Viscosin synthesis influence *Pseudomonas fluorescens* SBW25 colonization and microbial assembly at the wheat rhizoplane in response to plant genotype

Running title: Viscosin influence colonization and microbial assembly

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Abstract

The importance of microbial functions for plant health and performance is unquestioned. Beneficial rhizosphere bacteria such as *Pseudomonas* spp. promote plant growth and protect against pathogens by producing a range of bioactive compounds, including cyclic lipopeptides (CLPs) known for their antimicrobial activities. However, the natural roles of CLPs during interactions between bacteria and plants remain underexplored especially in natural systems. Here, *Pseudomonas fluorescens* SBW25 producing the CLP viscosin was used to unravel the impact of viscosin on bacterial colonization potential and microbiome assembly at the plant root. Two varieties of winter wheat were inoculated with SBW25 or its viscosin-deficient mutant. Viscosin was shown to improve colonization potential and have a significant effect on assembly of both bacterial and protist communities at the root-soil interphase. This effect was shown to be plant cultivar dependent and pinpoints the importance of microbe-microbe interactions in colonization of plant roots. This study provides new insights into the natural role of viscosin and specifically the importance of viscosin for the bacterial colonization of plant roots and in shaping the microbial communities associated with different wheat varieties.

Keywords: Cyclic lipopeptides, secondary metabolites, *Pseudomonas* sp., rhizoplane colonization, microbiome assembly, protists.

42 **INTRODUCTION**

43 Microorganisms associated with plant roots can influence plant health positively through
44 multiple mechanisms e.g. nutrient acquisition, pathogen control and induction of plant
45 defense responses [1]. While promising results on exploiting plant growth promoting
46 bacteria to replace or reduce the use of fertilizers and pesticides in agriculture have been
47 obtained [2, 3, 4], the translational power from laboratory studies to field performance is
48 currently low [5]. One major obstacle is our inadequate understanding of the parameters
49 governing bacterial colonization and microbial community assembly in natural systems.
50 Plants and microbes have evolved in complex settings for millions of years giving rise to
51 intimate interactions involving secondary metabolites used for both chemical combat as
52 well as mediators in specific interactions [6, 7]. Obtaining fundamental knowledge on
53 these microbe-microbe-plant interactions and the impact of the metabolic interplay on
54 community assembly under natural soil conditions is hence essential for full exploitation
55 of the plant growth promoting potential of bacteria in agricultural settings.

56 Cyclic lipopeptides (CLP) produced by *Pseudomonas* sp. are secondary metabolites
57 reported to be involved in root colonization by influencing traits like motility and biofilm
58 formation [8, 9]. Furthermore, the CLP viscosin has antimicrobial activity against
59 protozoans and has been shown to protect *Pseudomonas fluorescens* SBW25 from
60 protozoan predation by *Naegleria americana in vitro* as well as superior persistence in
61 soil compared to viscosin-deficient mutants [10]. Moreover, viscosin has lytic activity
62 against zoospores of the economically important plant pathogen *Phytophthora infestans*
63 [11], and can protect germinating seedlings of sugar beet in *Phytium* infested soils [12].

64 Recently, viscosin-producing *Pseudomonas* strains were shown to be enriched in the
65 rhizoplane of winter wheat in a plant-cultivar dependent manner (Herms et al.,
66 unpublished). This suggests a role of viscosin in plant-bacterial interactions dependent
67 on plant genotype. Such plant-genotypic differences in the presence of viscosin-
68 producing pseudomonads, could have a direct effect on the microbial community
69 assembly on the roots, as well as an indirect effect through alternation of the protist
70 community. While bacterial and fungal community composition in the root-zone of diverse
71 crops has been extensively described at higher taxonomic levels by amplicon sequencing
72 [13, 14, 15], there is a lack of knowledge at the bacterial strain level. Such strain specific
73 knowledge is important due to high diversity of microbes even between strains of the
74 same species [16, 17]. Especially the impact and importance of specific bacterial
75 phenotypes e.g., production of secondary metabolites, on the overall assembly of the root
76 microbiome and plant performance makes these studies highly needed. In addition, root
77 colonization has primarily been explored in sterile root systems that lack indigenous soil
78 microorganisms [18, 19, 20]. This ignores the three-way interaction among the inoculant,
79 the root and the indigenous soil community, and competition for colonization space
80 operating under natural soil conditions [21, 22, 23]. Protist communities in the rhizosphere
81 are less well described, but studies have indicated major differences in the protist
82 community composition in different rhizosphere compartments [24]. Protists play an
83 important role in the rhizosphere because of their effect on nutrient availability in the soil
84 [25] and can impact the structure of the microbial communities [26]. However, knowledge
85 on specific predator-prey interactions and their effect on the microbial community
86 composition is scarce.

87 To unravel these intimate interactions at the root-soil interface and the importance of
88 secondary metabolites for early root colonization and microbiome assembly under
89 complex conditions, we used *P. fluorescens* SBW25 as a viscosin-producing model strain
90 in combination with two winter wheat cultivars, Sheriff and Heerup (Heerup naturally
91 enriching for viscosin producing pseudomonads compared to Sheriff; Herms et al,
92 unpublished). We inoculated wildtype *P. fluorescens* SBW25 (SBW25 WT), as well as its
93 corresponding mutant deficient in viscosin production (Δ*viscA*) on wheat seedlings and
94 evaluated their colonization potential and impact on bacterial and protist community
95 assembly in the rhizoplane. We propose two hypotheses: 1) the ability to produce viscosin
96 increases root colonization in a plant-genotype dependent manner and 2) the viscosin-
97 producing strain impacts the protist community by lowering the relative abundance of
98 potential plant pathogenic oomycetes because of the antiprotist properties of viscosin. In
99 addition, we ask the question whether the ability to produce viscosin has a plant-genotype
100 dependent impact on the microbial community assembly. Finally, we evaluated the plant
101 response to the microbial inoculation, as an important link of knowledge to an agricultural
102 setting.

103

104

105 **MATERIALS AND METHODS**

106 **Strain construction and culture conditions**

107 Bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas*
108 *fluorescens* SBW25 wild type (SBW25 WT) [27] and the viscosin-deficient mutant *P.*
109 *fluorescens* SBW25 (Δ*viscA*) [11] was routinely grown in Luria Broth at 28□C, shaking at
110 180 rpm (1% tryptone, 0.5% yeast extract and 1% NaCl). When required, antibiotics were
111 supplemented at final concentrations of 50 µg ml^{-1} kanamycin or 10 µg ml^{-1} gentamicin.
112 Both strains were tagged with mCherry by introducing the mCherry delivery plasmid
113 pME9407 and the helper plasmid pUX-BF13 by electroporation and selection by 10 µg
114 ml^{-1} gentamicin as previously described [28].

115

116 **Soil collection and properties**

117 Soil was collected from the long-term nutrient depletion trial at the experimental farm of
118 the University of Copenhagen in Taastrup, Denmark (55° 40′N, 12° 17′E) [29]. The soil is
119 a sandy loam (164 g clay kg^{-1}, 173 g silt kg^{-1}, 333 g fine sand kg^{-1}, 312 g coarse sand
120 kg^{-1}, and 17 g soil organic matter kg^{-1}) and is classified as a Luvisol. The chemical
121 properties of the soil are shown in Table S1. Prior to experiments, the soil was air-dried
122 and sieved through a 2 mm mesh.

123

124 **Plant experiment setup**

125 Two cultivars of winter wheat, Heerup and Sheriff (Sejet Plant Breeding, Horsens,
126 Denmark), were grown in PVC pots (19 cm high and 3.5 cm in diameter). Soil was
127 thoroughly mixed in the ratio 3:2 with 0.4 - 0.9 mm particle size sand (DANSAND,

128 Brædstrup, Denmark) in a plastic bag. The pots were sealed in the bottom with 50 μm
129 polyamide mesh (Sintab, Oxie, Sweden) before transferring the soil-sand mixture into the
130 pots.
131 Seeds were soaked in sterile Milli-Q water for 1 h and then transferred to Petri dishes with
132 2 layers of sterile filter paper, moistened with 5 ml of sterile Milli-Q water. Seeds were
133 kept in the dark at room temperature for 3 days for optimal germination. Overnight *P.*
134 *fluorescens* cultures were washed twice with sterile 0.9% NaCl, and OD_{600nm} was adjusted
135 to 1.0 (equivalent to approximately 5×10^8 CFU ml^{-1}) for seed coating. Germinating seeds
136 with primary roots of 3-4 cm in length were selected and soaked in a Petri dish with
137 bacterial or control solution for 1 hour and immediately transferred to the PVC pots, one
138 seed per pot. The germinated seeds of each wheat species were inoculated as follows:
139 (1) *P. fluorescens* SBW25 mCherry (SBW25 WT); (2) *P. fluorescens* SBW25 Δ*viscA*
140 mCherry (Δ*viscA*) and (3) control treatment with 0.9% NaCl. Eight replicates were set up
141 for each treatment: 5 for DNA extraction and 3 for CFU counting.
142 The water content of the soil-sand mixture was initially adjusted to 17% (w/w). Each pot
143 was supplemented with 0.83 ml of plant nutrient solution (Drivhusgødning, Park®,
144 Schmees, Twistringen, Germany) (NPK 3-1-4), containing 2.04% nitrate-nitrogen (N),
145 0.3% ammonium-N, 0.46% amide-N, 0.69% phosphorus (P), 4.38% potassium (K),
146 0.08% sulfur (S), 0.06% magnesium (Mg), 0.033% iron (Fe), 0.013% manganese (Mn),
147 0.002% copper (Cu), 0.002% zinc (Zn), 0.0006% molybdenum (Mo), and 0.004% boron
148 (B). The plants were grown in a growth chamber under the following conditions:
149 temperature 19/15°C (day/night), relative air humidity 60%/60% (day/night) and
150 photosynthetically active radiation 600/0 μmol m^{-2} s^{-1} (day/night) with a photoperiod of

151 16h/8h. The pots were watered with deionized water every second day by weighing and
152 watering up to moisture content of 15% throughout the experimental period.
153 Sampling was performed after 14 days of growth. Root sample collection and
154 compartment processing were performed as described by Zervas et al. [30] to obtain
155 rhizosphere (soil adhering to the root; exclusively used for confirmation of proper
156 sampling of the compartments as tested by 16S rRNA amplicon sequencing (see below))
157 and rhizoplane (the root surface) associated microbes, respectively. Samples for DNA
158 extraction were immediately flash frozen in liquid nitrogen and kept on dry ice until storing
159 at -80°C. Samples were freeze-dried (CoolSafe 100-9 Pro freeze dryer, LaboGene,
160 Lynge, Denmark). All freeze-dried samples were stored at -20°C until DNA extraction.
161 Root and shoot length were recorded at sampling, and roots were scanned to determine
162 total root length and root diameter. Following sampling, roots and shoots were dried at
163 60□C for 3 days before determining the dry weight.
164

