 as a consequence of seed priming. This was noticed, in particular, for genotypes GL Rustikal and Line D. Cells expressing the fluorescent protein were observed densely associated in colonies but also in loosened colonisation structures (Figure 63).

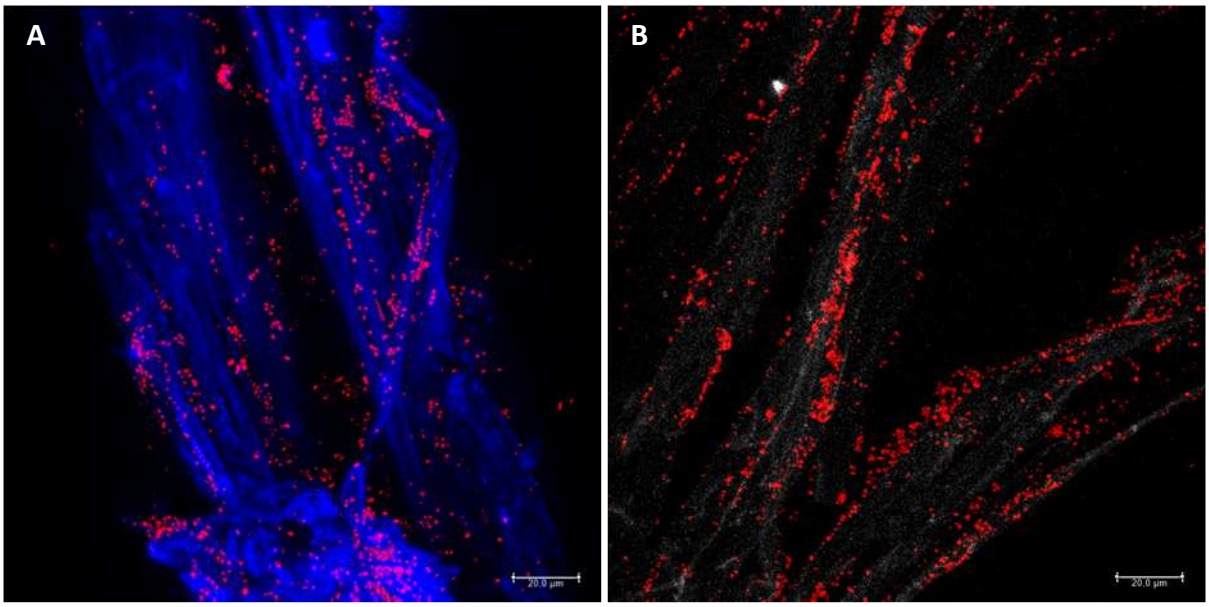

Figure 63: Hairy roots (blue and grey signal) of GL Rustikal (A) and Line D (B) colonised with 3Rp8-DsRed2 (red signal) twelve days post priming and sowing, visualised by CLSM (maximum projection, z=10/29).

Seed priming with 3Re4-18-mNeptune resulted in a looser and less dense colonisation pattern of hairy roots of *C. pepo* genotypes in the cases of GL Rustikal and Line D (Figure 64). On roots of Gl. Diamant some dense colonies were observed as well. The lower colonisation density could, on the one hand, be due to a poorer ability of colonisation of the *S. plymuthica* strain, but also to the visualisation technique, since mNeptune was excited with the same laser settings as DsRed2.

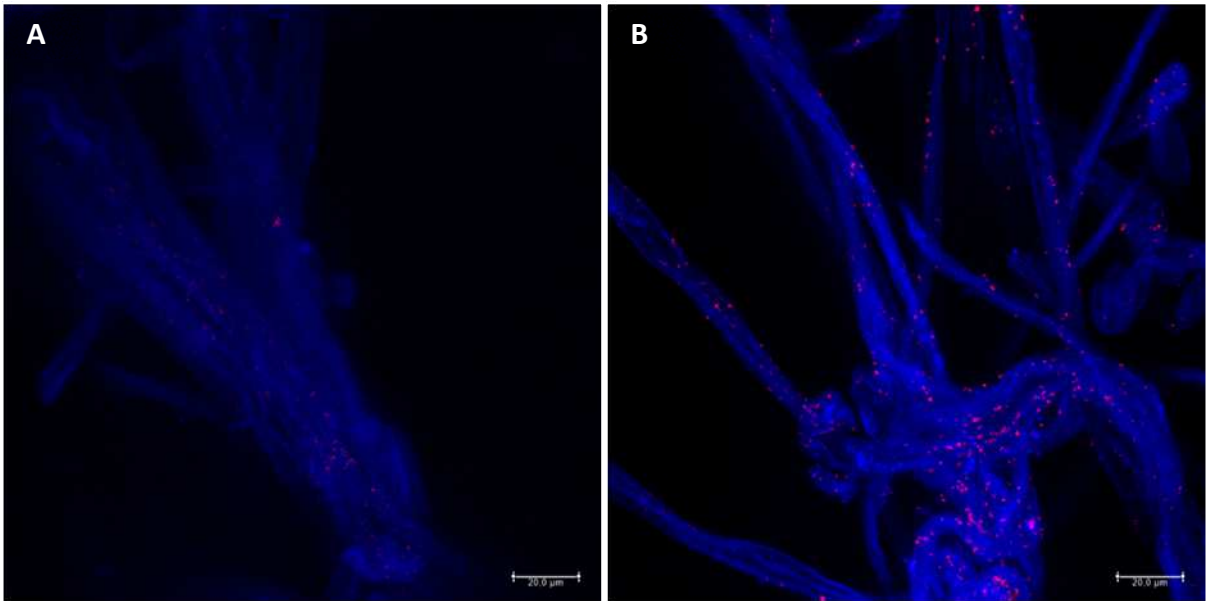

Figure 64: Hairy roots (blue signal) of GL Rustikal (A) and Line D (B) colonised with 3Re4-18-mNeptune (red signal) twelve days post priming and sowing, visualised by CLSM (maximum projection, z=16/20).

An intense and noticeable colonisation of hairy *C. pepo* roots could be demonstrated upon seed priming with *S. plymuthica* S13-GFP. Especially hairy roots of Line D were colonised with a lot of dense cell patches. But also roots of GL Rustikal and Gl. Diamant were densely colonised with colonies (Figure 65).

