Linking microbial community structure with function using functional amplicon sequencing of the NRPS genes around wheat roots during drought stress

Ying Guan[1a], Edmond Berne[2], Rosanna Catherine Hennessy[1], Paolina Garbeva[3], Mette Haubjerg Nicolaisen [1*#], Frederik Bak[1#],

[1] Department of Plant and Environmental Science, University of Copenhagen, Frederiksberg C, Denmark

[2] Bioengineering Department, Polytech Nice Sophia, University de Cote d'Azur, Nice, France

[3] Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, Netherlands

[a] Present address: College of Resources and Environmental Science, Nanjing Agricultural University, Nanjing, China

* Correspondence: meni@plen.ku.dk

# These two authors contributed equally

Abstract

Secondary metabolites are an important group of compounds, mediating interactions between microorganisms and plants. One class of these secondary metabolites includes cyclic lipopeptides and siderophores produced by nonribosomal peptide synthetases (NRPSs) are essential for bacterial root colonization. However, due to the vast array of secondary metabolites, there are still a lot of compounds for which their role in plant root colonization is unknown. This is even more noticeable during drought. To unravel some of these secondary metabolites' role, we used functional amplicon sequencing of the adenylation domain of the NRPSs and linked this with amplicon sequencing of the 16S rRNA gene from roots of wheat in controlled conditions or under drought stressed. Drought had a transient impact on the bacterial community composition, which was also seen for the NRPS composition. We found that drought selected for distinct groups of siderophores from different taxonomical groups, enriching for *Streptomyces* and depleting *Pseudomonas* siderophores. In addition, drought selected for a *Pseudomonas* NRPS gene encoding the cyclic lipopepeptide viscosin hinting towards this compound providing a competitive advantage in root colonization during drought. We confirmed this by a follow up experiment using the viscosin producing *P. fluorescens* SBW25 and its Δ*viscA* mutant. In summary, our work demonstrates the potential for functional amplicon sequencing of NRPS genes coupled with in planta experiment to elucidate their importance in root colonization.

Importance

To exploit beneficial plant-microbe interactions for improved plant growth under drought stress, we need to improve our understanding of factors important for successful root colonization. Functional amplicon sequencing can leverage our understanding of plant-microbe interactions and provide the background knowledge for experimental testing. Secondary metabolites (SM) are important in plant-microbe interactions, and thus, targeting these genes provide the potential to increase translational power from lab experiments to field testing. Here, we show that a group of secondary metabolites, the siderophores, respond differently to drought stress depending on taxonomic affiliation and that the potential to synthesize viscosin increases root colonization during drought. This work demonstrates that the resolution provided by functional amplicon sequencing can be used to identify key genes in plant-microbe interactions around the roots.

Introduction

Microorganisms live and interact in close contact with plants around the roots. The area around the roots is the rhizosphere which provides a plethora of nutrients for microorganisms through root exudates The microorganisms are important for plant health and development, as they provide beneficial functions such as nutrient acquisition, disease suppression, or reduce abiotic and biotic stresses (Mendes et al., 2013). One way for microorganisms to provide these functions, are through secondary metabolites. This group represents a vast diversity of metabolites which are not only involved in plant beneficial actions, but also in root-colonization and microbe-microbe interactions.

Microorganisms produce a broad array of secondary metabolites (SMs) that are crucial for shaping microbial community dynamics and regulating plant-microbe interactions (Philippot *et al.*, 2013; Tyc *et al.*, 2017). An important class of secondary metabolites are the non-ribosomal peptides (NRPs), synthesized by non-ribosomal peptide synthetases (NRPSs). NRPs have a variety of functions in antimicrobial activity, bacterial motility (de Bruijn *et al.*, 2007, 2008), biofilm formation (de Bruijn *et al.*, 2008; D'aes *et al.*, 2014), root colonization (Tran *et al.*, 2007; Guan *et al.*, 2024) , and the induction of systemic resistance (ISR) in plants (Ma *et al.*, 2016; Omoboye *et al.*, 2019).

We have recently shown that the potential to produce the NRP viscosin enhanced the colonization ability of *P. fluorescens* SBW25 on wheat roots and shape the assembly of rhizosphere microbial communities (Guan *et al.*, 2024). Furthermore, the rhizosphere microbiome hosts a distinct composition of NRPSs different from the neighboring bulk soil and other environments (Dror *et al.*, 2020) pointing towards a role for these compounds in plant microbe and microbe-microbe interactions around the roots. Despite this, little is known about the composition of NRPS genes in the rhizosphere. There are studies on tomato, lettuce, cucumber, potato, and populus (Aleti *et al.*, 2017; Blair *et al.*, 2018; Dror *et al.*, 2020, 2022), but so far the composition of the NRPS on the rhizoplane has to our knowledge not been determined. Furthermore, our knowledge of the temporal dynamics of the composition of NRPS genes is limited to a single study on potato (Aleti *et al.*, 2017).

Beneficial rhizosphere microorganisms are crucial for enhancing plant drought tolerance. Studies suggest that drought stress typically reduces soil microbial diversity. However, through complex interactions with their microbiome, plants may recruit drought-tolerant microbes, enhancing their response to drought. Early research showed that microbial

exopolysaccharides, associated with biofilm formation, can enhance soil water retention and thus increase plant drought tolerance. While this shows that SMs are important in plant-microbe interactions during drought, there are no studies that have addressed the effect of drought stress on the development in composition of the NRPSs around the roots.

Understanding the roles of the NRPSs in the environment is challenged by the lack of cultivated bacteria and studies targeting the mechanisms of these compounds in environmental settings. Recently, culture-independent approaches, and in particular amplicon sequencing of certain parts of the biosynthetic gene clusters, sheds light on how these compounds shape the microbial communities in the rhizosphere. Amplicon sequencing has not been limited to NRPS, but includes polyketide synthases, and have been used to describe the composition of these groups in lettuce and tomato (Dror *et al.*, 2020), and in different environments (Geers *et al.*, 2023).

During NRP synthesis by NRPSs, an adenylation (A) domain selects and activates specific amino acids. The specified amino acid is then adenylated and transferred to the phosphopantetheine carrier on the thiolation (T) domain. This process determines the amino acid composition of the peptide, including the incorporation of modified or unusual amino acids, such as D-amino acids. The specificity of the A domain is a key determinant in peptide sequence and function.

Here, we studied the root associated bacterial community and NRPS composition during different growth stages of wheat under drought stress, using amplicon sequencing of the 16S rRNA gene and the NRPS A domain. Specifically, we focused 1) on the diversity and composition of the NRPSs in the rhizoplane compared to the rhizosphere, 2) the temporal dynamics of NRPS composition in the rhizoplane and 3) the effect of drought on the NRPS composition. We found that drought stressed plants depleted NRPS genes encoding for pseudomonads siderophores. In contrast, drought increased the relative abundance of biosynthetic genes coding for viscosin, a CLP produced by pseudomonads. Therefore, we hypothesized that viscosin confers an advantage in root colonization under drought stress. This was experimentally validated using a natural soil system to compare colonization and drought stress tolerance of a viscosin producing wild-type model strain *P fluorescens* SBW25 compared to a viscosin-deficient mutant.

Results

Drought transiently affects bacterial community composition and diversity

The microbial communities colonizing the root surface (rhizoplane) differ in community structure from those colonizing the rhizosphere, and both compartments are affected by drought stress. By examining the NRPS composition's response to drought can reveal potential keystone metabolites required for the successful colonization of roots during drought. We grew winter wheat plants (cv. Sheriff) for a duration of 5 weeks under controlled conditions in a growth chamber. The soil was collected from a nearby agricultural field and mixed with sterile sand (3:1, from here on "soil"). Drought was imposed on 2-weeks old wheat seedlings, by not watering the plants for two weeks, where after water was added to alleviate the drought stress (Fig S1). Control plants received continued watering throughout the entire experiment. The drought-stressed plants had reduced shoot length, shoot fresh weight and root dry weight 4 weeks after sowing (Fig 1A-C) as well as less chlorophyll content, higher peroxidase dismutase and superoxide dismutase activity (Fig S2). At 5 weeks, after one week of re-watering, drought-stressed plants did not grow to the same height or and had a lower weight than control plants (Fig 1A-D). To determine the effect of the drought stress on bacterial rhizoplane communities, we performed 16S rRNA gene amplicon sequencing targeting the V5-V7 regions. Collectively, we obtained 2,146 Amplicon Sequence Variants (ASVs) with a median sequencing depth of 19,328 reads per sample, with a range of 12,905 to 28,215 reads per sample. Rarefaction curves indicated that sufficient sequencing depth was reached (Fig. S3). Drought changed the community composition (Fig 1E, Table S1), but this change was transient as communities after 5 weeks clustered together, as indicated by the positive interaction between time and water level in the PERMANOVA ($R^2 = 0.11$, $p = 0.001$, Table S1). Concomitantly, drought resulted in higher Shannon diversity in the rhizoplane community compared to control plants (Wilcoxon rank test, $p = 0.0079$) after 4 weeks, whereas no difference was observed after 5 weeks (Fig. 1D).