165 **DNA extraction**

166 Genomic DNA was extracted from 0.5 g sample using a FastPrep-24™ 5G beadbeating
167 system (MP Biomedicals, Irvine, CA, USA) at 6.0 m/s for 40 s and the FastDNA™ SPIN
168 Kit for soil (MP Biomedicals) following the manufacturer's instructions. The DNA extract
169 was stored at −20°C until further processing for qPCR analysis and rRNA gene amplicon
170 sequencing.
171

172 **Quantitative PCR analysis of root colonization ability**

173 Quantification of SBW25 WT and Δ*viscA* was performed by qPCR targeting the mCherry
174 gene using the AriaMx Real-Time PCR (Agilent Technologies, Santa Clara, CA, USA).
175 The primers used are presented in Table 1. Twenty-microliter reactions were prepared
176 with 1 □ Brilliant III Ultra-Fast SYBR® Green Low ROX qPCR Master Mix (Agilent
177 Technologies, Santa Clara, CA, USA), 1 µg $µl^{-1}$ bovine serum albumin (New England
178 Biolabs® Inc., Ipswich, MA, USA), 0.2 µM of each primer and 2 µl of template DNA.
179 Thermal cycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C
180 for 20 s and 56°C for 30 s. A dissociation curve was generated at the end of the qPCR
181 program by including a cycle of 95°C for 1 min, 55°C for 30 s, and finally reaching 95°C
182 by increments of 0.5°C s^{-1}, each increment followed by a fluorescence acquisition step.
183 Absolute abundance was calculated based on a standard curve for the mCherry target
184 gene [31]. Standard curves used for quantification were based on a ten-fold serial dilution
185 of DNA from mCherry-tagged *P. fluorescens* SBW25. The standard curve was run with
186 three technical replicates per dilution, and had a dynamic range from 10^2 to 10^8 copies/µl.
187 The efficiency ranged from 99.0 to 99.6%, and R^2 values were > 0.99 for all standard
188 curves.
189
190 **CFU analysis of root colonization ability**
191 Colony forming units (CFUs) were determined for rhizoplane samples. Ten-fold dilutions
192 were inoculated on Gould's S1 [32] agar plates supplemented with 10 µg ml^{-1} gentamicin
193 to select for tagged SBW25 WT and Δ*viscA* cells. Plates were incubated at 28°C in the
194 dark for 48 h before recording CFUs.
195

196 **Root imaging and analysis**

197 Prior to root imaging, all roots were thoroughly rinsed to remove soil and sand particles,
198 and stored in distilled water at 4°C. Upon imaging, roots were untangled and arranged in
199 a shallow acrylic dish with distilled water and imaged using an Epson Perfection V700
200 Photo scanner (Epson, Suwa, Japan) in 8-bit greyscale mode with 400 dpi resolution,
201 subsequently converted to 8-bit JPG for software compatibility. Root length in diameter
202 size classes and average root diameter were determined using the skeletonization
203 method in WinRhizo v. 2016a (Regent Instruments, Quebec, Canada). Roots were
204 divided into 10 size classes between 0 mm diameter and 4.5 mm diameter in 0.5 mm
205 increments and the total root length in each size class was calculated. Total root length,
206 total root surface area, average root diameter and total root volume were also calculated.
207 Size of class fractions were calculated by dividing the root length in a size class by the
208 total root length for each sample pot. Fine roots were determined as those with diameter
209 < 0.5 mm.

210

211 **16S rRNA and 18S rRNA gene amplicon sequencing**

212 The V5–V7 region of the bacterial 16S rRNA gene was amplified with primers 799F [33]
213 and 1193R [34] (Table 1). This primer pair was found to amplify relatively low proportions
214 of plant mitochondria and chloroplast DNA compared to bacterial DNA [35]. The purity
215 and concentration of DNA were determined using a NanoDrop ND-1000
216 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and a Qubit 2.0
217 fluorometer (Thermo Fisher Scientific). The ZymoBIOMICS Microbial Community DNA
218 Standard (Zymo Research, Irvine, CA, USA), pure culture SBW25 DNA, and two water

219 controls were included. A two-step dual indexing strategy for Illumina MiSeq (Illumina,
220 San Diego, CA, USA) sequencing was used. First, PCR amplicons were generated in a
221 25-µl setup using 0.8 U Platinum II Taq (Thermo Fisher Scientific), 1 □ Platinum II PCR
222 Buffer, 1 mM dNTP mix, 0.2 µM primer 799F, 0.2 µM primer 1193R, and 5 µl DNA
223 template. The PCR thermocycler program included an initial denaturation temperature of
224 95°C for 2 min, then 33 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s and a
225 final elongation step of 72°C for 5 min. The PCR amplification was confirmed by gel
226 electrophoresis on 1.5% agarose gels. PCR products were purified using AMPure XP
227 beads (Beckman Coulter Inc. Brea, CA, USA). The following library construction and
228 Illumina MiSeq sequencing (2 × 300 bp) were performed by Eurofins Genomics
229 (Ebersberg, Germany).
230 The 1380F and 1510R primer set (Table 1) [36] targeting the V9 region of the 18S rRNA
231 gene was used to evaluate the protist community composition. A two-step dual-indexing
232 strategy for Illumina NextSeq sequencing using a 2-step PCR was used. First, PCR
233 amplicons were generated in 25 µl-reactions with 1 □ PCRBIO Ultra Mix
234 (PCRBIOSYSTEMS), 0.2 µM of each primer, 0.4 µg $µl^{-1}$ bovine serum albumin (New
235 England Biolabs® Inc., Ipswich, MA, USA) and 5 µl DNA template. The PCR thermocycler
236 program included an initial denaturation temperature of 95°C for 2 min, then 33 cycles of
237 95°C for 15s, 55°C for 15s, and 72°C for 40 s and finally a final elongation step of 72°C
238 for 4 min. Each reaction in the first PCR was done in duplicates, which were pooled, and
239 used for dual indexing in the second PCR. The second PCR was run with 5 µl of
240 amplicons produced in the first PCR, primers with Illumina adaptors and unique index
241 combinations (i7 and i5) using the reaction conditions described for the first PCR. The

242 second PCR was run using 98°C for 1 min, then 13 cycles of 98°C for 10 s, 55°C for 20
243 s and 72°C for 40 s and a final elongation of 72°C for 4 min. The PCR amplification was
244 confirmed using 1.5% agarose gels. Subsequently, the amplicon product was cleaned
245 using HighPrep™ magnetic beads (MagBio Genomics Inc. Gaithersburg, MD, USA),
246 according to the manufacturer's instructions. Finally, DNA concentrations were measured
247 using Qubit 4.0 fluorometer using the High-Sensitivity DNA assay (Thermo-Fischer
248 Scientific). Samples were then equimolarly pooled and sequenced on an Illumina Nextseq
249 sequencer.
250
251 **Sequence processing**
252 Raw amplicon reads were processed using the DADA2 pipeline v. 1.14.1 [37]. In brief,
253 reads were quality checked and primers were removed using trimLeft in the filterAndTrim
254 function. According to the sequence quality, the 16S rRNA gene reads were filtered using
255 default parameters except for trimRight and minLen (the reads were filtered by truncating
256 the last 20 bp of the forward reads and the last 128 bp of reverse reads using trimRight
257 and minLen = 150 to avoid poor quality and ambiguous sequences). Chimeras were
258 removed after merging denoised pair-end sequences. Each unique amplicon sequence
259 variant (ASV) was assigned to taxa according to SILVA database v. 138.1 [38] and PR^2
260 database v. 4.14.0 [39] for the 16S rRNA gene and the 18S rRNA gene, respectively. For
261 bacteria, non-bacterial ASVs including chloroplasts and mitochondrial reads were
262 removed. Similarly, for protist, we discarded plant (Streptophyta), animal (Metazoa), and
263 fungal reads to generate the retained and conservative protist ASV table. To reduce the

264 amount of spurious ASVs, ASVs with a relative abundance below 0.1% in each sample
265 were removed from the dataset [40].
266
267 **Data analysis and statistics**
268 Statistical analysis of the plant physiology experiments was performed using GraphPad
269 Prism v. 8.3.0. Differences between two groups were analyzed by unpaired *t*-test ($P <$
270 0.05). Multiple comparisons were analyzed by one-way analysis of variance (ANOVA) via
271 Tukey's HSD test (*$P < 0.05$ and **$P < 0.01$).
272 The 16S rRNA and 18S rRNA datasets were analyzed in R version 4.1.3 [41]. For
273 microbiome diversity and composition analyses, we used the R packages phyloseq v.
274 1.38.0 [42], ampvis2 v. 2.7.17 [43] and microeco v. 0.11.0 [44]. The amp_rarecurve
275 function in the ampvis2 package was used to generate rarefaction curves (number of
276 reads vs number of observed ASVs) for each sample. For 16S rRNA, we excluded the
277 samples "YG6" (Sheriff), "YG34" (Heerup) and "YG51" (Heerup) from the analysis due to
278 their low read number. A sample overview is provided in Table S2. The alpha diversity
279 was estimated using Shannon diversity in Divnet v. 0.4.0 [45] with default parameters.
280 This method allows for cooccurrence of taxa in contrast to other methods, which estimate
281 the diversity based on multinomial models. Significance testing of the Shannon diversity
282 was done using beta function in breakaway v. 4.7.6 [46]. Samples were not rarefied prior
283 to downstream analyses to avoid discarding information [47].
284 Principal component analysis (PCA) based on Aitchison distance was performed using
285 the R function 'prcomp' and permutational multivariate analysis of variance
286 (PERMANOVA) was used to test the effect of the inoculant treatments, compartment and

287 plant genotype on the beta diversity of the microbial community in vegan v.2.6.2 [48].
288 Analysis of shared and unique ASVs between groups was done using the trans_venn
289 function in microeco package v. 0.11.0 [44].
290 The differential abundance of ASVs between inoculant treatments (SBW25 WT vs.
291 viscosin deficient mutant-treated, SBW25 WT-treated vs. control, and viscosin deficient
292 mutant-treated vs. control) were analyzed while controlling for the compartment of the two
293 wheat cultivars. The differential abundance was determined using beta-binomial
294 regression with the corncob package v. 0.2.0 [49]. Only ASVs that had an estimated
295 differential abundance of < -1 or >1, and P-values adjusted for multiple testing < 0.05
296 (FDR < 0.05) were considered significant.
297
298 **Data availability**

299 All raw sequencing data used in this study has been deposited in the NCBI Sequence
300 Read Archive (SRA) database under project accession numbers PRJNA928659 (16S
301 rRNA) and PRJNA931264 (18S rRNA).