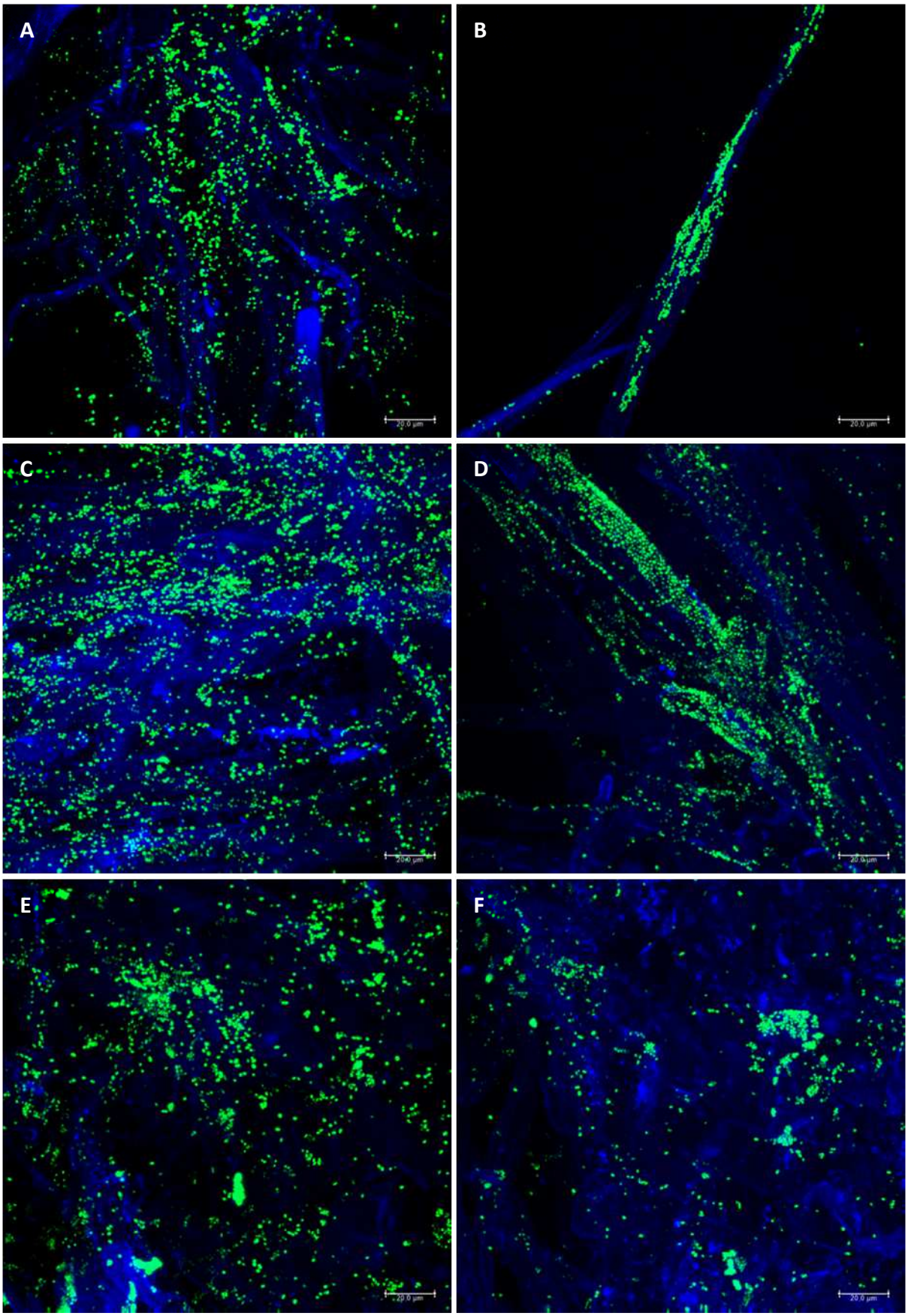

Figure 65: Hairy roots (blue signal) of GL Rustikal (A, B), Gl. Diamant (C, D) and Line D (E, F) colonised with S13-GFP (green signal) twelve days post priming and sowing, visualised by CLSM (maximum projection, z=78/25/37/58/76/61).

In contrast, seed priming with a mixture of *S. plymuthica* 3Rp8-DsRed2, 3Re4-18-mNeptune and S13-GFP resulted in a loose colonisation of roots (Figure 66). This could be due to some kinds of competition among the strains.

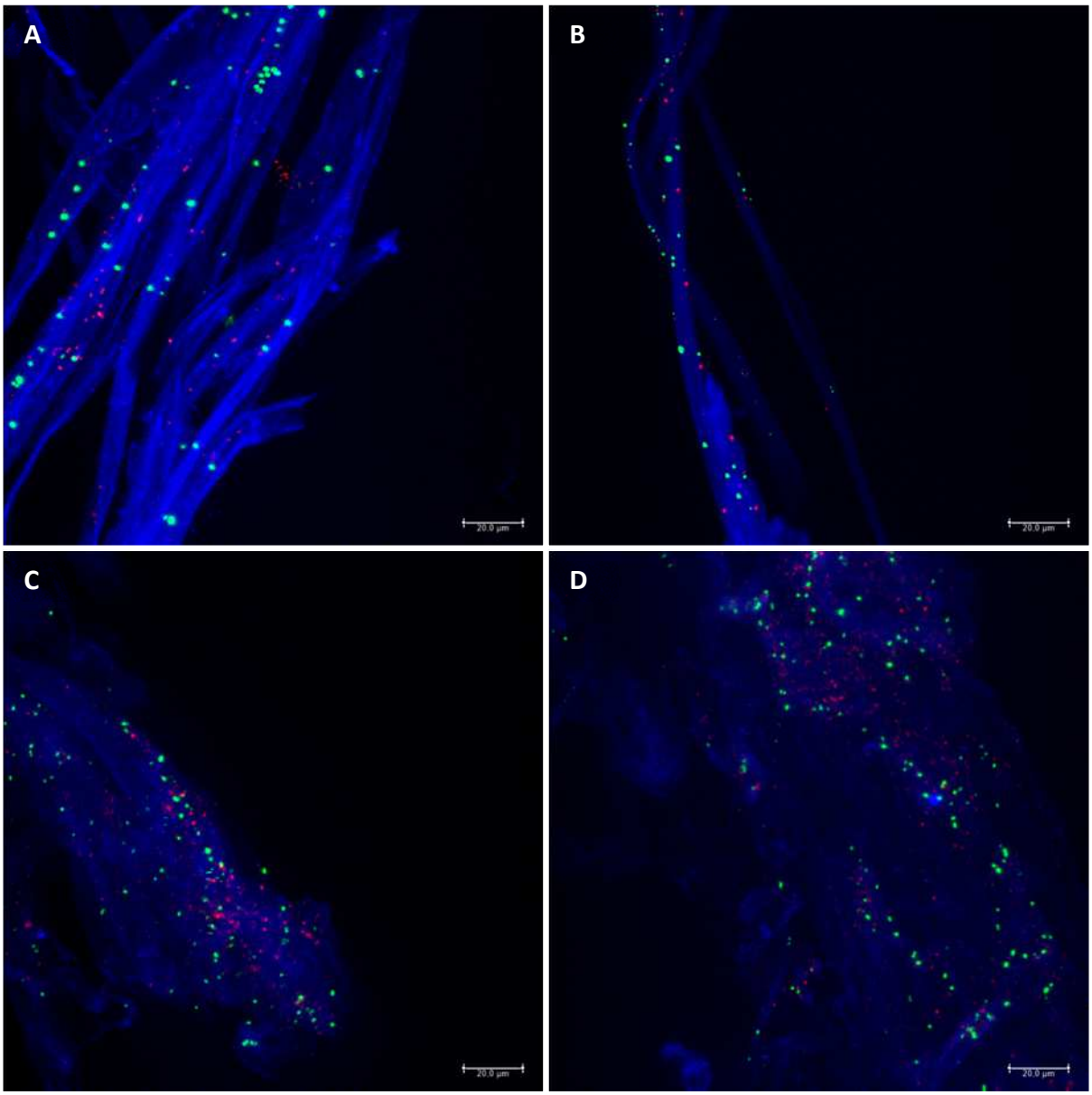

Figure 66: Hairy roots (blue signal) of GL Rustikal (A), Gl. Diamant (B) and Line D (C, D) colonised with S13-GFP (green signal), 3Rp8-DsRed2 and 3Re4-18-mNeptune (red signal) twelve days post priming and sowing, visualised by CLSM (maximum projection, z=38/57/60/85).

3.6.2 Colonisation of leaves

A loose distribution of *S. plymuthica* S13-GFP was found in leaf tissues of a Line D plant, which was primed with S13-GFP (Figure 67). This leads to the assumption that bacteria also colonise interior plant tissues.

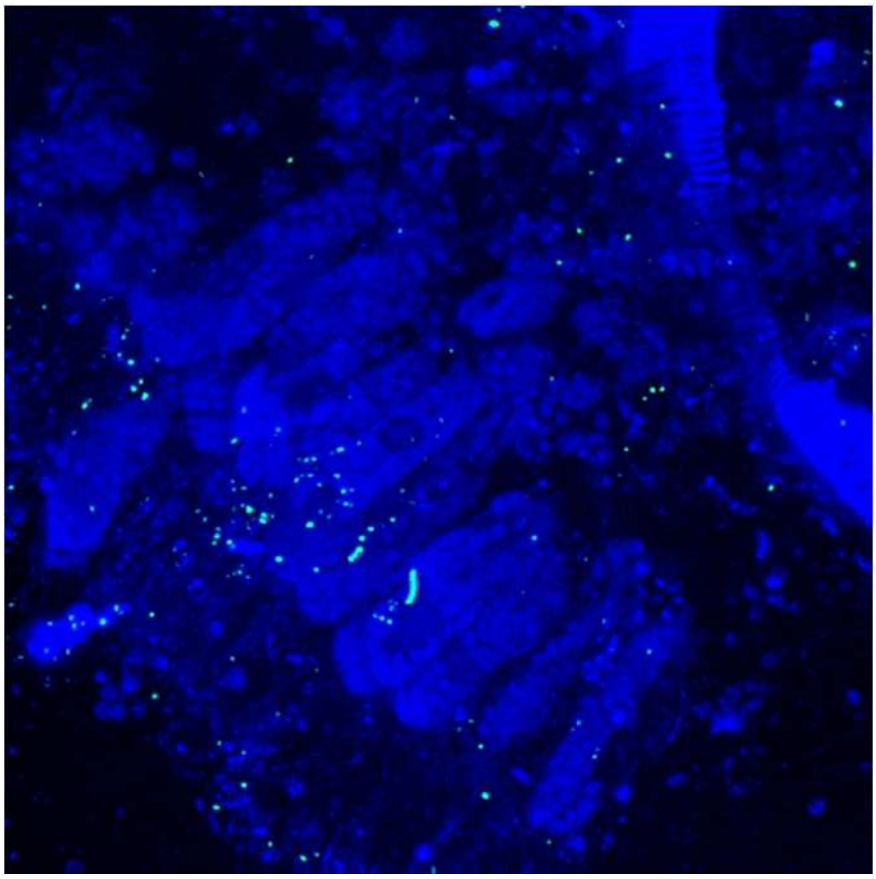

Figure 67: Cross-section of a Line D leaf (blue signal) colonised with S13-GFP (green signal) twelve days post priming and sowing, visualised by CLSM (maximum projection, z=28).

