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Persistence Control
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Q3 Target: Provide a summary of methods used to characterize and test modified microorganisms for secure use.

Executive Summary

As PNNL's contribution to the Secure Biosystems Design program, the Persistence Control (PerCon) Science Focus Area (SFA) aims to use high-throughput genetic and bioinformatic tools to understand, predict, and control plant-microbe interactions in the rhizosphere to benefit the bioenergy crop sorghum. Specifically, we investigate how microbes survive in the rhizosphere to engineer metabolic dependence on root exudate compounds for bacterial isolates native to the sorghum rhizosphere. These metabolic addiction phenotypes will promote growth and activity in the plant rhizosphere and prevent proliferation away from the plant. Pursuing the secure biocontainment aims of our research will position the US to responsibly harness the full potential of rhizosphere microbiomes to support ecosystems that fulfill national goals for a resilient bioeconomy through sustainable bioenergy cropping systems with potential to durably deposit atmospheric carbon in soils.

In the inaugural funding cycle of the SFA, we have advanced methods to characterize and test modified microorganisms for secure use by establishing persistence control testbeds using synthetic microbial communities derived from sorghum-associated soil. These advances summarized as follows will advance the testing of secure biosystems:

1. We naturally evolved a synthetic microbial community that grows on a synthetic sorghum exudate medium and supports the colonization of an engineered non-native plant-growth promoting bacterium.

2. We established a defined synthetic microbial community that promotes the growth of sorghum in laboratory and field settings [1].

Background

The potential to employ emerging genetic and computational tools to design and securely deploy synthetic rhizosphere microbiomes, defined communities of microbes that colonize plant roots and benefit plant growth, offers tremendous opportunity to realize highly productive and stress-tolerant biomass cropping systems. A critical obstacle to realizing this vision is understanding fundamental principles of microbiome persistence in complex environments, notably amid the dynamics of root exudation and microbial colonization. Further, we need predictive tools to assess the risks associated with the deployment or unintended release of engineered microbes in plant and soil ecosystems and to mitigate those risks through effective strategies to control persistence. To meet these challenges, high-throughput genetic manipulation and bioinformatic tools provide a platform to elucidate the genetic elements underpinning rhizosphere community function, to create beneficial communities, and to control their environmental persistence.

The Persistence Control SFA investigates approaches to reshape the environmental niche of native sorghum rhizosphere bacterial isolates by creating an engineered metabolic addiction to root exudate compounds. We aim to understand the genes and networks that underpin environmental persistence of microbiomes to create dependencies of engineered microbes on plants such that the microbes can no longer persist in the environment in the absence of the target plant (Figure 1). Our strategy to control the environmental persistence of engineered microbes is to establish metabolic addiction to plant root exudate compounds by installing compound-specific catabolic pathways while removing pathways used for scavenging nutrients in soil microbiome environments. This strategy requires exquisite control of genome content to, for instance, eliminate metabolic niches that allow survival cross-feeding of nutrients by other microbiome members as well as to introduce complete pathways to thrive in the plant rhizosphere, but not in the rhizosphere of other plants. It also demands a deep understanding of plant or microbe interactions that could overcome control through complementation.