302 **RESULTS**

303 **Colonization ability of *P. fluorescens* SBW25 and its viscosin-deficient mutant**

304 To elucidate the importance of viscosin production for the ability of *P. fluorescens* SWB25
305 to colonize wheat roots, we inoculated seedlings with mCherry-tagged *P. fluorescens*
306 SBW25 wild type (SBW25 WT) or its viscosin-deficient mutant (Δ*viscA*). Samples taken
307 immediately after seedling inoculation showed similar colonization potential independent
308 of the ability to produce viscosin, as measured by qPCR and CFU counts, respectively
309 (Fig. 1). After two weeks of seedling growth, qPCR data showed higher abundance of
310 SBW25 WT in the rhizoplane of both cultivars compared to Δ*viscA*, although significant
311 differences were observed only in the Sheriff cultivar. The CFU assay only detected
312 SBW25 WT in the rhizoplane samples from Heerup and Sheriff, whereas no CFUs were
313 observed for Δ*viscA* at the measured dilution, pointing to at least 10 times higher
314 colonization by SBW25 WT compared to Δ*viscA* (Fig. 1).

315

316 **Wheat genotype dependent effects of inoculation on plant growth**

317 To evaluate the effect of SBW25 WT and Δ*viscA* on plant growth, we measured shoot
318 length as well as shoot and root biomass and performed image analysis of the roots. In
319 Sheriff, SBW25 WT and Δ*viscA* showed similar effects on seedlings, with significantly
320 shorter shoot length ($P < 0.05$) and significantly higher root dry weight ($P < 0.01$) as
321 compared to control treatment (Fig. 2). No effect on root length nor shoot dry weight was
322 observed for any of the inoculations in Sheriff as compared to the control treatment (Fig.
323 2). In Heerup, the SBW25 WT and Δ*viscA* had contrasting effects on root dry weight,
324 where Δ*viscA* induced a significantly higher root dry weight than both the SBW25 WT (*P*

325 < 0.05) and the control treatment ($P < 0.01$). In contrast, neither shoot length, root length
326 nor shoot dry weight were significantly impacted by inoculation with SBW25 WT or Δ*viscA*
327 in Heerup, as compared to the control treatment.
328
329 The results of the root image analysis (Fig. S1A) showed only minor impact on plant
330 parameters by inoculation treatments. The only significant difference observed was a
331 significantly higher number of root tips in the Heerup cultivar ($P < 0.05$) when inoculated
332 with either SBW25 WT or Δ*viscA* as compared to the control (Fig. S1B, Table S3). Taken
333 together, the impact of the ability to produce viscosin on plant parameters were
334 dependent on the plant cultivar, as impacts on Sheriff were found to be independent,
335 whereas impacts on Heerup were dependent on the ability to produce viscosin.
336
337 **Effects on bacterial community assembly**
338 To investigate the effects of SBW25 WT and Δ*viscA* on the rhizoplane bacterial
339 microbiome assembly we used 16S rRNA gene amplicon sequencing. The sequencing
340 depths of all samples were sufficient since the number of ASVs was saturated for each
341 sample in the rarefaction curves (Fig. S2A). There were 57 samples with 3 263 907 reads
342 in the total dataset after filtering. The sample sizes ranged from 41 099 to 80 057 reads,
343 with a median of 56 564. The dataset consisted of 449 ASVs.
344 A clear separation of the bacterial communities in the rhizosphere samples from that in
345 the rhizoplane samples (PERMANOVA, $P < 0.001$) confirmed a successful separation of
346 the compartments during sampling (Fig. S3A, Table S4).

347 Inoculation with Δ*viscA* significantly increased the Shannon diversity (Breakaway, $P <$
348 0.001) in the rhizoplane of the Heerup cultivar as compared to the SBW25 WT and the
349 control treatments (Fig. S4A). In the rhizoplane of Sheriff, the Shannon diversity was
350 significantly higher upon inoculation with both the SBW25 WT and Δ*viscA* as compared
351 to the control treatment ($P < 0.001$). Hence, the influence on bacterial diversity reflects
352 the impact observed on the shoot length and root dry weight (Fig. 2A and 2E), with a
353 similar impact from the SBW25 WT and Δ*viscA* on the Sheriff cultivar, and a differential
354 impact of the two strains on the Heerup cultivar as compared to the control treatment.
355 In addition, inoculation had a significant effect on the bacterial community composition in
356 the rhizoplane (PERMANOVA, $R^2 = 0.12$, $P = 0.001$; Fig S5A, Table S5). Further, the
357 interaction between cultivar and inoculation treatment was also significant ($R^2 = 0.09$, P
358 < 0.05; Table S5), indicating a cultivar dependent impact of the inoculation treatments on
359 the community composition. This was confirmed by a significant but differential effect of
360 inoculation treatments on the rhizoplane bacterial community structure in Sheriff ($R^2 =$
361 0.20, $P = 0.003$) and Heerup ($R^2 = 0.25$, $P = 0.006$) cultivars, respectively (Fig. 3A and
362 3B).
363

364 **Bacterial community composition response**

365 The rhizoplane communities in both cultivars were dominated by Proteobacteria,
366 Actinobacteriota, and Firmicutes independent of treatment (Fig. S3C). Additionally,
367 *Massilia*, *Paenibacillus*, *Bacillus*, *Dyella*, and *Noviherbaspirillum* were the five most
368 abundant genera across all samples (Fig. S6A).

369 In the Sheriff rhizoplane, inoculation of SBW25 WT significantly (beta-binomial model, P
370 < 0.05) increased the relative abundance of 14 ASVs and decreased the relative
371 abundance of 11 ASVs compared to the control treatment (Fig. 4A). In contrast,
372 inoculation by Δ*viscA* significantly ($P < 0.05$) affected only seven ASVs, leading to
373 increased relative abundance of three ASVs, and decreased relative abundance of four
374 ASVs compared to the control treatment (Fig. 4B). Comparing the effects of Δ*viscA*
375 inoculation with SBW25 WT inoculation, the relative abundance of five ASVs decreased,
376 whereas the relative abundance of only one ASV significantly increased ($P < 0.05$) (Fig.
377 4C). Taken together, this points to both a general impact of inoculation with *P.*
378 *fluorescence* SBW25, and a specific impact from the ability to produce viscosin in the
379 Sheriff rhizoplane.
380 In the rhizoplane of Heerup, no significant difference in ASV abundance was observed
381 between the SBW25 WT treatment and the control treatment. In contrast, inoculation with
382 Δ*viscA* significantly ($P < 0.05$) increased the relative abundance of 39 ASVs and
383 decreased the relative abundance of three ASVs when compared both to the SBW25 WT
384 and the control treatment (Fig. 5). An additional four ASVs significantly ($P < 0.05$)
385 increased and five ASVs decreased in relative abundance when seedlings were
386 inoculated with Δ*viscA* compared to inoculation with SBW25 WT (Fig. 5A).
387 For both culitvars, ASVs belonging to the genera *Bacillus* and *Massilia* were affected. In
388 the Sheriff rhizoplane, inoculations reduced the relative abundance of *Bacillus* and
389 increased the relative abundance of most *Massilia* compared to the control treatment,
390 however more pronounced for SBW25 WT (Fig. 4). In Heerup, Δ*viscA* increased the
391 relative abundance of *Bacillus* and decreased the relative abundance of *Massilia*

392 compared to the SBW25 WT and control treatment. (Fig. 5). Hence, visocosin seems to
393 have a generally negative effect on *Bacillus* ASVs, and a positive effect on the
394 colonization potential of *Massilia* ASVs.
395
396 **Effects on protist community assembly**
397 The protist communities were analyzed using 18S rRNA gene amplicon sequencing. The
398 sequencing depth of all samples was sufficient since the number of ASVs was saturated
399 for each sample in the rarefaction curves (Fig. S2B). There were 60 samples with 2 342
400 857 reads in the total dataset after filtering. The read sizes ranged from 19 495 to 70 412,
401 with a median of 37 461. The dataset consisted of 599 ASVs. Regarding the communities
402 of protists, the compartment was the most important factor for differences in community
403 composition (PERMANOVA, R^2 = 0.086, P = 0.001) (Fig. S3B, Table S4), again
404 supporting a reliable sampling strategy for obtaining specific compartment samples.
405 For the Sheriff rhizoplane community, the Shannon diversity was lower in SBW25 WT-
406 treated plants than Δ*viscA*-treated plants and control plants (P < 0.001) (Fig. S4B). In
407 contrast, inoculation with SBW25 WT or Δ*viscA* had no effect on the Shannon diversity in
408 the rhizoplane communities of the Heerup cultivar.
409 Inoculation treatment had a significant effect on the protist community composition in the
410 rhizoplane (PERMANOVA, R^2 = 0.09, P = 0.007) (Fig. S5B; Table S5). When analyzed
411 separately, the inoculation treatments significantly impacted the rhizoplane protist
412 community in the Sheriff (R^2 = 0.18, P = 0.005) cultivar, whereas no significant effect (R^2 =
413 0.15, P = 0.292) was observed in the Heerup cultivar (Fig. 3C and 3D).
414

415 **Protist community composition response**

416 At the division level, the rhizoplane community in both cultivars were dominated by
417 Oomycota (also referred to as Pseudofungi), Cercozoa, and Chlorophyta (Fig. S3D). At
418 the genus level, the five most abundant genera across all samples were *Phytophthora*
419 and four unclassified Oomycota (Fig. S6B). In the rhizoplane of Sheriff, the Cercozoa
420 *Group-Te* was significantly ($P < 0.05$) reduced after inoculation with SBW25 WT
421 compared to the other two treatments. In addition, inoculation of SBW25 WT significantly
422 ($P < 0.05$) reduced the relative abundance of an unclassified Oomycota (Fig. S7) as
423 compared to the control treatment. No significant difference in ASVs was found between
424 treatment with the Δ*viscA* as compared to the control treatment.

425 In the rhizoplane of Heerup, only one ASV belonging to the Amoebozoa *Leptomyxidae*
426 was significantly ($P < 0.05$) increased after inoculation with Δ*viscA* compared to the
427 control treatment (Fig. S7). Furthermore, no ASVs were identified to exhibit significant
428 differences in their abundance between the treatment by SBW25 WT and the control
429 treatment, as well as between the treatment by SBW25 WT and the treatment by the
430 Δ*viscA*.