3.6.3 Colonisation of seeds

A very dense colonisation was observed in germinating seeds primed with *S. plymuthica* S13-GFP. The surface of embryonic cells in multicellular stage was colonised with S13-GFP in a loose pattern (Figure 68). In contrast, a cross-section of developing radicle showed a very dense settlement (Figure 69).

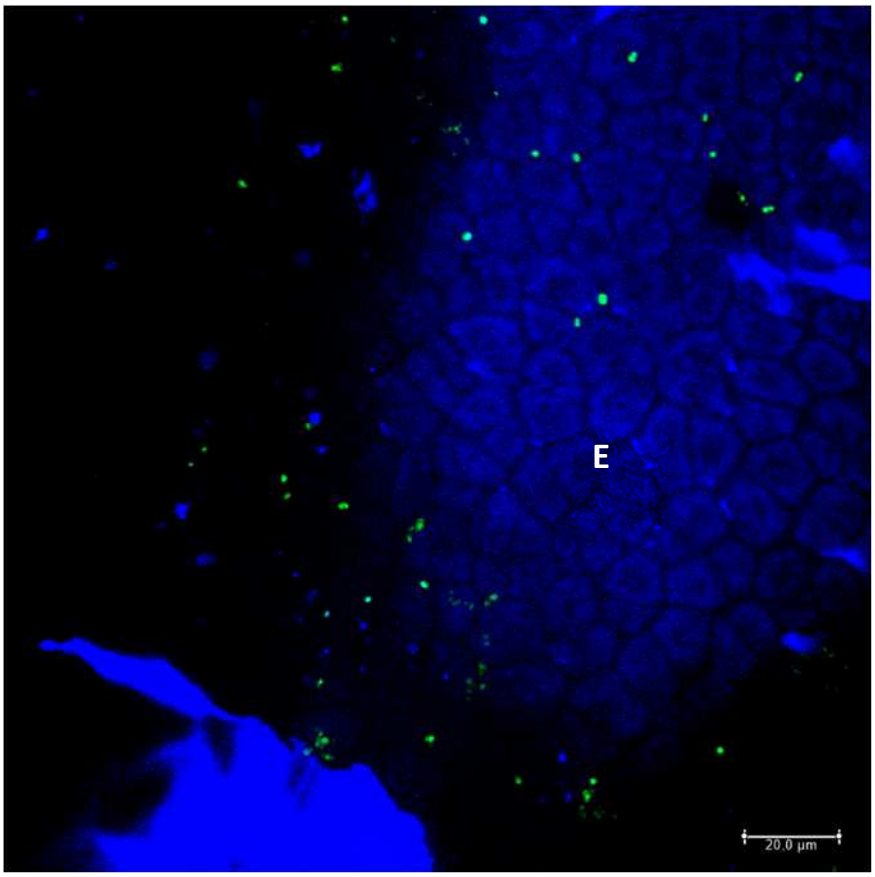

Figure 68: Surface of GL Rustikal hypocotyl and plumule section of embryo (E) in multicellular stage two days post priming with *S. plymuthica* S13-GFP and sowing, visualised by CLSM (maximum projection, z=40). Blue: Autofluorescence of plant tissue. Green: S13-GFP cells.

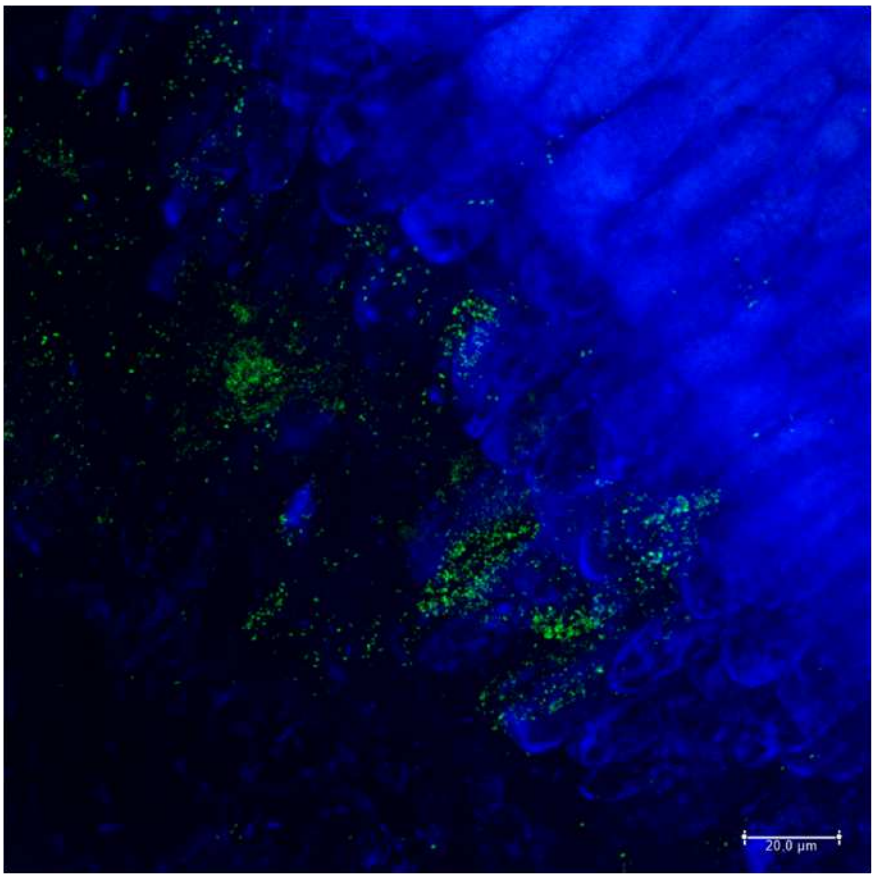

Figure 69: Cross-section of GL Rustikal radicle two days post priming with *S. plymuthica* S13-GFP and sowing, visualised by CLSM (maximum projection, z=51). Blue: Autofluorescence of plant tissue. Green: S13-GFP cells.

Different layers of the seed coat were examined as well. Especially in vascular bundles of chlorenchyma layer dense colonies of S13-GFP were observed. A superficial settlement was also observed on remainings of the nucellus of the seed coat (Figure 70).