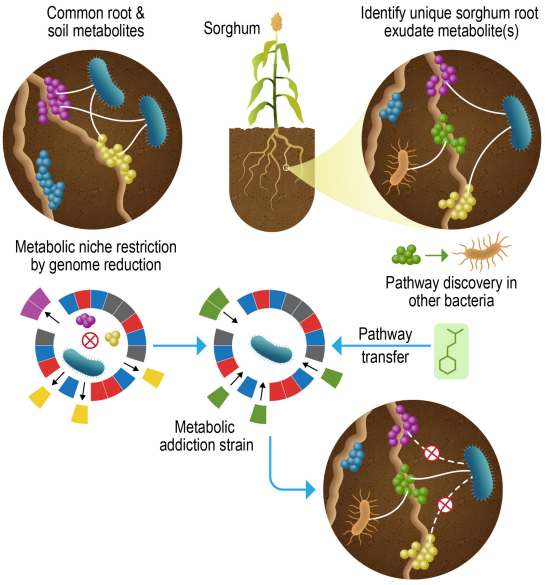


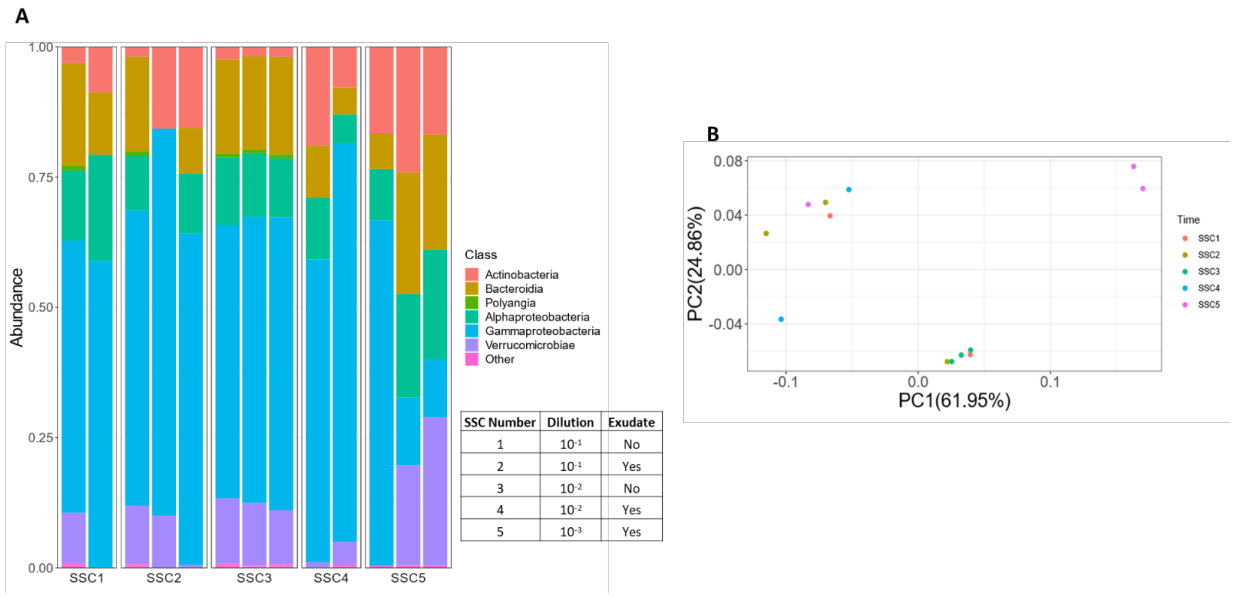
Figure 1. The Persistence Control SFA aims to control the proliferation of engineered microbes by establishing metabolic addiction of microbe to a bioenergy crop supported by the engineered microbiome.

To help accomplish the goals of the Secure Biosystems Design program, the PerCon SFA team draws expertise from across three DOE Office of Science National Laboratories – Pacific Northwest National Laboratory, Lawrence Berkeley National Laboratory, and Oak Ridge National Laboratory – and three Universities: the University of California Berkely, the University of California Santa Barbara, and the University of Washington. To understand the genes and networks that control environmental fitness we have developed and integrated research strategies that draw from our team's expertise in functional genomics, synthetic biology, microbial ecology, chemical biology, bioinformatics, machine learning, and plant-microbe interactions. In this report, we share a selection of our approaches for persistence control to securely design new functions into organisms for bioenergy and bioeconomy applications.

Selective colonization of engineered bacteria in synthetic microbial consortia

Understanding how cell-cell interactions govern the colonization dynamics of an introduced species in the rhizosphere is a monumental challenge due to the complexity of soil microbiomes. One effective approach to investigate these dynamics is to reduce community complexity by developing simple, experimentally tractable microbial communities [2, 3]. Reduced-complexity communities are amenable to multi-omic analyses and interpretation due to their simplicity, and they retain taxonomic and functional representation of the native community because they are naturally evolved from the complete soil microbiome. Here we describe the development and the colonization potential of a set of reduced-complexity communities derived from a sorghum field site. We term these consortia Synthetic Sorghum Communities (SSCs). We developed five distinct SSCs and assess for each SSC the colonization potential of an introduced model microbial species, *Pseudomonas fluorescens* SBW25. We found SBW25 stably colonized only one of five SSCs sourced from the same soil and we identified taxonomic drivers of community inclusion or rejection. Our results provide new model soil communities to investigate microbial interaction dynamics and begin to reveal factors that drive colonization efficiency, while contributing to a growing knowledgebase aiming to develop, store, and analyze reduced-complexity soil communities.