At the ASV level, nine ASVs were more abundant in control plants, while 48 increased in relative abundance during drought (Corncob, FDR < 0.05, Fig. S4). Several bacterial genera, including *Streptomyces* and *Kribella* increased in relative abundance on the rhizoplane in the drought-stressed plants. Notably, three genera, *Massilia, Flavobacterium,* and *Arenimonas,* decreased in relative abundance during drought (Fig. S5).

In conclusion, drought caused effects on the plants lasting through the experiment, while the effect on bacterial communities was transient, as the bacterial communities converged between the two treatments at week 5.

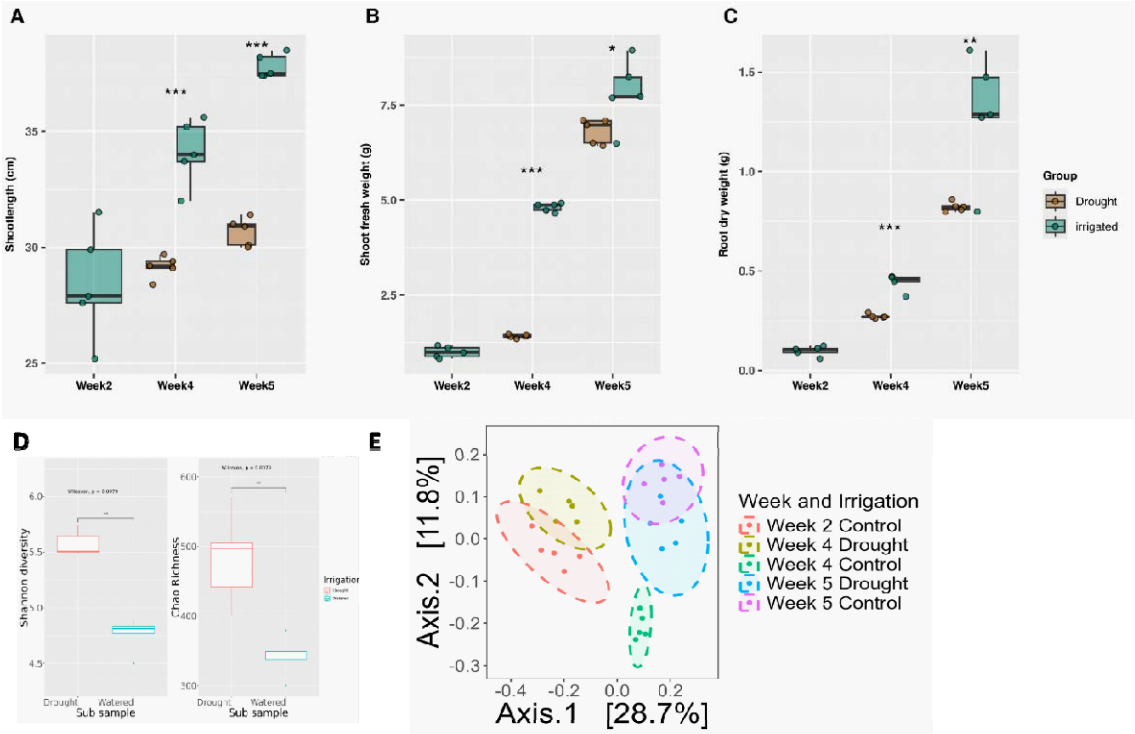

Figure 1. The effect of drought on plant growth before, during, and after drought. The shoot length (**A**), shoot fresh weight (**B**) and root dry weight (**C**) at different sampling times after sowingIrrigated: well-watered plants, Drought: drought stress plants. (**D**) **Shannon diversity and richness at week 4.** . Each box plot represents data from five technical replicates. Each dot represents a sample point. Asterisks in A-C indicate a statistically significant difference between the two groups (Drought and irrigated), which was derived from the t-test: *p < 0.05, **p < 0.01 and ***p < 0.001. he horizontal bars within boxes represent medians. The tops and bottoms of boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5× the interquartile range from the upper edge and lower edge of the box, respectively. (**E**) PCoA of Bray Curtis dissimilarities of the bacterial community based on 16S rRNA amplicon sequencing.

Developmental stage is a more important driver of NRPS composition than compartment

Since 16S rRNA amplicon analysis only provide limited information on functional potential, we performed amplicon sequencing of the NRPS A domains from the rhizoplane and the rhizosphere of wheat as previously described (Tracanna *et al.*, 2021). While it is well established that these two compartments host distinct bacterial communities (e.g. (Guan *et al.*, 2024)), this is unknown for the composition of NRPS. Filtered NRPS sequences were clustered into Amplicon Clusters (ACs) as in (Tracanna *et al.*, 2021).

Our data contained 50 samples and provided us with 5,090,907 reads, and 97,271 ACs. The median number of reads was 83,886 per sample, with a range of 20,133-293,792 reads. Rarefaction curves showed that the majority of the diversity was captured at around 40,000 reads per sample (Fig. S6). The reads were annotated using MiBig and Antismash databases in the dom2bgc pipeline (Tracanna *et al.*, 2021). Despite, using the most recent databases, only 15% and 14% of the reads were annotated in the rhizoplane and rhizosphere, respectively.

We have previously shown that the bacterial communities differ between the rhizosphere and rhizoplane in the Sheriff cultivar (Guan *et al.*, 2024), and we aimed to determine if this was the case for the NRPS amplicons as well. However, neither the alpha diversity (Shannon index) nor the richness differed between the two compartments at any of the three sampling points in the control plants (Fig. 2).

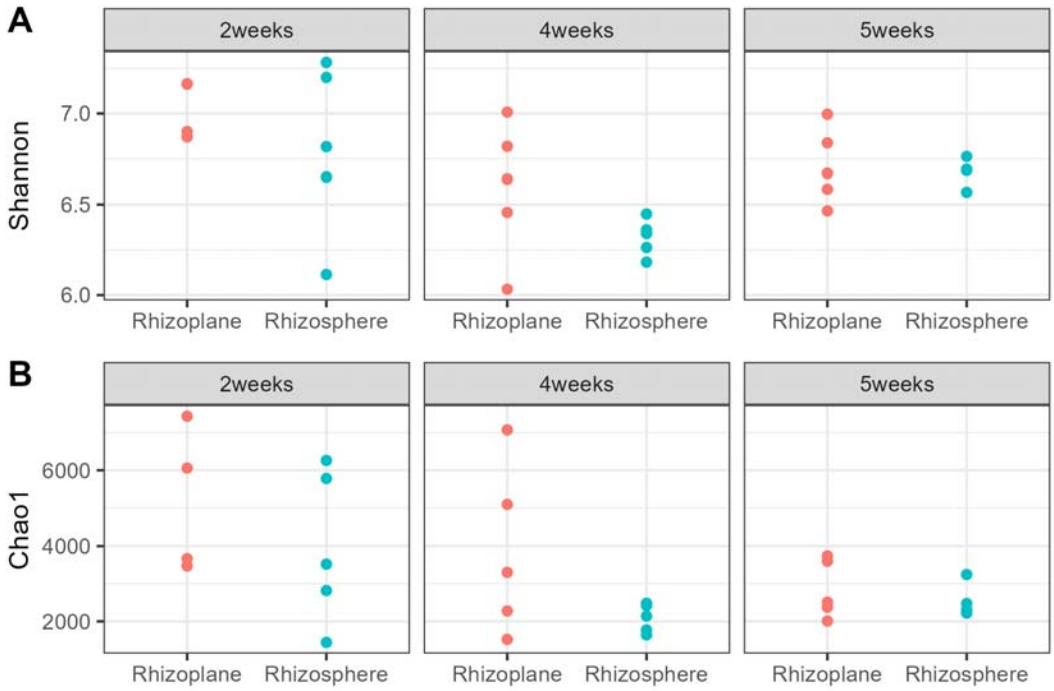

Figure 2. A) Shannon index and B) Chao1 richness of the rhizosphere and rhizoplane samples from control plants.