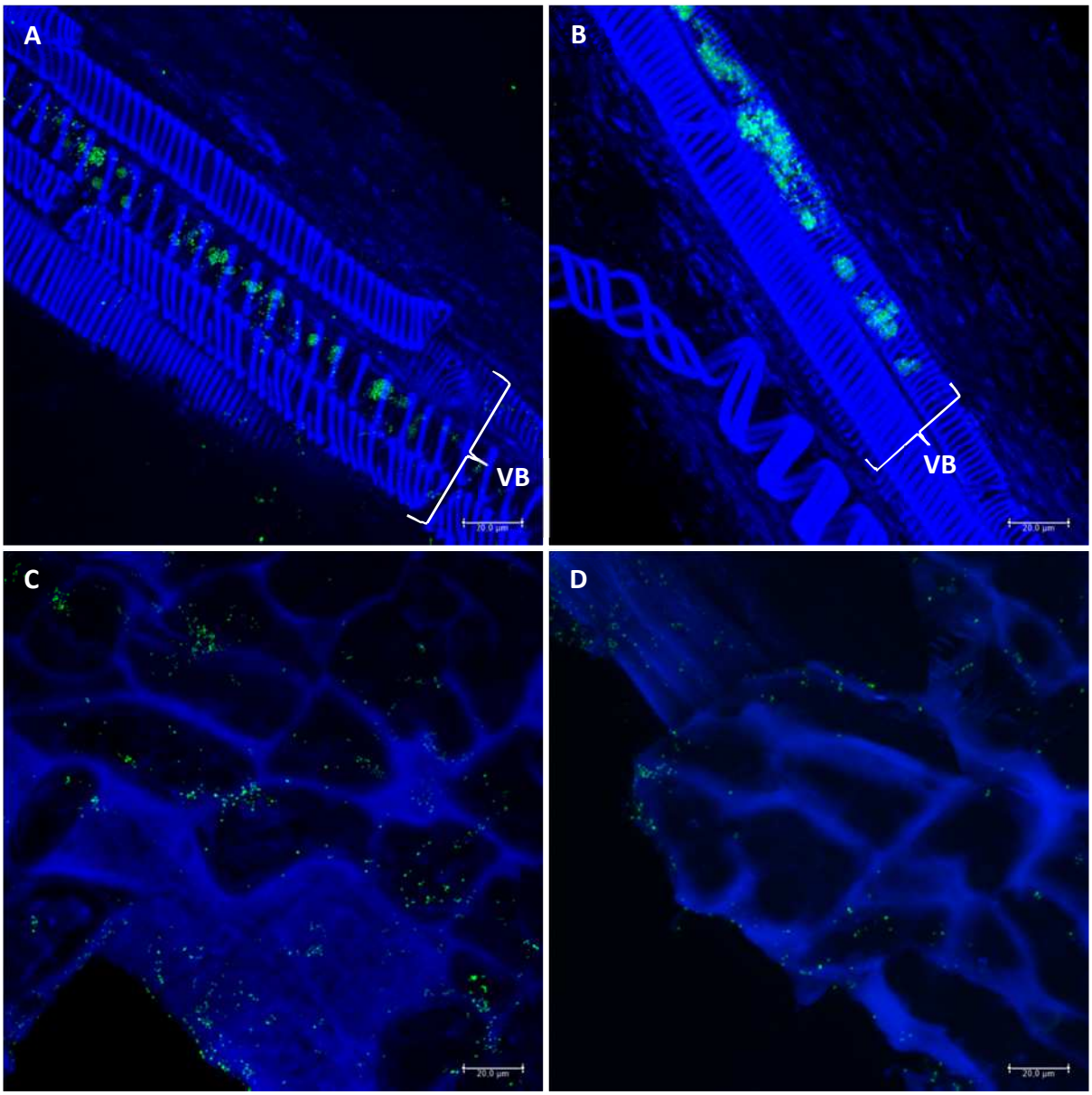

Figure 70: Cross-section of GL Rustikal chlorenchyma layer of the seed coat (blue signal) with *S. plymuthica* S13-GFP cells (green signal) distributed inside vascular bundles (VB) (A, B) and *S. plymuthica* S13-GFP cells (green signal) colonising remainings of the nucellus of the seed coat (blue signal) (C, D) three days post priming with *S. plymuthica* S13-GFP and sowing, visualised by CLSM (maximum projection, z=59/52/81/104).

Confocal laser scanning microscopy of *C. pepo* cotyledons in germinating seeds showed a superficial colonisation (Figure 71). Bacteria were presumably transferred from primed seeds to above-ground plant parts during germination and plant growth.

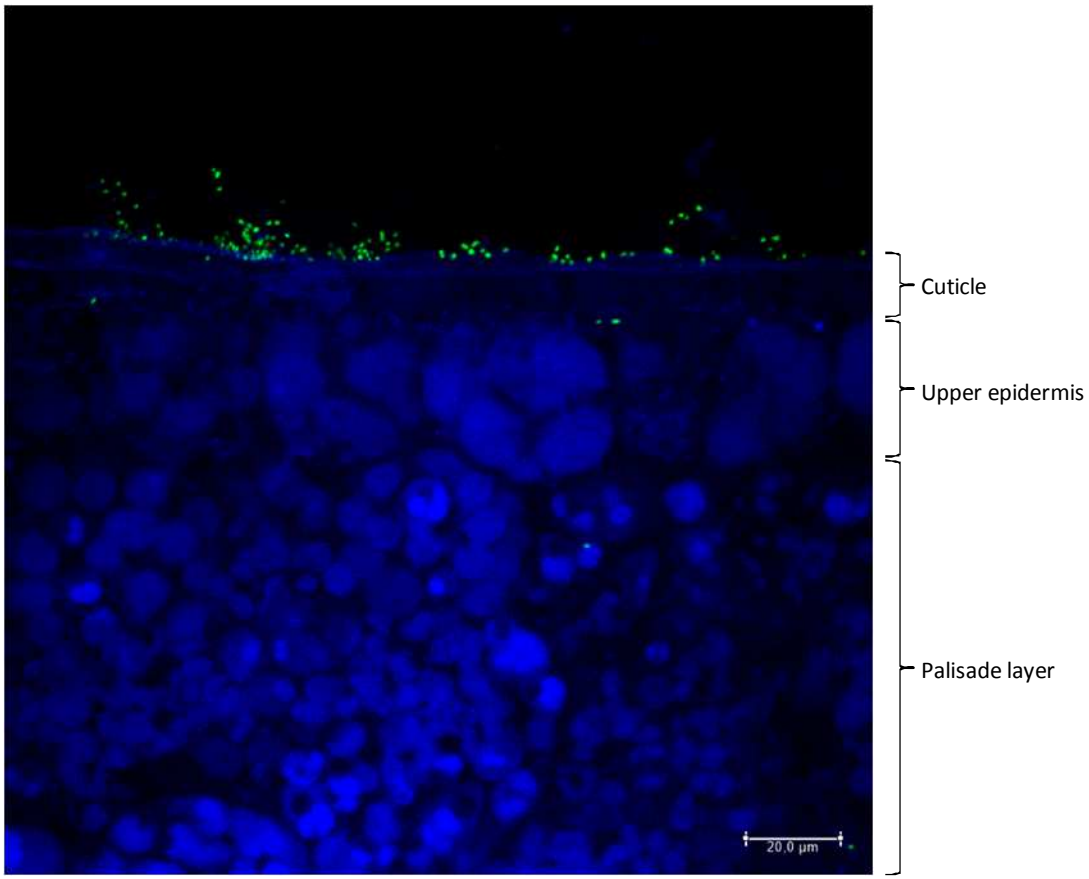

Figure 71: Cross-section of a GL Rustikal cotyledon three days post priming *S. plymuthica* S13-GFP and sowing, visualised by CLSM (maximum projection, z=38). Blue: Autofluorescence of plant tissue. Green: S13-GFP cells.

4 Discussion and Conclusions

The great importance of the Styrian oil pumpkin cultivation in Austria under changing and challenging climatic conditions and the high susceptibility to a large set of pathogenic bacteria and fungi led to the high interest in the constitution of the plant-associated microbiome. But also the differing and inconsistent responses to applied biological control agents, especially in the field, forced this analysis. The promising findings of the wide-ranging plant-associated microbiome analysis subsequently raised the assumption that genotype-specificity of plants on cultigen level also applies for the treatment with biological control agents (BCAs). In the following paragraphs the results of the analysis of the plant-associated microbiome constitution and the genotype-specific response to BCAs are comprehensively discussed and essentials highlighted.

The microbiome analyses and experiments confirmed the assumption of a genotype-specific microbiome and genotype-specific plant-microbe interactions. They revealed a strong impact of the *C. pepo* cultigen on the composition of the seed microbiome, but not on the rhizosphere microbiome. The seed microbiomes were further characterised by a low diversity in comparison to rhizospheres and bulk soil. Remarkably, potential phytopathogens of *C. pepo* belonging to Enterobacteriaceae family like *Erwinia* and *Pectobacterium* were also part of the seed communities. In general, it has to be taken into account that results of microbial communities´ analyses, which were derived by a partial 16S rRNA gene amplicon sequencing approach, allow only cautious interpretation.

The rhizosphere effect entitles the effect that the rhizosphere harbours less diverse bacteria, which are selectively enriched, in contrast to the surrounding soil (Bulgarelli et al. 2012, Lundberg et al. 2012). This trend was also observed in the analysed *C. pepo* rhizosphere bacterial communities. Several taxa such as Bacilli, Actinobacteria, Saprospirae, Alpha-, Beta- and members of the Gammaproteobacteria were enriched in the rhizosphere microbiome, while Thaumarchaeota (phylum of Archaea), Acidobacteria and Chloroflexi comprised higher proportions in the soil. These results support the assumption that Gammaproteobacteria, Betaproteobacteria and Firmicutes contribute to disease suppressive microbiome and that plants selectively select beneficial microorganisms (Berendsen et al. 2012). In contrast to seed-associated consortia, the bacterial communities of the rhizospheres of the different

genotypes were similar. The influence of the soil might have been responsible for a normalisation of the root-associated microbiomes as it is proposed that plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere (Berg and Smalla 2009). Beside the rhizosphere, plants constitute several compartments including the spermosphere, which comprises all compartments of a seed (Nelson 2004). Similar to the rhizosphere effect, the spermosphere or seed effect is also defined by a selective enrichment of specific microorganisms of which they are presumably useful for germination and plant growth and health (Adam et al. 2016b).