To establish SSCs, we cultivated and passaged soil samples from a sorghum field site on nutrient agar. In order to emulate a rhizosphere-like environment in a simple cultivation system, we created a synthetic sorghum medium (SSM) that is comprised of metabolites detected in metabolomics data collected from field sorghum plants. The medium uses a MOPS Media Enhanced (MME) buffer and contains trace minerals and 20 readily procured carbon and nitrogen compounds, including sugars, sugar alcohols, amino acids, organic acids, and osmolites.



SSC Number	Dilution	Exudate
1	10^{-1}	No
2	10^{-1}	Yes
3	10^{-2}	No
4	10^{-2}	Yes
5	10^{-3}	Yes

Figure 2. Taxonomic makeup of synthetic sorghum communities (SSC). (A) Relative abundance of each Class at Week 12 for the five SSC. Inset table shows SSC labeled by dilution and presence or absence of plant root exudates. (B) PCA showing variation on SSC community composition.

We cultivated the soil samples over twelve weekly passages, storing each passage and measuring the taxonomic diversity using 16S rRNA profiling. After 1 week of growth the number of detected amplicon sequence variants (ASVs) in the communities was 250-400, independent of multiple soil inoculation densities. Community complexity was also independent of whether the agar plates contained additional sorghum exudates we included in some samples we collected from hydroponic extractions of sorghum. By the third passage, we observed a significant simplification of the measured microbial communities, with ASV counts dropping and eventually stabilizing after twelve passages to between 50 and 140 ASVs, representing five to seven phyla and 27 to 75 genera (**Figure 2**).

Differential analysis of bacterial taxa distributions from Week 1 to Week 12 revealed variable shifts in abundance at the Phylum level. Most of the detected taxa decreased in abundance through passaging. *Actinobacteriota*, *Firmucutes*, *Acidobacteriota* showed the largest decreases in abundance. In contrast, *Verrucomicrobiota* and *Bacteroidota* generally showed increased in abundance with some *Verrucomicrobiota* ASVs showing large increases. The *Proteobacteria* were the largest phyla by ASV counts and showed a more varied response to passaging with some *Proteobacteria* increasing others decreasing. With *Proteobacteria* we also noticed an early response to growth on plates including hydroponic exudates vs. those without. Plates with natural exudates had much higher initial levels of *Proteobacteria* but this difference in plate type was not apparent after 12 weeks of growth.

Reproducible Science through Cold Storage of Sorghum Communities

To determine how SSC can be used to conduct reproducible science, we assessed SSC composition after reconstitution from cold storage. We explored two measures of surviving storage. One measurement was the shift in taxonomic makeup as a result of freezing and replating. All SSC showed taxonomic shifts because of storage and reconstitution suggesting certain species within the communities do not survive a freeze and re-thaw cycle. This varied depending on the method of storage. Generally, SSC stored using lyophilization or DMSO showed less distance from the parent community compared to SSC stored with glycerol (**Figure 3**).

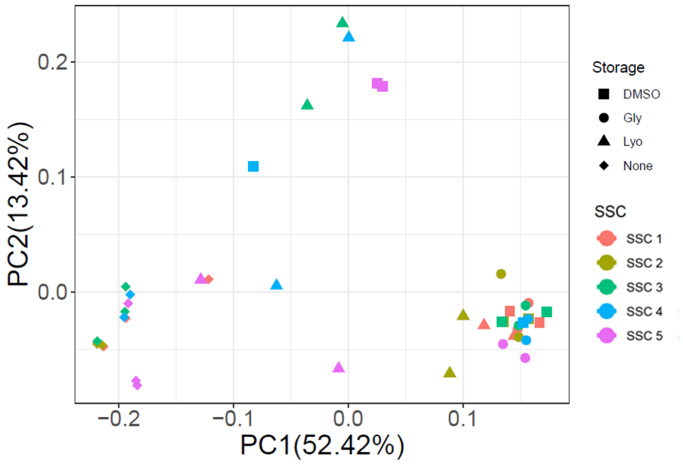


Figure 3. Storage methods of SSC. A PCA plot of two replicates of each SSC is shown. Storage methods are indicated by shapes with "None" indicating the parent SSC before any storage or freeze/thaw cycle.