Further, the NRPS composition for the control plants showed that time was the most important factor in shaping the composition (R^2 = 0.32, p = 0.001, PERMANOVA, Table S2), while compartment was of less importance (R^2 = 0.046, p = 0.04, PERMANOVA) (Fig.

3). This is in contrast to the compartment effect for the bacterial community, where it explained 20% of the variation (Guan *et al.*, 2024).

We then zoomed in on the rhizoplane. As for the 16S rRNA amplicons in the rhizoplane, the composition of the NRPS domains changed over time (PERMANOVA, $R^2 = 0.23$, p =0.001, Table S3), while treatment also changed the composition of the NRPS genes ($R^2 = 0.08$, p = 0.005). As observed for the 16S rRNA gene, the interaction was also significant ($R^2 = 0.09$, p = 0.001), and based on the PCoA, this indicates that the effect of drought on the NRPS domain composition was transient, as the drought and control samples clustered together at week 5 (Fig. 4).

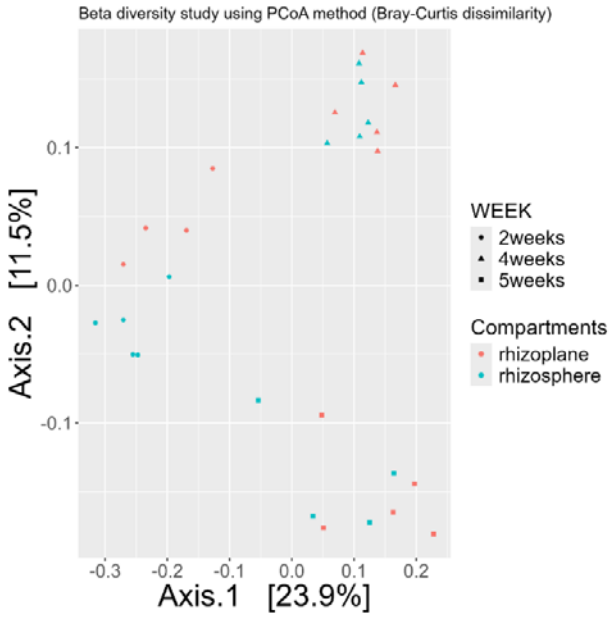

Figure 3. PCoA of the NRPS ACs from watered plants, separated by compartment (color).

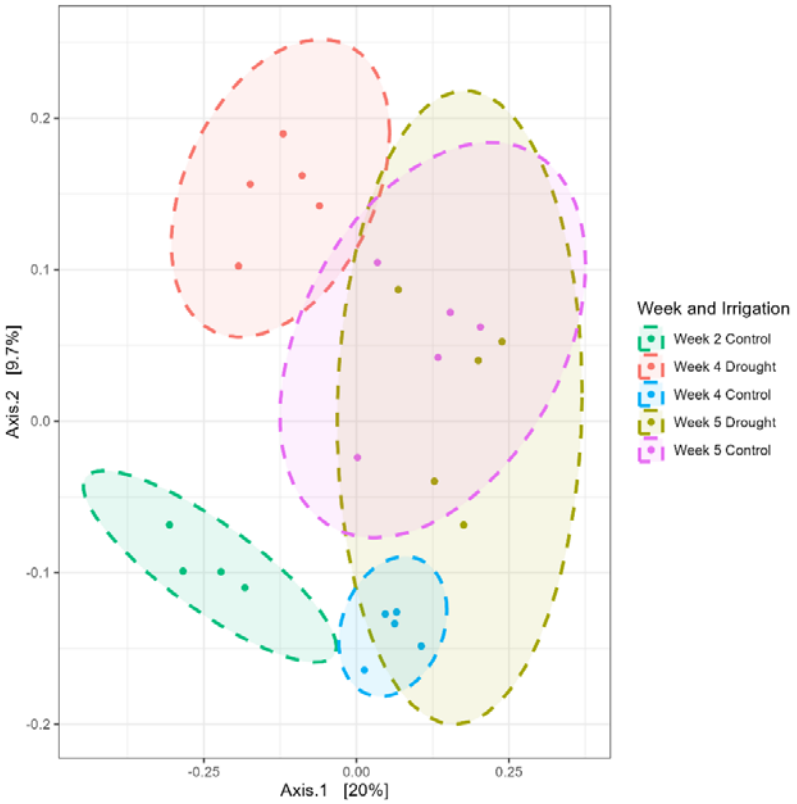

Figure 4. PCoA of rarefied NRPS amplicon clusters from the rhizoplane using Bray-Curtis dissimilarity.

Drought changes the composition of NRPS genes to a lower extent than bacterial composition

As the 2-week drought period changed the bacterial community in the rhizoplane, we asked whether this was also the case for the NRPS composition. Drought did not change the Shannon diversity or the richness either at week 4 or week 5 (Fig. 5), contrasting the findings from the 16S rRNA amplicons. In the rhizoplane, 15.2% of the ACs were annotated as NRPS in the control plants compared to 16.4% in the drought treated plants across the three sampling times. They were taxonomically assigned to seven different phyla, with the majority (65-68%) of the ACs assigned to taxonomy belonged to Actinomyceota, with Proteobacteria and Myxococcota comprising 18-24% and 10-13% depending on the watering regime, respectively (Fig 6A). NRPS genes annotated to Planctomyceota were only detected in the drought-stressed plants.

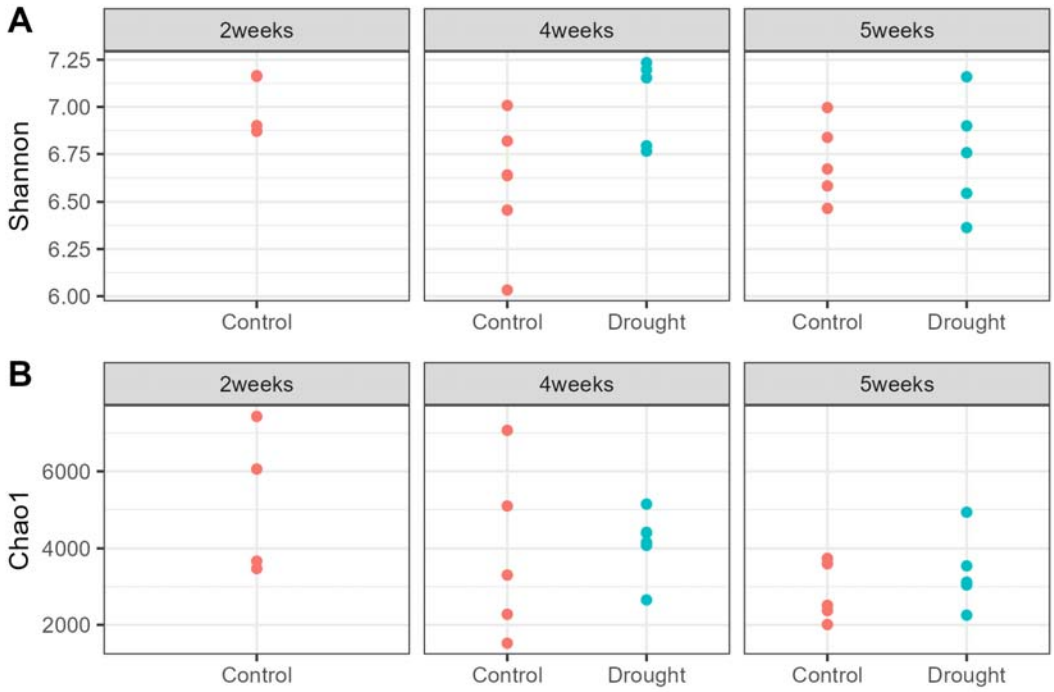

Figure 5. The effect of drought on the alpha diversity (Shannon Index) (A) and richness (Chao1) (B) in the rhizoplane. Each dot represents a sample. There were no significant differences between treatments at any timepoint (Wilcoxon rank test).