The transmission of phytopathogens from seeds to above-ground plant parts could be responsible for a severe infestation, e.g. in the case of *C. pepo* regarding the fruit rot disease. For example, *Pectobacterium carotovorum*, an important pathogen causing fruit rot on the Styrian oil pumpkin, was part of the seed microbiome in all genotypes, with the exception of GL Rustikal. The genus *Erwinia* was enriched in two genotypes, in the inbred Line D as well as in GL Classic. Moreover, the genus *Pseudomonas* was present in the seed microbiome of all genotypes, which comprises a number of beneficial species (Avis et al. 2008), but also includes species which can cause leaf necrosis in *Cucurbita pepo*, such as *P. vridiflava* (Grube et al. 2011, Huss and Mavridis 2007). Representatives of *Xanthomonas campestris* and *P. syringae*, for example, have been shown to be seed-borne pathogens in the Cucurbitaceae family (Babadoost and Zitter 2009, Robinson and Decker-Walters 1997, Zitter et al. 1996, Blancard et al. 1994).

Besides possible phytopathogens, possible beneficials like *Lysobacter*, *Paenibacillus* and *Lactococcus* were also part of the seed microbiomes. Seed treatments with strains of *Lysobacter gummosus* L101 and *Paenibacillus polymyxa* PB71 resulted in significant effects on harvest yields (Fürnkranz et al. 2012). Based on the results of this study, *Lactococcus* species can also be taken into consideration as biological control agents for the Styrian oil pumpkin. This genus was highly abundant in some genotypes, i.e. in four out of eight cultivars bred in Austria, comprising Line B, Line D, GL Opal and GL Rustikal. Shrestha et al. (2014) already reported good effects of lactic acid bacteria against *Pectobacterium carotovorum* as well as *Xanthomonas campestris* on pepper in the greenhouse and in field experiments. The genus *Serratia* includes potential biocontrol species as well. Members of this genus are able to substantially enhance germination rates of chemically untreated

C. pepo seeds in field experiments (Fürnkranz et al. 2012), but this genus was detected in extremely minor abundances in seed and rhizosphere samples. In bulk soil samples this taxon was not detected. This might be an indication for a plant-associated occurrence, but because of the very low proportions in seed and rhizosphere communities this assumption needs to be taken with caution and requires validation. But due to promising results of Adam (2015), especially regarding *S. plymuthica* strains 3Rp8 and S13, targeted greenhouse experiments and microscopic analyses of different *S. plymuthica* strains as BCAs were carried out with three *C. pepo* genotypes: GL Rustikal and its parental components Gl. Diamant and Line D. Adam (2015) and Fürnkranz et al. (2012) have previously described a positive effect of 3Rp8 and S13 on plant growth promotion and germination rate enhancement, but no studies on different responses among cultivars were performed yet. Basically, a genotype-specific effect depending on the breeding level was observed, as hybrids achieved higher tissue weights due to heterosis in comparison to the inbred line, which suffered from a lower germination capacity which might be due to inbreeding depression effects. A promoting effect on fresh weight of plants grown in germination pouches and in sterile soil was observed upon seed priming with 3Rp8 especially in combination with GL Rustikal. Impressively, a genotype dependent effect of *S. plymuthica* strains was observed by means of reisolation of transformed bacteria out of *C. pepo* root and leaf tissues. 3Rp8 and S13 applied as single strains dominated *C. pepo* roots and leaves, but the first on hybrids and the latter to the same extent as 3Rp8 on the inbred line. Adam (2015) has already shown the impressive positive effects on germination and fresh weight of 3Rp8 on GL Rustikal followed by S13. Genotype-specificity of plant-associated bacterial communities has already been shown for *Arabidopsis thaliana* by Haney et al. (2015) and Lundberg et al. (2012) and for *Brassica napus* by Rybakova et al. (2017). Consequently, also a genotype-specificity of biocontrol strains is assumed (Drogue et al. 2012). After treatment with the mixture of 3Rp8, S13 and 3Re4-18 only a lower number of bacteria could be reisolated, although the dominant 3Rp8 and S13 were part of it. Studies like those of Stockwell et al. (2011), Sarma et al. (2015) and Hays et al. (2015) showed that bacteria are more efficient for biocontrol of pathogens and perform better in their network mode of activity when they are applied in consortia. However, the lower CFU number of the applied mixture in comparison to the single strain seed treatment could be due to some kind of competition of the strains since they have similar requirements and occupy the same niche. A similar effect was observed

upon microscopy since 3Rp8 and S13 applied as single strains colonised root surfaces in a dense way, whereas a mixture of those with 3Re4-18 resulted in a weaker colonisation. For HRO-C48 a cell density-dependent effect on plant growth promotion has been shown by Kurze et al. (2001), which supports the relation of surveyed colonisation and cell density derived by reisolation. According to Scher et al. (1984) a crucial population density for HRO-C48 in the order of magnitude of 10^4 CFU per gram root fresh weight of maize is necessary for successful biocontrol. A certain cell density is, among other factors, required for quorum sensing, which is a prerequisite for the regulation of the production of antimicrobial compounds (Liu et al. 2007). In general, the plant microbiome can help plants to fend off diseases, promote stress resistance, stimulate growth and occupy space that would otherwise be taken up by pathogens. They influence crop yield and quality as well (Berg et al. 2013). Plant growth promotion can be achieved by direct and indirect mechanisms, by production of signal molecules which initiate modification of root system, by providing nutrients, altering functions and influencing plant physiology, for example by initiating systemic resistance (Vacheron et al. 2013, Pang et al. 2009, Richardson et al. 2009). Upon greenhouse experiments under gnotobiotic conditions this threshold could be reached by all genotypes and BCA treatments, but under arable field conditions the colonisation efficiency could be different. Adam (2015) already showed that the effects of BCA application on the Styrian oil pumpkin under controlled and aseptic conditions are hardly achievable under harsh conditions in the field, in heavily infested soils. Nevertheless, biocontrol and exploitation of plant-microbe interactions open up a promising possibility for conventional and organic agriculture worldwide to produce in an environmentally friendly way (Berg 2009).

Genotype-specificity was also observed in terms of plant weight. 3Rp8 followed by 3Re4-18 enhanced fresh and dry weight of hybrids GL Rustikal and Gl. Diamant and S13 the weight of the inbred Line D. Adam (2015) only showed a neutral behaviour of 3Re4-18 on GL Rustikal in terms of plant growth promotion. In this context, the assumption arises that genotype-specificity of microorganisms is also related to the breeding level, which is defined by the genetic variation and degree of inbreeding of a genotype or cultivar. Concerning the lettuce cultivar Gelber Winter the *S. plymuthica* strains 3Rp8 and S13 as well were positive for plant weight, but HRO-C48 priming enhanced germination compared to the control and other treatments. HRO-C48 is the basic component of the commercially available biocontrol

product RhizoStar® and known for its growth promotion effect besides its antagonistic activity (Berg et al. 1999, Kalbe et al. 1996).

In contrast to beneficial BCAs like 3Rp8 and S13 the strain 4Rx13 suppressed germination of *C. pepo*, albeit it's high genomic similarity with beneficial *S. plymuthica* strains (Adam 2015, Neupane 2013). According to Neupane (2013) this could be due to mutations in one or more putative genes for growth and antagonism. 4Rx13 is known for its antagonistic activity (Berg et al. 2002) and its production of high amounts of sodorifen, a newly discovered and unusual volatile organic compound (Weise et al. 2014). The high germination and growth capacity of all *C. pepo* genotypes despite a treatment with 4Rx13 grown in sterile soil in comparison to germination pouches, in which the germination was suppressed totally, could be related to the soil environment and to nutrient supply in sterile soil. In contrast, the seedlings in the germination pouches were exposed to air, only watered and not supplied with any additional nutrients. Various micronutrients like molybdenum (Datta et al. 2011), nickel (Brown et al. 1987), zinc and boron (Johnson et al. 2005) are essential for germination. Upon growth in germination pouches young *C. pepo* plants lack nutrients and are susceptible to the aborting influence of 4Rx13.