The second metric examined how different individual reconstitutions of the same SSC stored using the same method were different from one another. That is, what is the reproducibility regarding reconstituting different replicates stocks of SSC? For each method and SSC there were two replicate stocks reconstituted and the distance between them was examined. When

examining this second question we found that both DMSO and glycerol storage methods led to SSC that could be reliably reconstituted (**Figure 3**). However, SSC stored using lyophilization showed a large degree of variability between replicates. A direct comparison of SSC stored via glycerol vs. DMSO suggests that glycerol might be the better storage method, though this is primarily driven by one SSC that showed high variability when stored using DMSO. Not only were replicates stocks of the same SSC similar to each other but stocks stored using glycerol or DMSO were also similar suggesting that the same sets of species in each survived the initial freeze thaw cycle. At this point, several stocks were made of each of the five SSC and stored using all three methods. Because lyophilization showed a large amount of variability in reconstitution and one ESC showed large variation when using DMSO we focused on using glycerol stored stocks for all future work. The success in using glycerol as a storage method for evolved communities was also found in a previous analysis looking at chitin degrading communities derived from soil in Prosser, WA [2].

Colonization of Synthetic Sorghum Communities by an engineered bacterium

The successful promotion of bioenergy crop growth through microbiome engineering will require the successful colonization of engineered microbes into complex communities. We do not have a clear understanding of what drives the colonization dynamics of communities by individual species, though there are many documented cases of metabolic cross-feeding antagonism. Studying colonization in low-complexity communities opens a window of understanding that we intend to apply to persistence control engineering.

To assess colonization dynamics of an introduced species with sorghum-specific communities, we added fluorescently tagged SBW25 [4, 5] to each SSC and observed colonization dynamics on SSM agar medium using flow cytometry and 16S rRNA sequencing. We estimated colonization efficiency by calculating and tracking the ratio of fluorescent cells (SBW25) to non-fluorescent cells (SSC members) (**Figure 4**). The initial SBW25 ratios varied from 0.5% to 10% for all SSCs, but most SSCs suppressed SBW25 growth by the third weekly passage. SBW25 was detected in only SSC1 and SSC2 by the fourth passage, and it was stably maintained in only SSC1 after five weekly passages at a level approximately 1% of the population fraction.

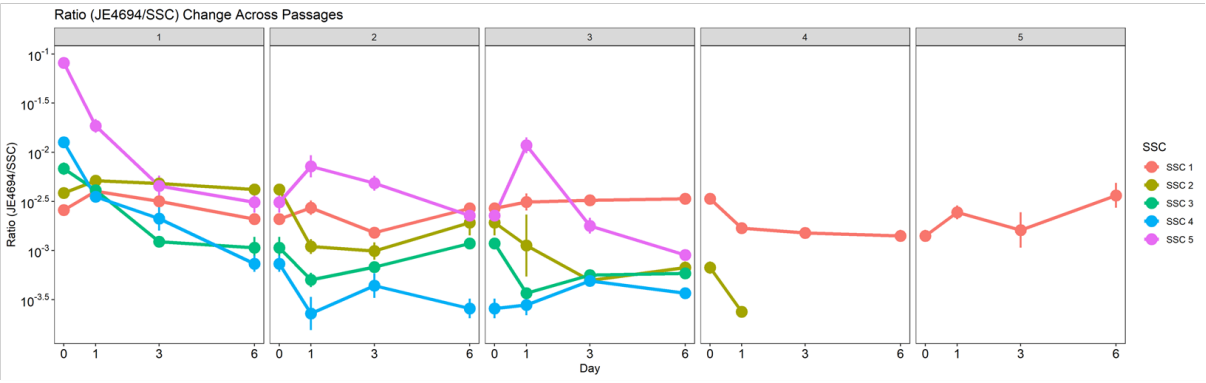


Figure 4. Differential colonization of SSC by SBW25. Ratios of fluorescently tagged SBW25 cells against all cells when SBW25 was cultured with ESC. Each box represents a separate passage (a cycle of plate growth followed by replating) and days when samples were taken within a passage are indicated on the x-axis. Lines indicate levels of SBW25.