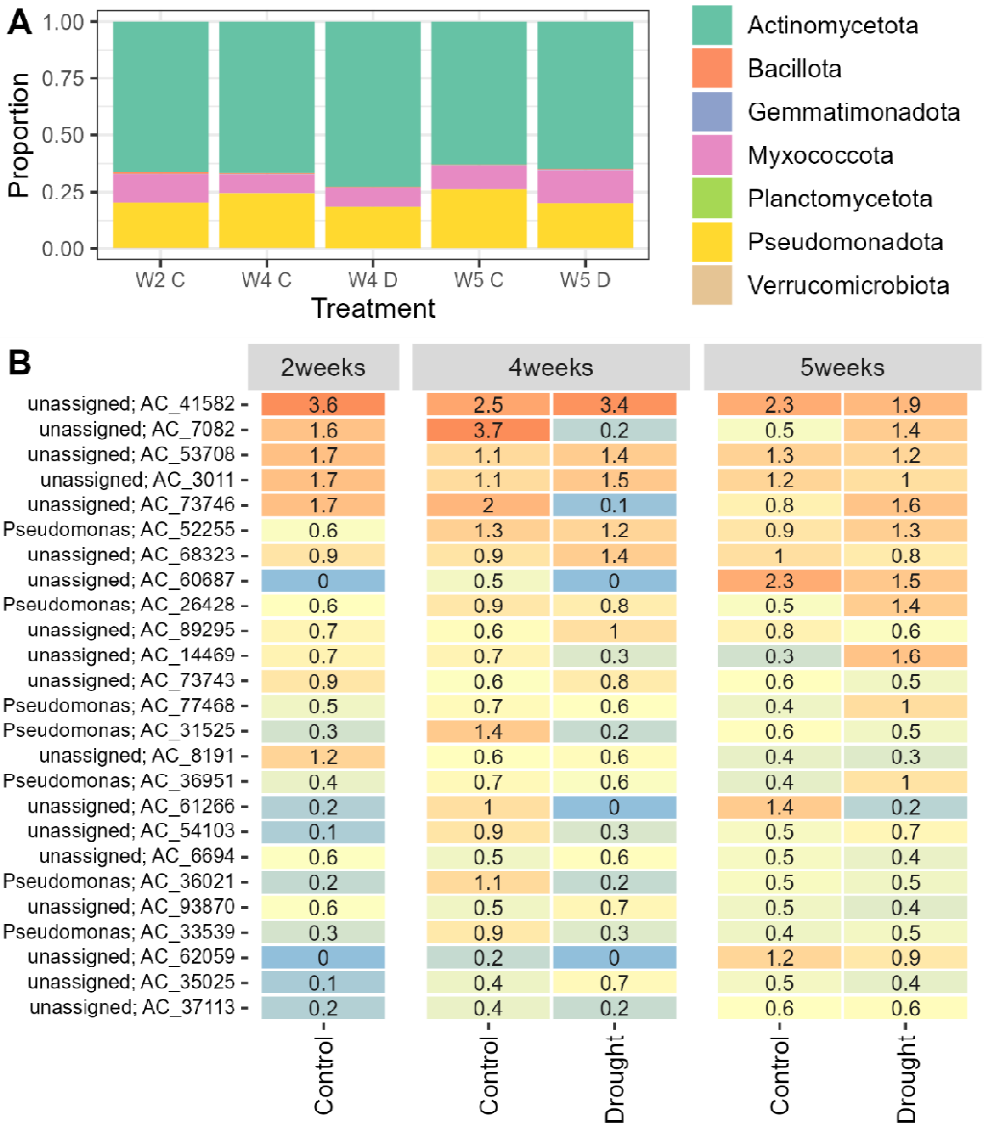

Figure 6. A) Taxonomic assignment at the phylum level of the ACs in drought stressed plants and control plants. B) Relative abundance of the 25 most abundant ACs across all sampling times and treatments in the rhizoplane.

Of the 25 most abundant NRPS ACs in the rhizoplane, seven were taxonomically assigned to *Pseudomonas* (Fig. 6B) and functionally classified as NRPS, while the rest were unassigned. Due to the low proportion of assigned amplicon clusters, we used blastp to search the 25 most abundant ACs in the MiBig database v.3.1 (Table S4). The most abundant (amplicon_cluster_41582) was classified as Pf-5 pyoverdine. Of the *Pseudomonas*, six of seven were assigned to pyoverdine-related "compounds" (Table S4). Despite our effort, 10 of

the 25 most abundant amplicon clusters did not represent known biosynthetic gene clusters indicating a potentially high and untapped abundance of novel compounds in the rhizoplane.

Drought reduces the relative abundance of *Pseudomonas* pyoverdine related NRPS genes in the rhizoplane

We then asked which ACs were enriched or depleted when plants were exposed to drought. To determine this we tested differentially abundant ACs using corncob (Martin *et al.*, 2020). Drought stress enriched 64 ACs and depleted 37 ACs in week 4 (Fig. 7). At week 5, only one unassigned AC was enriched during drought and one unassigned AC was depleted during drought. The majority of ACs were unassigned using the dom2bgc pipeline among the differentially abundant ACs at week 4. However, we identified eight ACs taxonomically assigned to *Streptomyces*, one *Rhodococcus*, one *Lentzea*, and two *Sacchaotrix* among the ACs enriched in the drought stressed plants at week 4. Contrastingly, we found seven *Pseudomonas* assigned ACs, one *Mycolibacterium* and two *Pseudoxanthamonas* that were depleted during drought (Fig. 8). They were functionally annotated with a blastp search against the MiBig database, and we identified genes encoding for bacillibactin, peuchelin and potashchelin as ACs with highest prevalence among the differentially abundant ACs in the drought-stressed rhizoplane samples (Table S5). These compounds are siderophores taxonomically assigned to *Bacillus*, *Streptomyces* and *Halomonas*. Conversely, *Pseudomonas* genes coding for siderophores, such as pyoverdine, and histicocorrugatin decreased in relative abundance during drought (Table S5). To complement this analysis, we identified core ACs that were only present in drought-stressed or control plants (unique ACs). Here, we identified that the drought-stressed plants hosted a higher number of unique ACs (10) compared to the control/watered plants (3 unique ACs) at the end of the drought period (week 4) (Table 1). Cooperating the results from the differential abundance test, coechelin encoding NRPS gene was also among the unique ACs in the drought-stressed plants at week 4. Coechelin is a siderophore encoded by *Streptomyces*. Together, this shows that siderophores produced by *Streptomyces*, *Bacillus* and *Halimonas* are enriched during drought while *Pseudomonas* produced siderophores are depleted. This demonstrates that iron scavenging strategies are important for success in root colonization during drought.