431 Inoculation with SBW25 WT showed a trend of decreased relative abundance of *Pythium*
432 as compared to Δ*viscA* and control treatments in the Sheriff rhizoplane (Fig. 6). For the
433 Heerup rhizoplane, the trend was an increase in *Phytium* when inoculated with Δ*viscA* as
434 compared to SBW25 and control treatments, with no difference between the SBW25 and
435 control treatments.

436

437 **DISCUSSION**

438 Understanding drivers for microbial assembly at the root-soil interphase is fundamental
439 for the ability to engineer a beneficial root microbiome for improved plant health and
440 growth with a reduced environmental footprint – independent on whether the approach
441 is based on microbial inoculants or plant breeding for below ground interactions.
442 Further, high-resolution studies in natural soil with a high microbial complexity, are
443 highly needed for in-depth analysis of the three-way interactions between plant,
444 microorganisms and soil.
445 In the present study we addressed this challenge by growing wheat in natural soil with
446 the viscosin producing *P. fluorescence* SBW25 WT or its viscosin-deficient mutant
447 (Δ*viscA*), to study the effect of the ability to produce viscosin on colonization potential
448 and microbial community assembly in two wheat cultivars, Heerup and Sheriff.

449 The ability to produce viscosin enhanced the colonization potential of SBW25 WT
450 compared with Δ*viscA* in both cultivars. Viscosin has previously been shown to be
451 involved in surface spreading on sterilized sugar beet roots in potting compost [12] and
452 our results expand this finding by showing the importance of viscosin for wheat root
453 colonization in a natural soil environment with competition from other microorganisms.
454 Further, enhanced colonization by a CLP producing strain is in line with previous findings
455 for massetolide A and amphisin producing strains [8, 9] pointing towards a general role
456 of CLPs in root colonization. For viscosin, and CLPs in general, this associated
457 colonization phenotype could be due to the amphiphilic properties of the compound,
458 altering the surface charge of the bacteria or the root surface for an improved colonization
459 [50]. In contrast to these findings, Yang et al. [51] did not find any impact on colonization

460 potential in wheat based on the ability to produce viscosin when comparing *P. fluorescens*
461 HC1-07 and its viscosin-deficient mutant. However, the *P. fluorescens* HC1-07 strain was
462 mutated in the *viscB* gene [51], whereas the impairment of viscosin production in *P.*
463 *fluorescens* SBW25 used in this study, was due to a mutation in the *viscA* gene [28].
464 Whether these contrasting results are mutant generation, soil condition, plant variety or
465 chemically determined is still to be elucidated.

466 The abundance of SBW25 and Δ*viscA* on plant roots decreased 100-fold over the
467 course of the experiment and they comprised less than 1% of the total community after
468 two weeks. This is in line with other work on wheat seed inoculation with growth-
469 promoting *Bacillus* strains in the field [52] and demonstrates that the assembly of the
470 rhizoplane community is highly deterministic leaving only a small part available for
471 exchange of bacteria. This might explain the often-low transitional power observed
472 when going from simple testing in greenhouse systems to field performance of bacterial
473 inoculants [5, 53].
474 Amplicon sequencing of the 16S rRNA gene showed a general colonization of the roots
475 by Proteobacteria, Actinobacteriota, and Firmicutes. This is in line with other studies of
476 the wheat microbiome under natural conditions [54, 55, 56], and supports that microbial
477 community assembly in our system mimics the assembly at natural soil conditions. Based
478 on culture-dependent work showing that viscosin producers were preferentially isolated
479 from Heerup over Sheriff (Herms et al., unpublished), we hypothesized that the ability to
480 produce viscosin would increase root colonization, but in a plant-genotype dependent
481 manner. However, no difference was observed between the two wheat cultivars in the
482 number of SBW25 colonizing the rhizoplane, and hence, only the first half of the

483 hypothesis was supported by our data. This may be explained by the inoculation strategy
484 applied in our experiment, where inoculants were added by root dipping, giving SBW25
485 a head-start for colonization compared to bacteria colonizing from the soil community.
486 Despite the similar colonization potential of the two wheat cultivars observed for SBW25
487 and Δ*viscA*, respectively, viscosin was found to impact plant root architecture parameters
488 dependent on plant genotype. It has previously been shown that inoculation of plants with
489 plant beneficial bacteria alters root colonization [57, 58, 59], but to our knowledge this is
490 the first time, that is has been shown to be cultivar-dependent. The observed differential
491 effect on plant root parameters could be the result of differential community assembly,
492 dependent both on plant genotype and/or the ability of the inoculant to produce viscosin.
493 This further supports the need for detailed studies on plant-microbe interactions for
494 optimized agricultural output, and the prospect of breeding for specific plant-microbe
495 interactions.

496 Despite the equal colonization potential, a pronounced difference in the microbial
497 community assembly, measured by ASVs significantly changing between treatments,
498 was observed between the two cultivars. The multitude of ASVs changing in relative
499 abundance, regardless of whether SBW25 WT or Δ*viscA* was introduced in the Sheriff
500 rhizoplane, hint to a general effect of *P. fluorescens* SBW25 independent of viscosin
501 production, on the Sheriff microbiome assembly. Additionally, a specific effect of
502 SBW25 WT was observed, with four ASVs from the genera *Paenibacillus,*
503 *Rhodanobacter, Streptomyces* and unclassified Rhodanobacteriaceae consistently
504 increasing in relative abundance after the addition of SBW25 WT in comparison to
505 inoculation with Δ*viscA*. These ASVs can be hypothesized to specifically respond to the

506 viscosin produced by SBW25 WT. However, to fully elucidate that, it is needed to show
507 that viscosin is being synthesized in the rhizoplane habitat, which currently is not
508 technically possible at relevant concentrations in soil systems.
509 In contrast to the *P. fluorescens* SBW25 and presumed viscosin effects on the bacterial
510 community assembly identified in the Sheriff cultivar, no ASVs changed significantly in
511 the Heerup rhizoplane as a response to SBW25 WT inoculation. However, inoculation
512 with the mutant revealed an almost identical impact on the microbial community as
513 compared to the control and the SBW25 WT. Interestingly, the majority of affected
514 ASVs increased in relative abundance, hinting to a competitive advantage of harboring
515 the viscosin gene. One explanation for the lack of impact seen for the SBW25 WT could
516 thus be that the Heerup rhizoplane is naturally colonized by viscosin-producing
517 pseudomonads, and that the presence of these strains are drivers for the overall
518 microbial assembly at the roots of this cultivar. Hence, it can be speculated that the
519 viscosin producers are first colonizers of the Heerup rhizoplane under natural
520 conditions, and independent on whether it is naturally soil-derived viscosin producers, or
521 artificially added viscosin producing inoculants, they play a defined role in the further
522 assembly of the Heerup rhizoplane microbiome. These observations call for further
523 research to evaluate the importance of this phenotype for microbiome assembly in
524 general in Heerup, and whether such an intimate interaction would be dependent on
525 root architecture, root exudate composition or root morphology [60].

526 *Bacillus* and *Massilia*, genera previously shown to be associated with the wheat
527 rhizosphere [61, 62], were observed to be specifically impacted by the inoculations in a
528 wheat cultivar and inoculation dependent manner, collectively accounting for 50% and

529 25% of the impacted ASVs in Sheriff and Heerup, respectively. *Bacillus* decreased
530 significantly as a response to *P. fluorescence* (both SBW25 WT and the viscosin-
531 deficient mutant) in the Sheriff rhizoplane, however with a more pronounced effect by
532 SBW25 WT. *Bacillus* was found to have a significantly higher relative abundance in the
533 rhizoplane of Heerup when Δ*viscA* was inoculated, as compared to both the control and
534 the SBW25 WT treatment. This suggests a direct impact of SBW25 WT on the *Bacillus*
535 community. *Bacillus* is well known for its plant-growth promoting abilities [63]. Further, it
536 is a preferred genus for microbial products for plant growth promotion, due to the ability
537 to produce spores, and hence specific knowledge on the colonization and microbial
538 assembly dynamics is key for product development and application range.

539 In contrast to *Bacillus*, *Massilia* increased in relative abundance by introduction of
540 SBW25 WT and to a lesser extent Δ*viscA*. Strains belonging to the genus *Massilia* are
541 known as copiotrophic root colonizers [62, 64, 65], and have been proposed as a hub
542 taxon in the wheat root microbiome [62]. Hence, our study provides basic knowledge on
543 how the abundance of hub taxons can be dependent on other community members,
544 and thereby might be prone for microbial engineering using inoculants. Furthermore,
545 *Massilia* species are known to colonize the endophytic compartment of wheat, and
546 hence may in several ways play an important role for early stage development and
547 microbial assembly [61].

548 These cultivar specific impacts illuminate the very distinct interactions present in the
549 rhizoplane of plants, and how impacts might be modulated by a single metabolite.
550 Moreover, it demonstrates the need for functional studies of microbes in natural
551 systems, as major impacts can be observed based on a single change in the ability to

552 produce a single specialized metabolite. It further emphasizes the need to understand
553 microbial interactions at the early stage of plant development, for predictive value in
554 microbiome assembly and plant impact throughout the plant growth cycle.

555 The resilience in the bacterial community of the Heerup rhizoplane was in agreement
556 with the phenotypic response of the plant. Hence, the root dry weight significantly
557 increased when the plant was challenged with the mutant, which also caused significant
558 changes in the microbiome, as compared to the SBW25 WT and water treatment. For
559 the Sheriff cultivar, both the SBW25 WT and Δ*viscA* caused a significant shift in the
560 microbiome coinciding with a significant increase in root dry weight for both treatments
561 compared to the control. Whether there is a direct link between viscosin production and
562 root dry weight is currently not known, as it could also be caused by secondary effects
563 from a changing microbiome.

564 The inoculation with *P. fluorescens* had only a minor effect on the protist communities,
565 with few ASVs being significantly affected. In the Sheriff cultivar, we observed a
566 decrease of the Cercozoa *Group-Te* in the SBW25 WT treated rhizoplane. *Group-Te* is
567 found in the rhizosphere of multiple crops and model plants, e.g. maize, Arabidopsis,
568 potato [66, 67], but the ecology of this organism is unknown. In contrast to the Sheriff
569 cultivar, SBW25 WT did not result in any changes of any protist ASVs in the rhizoplane
570 of the Heerup cultivar, in line with our results of the bacterial community.

571 This also agrees with observations on the bacterial community assembly, and further
572 supports the proposed explanation that the Heerup rhizoplane naturally recruits viscosin
573 producers to a specific carrying capacity, and hence the addition of SBW25 WT does
574 not have any significant impact. Together with the relatively low abundance of the

575 SBW25 WT, our results indicate that there is a high degree of predetermined factors
576 that regulate microbiome assembly in the wheat rhizoplane.