To assess the drivers of community colonization, we conducted network inference analyses of 16S rRNA profiles from the engraftment assays. SBW25 showed relatively strong negative or positive correlation to nine separate ASVs (defined as within the top ten ASVs showing co-abundance with SBW25 in at least 3 out of 4 network inference methods used). Two of these ASVs were additional *Pseudomonas* species but the others were taxonomically diverse. One *Pseudomonas* species and one *Klebsiella* species were correlated positively with SBW25 under all four network inference methods (Pearson correlation coefficient, Spearman correlation coefficient, Context Likelihood of Relatedness [6] and GENIE3 [7]).

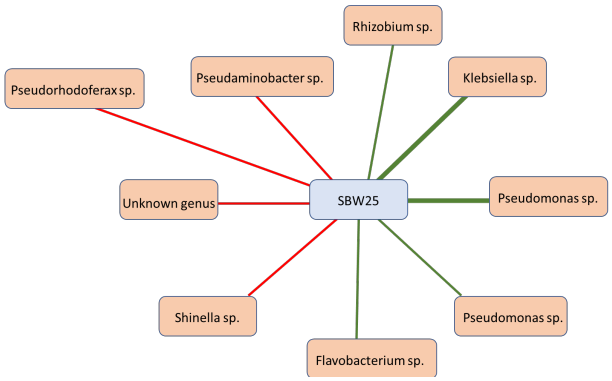


Figure 5. Network analysis of SSC during colonization. Genus-level ASVs that showed the top positive or negative correlations to SBW25 abundance are shown. Green lines indicate positive correlations and red lines negative correlation. The thickness of the lines indicates the strength of the interaction.

Our emerging hypothesis from this analysis is that species shown to be linked to SBW25 have positive of negative interactions with SBW25 that may drive its colonization dynamics in ESC. To explore this further we examined the abundance of these species in SSC that supported colonization of SBW25 (SSC1, SSC2) versus those that did not (SSC3-5). The *Klebsiella*, *Flavobacterium* and one of the *Pseudomonas* sps. (all positively associated with SBW25) were between 2.8 and 3.8-fold higher in ESC that allowed engraftment versus those that did not. The remaining *Pseudomonas* species was present at lower levels but the other species were not even detected in ESC that did not support engraftment. When examining species that were negatively associated with SBW25 we found that the *Pseudaminobacter*, *Pseudorhodoferax* and *Shinella* sp. were 2 to 10-fold less abundant in SSC that supported colonization compared to communities that rejected SBW25.

Synthetic microbial community promotion of sorghum growth in laboratory and the field

The rhizosphere represents a dynamic and complex interface between plant hosts and the microbial community found in the surrounding soil. While it is recognized that manipulating the rhizosphere has the potential to improve plant fitness and health, engineering the rhizosphere microbiome through inoculation has often proved challenging. This is in large part due to the competitive microbial ecosystem in which the added microbes must survive, and lack of adaptation of these added microbes to the specific metabolic and environmental pressures of the rhizosphere. Here, we constructed an inoculation formula using a defined synthetic community (dSynCom) approach that we hypothesized would improve engraftment efficiency and potentially the relationship with the host plant, *Sorghum bicolor*. The dSynCom was assembled from bacterial isolates that were either: 1) identified to potentially play a role in community cohesion through network analysis, or 2) identified to benefit from host-specific exudate compounds. Growth of the dSynCom was evaluated *in vitro* on solid media, *in planta* under gnotobiotic laboratory conditions, and using sorghum plants grown in the field. The dSynCom performs best in terms of maintaining diversity when grown in the presence of the plant host in lab conditions, and that many lineages are lost from the community when grown either *in vitro* or in a native field setting. Finally, we demonstrate that the dSynCom promotes growth of above-

and below- ground plant phenotypes compared to uninoculated controls, both in the lab and when applied to plants grown in the field. These results demonstrate the potential utility of synthetic communities to support crop performance even in the absence of persistence and the need for a deeper mechanistic understanding of community control of host fitness in agricultural contexts.