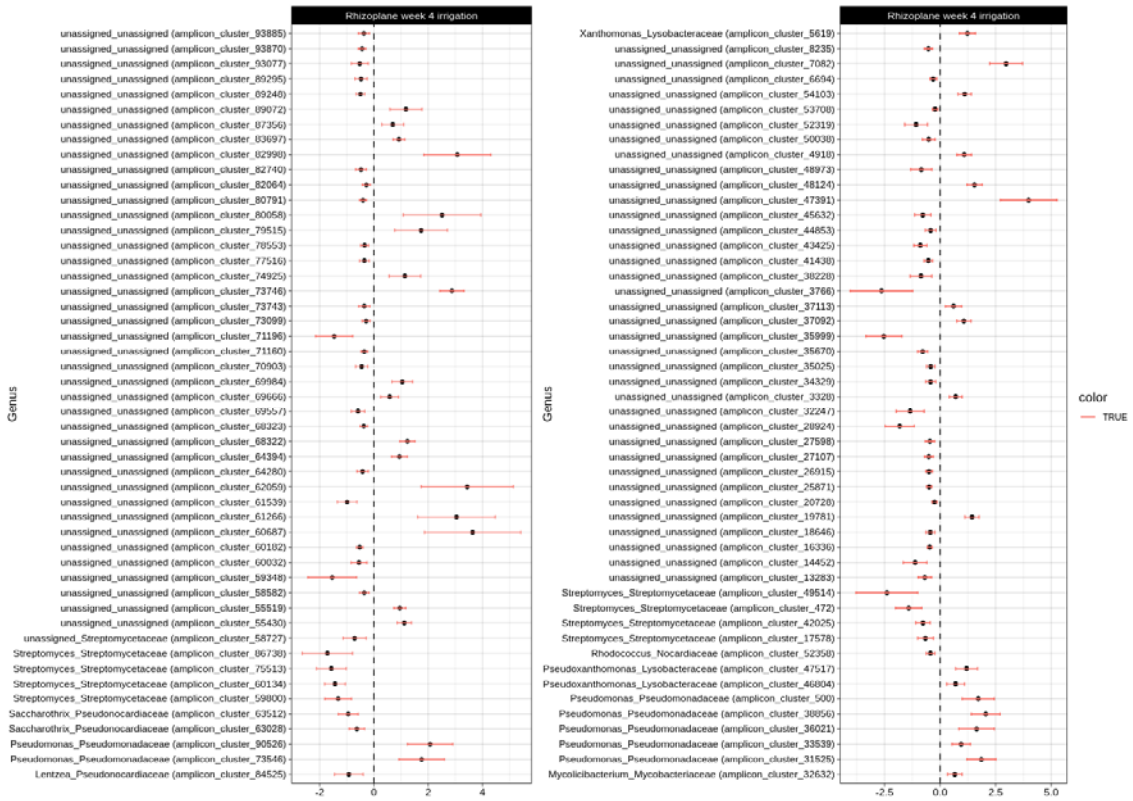

Figure 7. Differential abundance test (corncob, FDR < 0.05), right side is the ACs depleted during drought, and left side is the ACs enriched during drought.

Table 1. MiBig annotation of core ACs unique to either the drought stressed or control plants at week.

Drought	Control
Scytocyclamide	Myxothizaol
Unknown	Thiocoraline
Coelichelin	Necroxime
Aurantimycin	
Polyoxypeptin	
Ohmyungsamycin	
Pepticinnamin	
Myxochromide	
Althiomycin	
Rotihibin	

The CLP viscosin produced by Pseudomonas enhances root colonization under drought stress

NRPs previously reported to play key roles in root colonization are cyclic lipopeptides produced by *Bacillus* and *Pseudomonas* (Bais *et al*., 2004; Tran *et al.*, 2007; Guan *et al.*, 2024). However, to our knowledge this has only been demonstrated during well-watered normal conditions. To determine whether CLPs provide an advantage in root colonization under drought stress we searched the NRPS amplicons for CLPs produced by *Pseudomonas* (Zhou *et al.*, 2024).

Unsurprisingly, *Pseudomonas* comprised the highest proportion of the identified CLPs with a taxonomic assignment in the rhizoplane at all sampling times and water treatments (Fig. S8). The control plants harboured 2.5 times higher proportion of *Pseudomonas* CLP-related ACs than drought stressed plants at 4 weeks, while this difference was reduced to 0.5 times higher at week 5. This suggests that on a general note, CLPs are not selected for under drought stress. As we previously showed that the potential to synthesize viscosin provides an advantage in root colonization for the *Pseudomonas* SBW25, we were particularly interested in the NRPS ACs coding for this compound. Viscosin was, together with viscosinamide, one of the most relative abundant *Pseudomonas* CLPs found across all samples in the rhizoplane (Fig. 8) and the most abundant at week 2. While viscosin coding ACs proportion of all ACs were comparable between treatments at week 4, viscosin coding ACs comprised 10% (0.048/0.47) of the *Pseudomonas* CLP-encoding ACs in the control plants and 22% (0.044/0.20) in the drought stressed plants. This difference was even more noticeable after 5 weeks, where the viscosin coding ACs comprised 26% (0.045/0.18) of the *Pseudomonas* CLP encoding ACs in the drought-stressed plants compared to 5.6% (0.15/0.27) of these CLPs in the control plants. The increased proportion of viscosin-related ACs in drought stressed plants indicates that *Pseudomonas* harbouring the viscosin biosynthetic gene cluster colonize the rhizoplane better than other CLP producing pseudomonads. Thus, we hypothesized that the potential to produce viscosin would increase a strains' root colonization during drought.

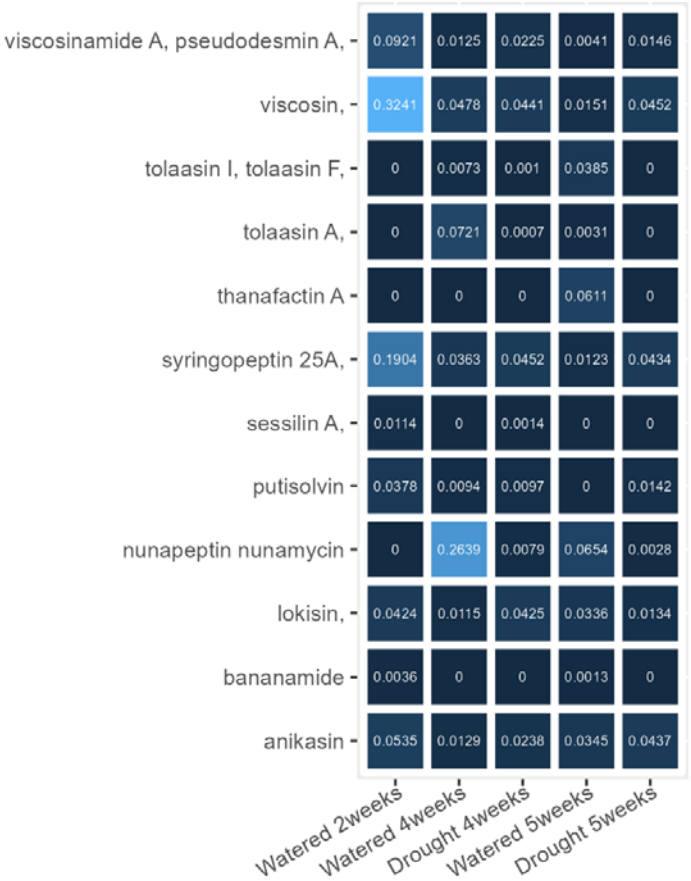

Figure 8. Proportion of reads assigned to *Pseudomonas* CLPs out of the total number of reads in the NRPS amplicon data. Values are mean (n=5).

To test this hypothesis, we inoculated surface-sterilised seedlings with a viscosin producing strain *P. fluorescens* SBW25 (WT) and a *viscA* deficient mutant (*ΔviscA*) unable to synthesize viscosin and grew the plants as described above. We have previously shown that the WT colonizes wheat roots better under well-watered conditions (i.e. control plants) using both qPCR, CFU counts and microscopy (Guan *et al.*, 2024). Both WT and Δ*viscA* were chromosomally tagged with mCherry, which we targeted with qPCR. In both control and drought stressed plants, we found a higher abundance of mCherry genes per gram of rhizoplane soil in the WT inoculated plants compared with plants inoculated with the *ΔviscA* mutant after 4 weeks in drought-stressed and control plants, respectively (Fig. 9). The number of mCherry genes decreased from week 4 to week 5, but the WT inoculated plants still showed higher colonization compared to the *ΔviscA* in drought stressed plants. At both sampling points, the mCherry abundances suggest a higher colonization of the WT in the drought stressed plants compared to the control plants. Overall, this experiment supports the hypothesis that the ability to produce viscosin provides an advantage in root colonization

during drought, and consolidate the general importance of viscosin for plant root colonization. Furthermore, this ability provides an advantage even after watering has been resumed. Additionally, we conclude that not all CLPs results in an improved colonization during drought.