577 In the Sheriff rhizoplane SBW25 WT decreased the relative abundance of a single
578 oomycete ASV (ASV 25) in support of hypothesis two of viscosin producing bacteria
579 reducing abundance of oomycetes. While no single *Pythium* ASV changed significantly
580 between the treatments in Heerup, a trend of increased *Phytium* abundance when
581 plants were inoculated with Δ*viscA* as compared to SBW25 WT and the control
582 treatment was observed. This further supports hypothesis two. Oomycetes such as
583 *Phytopthora* and *Pythium* are abundant in the plant-soil habitat. While many species are
584 saprophytes, there are also several plant pathogenic species. Higher root dry mass is
585 Sheriff in the presence of SBW25 could be associated to a disease suppression ability,
586 however, viscosin production by microbial inhabitants of the rhizoplane might also be
587 important for decreasing abundance of this genus.

588 In conclusion, the ability to produce viscosin enhances root colonization and
589 significantly alters the relative abundance of several microbial community members with
590 limited effects on the protist communities. Surprisingly, and in contrast to our hypothesis
591 of cultivar-dependent root colonization, *P. fluorescens* SBW25 colonized both cultivars
592 to the same extent. This opens new questions on the importance of viscosin for root
593 colonization, and what governs the stability of the microbial assembly observed in the
594 Heerup rhizoplane.

595 This work shows how a specific secondary metabolite might impact the microbial
596 assembly in the rhizosphere, and further that such a response is plant cultivar
597 dependent. This knowledge is important for an improved understanding of microbial

598 ecology in the plant-soil interphase, and further provides valuable information for
599 developing microbial inoculants for plant growth promotion. The future bio-based society
600 might thus have to develop cultivar personalized inoculants for agriculture as a parallel
601 to the personalized medicine concept used in humans.
602
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612
613 **Competing Interest Statement**
614 The authors declare no competing interests.
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619 **REFERENCES**

620 1. Harman G, Khadka R, Doni F, Uphoff N. Benefits to Plant Health and Productivity
621 From Enhancing Plant Microbial Symbionts. Frontiers in Plant Science.
622 2021;11:610065.

623 2. Zandi P, Basu SK. Role of Plant Growth-Promoting Rhizobacteria (PGPR) as
624 BioFertilizers in Stabilizing Agricultural Ecosystems. Sustain Dev Biodiver.
625 2016;9:71-87.

626 3. Faltin F, Lottmann J, Grosch R, Berg G. Strategy to select and assess
627 antagonistic bacteria for biological control of Rhizoctonia solani Kuhn. Can J
628 Microbiol. 2004;50:811-820.

629 4. Elnahal ASM, El-Saadony MT, Saad AM, Desoky ESM, El-Tahan AM, Rady MM,
630 et al. The use of microbial inoculants for biological control, plant growth
631 promotion, and sustainable agriculture: A review. Eur J Plant Pathol.
632 2022;162:759-792.

633 5. Raymond NS, Gómez-Muñoz B, van der Bom FJT, Nybroe O, Jensen LS, Müller-
634 Stöver DS, et al. Phosphate-solubilising microorganisms for improved crop
635 productivity: a critical assessment. New Phytologist. 2021;229:1268-1277.

636 6. Berendsen RL, Pieterse CMJ, Bakker PAHM. The rhizosphere microbiome and
637 plant health. Trends in Plant Science. 2012;17:478-486.

638 7. Venturi V, Keel C. Signaling in the Rhizosphere. Trends in Plant Science.
639 2016;21:187-198.

640 8. Tran H, Ficke A, Asiimwe T, Hofte M, Raaijmakers JM. Role of the cyclic
641 lipopeptide massetolide A in biological control of *Phytophthora infestans* and in
642 colonization of tomato plants by *Pseudomonas fluorescens*. New Phytol.
643 2007;175:731-742.

644 9. Nielsen TH, Nybroe O, Koch B, Hansen M, Sorensen J. Genes involved in cyclic
645 lipopeptide production are important for seed and straw colonization by
646 *Pseudomonas* sp strain DSS73. Appl Environ Microb. 2005;71:4112-4116.

647 10. Mazzola M, de Bruijn I, Cohen MF, Raaijmakers JM. Protozoan-induced
648 regulation of cyclic lipopeptide biosynthesis is an effective predation defense
649 mechanism for *Pseudomonas fluorescens*. Appl Environ Microbiol.
650 2009;75:6804-6811.

651 11. de Bruijn I, de Kock MJ, Yang M, de Waard P, van Beek TA, Raaijmakers JM.
652 Genome-based discovery, structure prediction and functional analysis of cyclic
653 lipopeptide antibiotics in *Pseudomonas* species. Mol Microbiol. 2007;63:417-428.

654 12. Alsohim AS, Taylor TB, Barrett GA, Gallie J, Zhang XX, Altamirano-Junqueira
655 AE, et al. The biosurfactant viscosin produced by *Pseudomonas fluorescens*
656 SBW25 aids spreading motility and plant growth promotion. Environmental
657 Microbiology. 2014;16:2267-2281.

658 13. Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, et al.
659 Defining the core *Arabidopsis thaliana* root microbiome. Nature. 2012;488:86-90.

660 14. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S,
661 et al. Structure, variation, and assembly of the root-associated microbiomes of
662 rice. Proceedings of the National Academy of Sciences. 2015;112:E911-E920.

663 15. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and
664 heritability of the maize rhizosphere microbiome under field conditions.
665 Proceedings of the National Academy of Sciences. 2013;110:6548-6553.

666 16. Wang JN, Mei XL, Wei Z, Raza W, Shen QR. Effect of bacterial intra-species
667 community interactions on the production and activity of volatile organic
668 compounds. Soil Ecol Lett. 2021;3:32-41.

669 17. James GA, Beaudette L, Costerton JW. Interspecies Bacterial Interactions in
670 Biofilms. J Ind Microbiol. 1995;15:257-262.

671 18. Vesga P, Flury P, Vacheron J, Keel C, Croll D, Maurhofer M. Transcriptome
672 plasticity underlying plant root colonization and insect invasion by *Pseudomonas*
673 *protegens*. ISME J. 2020;14:2766-2782.

674 19. Rochat L, Pechy-Tarr M, Baehler E, Maurhofer M, Keel C. Combination of
675 Fluorescent Reporters for Simultaneous Monitoring of Root Colonization and
676 Antifungal Gene Expression by a Biocontrol Pseudomonad on Cereals with Flow
677 Cytometry. Mol Plant Microbe In. 2010;23:949-961.

678 20. Tovi N, Orevi T, Grinberg M, Kashtan N, Hadar Y, Minz D. Pairwise Interactions
679 of Three Related *Pseudomonas* Species in Plant Roots and Inert Surfaces. Front
680 Microbiol. 2021;12:666522.

681 21. Singh P, Singh RK, Zhou Y, Wang J, Jiang Y, Shen N, et al. Unlocking the
682 strength of plant growth promoting *Pseudomonas* in improving crop productivity
683 in normal and challenging environments: a review. Journal of Plant Interactions.
684 2022;17:220-238.

685 22. Lloyd DP, Allen RJ. Competition for space during bacterial colonization of a
686 surface. Journal of The Royal Society Interface. 2015;12:20150608.

687 23. Hawkes CV, DeAngelis KM, Firestone MK. CHAPTER 1 - Root Interactions with
688 Soil Microbial Communities and Processes. In: Cardon ZG, Whitbeck JL (eds).
689 The Rhizosphere. Academic Press, Burlington, 2007. pp 1-29.

690 24. Ceja-Navarro JA, Wang Y, Ning DL, Arellano A, Ramanculova L, Yuan MM, et al.
691 Protist diversity and community complexity in the rhizosphere of switchgrass are
692 dynamic as plants develop. Microbiome. 2021;9.

693 25. Clarholm M. Interactions of Bacteria, Protozoa and Plants Leading to
694 Mineralization of Soil-Nitrogen. Soil Biol Biochem. 1985;17:181-187.

695 26. Rosenberg K, Bertaux J, Krome K, Hartmann A, Scheu S, Bonkowski M. Soil
696 amoebae rapidly change bacterial community composition in the rhizosphere of
697 *Arabidopsis thaliana*. Isme J. 2009;3:675-684.

698 27. Rainey PB, Bailey MJ. Physical and genetic map of the *Pseudomonas*
699 *fluorescens* SBW25 chromosome. Molecular Microbiology. 1996;19:521-533.

700 28. Bonnichsen L, Bygvraa Svenningsen N, Rybtke M, de Bruijn I, Raaijmakers JM,
701 Tolker-Nielsen T, et al. Lipopeptide biosurfactant viscosin enhances dispersal of
702 *Pseudomonas fluorescens* SBW25 biofilms. Microbiology. 2015;161:2289-2297.

703 29. van der Bom F, Magid J, Jensen LS. Long-term P and K fertilisation strategies
704 and balances affect soil availability indices, crop yield depression risk and N use.
705 European Journal of Agronomy. 2017;86:12-23.

706 30. Zervas A, Ellegaard-Jensen L, Hennessy Rosanna C, Bak F, Guan Y, Horn
707 Herms C, et al. Diversity and Structure of Bacterial Communities in Different
708 Rhizocompartments (Rhizoplane, Rhizosphere, and Bulk) at Flag Leaf
709 Emergence in Four Winter Wheat Varieties. Microbiology Resource
710 Announcements. 2022;11:e00222-00222.

711 31. Shaner NC, Campbell RE, Steinbach PA, Giepmans BNG, Palmer AE, Tsien RY.
712 Improved monomeric red, orange and yellow fluorescent proteins derived from
713 Discosoma sp red fluorescent protein. Nat Biotechnol. 2004;22:1567-1572.

714 32. Gould WD, Hagedorn C, Bardinelli TR, Zablotowicz RM. New Selective Media for
715 Enumeration and Recovery of Fluorescent Pseudomonads from Various
716 Habitats. Appl Environ Microb. 1985;49:28-32.

717 33. Chelius MK, Triplett EW. The Diversity of Archaea and Bacteria in Association
718 with the Roots of Zea mays L. Microbial Ecology. 2001;41:252-263.

719 34. Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N,
720 Assenza F, et al. Revealing structure and assembly cues for *Arabidopsis* root-
721 inhabiting bacterial microbiota. Nature. 2012;488:91-95.

722 35. Anguita-Maeso M, Haro C, Navas-Cortés JA, Landa BB. Primer Choice and
723 Xylem-Microbiome-Extraction Method Are Important Determinants in Assessing
724 Xylem Bacterial Community in Olive Trees. Plants. 2022;11:1320.

725 36. Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM. A Method for
726 Studying Protistan Diversity Using Massively Parallel Sequencing of V9
727 Hypervariable Regions of Small-Subunit Ribosomal RNA Genes. Plos One.
728 2009;4:e6372.