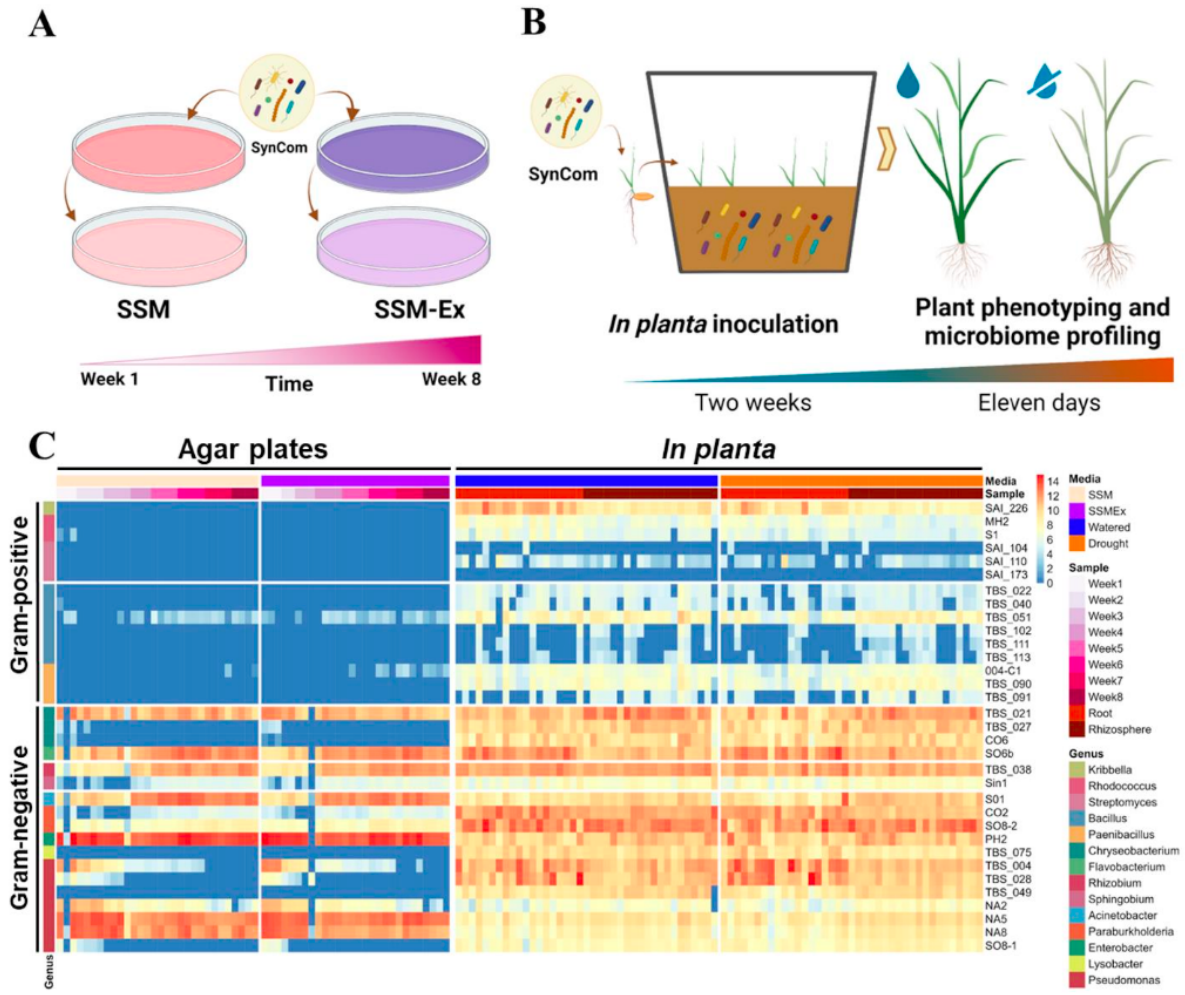


Figure 6. dSynCom colonizes the rhizosphere and roots of sorghum. (A) Schematic diagram of the dSynCom stability analysis on agar plates. Dilutions of the dSynCom were plated on agar plates with Synthetic Soil Media (SSM) or SSM with sorghum exudates (SSM-Ex) and passed over eight weeks. 16S rRNA profiling was performed of at least two replicas across passages. B) Schematic representation of the lab-based dSynCom colonization analysis. Surface disinfected sorghum seeds (cultivar RTx430) were germinated and treated with mock or dSynCom in 5L-capacity microboxes under a sterile environment. Plants were irrigated every three days (26 days) or drought stressed (no irrigation added, 11 days). Plant phenotyping and 16S rRNA profiling were performed at the time of harvest. C) Heat map of the abundance of the dSynCom members identified across treatments *in vitro* and *in planta* experiments. The abundance is presented in log2 scale for n=174 samples for *in vitro* experiments and n=154 samples for the *in planta* experiment.