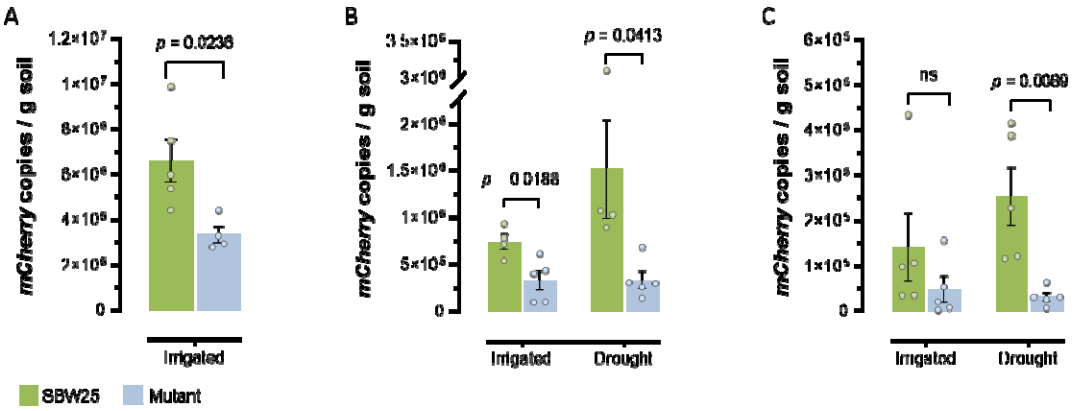

Figure 9. Absolute abundances of mCherry gene copies in the rhizoplane, at week 2(A), week 4 (B) and week 5(C). Each point represents a sample. The height of the bars represents the mean value . Error bars indicate SE based on five biological replicates, and each point represents a sample. Statistically significant values ($P < 0.05$, T-test) were shown (ns = no statistical difference).

Discussion

Bacterial produced NRPs play important roles in plant-microbe interactions (Jacoby *et al.*, 2021). So far, research on their roles have primarily focused on their growth promoting activities or their biocontrol activity (Zhou *et al.*, 2024). However, their role in root colonization and specifically during drought have received limited attention, and to our knowledge this is the first study directly targeting NRPS genes in the rhizoplane during drought.

Overall, we found a minor difference in NRPS composition between the rhizoplane and rhizosphere of winter wheat, although this difference did not result in a change in the diversity. Hence, the proximity to the roots is less important for the NRPS composition than for the bacterial community (Guan *et al.*, 2024). For both compartments, the NRPS composition changed over time. This has also been shown in the rhizosphere of potato (Aleti *et al.*, 2017) and reflects the change in overall community composition and changes in root exudates which attracts bacteria. Still, the change in NRPS composition also indicates that the

genes required for root colonization changes over time, either due to environmental changes or differences in community.

A 2-week drought period changed the bacterial community, both at the taxonomic level (16S rRNA gene) and the functional (NRPS, A-domain). Yet, the alpha diversity and richness of the NRPS genes did not differ between treatments. Most of these NRPS-genes were unannotated, as also demonstrated in other studies applying NRPS or PKs amplicon sequencing in environments such as soil and rhizosphere (Dror *et al.*, 2020; Tracanna *et al.*, 2021). Due to the large proportion of unassigned ACs in the dataset using the dom2bgc pipeline, we relaxed the cut-off criteria using blastp against the Mibig database. Through this we identified two groups of NRPS related gene groups that were affected by drought.

The first group of NRPS genes identified following analysis using the relaxed cut-off criteria were those encoding iron-scavenging siderophores. *Pseudomonas*-encoded pyoverdines decreased during drought, whereas *Streptomyces* encoded coechelin, but also siderophores encoded by *Halimonas* and *Bacillus* increased as a result of the drought. The latter coincided with an increase in relative abundance of *Streptomyces* in the 16S rRNA amplicons. A longer drought period increases the aerobic conditions in the soil, which in turn oxidises the iron to form insoluble iron oxides (Bouskill *et al.*, 2013), reducing the availability for plants and microorganisms. The decrease of *Pseudomonas* genes encoding for siderophores then suggests that they are not able to dissolve these insoluble iron oxides, whereas the other groups might. In addition, *Pseudomonas* can scavenge iron from phytosiderophores (Jurkevitch *et al.*, 1993), which are reduced by plants during drought. This has led Xu et al. (2021) to propose that the reduction of plant phytosidereophores during drought favours *Streptomyces* (or Actinobacteria in general) (Xu *et al.*, 2021). This might be at play around the roots of wheat as well during drought indicating that this is a common phenomenon. While their work was performed using shotgun metagenomics, we here demonstrate the potential of using NRPS amplicons for inferences of the ecology in the root zone. Furthermore, this suggests that a relaxed cut-off criteria during blastp was reasonable.

The second group of NRPS genes were CLPs, a group of metabolites important in plant-microbe interactions are those encoding CLPs. Species within *Pseudomonas* are known to produce a vast collection of these compounds, as they have a role in biofilm dispersal, as antimicrobials and in root colonization (Zhou *et al.*, 2024). Here, our analyses of the NRPS

indicated that the ability to produce the CLP viscosin is an important trait for root colonization of wheat by *Pseudomonas* during drought. This was supported by the higher abundance of *P. fluorescens* SBW25 in the rhizoplane during and after exposure to drought compared to the *ΔviscA*. In this work, we determined the abundance using qPCR targeting the mCherry gene, but in a recent study, we demonstrated that this detection method was supported by CFU counts and microscopy images (Guan *et al.*, 2024). It has been proposed that CLPs also function as wetting agents capable of enhancing nutrient solubility and that of hydrophobic substrates. For example, surface-active *P. fluorescens* and *P. putida* strains can increase leaf wetness while syringafactins from the plant pathogen *P. syringae* can attract water vapor, thereby alleviating water stress on dry leaves. Thus, we speculate that *P. fluorescens* SBW25 uses viscosin as a wetting agent to increase nutrient availability. Whether this is also beneficial to the plant needs to be determined.

On a broader level the composition of 16S rRNA and NRPS amplicons converged one week after stopping the drought period. As plants relatively quickly changes its root exudates, this support the "cry for help" hypothesis (Mendes *et al.*, 2013) as it is now called off and the normal microbiome can once again assemble. However, at a finer resolution, we still saw a "legacy" effect of the viscosin encoding ACs which were relatively more abundant one week after stopping the drought.

As our data revealed, a lot of ACs were unassigned underpinning the need for more work in this area to improve the databases. In addition, the primers are biased towards what is already known from cultures, reflected in the low diversity detected at the phylum level using the dom2bgc pipeline. However, an increase in annotated ACs will not reveal the role of the compounds in plant-microbe interactions. Despite this inherent limitation, our study demonstrate the potential of amplicon sequencing of NRPS genes to reveal specific genes involved in root colonization and in relation to drought stress which can be supported with experimental work.

Conclusion

Overall, our study shows that the root microbiome comprises a wide range of NRPS-genes that change in composition during plant growth and when plants are exposed to drought. Specifically, the differences in siderophore encoding genes highlights the importance of zooming in on specific molecules to provide a detailed understanding of the ecosystem.

Furthermore, we demonstrated the potential of using NRPS amplicons to making biological inferences, followed up by experimental work confirming the results. This allowed us to show that the ability to synthesize viscosin provides an advantage in root colonization, not only in normal conditions but also during drought. Further expanding our repositories of NRPS containing genes will leverage the use of these amplicons to resolve important ecological questions on the role of NRPs in shaping root colonization patterns and mediating plant-microbe interactions in the rhizosphere.

Materials and Methods

Plant materials and growth conditions

The winter wheat cultivar Sheriff (Sejet Plant Breeding, Horsens, Denmark) seeds were used in this study. Initially, the seeds were submerged in sterile Milli-Q water for an hour and subsequently relocated to Petri dishes with dual layers of sterile filter paper, saturated with 5 ml of sterile Milli-Q water for germination. For optimal germination, the seeds were kept in the dark at room temperature for three days. Following this, the three-day-old germinated seedlings were root-dipped in sterile saline solution (0.9% NaCl) and transplanted into polyvinyl chloride (PVC) pots (24 cm in height and 7 cm in diameter) with drainage holes, with each pot accommodating one plant. The pots were filled with approximately 1000g of non-sterile field soil premixed with sterile sand (DANSAND, Brædstrup, Denmark) at a 3:2 ratio ("soil" from here on). The field soil was gathered from the plough layer (0 to 25 cm) at the University of Copenhagen's experimental farm in Taastrup, Denmark (55° 40′N, 12° 17′E).