Since transformed bacteria, which were applicated on seeds by priming, were observed on subterranean and above-ground plant parts, a transmission during the process of plant tissue growth must have been occurred. The strong colonisation, especially on roots, could be related to the fact that Proteobacteria are among dominant phyla including Actinobacteria, Bacteroidetes and Firmicutes that colonise plant tissues (Bulgarelli et al. 2013). The potential dominance of Proteobacteria could be exploited in a way considering competition in a niche by members of the same taxon. Based on the assumption that the existing evenness of a root microbiome is maintained, seed-borne pathogens could be suppressed by seed priming with strongly colonising BCAs, as observed by 3Rp8 and S13. But the evenness can of course be influenced. If there is a too strong shift, the uniform distribution of the taxa could be impaired and thus also the diversity can be reduced. Hibbing et al. (2010) stated that interactions within and between bacterial species can have a substantial impact on the outcome of competition. Bacteria can even affect similar or closely related strains (Be'er et al. 2009). Also antagonistic effects of bacteria and fungi in the same niche were shown by Rousk et al. (2008).

Due to, compared to other crops, a relatively short breeding history, the genetic differences between the *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* genotypes are small, as this thin-coated seed segregant first appeared in the late 19th century (Teppner 2004). The commercial breeding program for the Styrian oil pumpkin in Austria at Saatzucht Gleisdorf GmbH started in 1960 and was intensified 20 years ago. Thus, it is remarkable that cultivar dependent differences in the seed microbiomes were found within this narrow gene pool. These results contrast with the findings of Klaedtke et al. (2016), who revealed that the microbial assemblages of bean seeds were shaped by the seed production site rather than by the genotype. Based on this study, the influence of field site origin of seeds is minor compared to the genotype. According to Thomson et al. (2010), field vegetation in the sense of plant-derived organic matter inputs affects the abundances of dominant soil bacterial taxa, which subsequently impacts rhizosphere composition. But this assumption was not addressed in this study.

Within the components of the GL Rustikal pedigree, the seeds of Gl. Diamant were colonised by a significantly more diverse microbiome than the other genotypes, whereas for example GL Rustikal seeds were characterised by a significantly greater level of colonisation of Leuconostocaceae than other genotypes. The seed core microbiome is dominated by high abundances of seven Enterobacteriaceae, one Pseudomonadaceae, one *Lactococcus* and one *Exiguobacterium* OTU.

The results of the seed analyses are of particular interest for the seed production industry, as the Styrian oil pumpkin is highly susceptible to various fungal and bacterial pathogens during germination making chemical or complex seed treatments inevitable. It remains to be investigated to which extent naturally occurring seed-borne bacteria influence germination and plant development. According to Berg et al. (2013), the phylum of Proteobacteria includes many plant growth promoting bacteria. Nevertheless, the dominance of the seed-associated microbiomes by Proteobacteria, and in particular Enterobacteriaceae, may contribute to disease susceptibility as the microbial richness and evenness of the microbial taxa are important for the maintenance of plant health (Bakker et al. 2012). The interactions of seed-borne microorganisms with indigenous soil populations of microorganisms may influence the expression of biological control traits or the subsequent colonisation of the rhizosphere (Nelson 2004). Therefore, the results of this study could have implications for

seed treatments or influence seed disinfection strategies that might replace commercial chemical treatments. The knowledge of genotype-specific promotion of certain *S. plymuthica* strains, as demonstrated with 3Rp8 for the hybrids GL Rustikal and Gl. Diamant and with S13 for the inbred Line D, can be used in a targeted way. A possible implication for breeding programs could be the selection of genotypes enriching less Enterobacteriaceae pathogens and/or expressing a higher microbial diversity in their seeds. The hologenome theory of evolution postulates that the host and its associated beneficial microbiome (holobiont) co-evolve as one unit to provide benefits to one another including defence mechanisms (Gopal and Gupta 2016, Zilber-Rosenberg and Rosenberg 2008). When postulating that breeding plants is some kind of directed evolution, it may be assumed that breeding under conventional conditions (for example with the use of chemical strippers and fungicides) leads to a loss of natural defence mechanisms originally provided by the holobiont system. The results of studies like this could contribute to a paradigm shift towards ecological breeding programs. Scher et al. (1984) showed that Captan seed treatment had no substantial effect on subsequent bacterial root colonisation. In contrast, Bikrol et al. (2005) analysed the influence of Thiram fungicide seed treatment on nitrogen fixation by *Glycine max* resulting in a dose-dependent effect on nitrogen fixation and crop yield. But as discussed above, even fungal and bacterial communities can affect each other (Rousk et al. 2008).

The already conducted analyses and experiments are just the beginning of versatile and complex research in order to implement the knowledge of plant-microbe interactions into an advanced breeding strategy of the Styrian oil pumpkin. The next steps will include the 16S rRNA gene amplicon sequencing of progeny seeds and rhizospheres of some selected genotypes as well as the analysis of the fungal communities by ITS amplicon sequencing. The selected genotypes comprise the GL Rustikal pedigree including the highly diverse Gl. Diamant, Line D, which was characterised by a high *Erwinia* occurrence in seeds, as well as Line A and B as parental components of Gl. Diamant. The open pollinated cultivar GL Classic, which was characterised by a high *Erwinia* abundance in its seeds as well, was also included. For the analysis of fungal diversity by means of the ITS amplicon sequencing the application of the designed and validated pepoPNA clamp is an essential to exclude non-target, plant-derived sequences. Further CLSM analysis, greenhouse experiments and field trials for the assessment of differences in plant-microbe interactions of *Cucurbita pepo*

genotypes could reveal deeper understandings of biological roles of these microbial communities and of BCAs in the development and life cycle of the Styrian oil pumpkin.

List of Abbreviations

3Re4-18	*Serratia plymuthica* strain 3Re4-18
3Rp8	*Serratia plymuthica* strain 3Rp8
4Rx13	*Serratia plymuthica* strain 4Rx13
APS	ammonium persulphate
BC primer	barcoded primer
BCA	biological control agent
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CFU	colony forming unit
CSLM	Confocal Laser Scanning Microscope
dps	days post seeding
DsRed2	fluorescent protein DsRed2
DW	dry weight
eBFP2	fluorescent protein eBFP2
EDTA	ethylenediaminetetraacetic acid
FW	fresh weight
GFP	fluorescent protein GFP
HCl	hydrochloric acid
HRO-C48	*Serratia plymuthica* strain HRO-C48
ITS	internal transcribed spacer
kb	kilobases
LB medium	Luria-Bertani medium
MDE	mutation detection enhancement gel solution
mNeptune	fluorescent protein mNeptune
NaCl	sodium chloride
NaOCl	sodium hypochloride
NaOH	sodium hydroxide
NegC	negative control
ONC	overnight culture
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PDA	potato dextrose agar
PNA	peptide nucleic acid
rpm	rounds per minute
S13	*Serratia plymuthica* strain S13 (DBLH13)
SDS	sodium dodecyl sulphate
SSCP	single strand conformation polymorphism
TBE	buffer consisting of Tris HCl, boric acid and EDTA
TGGE	temperature gradient gel electrophoresis
TEMED	tetramethylethylenediamine
T_m	melting temperature
Tp	trimethoprim
ZYMV	Zucchini Yellow Mosaic Virus

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Appendix

Genotype-specific analyses with BCAs in the greenhouse

In subsequent Appendix-Table 1 and Appendix-Table 2 the results of reisolation experiments, i.e. the CFU/g fresh weight of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in germination pouches, are summarised.