729 37. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP.
730 DADA2: High-resolution sample inference from Illumina amplicon data. Nat
731 Methods. 2016;13:581-583.

732 38. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA
733 ribosomal RNA gene database project: improved data processing and web-
734 based tools. Nucleic Acids Research. 2013;41:D590-D596.

735 39. Guillou L, Bachar D, Audic S, Bass D, Berney C, Bittner L, et al. The Protist
736 Ribosomal Reference database (PR2): a catalog of unicellular eukaryote Small
737 Sub-Unit rRNA sequences with curated taxonomy. Nucleic Acids Research.
738 2013;41:D597-D604.

739 40. Reitmeier S, Hitch TCA, Treichel N, Fikas N, Hausmann B, Ramer-Tait AE, et al.
740 Handling of spurious sequences affects the outcome of high-throughput 16S
741 rRNA gene amplicon profiling. ISME Communications. 2021;1:31.

742 41. R Core Team. R: A Language and Environment for Statistical Computing. 2022.

743 42. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive
744 Analysis and Graphics of Microbiome Census Data. Plos One. 2013;8:e61217.

745 43. Andersen KS, Kirkegaard RH, Karst SM, Albertsen M. ampvis2: an R package to
746 analyse and visualise 16S rRNA amplicon data. bioRxiv. 2018:299537.

747 44. Liu C, Cui Y, Li X, Yao M. microeco: an R package for data mining in microbial
748 community ecology. Fems Microbiol Ecol. 2021;97:fiaa255.

749 45. Willis AD, Martin BD. Estimating diversity in networked ecological communities.
750 Biostatistics. 2022;23:207-222.

751 46. Willis A, Martin BD, Trinh P, Teichman S, Clausen D, Barger K, et al. breakaway:
752 Species Richness Estimation and Modeling. 2021.

753 47. McMurdie PJ, Holmes S. Waste Not, Want Not: Why Rarefying Microbiome Data
754 Is Inadmissible. PLOS Computational Biology. 2014;10:e1003531.

755 48. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, et al.
756 vegan: Community Ecology Package. 2022.

757 49. Martin BD, Witten D, Willis AD. corncob: Count Regression for Correlated
758 Observations with the Beta-Binomial. 2021.

759 50. Knights HE, Jorrin B, Haskett TL, Poole PS. Deciphering bacterial mechanisms
760 of root colonization. Env Microbiol Rep. 2021;13:428-444.

761 51. Yang MM, Wen SS, Mavrodi DV, Mavrodi OV, von Wettstein D, Thomashow LS,
762 et al. Biological control of wheat root diseases by the CLP-producing strain
763 *Pseudomonas fluorescens* HC1-07. Phytopathology. 2014;104:248-256.

764 52. Nunes I, Hansen V, Bak F, Bonnichsen L, Su J, Hao X, et al. Succession of the
765 wheat seed-associated microbiome as affected by soil fertility level and
766 introduction of *Penicillium* and *Bacillus* inoculants in the field. Fems Microbiol
767 Ecol. 2022;98:fiac028.

768 53. Nicolaisen MH, Cuong ND, Herschend J, Jensen B, Loan LC, Van Du P, et al.
769 Biological control of rice sheath blight using hyphae-associated bacteria:
770 development of an *in planta* screening assay to predict biological control agent
771 performance under field conditions. BioControl. 2018;63:843-853.

772 54. Usyskin-Tonne A, Hadar Y, Minz D. Spike Formation Is a Turning Point
773 Determining Wheat Root Microbiome Abundance, Structures and Functions. Int J
774 Mol Sci. 2021;22.

775 55. Rossmann M, Pérez-Jaramillo JE, Kavamura VN, Chiaramonte JB, Dumack K,
776 Fiore-Donno AM, et al. Multitrophic interactions in the rhizosphere microbiome of
777 wheat: from bacteria and fungi to protists. Fems Microbiol Ecol. 2020;96:fiaa032.

778 56. Chen X, Xu D, Zhang Y, Zhang L, Li F. Field level comparison of rhizosphere
779 microbiome characteristics between wheat cultivars Jimai22 and Xiaoyan22.
780 Agronomy Journal. 2023;n/a.

781 57. Ou K, He X, Cai K, Zhao W, Jiang X, Ai W, et al. Phosphate-Solubilizing
782 Pseudomonas sp. Strain WS32 Rhizosphere Colonization-Induced Expression
783 Changes in Wheat Roots. Front Microbiol. 2022;13:927889.

784 58. Liu X, Jiang X, He X, Zhao W, Cao Y, Guo T, et al. Phosphate-Solubilizing
785 *Pseudomonas* sp. Strain P34-L Promotes Wheat Growth by Colonizing the
786 Wheat Rhizosphere and Improving the Wheat Root System and Soil Phosphorus
787 Nutritional Status. Journal of Plant Growth Regulation. 2019;38:1314-1324.

788 59. Cao Y-y, Ni H-t, Li T, Lay K-d, Liu D-s, He X-y, et al. *Pseudomonas* sp. TK35-L
789 enhances tobacco root development and growth by inducing *HRGPnt3*

790 expression in plant lateral root formation. Journal of Integrative Agriculture.
791 2020;19:2549-2560.

792 60. Herms CH, Hennessy RC, Bak F, Dresbøll DB, Nicolaisen MH. Back to our roots:
793 exploring the role of root morphology as a mediator of beneficial plant–microbe
794 interactions. Environmental Microbiology. 2022;24:3264-3272.

795 61. Schlatter Daniel C, Yin C, Hulbert S, Paulitz Timothy C. Core Rhizosphere
796 Microbiomes of Dryland Wheat Are Influenced by Location and Land Use History.
797 Appl Environ Microb. 2020;86:e02135-02119.

798 62. Simonin M, Dasilva C, Terzi V, Ngonkeu ELM, Diouf D, Kane A, et al. Influence
799 of plant genotype and soil on the wheat rhizosphere microbiome: evidences for a
800 core microbiome across eight African and European soils. Fems Microbiol Ecol.
801 2020;96:fiaa067.

802 63. Blake C, Christensen MN, Kovács ÁT. Molecular Aspects of Plant Growth
803 Promotion and Protection by *Bacillus subtilis*. Mol Plant Microbe In. 2020;34:15-
804 25.

805 64. Ofek M, Hadar Y, Minz D. Ecology of Root Colonizing *Massilia*
806 (Oxalobacteraceae). Plos One. 2012;7:e40117.

807 65. Li X, Rui J, Mao Y, Yannarell A, Mackie R. Dynamics of the bacterial community
808 structure in the rhizosphere of a maize cultivar. Soil Biology and Biochemistry.
809 2014;68:392-401.

810 66. Taerum SJ, Micciulla J, Corso G, Steven B, Gage DJ, Triplett LR. 18S rRNA
811 gene amplicon sequencing combined with culture-based surveys of maize

812 rhizosphere protists reveal dominant, plant-enriched and culturable community
813 members. Env Microbiol Rep. 2022;14:110-118.

814 67. Sapp M, Ploch S, Fiore-Donno AM, Bonkowski M, Rose LE. Protists are an
815 integral part of the *Arabidopsis thaliana* microbiome. Environmental Microbiology.
816 2018;20:30-43.

817 68. Bao Y, Lies DP, Fu H, Roberts GP. An improved Tn7-based system for the
818 single-copy insertion of cloned genes into chromosomes of gram-negative
819 bacteria. Gene. 1991;109:167-168.

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Tables

Table 1. Strains, plasmids and primers.

	Characteristics or sequence	Reference
Strains		
Pseudomonas fluorescens		
SBW25	WT, produces viscosin.	[27]
SBW25Δ*viscA*	Impaired in viscosin production: *viscA*::Tn*Mod* Km^r	[11]
Plasmids		
pME9407	Delivery plasmid for mini-Tn*7-mcherry*; pME3280a carrying *mcherry* placed under P_{tac} control; Ap^r Gm^r	[19]
pUX-BF13	Helper plasmid encoding Tn7 transposition functions; R6K-replicon; Ap^r	[68]
Primers		
mCherry_Fw	5'-GCCCCGTAATGCAGAAGAAG-3'	This study
mCherry_Rv	5'-GTGTAGTCCTCGTTGTGGGA-3'	This study
799F	5'-AACMGGATTAGATACCCKG-3'	[33]
1193R	5'-ACGTCATCCCCACCTTCC-3'	[34]
1380F	5'-GCCTCCCTCGCGCCATCAG-3'	[36]
1510R	5'-GCCTTGCCAGCCCGCTCAG-3'	[36]