The water content of the soil was maintained at 17% (w/w). Following the transplantation of the germinated seeds into the pots, the soil was supplemented with a nutrient solution Nitrogen – Phosphorous – Potassium (NPK 3-1-4) (Drivhusgødning, Park®). Each pot received 0.83 ml of the nutrient solution, comprising 2.04% nitrate nitrogen (N), 0.3% ammonium nitrogen (N), 0.46% amide nitrogen (N), 0.69% phosphorus (P), 4.38% potassium (K), 0.08% sulfur (S), 0.06% magnesium (Mg), 0.033% iron (Fe), 0.013% manganese (Mn), 0.002% copper (Cu), 0.002% zinc (Zn), 0.0006% molybdenum (Mo), and 0.004% boron (B). Subsequently, the pots were positioned in the growth chamber that was set at 600 μmol m^{-2} s^{-1} light intensity, 60% humidity, a 16/8 h light/dark photoperiod, and a temperature of 19°C during the day and 15°C at night.

Plant experiments under drought and well-watered

Twenty five plants were randomly divided into two distinct watering regimes: 10 plants for drought stress treatment and 15 for the well-watered control ("control" from here on). Sample collection took place at 14, 28, and 35 days post sowing, covering a pre-drought, a drought, and a post-drought time interval. The experimental timeline is presented in Figure S1. This experimental setup resulted in five biological replicates for each treatment and sampling time point. All plants were cultivated under well-watered conditions for 14 days, after which the drought group underwent a drought treatment for an additional 14 days by withholding water. During non-drought periods and for well-watered plants, distilled water was administered every other day to maintain a soil moisture content of 17% throughout the experiment. For plants subjected to drought stress, water was reintroduced to the PVC pots at the end of the drought period (28 dpi) to facilitate plant recovery.

Sampling of rhizosphere and rhizoplane-associated microbial communities

Root sample collection and compartment processing were performed as described by (Guan *et al.*, 2024) to obtain rhizosphere (soil adhering to the root; exclusively used for confirmation of proper sampling of the compartments as tested by 16S rRNA amplicon sequencing (see below)) and rhizoplane (the root surface) associated microorganisms. Samples for DNA extraction were immediately flash frozen in liquid nitrogen and kept on dry ice until storing at -80°C. Samples were freeze-dried (CoolSafe 100-9 Pro freeze dryer, LaboGene, Lynge, Denmark). All freeze-dried samples were stored at -20°C until DNA extraction. Root and shoot length were recorded at sampling. Subsequently, roots and shoots were dried at 60°C for 3 days before determining the dry weight.

DNA extraction

Genomic DNA was extracted from 500 mg of either rhizosphere or rhizoplane samples using the FastPrep-24TM 5G bead-beating system (MP Biomedicals, Irvine, CA, USA) set at 6.0 m/s for 40 seconds and the FastDNA^{TM} SPIN Kit for soil (MP Biomedicals), strictly following the manufacturer's instructions. The DNA extracted from all samples was eluted in 60 µl elution buffer. The DNA's purity and concentration were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). All DNA samples were stored at −20°C for subsequent quantitative PCR analysis and preparation of amplicon libraries.

Chlorophyll quantification

The chlorophyll content of leaves of both well-watered and drought-stressed plants was evaluated at 28 dpi using a modified version of Liang's method (Liang *et al.*, 2017). For the assessment, an exact weight of 0.1 g of leaf tissue was obtained, followed by finely chopping the tissue and immersing it in 10 ml of 80% acetone. These samples were then stored in darkness for 48 hours and subsequently centrifuged at a speed of 14,000 g for 5 minutes to achieve a clear mixture. The chlorophyll content was then calculated employing Arnon's classic equations (Arnon, 1949) with absorbance values measured at 663 nm and 645 nm. The equations used are as follows:

Total chlorophyll (mg/L) = 20.21 (A645) + 8.02 (A663)

Chlorophyll content (mg/g) = C×V/A×1000

Where:

C: chlorophyll concentration in mg/L

V: total volume of extraction solution in ml

A: fresh weight of the sample in g

Determination of Peroxidase (POD) and Superoxide Dismutase (SOD) activities in wheat leaves

SOD and POD activities were measured at 28 dpi, following a slightly modified version of the method previously described (Prochazkova *et al.*, 2001). An exact weight of 0.1 g of leaf material was ground into a homogenous suspension with 2 ml of cooled extraction medium (phosphate buffer with 1% polyvinylpyrrolidone, pH 7.8) in a chilled mortar. The extraction medium was used to rinse the mortar, bringing the final volume to 10 ml. After centrifuging the mixture at 4°C, 10000 rpm for 15 minutes, the resulting supernatant was utilized as the enzyme source. **SOD activity** was estimated by recording the reduction in the optical density of the nitro-blue tetrazolium (NBT) dye catalyzed by the enzyme. Three milliliters of the reaction mixture, comprising 130 mM methionine, 0.75 mM nitroblue tetrazolium chloride, 0.1 mM EDTA-Na_2, 50 mM phosphate buffer (pH 7.8), and 0.1 ml enzyme, was initiated by adding 20 μM riboflavine and placing the tubes under fluorescent lamps for 25 minutes. The reaction was halted by switching off the light and storing the tubes in darkness. A complete reaction mixture without the enzyme, which yielded the maximum color, was used as a

control, and a non-irradiated complete reaction mixture served as a blank. The optical density was documented at 560 nm, and a single unit of enzyme activity was considered as the quantity of enzyme that diminished the absorbance value by half relative to test tubes devoid of the enzyme. **POD activity** was assessed based on the increase in optical density resulting from the formation of tetra-guaiacol. The 2.5 ml reaction mixture contained 3.4 mM guaiacol, 18 mM H_2O_2, 0.2 M phosphate buffer (pH 6.0), and 25 μl enzyme. The optical density resulting from the generation of tetra-guaiacol was measured at 470 nm, and a single enzymatic unit (u) was characterized as a 0.01 increment in the absorbance measurement per minute.

Strains and growth conditions

The viscosin-producing model strain *P. fluorescens* SBW25, along with its mutant strain that is deficient in viscosin production, were labelled using mCherry in a previous study (Guan *et al.*, 2024). These strains were recovered from frozen glycerol stocks and cultured on Luria Broth Agar (LBA) plates, which consist of 1% tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% agar, all supplemented with 10 μg ml^{-1} gentamicin. The plates were then incubated at 28°C for 48 h. Subsequently, single colonies were transferred into 20 ml liquid LB culture tubes with 10 μg ml^{-1} gentamicin supplementation and incubated at 28°C with continuous shaking at 180 rpm in an orbital shaker.

Table 2. Primers used in the study.

Primers	Sequence	Reference
NRPS A3	5'-GCSTACSYSATSTACACSTCSGG -3'	(Ayuso-Sacido and Genilloud, 2005)
NRPS A7R	5'-SASGTCVCCSGTSCGGTAS -3'	(Ayuso-Sacido and Genilloud, 2005)
799F	5'-AACMGGATTAGATACCCKG-3'	(Chelius and Triplett, 2001)
1193R	5'-ACGTCATCCCCACCTTCC-3'	(Bulgarelli *et al.*, 2012)
mCherry-Fw	5'-GCCCCGTAATGCAGAAGAAG-3'	(Guan *et al.*, 2024)
mCherry-Rv	5'-GTGTAGTCCTCGTTGTGGGA-3'	(Guan *et al.*, 2024)

Preparation of bacterial suspension and inoculation

The bacterial cells from the overnight culture were obtained by centrifuging at 6000×g for five minutes to yield cell pellets. These pellets were subsequently washed twice and resuspended in 0.9% sterile NaCl. The optical density (OD) at 600 nm for each inoculum was standardized to 1.0, equivalent to approximately 5×10^8 CFU ml^{-1}, as determined by serial

dilutions with plate counts. Three-day-old germinated seedlings were removed from the Petri dishes and the roots were immersed in either the *P. fluorescens* SBW25 mCherry suspension (SBW25) or the *P. fluorescens* SBW25Δ*viscA* mCherry suspension (Mutant) for two hours. In the SBW25 inoculation experiment three-day-old seedlings were inoculated with SBW25, or Mutant. Subsequent to the inoculation, all plants were cultivated as detailed above.