Appendix-Table 1: Investigation of reisolated CFU/g fresh weight of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in germination pouches, 13 days post seeding. Experiment 1. NegC: Negative control. Five plants of a variant were grown in one pouch in one replicate. CFU/g FW was calculated as the mean of the two results of the two CFU counts per dilution

Strains and cultivars	Root										Leaf					
	Weight	CFU								CFU/g FW	Weight	CFU				CFU/g FW
3Rp8-DsRed2		Dil. 10^{-3}		Dil. 10^{-4}		Dil. 10^{-5}		Dil. 10^{-6}				Dil. 10^{-3}		Dil. 10^{-4}		
GL Rustikal	1.067	-	-	180	225	-	-	26	9	4.57E+09	3.013	-	-	110	94	1.69E+08
Gl. Diamant	0.850	-	-	171	153	-	-	-	-	9.53E+08	2.347	-	-	23	27	5.33E+07
Line D	0.787	-	-	-	-	268	336	-	-	1.92E+10	2.206	-	-	53	43	1.09E+08
S13-GFP																
GL Rustikal	0.680	-	-	44	48	-	-	-	-	3.38E+08	2.677	n.e.	125	33	24	5.32E+07
Gl. Diamant	1.151	-	-	53	29	-	-	-	-	1.78E+08	1.922	-	-	6	4	1.30E+07
Line D	1.183	-	-	17	14	-	-	-	-	6.55E+07	1.920	-	-	21	22	5.60E+07
4Rx13-eBFP2																
GL Rustikal	0.275	-	-	76	79	-	-	-	-	1.41E+09	-	-	-	-	-	-
Gl. Diamant	0.194	-	-	35	47	-	-	-	-	1.06E+09	-	-	-	-	-	-
Line D	0.195	-	-	45	47	-	-	-	-	1.18E+09	-	-	-	-	-	-
3Re4-18-mNeptune																
GL Rustikal	1.132	-	-	60	61	-	-	-	-	2.67E+08	2.021	177	205	9	10	4.73E+07
Gl. Diamant	0.763	-	-	48	54	-	-	-	-	3.34E+08	2.083	55	62	-	-	1.40E+07
Line D	1.246	-	-	52	80	-	-	-	-	2.65E+08	3.617	-	-	17	12	2.00E+07
Mix																
GL Rustikal	0.978	-	-	18	31	-	-	-	-	1.25E+08	2.424	126	77	11	0	2.09E+07
Gl. Diamant	0.981	-	-	29	95	-	-	-	-	3.16E+08	2.027	25	39	-	-	7.89E+06
Line D	0.826	113	116	10	25	-	-	-	-	1.06E+08	2.215	-	-	57	29	9.71E+07
NegC																
GL Rustikal	1.732	-	-	1	2	-	-	-	-	4.33E+06	1.539	-	-	1	3	6.50E+06
Gl. Diamant	1.934	-	-	32	27	-	-	-	-	7.63E+07	1.695	-	-	6	14	2.95E+07
Line D	1.273	-	-	1	0	-	-	-	-	1.96E+06	1.356	-	-	9	13	4.06E+07

Appendix-Table 2: Investigation of reisolated CFU/g fresh weight of *C. pepo* cultivars after seed priming with different *S. plymuthica* strains grown in germination pouches, 15 days post seeding. Experiment 2. NegC: Negative control. Five plants of a variant were grown in one pouch in three replicates. CFU/g FW was calculated as the mean of the two results of the two CFU counts per dilution

Strains and cultivars	Root								Leaf							
	Weight	CFU						CFU/g FW	Weight	CFU						CFU/g FW
3Rp8-DsRed2		Dil. 10^{-4}		Dil. 10^{-5}		Dil. 10^{-6}				Dil. 10^{-4}		Dil. 10^{-5}		Dil. 10^{-6}		
GL Rustikal	15.049	-	-	-	-	19	19	1.26E+09	15.018	-	-	37	38	6	4	2.50E+08
Gl. Diamant	12.014	-	-	-	-	16	19	1.46E+09	10.210	-	-	31	35	3	4	3.23E+08
Line D	12.386	-	-	99	89	5	13	7.59E+08	8.679	-	-	19	34	6	3	3.05E+08
S13-GFP																
GL Rustikal	19.030	-	-	90	110	7	12	5.25E+08	11.859	-	-	23	26	3	3	2.07E+08
Gl. Diamant	12.926	-	-	79	63	9	2	5.49E+08	9.749	69	57	9	1	1	2	6.46E+07
Line D	11.309	-	-	69	95	5	6	7.25E+08	7.787	-	-	21	18	2	3	2.50E+08
4Rx13-eBFP2																
GL Rustikal	0.501	25	29	2	0	0	0	5.39E+08	-	-	-	-	-	-	-	-
Gl. Diamant	0.499	21	23	0	3	0	0	4.41E+08	-	-	-	-	-	-	-	-
Line D	0.488	21	23	1	0	0	0	4.51E+08	-	-	-	-	-	-	-	-
3Re4-18-mNeptune																
GL Rustikal	16.458	47	29	2	2	0	1	2.31E+07	10.693	2	5	0	0	0	0	3.27E+06
Gl. Diamant	12.549	16	13	0	1	0	1	1.16E+07	11.397	2	5	0	0	0	0	3.07E+06
Line D	12.411	-	-	6	5	0	1	4.43E+07	8.740	2	3	0	0	0	0	2.86E+06
Mix																
GL Rustikal	15.841	-	-	46	27	1	3	2.30E+08	13.426	7	4	0	2	0	0	4.10E+06
Gl. Diamant	14.288	-	-	23	23	2	4	1.61E+08	9.577	6	6	0	3	0	0	6.27E+06
Line D	11.207	-	-	13	23	4	4	1.61E+08	8.130	1	4	0	0	0	0	3.08E+06
NegC																
GL Rustikal	17.317	0	0	0	0	0	0	0	13.443	0	0	0	0	0	0	0
Gl. Diamant	14.817	0	0	0	0	0	0	0	11.880	0	0	0	0	0	0	0
Line D	13.718	0	0	0	0	0	0	0	7.960	0	0	0	0	0	0	0

pepoPNA probe order: Certificate of analysis (PNA Report)

Certificate of Analysis (PNA Report)

Name : Jaejin Choi

Date : March. 21, 2016

Quality Assurance

PNA

Sample No(Name): 01 (PNA)
Lot No : 160542
N-term C-term
Sequence : tga tgc ct*a aac caa-KK
(*: miniPEG gamma modification)

Amount

502 µl

OD(260nm): 7.9 nmole: 50.2 Molecular Weight: 4429.4 Weight: 222.2 µg

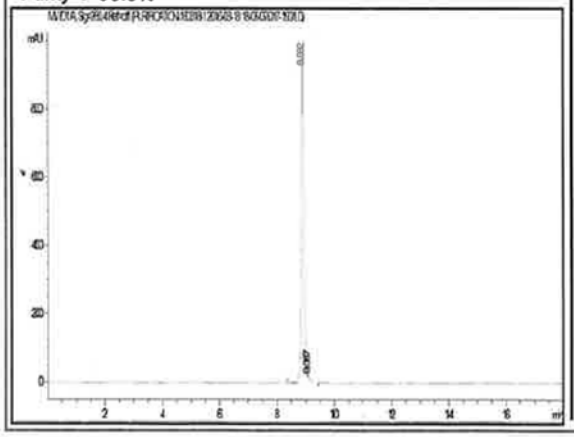
(Weight was calculated from OD value & Extinction coefficient at 260nm, ε_{260} *= 158.4 mL/(µmol x cm))

+ ε_{260}(A) = 13.7mL/(µmolexcm), ε_{260}(G) = 11.7mL/(µmolexcm), ε_{260}(C) = 6.6mL/(µmolexcm), ε_{260}(T) = 8.8mL/(µmolexcm),

Analysis

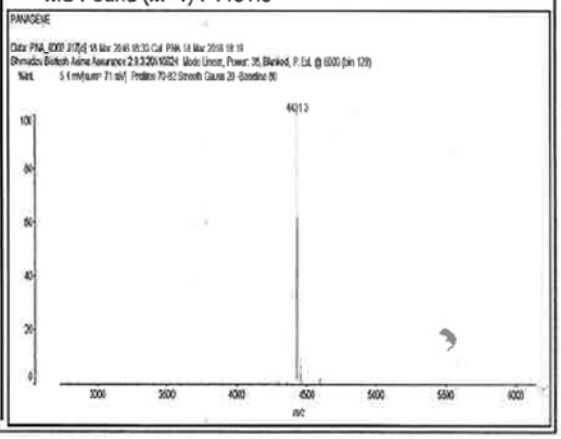
HPLC

Instrument : Agilent 1100 Series (Agilent Technologies)
Purity : 98.3%



MALDI MS

Instrument: AXIMA-Assurance (Shimadzu Biotech)
MS Found (M+1) : 4431.3



Special subjects & Remarks

Handling : When it is difficult to dissolve your PNA oligomers in water, we recommend heating the solution up to 60℃ for 10min and/or adding some organic solvents(0.1% TFA, 10-20% acetonitrile, DMF, NMP, etc.). For your information, PNA oligomers are usually dissolved into 20-100 uM in distilled water.
The best condition of dissolution should be optimized by user.