Figures

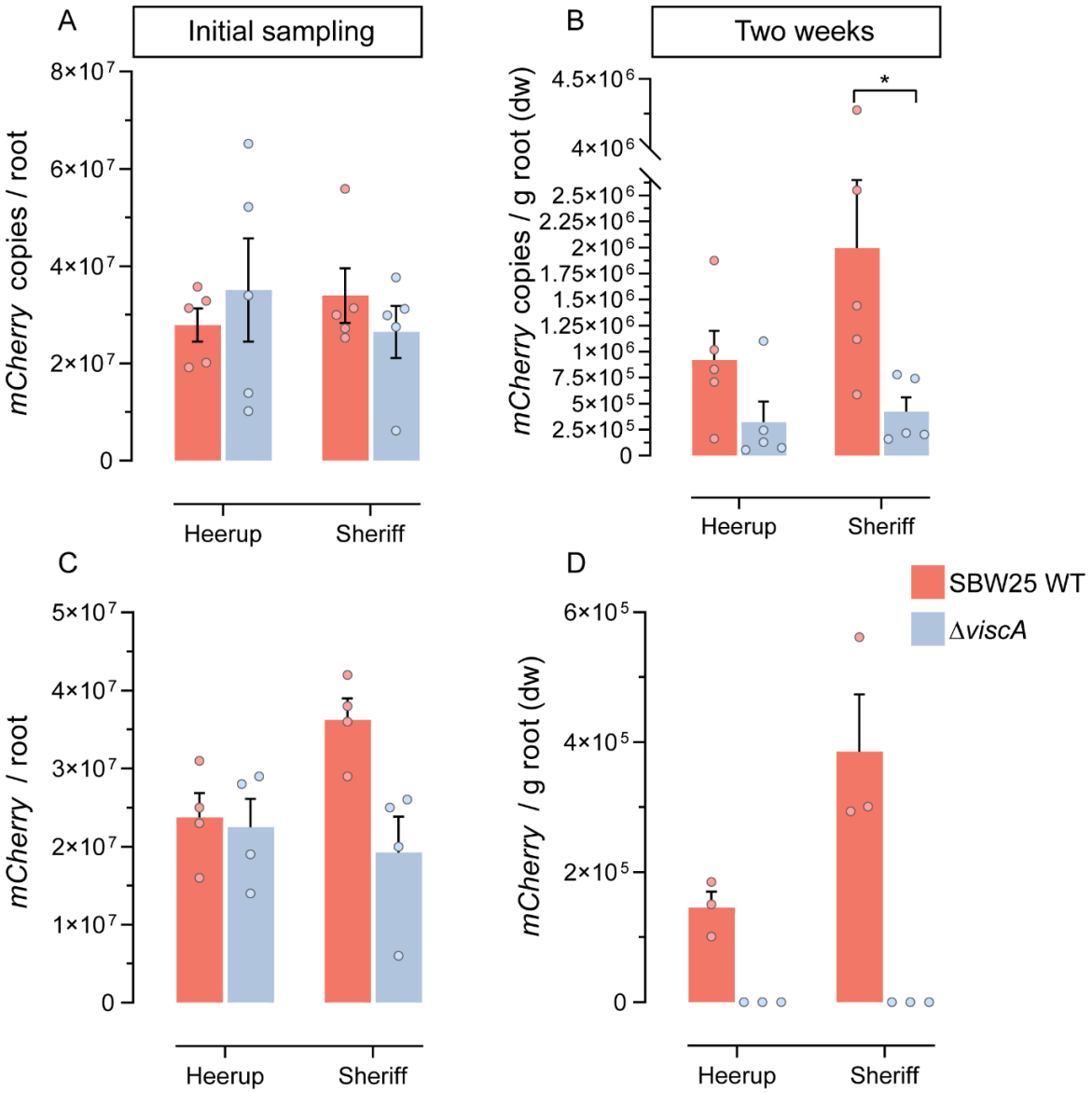

Fig.1 Colonization ability of *P. fluorescens* SBW25 WT and *P. fluorescens* SBW25 Δ*viscA*. (A) qPCR enumeration of inoculated bacteria immediately after inoculation. (B) qPCR enumeration of inoculated bacteria in the rhizoplane at two weeks after inoculation (n=5). (C) CFU enumeration of inoculated bacteria immediately after inoculation (n=4). (D) CFU enumeration of inoculated bacteria in the rhizoplane at two weeks after inoculation (n=3). Bars represent the mean + standard deviation, and each point represents a sample. Asterisks above histograms indicate whether two groups are significantly different (t-test, $p < 0.05$)

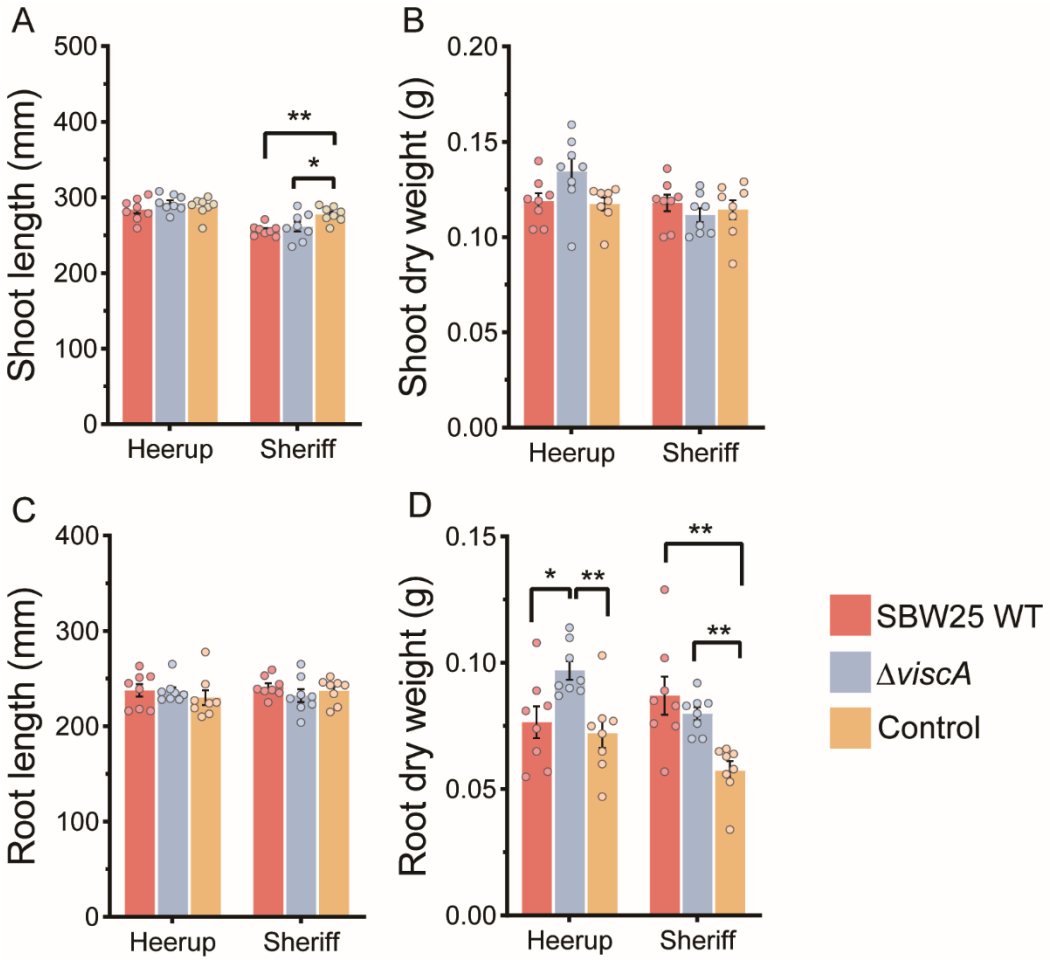

Fig.2 The effect of inoculation with *P. fluorescens* SBW25 WT or *P. fluorescens* SBW25 *ΔviscA* on plant growth (n = 8). Bars represent the mean + standard deviation, and each point represents a sample. Asterisks above histograms indicate whether two group are statistically significantly different as assessed by one-way ANOVA followed by a Tukey HSD test: *$P < 0.05$, **$P < 0.01$.

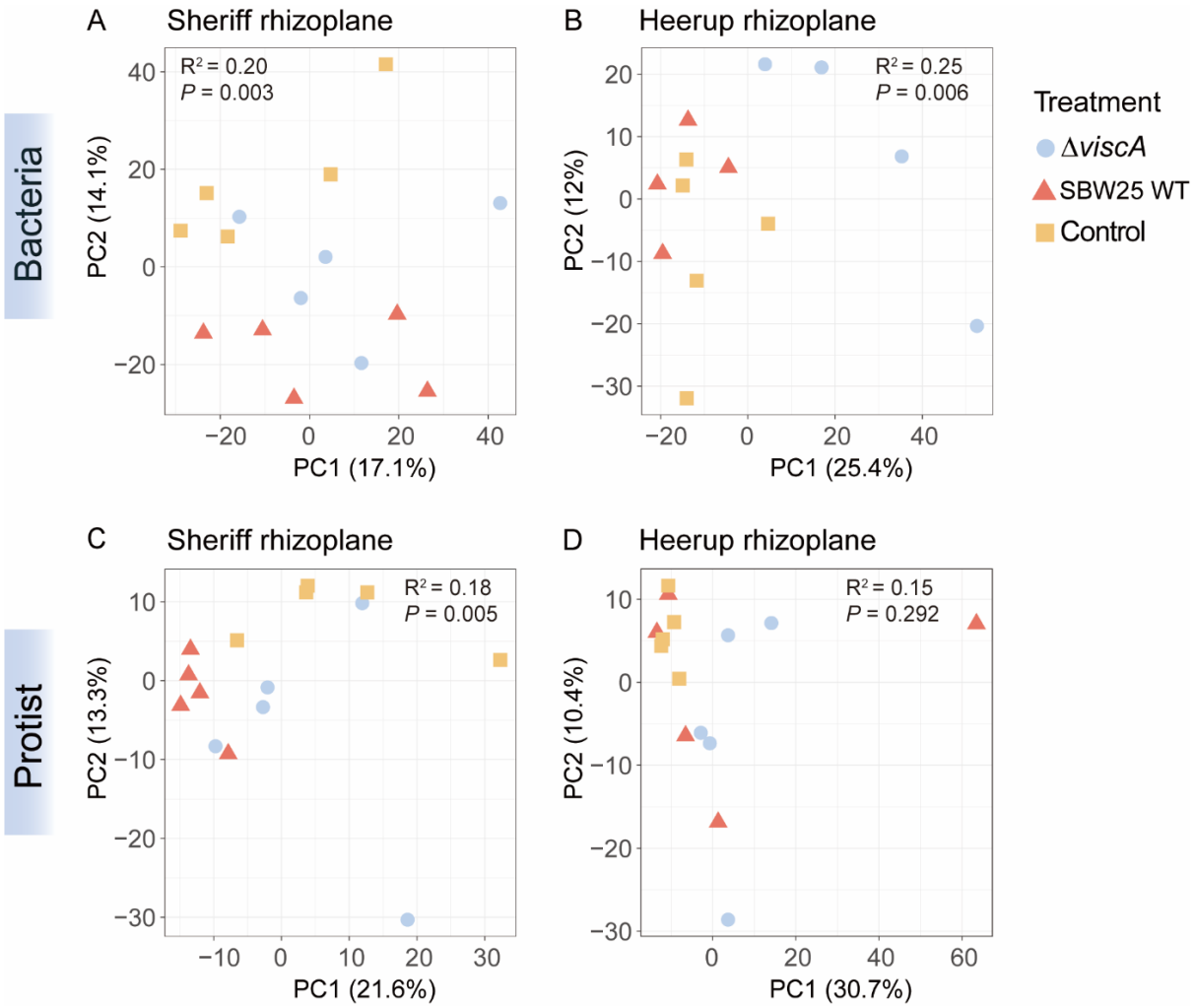

Fig.3 Principal Component Analysis (PCA) based on Aitchison distances of bacterial and protist communities in each subset. The models were validated using an ANOVA-like permutation test (999 permutations) as indicated by the P-value. R^2 is expressed as the proportion of the mean sum of squares obtained from PERMANOVA. Each symbol represents individual sample points and samples are colored for inoculation treatment.