Quantitative PCR analysis

The quantification of SBW25 and Mutant was executed using qPCR with the AriaMx Real-Time PCR system (Agilent Technologies, Santa Clara, CA, USA). The specific primers utilized are listed in Table 2. Each twenty-microliter reaction mixtures comprised 10 µl Brilliant III Ultra-Fast SYBR® Green Low ROX qPCR Master Mix (Agilent Technologies), 1 µl of bovine serum albumin (20 mg/ml) (New England Biolabs® Inc., Ipswich, MA, USA), 0.4 µl each primer (10 µM), 6.2 µl sigma water, and 2 µl of template DNA. The thermal cycling protocol consisted of an initial DNA polymerase activation at 95°C for 3 minutes, succeeded by 40 cycles of 95°C for 20 seconds and 56°C for 30 seconds. Upon completion of the qPCR procedure, a dissociation curve was generated to confirm the specific melting temperature of the amplified product. This was accomplished by including a series of steps: 1 minute at 95°C, 30 second at 55°C, and a 30-second ramp from 55°C to 95°C. The absolute abundance of the target *mCherry* gene was subsequently calculated based on a standard curve, derived from a ten-fold serial dilution of DNA from SBW25 mCherry cells. This standard curve exhibited a dynamic range spanning a ten-fold dilution series from 10^2 to 10^8 copies μl^{-1}, with three technical replicates for each dilution. In addition, the qPCR setup included three technical replicates of a non-template (nuclease-free water) negative control.

NRPS A domain and 16S rRNA amplicon sequencing

Primers A3F and A7R, detailed in Table 2, were used in a PCR reaction to amplify the NRPS A domains (Ayuso-Sacido and Genilloud, 2005). A two-step PCR and a dual-indexing approach were deployed for Illumina MiSeq sequencing. The PCR amplicons were produced using 15 µl of Platinum II Hot-start PCR Master Mix (Thermo Fisher Scientific), 0.6 µl of forward and reverse primers each (10 µM), 8.8 µl of nuclease-free water, and 5 µl of template. The PCR thermocycler protocol encompassed an initial denaturation phase at 95°C for 2 minutes, succeeded by 33 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds, with a final elongation step at 72°C for 5 minutes. The PCR amplification

was verified via 1.5% agarose gels with loading buffer, and the PCR products were subsequently purified using AMPure XP beads (Beckman Coulter Inc. Brea, CA, USA). The purified DNA concentration was reassessed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and a Qubit 2.0 fluorometer using the High-Sensitivity DNA assay (Thermo Fisher Scientific). Finally, the subsequent library construction and Illumina MiSeq sequencing (2×300 bp) were undertaken by the Erasmus Center for Biomics (Rotterdam, Netherlands).

The V5-V7 region of the bacterial 16S rRNA gene was amplified using primers 799F (Chelius and Triplett, 2001) and 1193R (Bulgarelli *et al.*, 2012) (Table 2). Notably, this primer pair results in minimal amplification of plant mitochondria and chloroplast DNA (Beckers *et al.*, 2016). The ZymoBIOMICS Microbial Community DNA Standard (Zymo Research, Irvine, CA, USA), pure culture SBW25 DNA, and water negative controls were also incorporated. A two-step dual-indexing approach was deployed for Illumina MiSeq sequencing. The PCR amplicons were generated with a mix of 15 µl Platinum II Hot-start PCR Master Mix (Thermo Fisher Scientific), 0.6 µl forward and reverse primers each (10 µM), 8.8 µl of nuclease-free water, and 5 µl of template DNA. The PCR thermocycler protocol consisted of an initial denaturation phase at 95°C for 2 minutes, followed by 33 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds, ending with a final elongation step at 72°C for 5 minutes. The PCR amplification was verified through 1.5% agarose gels with loading buffer, and the PCR products were subsequently purified using AMPure XP beads (Beckman Coulter). The purified DNA concentration was reassessed with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and a Qubit 2.0 fluorometer using the High-Sensitivity DNA assay (Thermo Fisher Scientific). Finally, the subsequent library construction and Illumina MiSeq sequencing (2×300 bp) were undertaken by Eurofins Genomics (Ebersberg, Germany).

Sequence processing

The raw amplicon sequence reads were processed using the DADA2 pipeline (version 1.30) (Callahan *et al.*, 2016). Aside from a few parameter alterations, all other settings were retained as default. Specifically, for 16S rRNA gene reads, based on sequence quality, filtering was applied using default parameters, except for ‘trimLeft’ and ‘truncLen’. Primers were removed using ‘trimLeft’ = 19/18, depending on primer length, and reads were

truncated using 'truncLen'=280/200 for the forward and the reverse reads, respectively avoid poor quality and ambiguous sequences. Chimeras were removed after merging denoised pair-end sequences. Each unique 16S rRNA amplicon sequence variant (ASV) was classified according to the SILVA database (version 138.1) (Quast *et al.*, 2013). Non-bacterial ASVs, including chloroplasts and mitochondrial reads, were filtered out, and the mock community and negative controls were checked for contamination. For NRPS A domains reads, solely the forward reads were employed, as recommended in (Tracanna *et al.*, 2021). Reads were truncated at 250 nt, and quality filtered using maxEE = 7. NRPS A domain reads were annotated using a modified version of the dom2bgc pipeline (Tracanna *et al.*, 2021). In brief, forward NRPS reads were translated into six frames and aligned the reads. Translated protein sequences were clustered based on Euclidean distances (Tracanna *et al.*, 2021) and annotated using the most recent versions of MiBig (v. 3.1) (Terlouw *et al.*, 2023) and antiSmash databases v.7.0.0 (Blin *et al.*, 2023).

In addition to the annotation performed using the dom2bgc pipeline, we performed a BlastP search of the NRPS ACs against the most recent MiBig database v.3.1 (Terlouw *et al.*, 2023) with a threshold of the E value (10^{-20}).

Data analysis and statistics

Statistical analysis was performed in R version 4.3 (R Core Team, 2020). For microbiome diversity and composition analyses, we used the R packages phyloseq version 1.46 (McMurdie and Holmes, 2013) and ampvis2 version 2.8.9 (Andersen *et al.*, 2018). The amp_rarecurve function in the ampvis2 package was used to generate rarefaction curves for each sample. For determination of alpha diversity, richness, beta diversity and unique NRPS ACs, data was rarefied to an even depth using the mean values of 100 iterations. The alpha diversity and richness for the 16S rRNA and NRPS amplicons were determined using the Shannon diversity and CHAO1 indices with phyloseq_estimate_richness. Significance testing of the Shannon diversity was done with a pairwise Wilcoxon-MannWhitney test with Bonferonni correction if necessary. For the NRPS A domain amplicons, samples I23.1095.D02 and I23.1089.E04 were excluded from the analysis after rarefying.

A Principle Coordinates Analysis (PcoA) was performed using ordinate() in phyloseq (based on vegdist() from vegan) with rarefied data and Bray-Curtis dissimilarity matrix. Permutational Multivariate Analysis of Variance (PERMANOVA) based on Bray-Curtis dissimilarity matrix was used to test the effects of different factors (Sampling time, watering

treatment, plant compartment) and their interactions on the beta diversity of communities in vegan version 2.6.6.1 (Oksanen *et al.*, 2020). Dissimilarity matrices for the rhizoplane of 16S rRNA amplicons and NRPS amplicons were compared with mantel test.

The differential abundance of ASVs between groups was determined using beta-binomial regression with the corncob package version 0.4.1 (Martin *et al.*, 2020). Only ASVs 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.

We searched for ACs unique to either the drought stressed or control plants among the core ACs in the rhizoplane. Here, we defined core ACs to be present in at least four out five replicates. The core ACs of one treatment were then compared to all ACs from the other to determine unique core ACs.

Data availability

All raw sequencing data used in this study had been deposited in the NCBI Sequence Read Archive (SRA) database under accession codes PRJNA987606 (16S rRNA) and PRJNA987694 (AD).

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Conflict of Interest

The authors declare no competing interests.

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