Storage : Lyophilized PNA oligo can be stored at room temperature. For long term storage, keep PNAs under -18℃

PNA Bio
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www.pnabio.com / TEL 800-764-0478 / Email order@pnabio.com

Publications

Publications related to this Master´s thesis:

Adam E, Bernhart M, Müller H, Winkler J and Berg G (2016b). The *Cucurbita pepo* seed microbiome: genotype-specific composition and implications for breeding. Plant Soil. DOI: 10.1007/s11104-016-3113-9.

Bernhart M, Adam E, Müller H, Winkler J, Berg G (2016). Cultivar specificity of the rhizosphere and seed microbiomes of the Styrian oil pumpkin. Poster Presentation at the 8th ÖGMBT Meeting. September 12th to September 14th 2016, Graz, Austria.

Bernhart M, Adam E, Müller H, Winkler J, Berg G (2016). Analysis of plant-microbe interactions on the Styrian oil pumpkin as basis for an advanced breeding strategy. Presentation at DPG-Arbeitskreis Biologische Bekämpfung von Pflanzenkrankheiten Jahrestagung. March 17th to March 18th 2016, Tulln, Austria.

Adam E, Bernhart M, Müller H, Winkler J, Berg G (2015). Screening concept for plant-microbe interactions on the Styrian oil pumpkin as basis for an advanced breeding strategy. Presentation at EUCARPIA meeting: Workshop on implementing plant-microbe interaction in plant breeding. June 25th to June 26th 2015, Weihenstephan, Germany.

Adam E, Bernhart M, Müller H, Winkler J, Berg G (2015). Screening concept for plant-microbe interactions on the Styrian oil pumpkin as basis for an advanced breeding strategy. Poster Presentation at EUCARPIA meeting: Workshop on implementing plant-microbe interaction in plant breeding. June 25th to June 26th 2015, Weihenstephan, Germany.

Poster presented at ÖGMBT Meeting 2016 in Graz

Cultivar specificity of the rhizosphere and seed microbiomes of the Styrian oil pumpkin

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Introduction

Plant breeding activities shape the rhizosphere microbiome but less is known about the relationship with the seed microbiome. To support a concept involving beneficial plant-microbe interactions in breeding activities of Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.), the microbiomes of both habitats of different genotypes as well as the bulk soil were analysed.

Materials & Methods

The seed and rhizosphere microbiomes of 14 genotypes of the Styrian oil pumpkin, including hybrids and their pedigree components (‚GL Rustikal' pedigree shown in Fig. 1A) as well as a population cultivar, segregating lines and a squash hybrid were evaluated using a 16S rRNA amplicon sequencing approach, which was assessed by bioinformatics and statistical methods.

Results & Discussion

- The microbial diversity in the soil and rhizosphere was significantly higher than in seeds (Shannon diversity index H' 10.2, 9.3 and 5.3 respectively).
- A strong cultivar specificity was detected for the seed-associated microbial communities, but not for the rhizosphere.
- The seed microbiomes of all cultivars were dominated by ***Enterobacteriaceae*** including potential pathogens (***Erwinia***, ***Pectobacterium***).
- The taxonomic network analysis (Fig. 1B) of the 'GL Rustikal' pedigree revealed that the diverse seed microbiome of the 'Gleisdorfer Diamant' (Shannon diversity index H' 7.9) was comprised of more than 50% unique taxa.
- 'Gleisdorfer Diamant' and Line D as parental components shared less operational taxonomic units (OTUs) (Fig. 1C) with 'GL Rustikal' than the genetically more distant components 'Gleisdorfer Diamant' and Line D shared with each other. The core microbiome of all five genotypes investigated comprised 14% of the OTUs.

→The high abundance of common ***Enterobacteriaceae*** could indicate an essential function or that the inheritance of the microbiome is focused on certain taxa. There may be a correlation of the higher bacterial diversity in 'Gleisdorfer Diamant' with the displacement and competition of ***Enterobacteriaceae*** in its seeds (Adam et al. 2016).

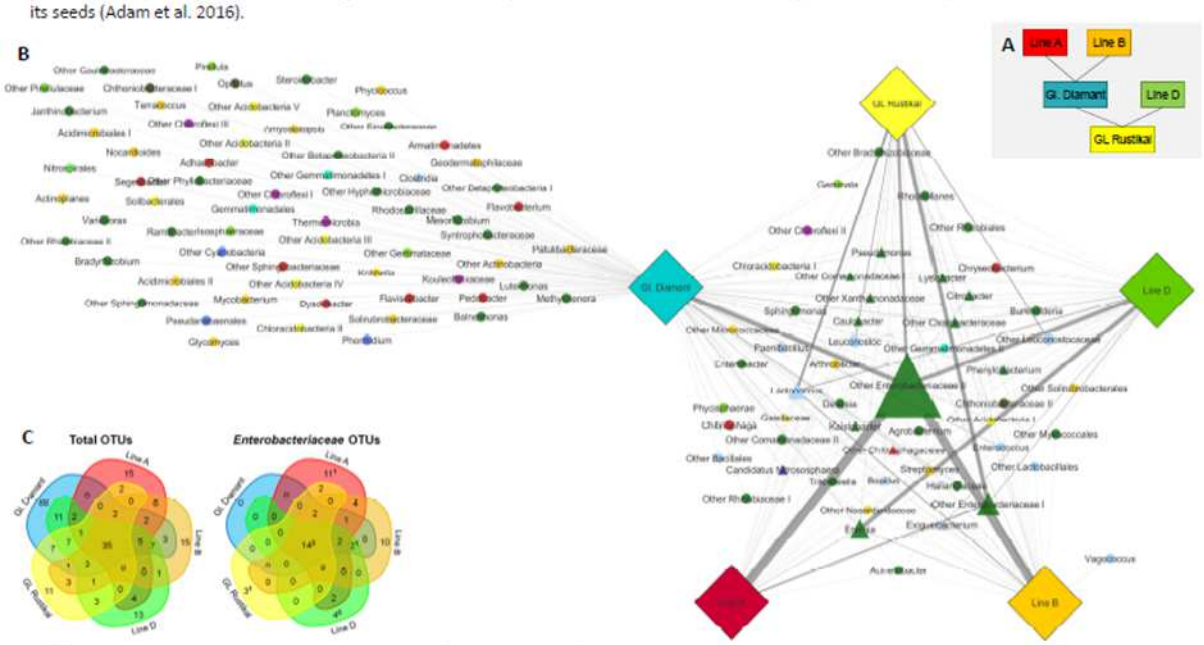

Fig. 1 (A) Taxonomic interaction network created with Cytoscape (Shannon et al. 2003) at genus level of the seed associated bacterial communities of the 'GL Rustikal' pedigree with **(B) the pedigree relationships. (C) Distribution of total and of *Enterobacteriaceae* OTUs** within the seed microbiomes (Adam et al. 2016/submitted). The squares in the network illustrate the *C. pepo* genotypes. Seed associated bacterial taxa with a relative abundance of at least 0.1% are connected with the corresponding genotype by a grey line. The line width correlates with the relative abundance of each taxon connected with the respective genotype. The size of the shape next to the bacterial taxa corresponds to the mean of the relative abundance of all 118 bacterial taxa analyzed in this network. The 20 taxa belonging to the core microbiome are shown as triangles. Taxa that are shared by four, three or two genotypes, or that are unique in a genotype are illustrated as circles. Taxa of the same phylum are depicted in the same shape color.

Perspectives

- A strong cultivar specificity was detected for the seed-associated microbial communities, including potential pathogens .
- The results of the seed samples are of particular interest, as the Styrian oil pumpkin is highly susceptible to various fungal and bacterial pathogens during germination. Thus, the findings are interesting for **seed production** and could imply the design of **tailored biological seed treatments**.
- A possible implication for **breeding programs** could be the selection of genotypes enriching less enterobacteriaceal pathogens, expressing a higher microbial diversity in their seeds or with a higher capacity of exploiting beneficial indigenous microbial communities.

References:
Adam E, Bernhart M, Müller H, Winkler J, Berg G (2016) The Rhizosphere and Seed Microbiomes of Oil Pumpkin Breeding Lines, Hybrids and a Population Cultivar. Proceedings of Cucurbitaceae 2016: 34-37
Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Research 13: 2498-2504. doi:10.1101/gr.1239303

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