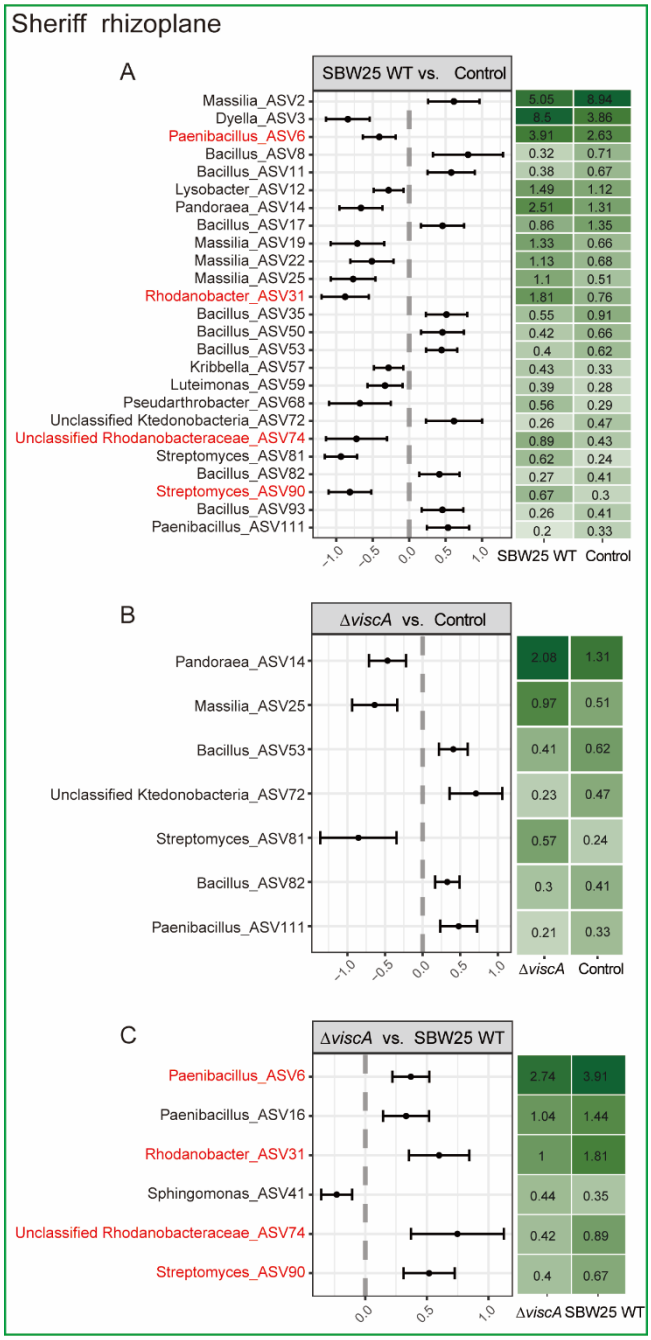

Fig.4 The bacterial ASVs/genera with significant differences in relative abundance between inoculation treatments in Sheriff. The differential abundance was determined using beta-binomial regression with the corncob. Only ASVs having an estimated differential abundance of < -1 or > 1 and P-values adjusted for multiple testing < 0.05 (FDR < 0.05) were considered significant. The red color indicates that inoculation with SBW25 WT showed a significant increase in ASVs compared to both control and mutant treatment.

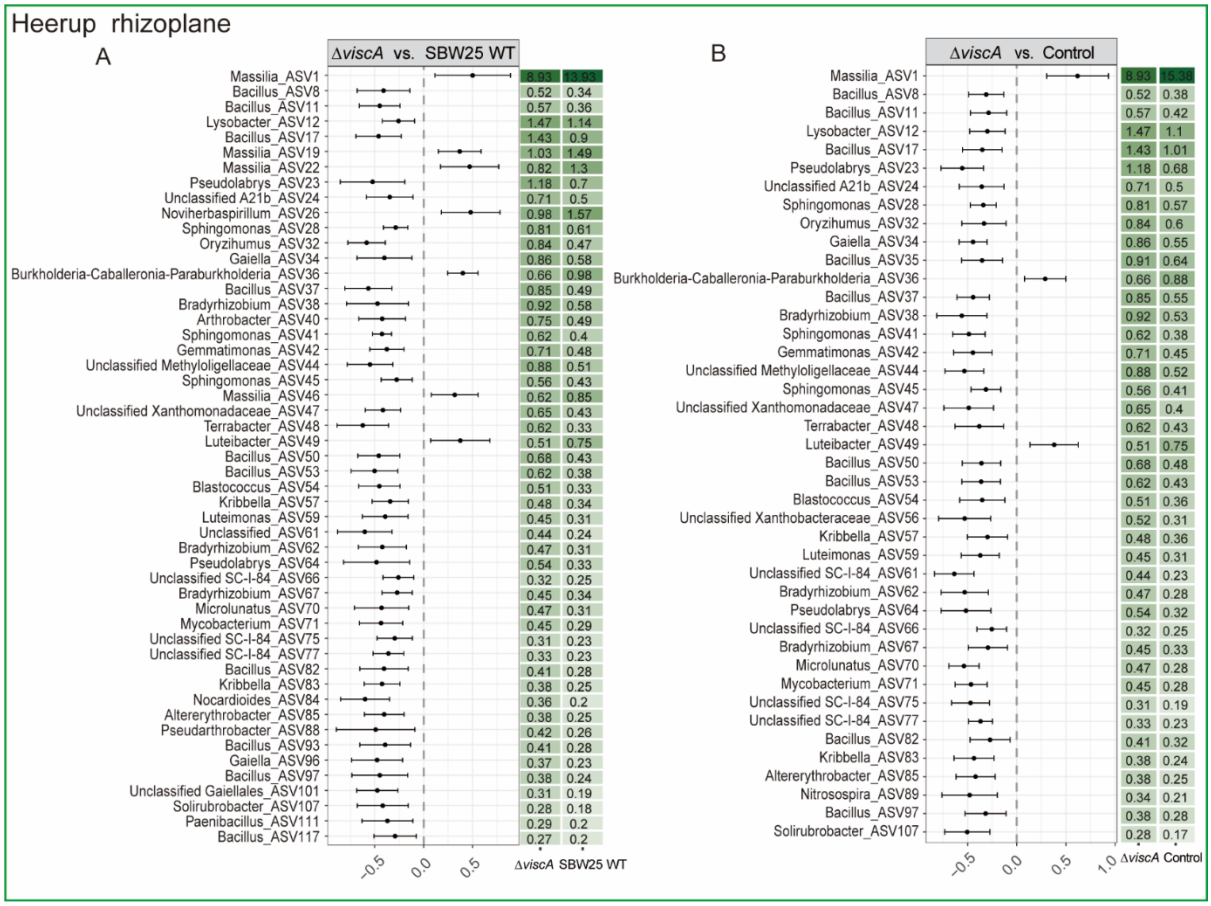

Fig.5 The bacterial ASVs/genera with significant differences in relative abundance between inoculation treatments in Heerup. No significant difference in ASVs was found between SBW25 WT treatment and control treatment in Heerup rhizoplane. The differential abundance was determined using beta-binomial regression with the corncob. Only ASV that had an estimated differential abundance of < -1 or > 1 and P-values adjusted for multiple testing < 0.05 (FDR < 0.05) were considered significant.

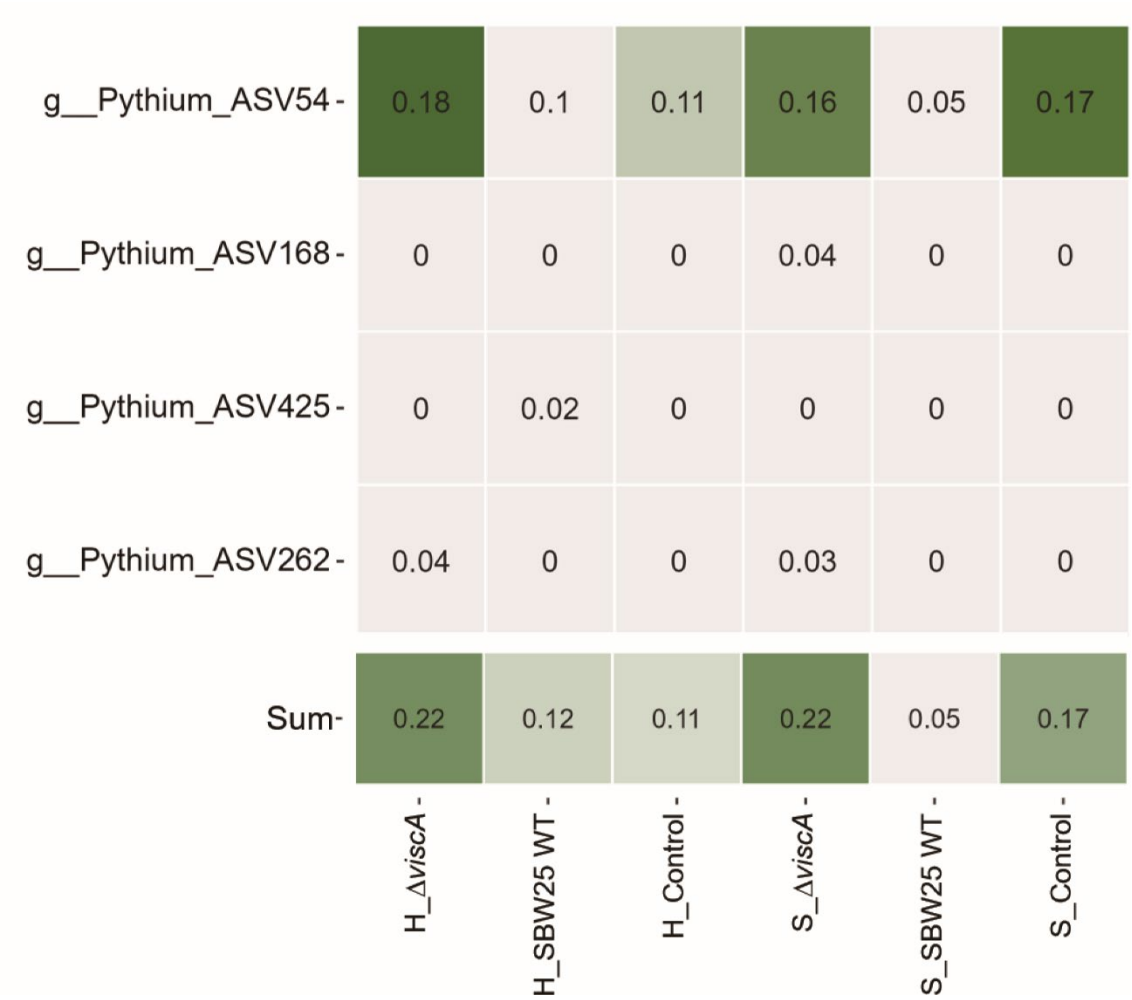

Fig.6 The relative abundance of Pythium in the rhizoplane was analyzed in each group. The mean relative abundance of all Pythium in each treatment group (n=5). Sum represents the abundance of these four ASVs for each group. H: Heerup; S: Sheriff.