We found that while many members of the dSynCom were not stably maintained in assays with *in vitro* media, cultivation with lab-grown plants enabled persistence of most introduced

strains (**Figure 6**). In addition to a more even representation of the community members when grown with the sorghum plants, we observed improved plant growth (shoot fresh weight and dry

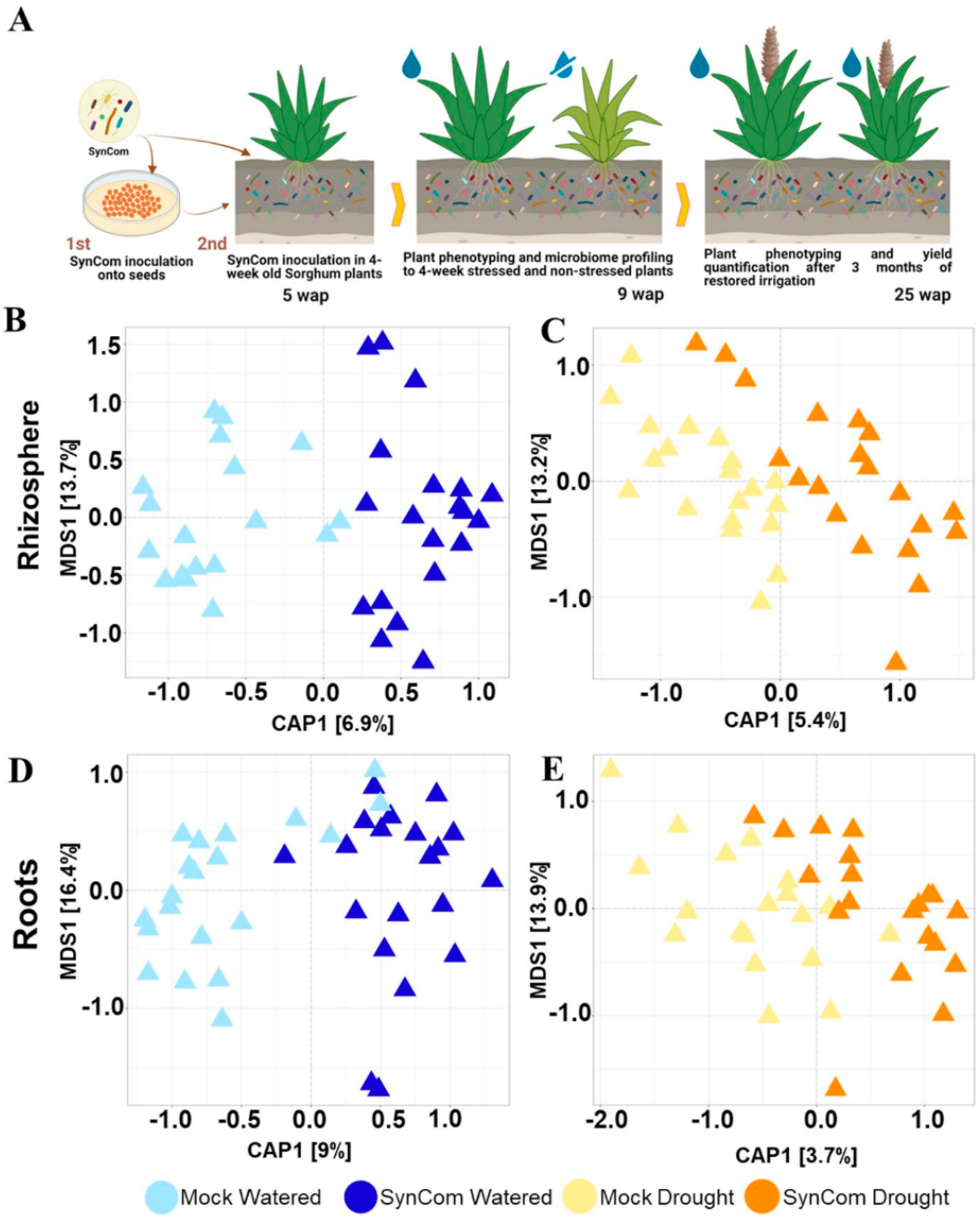


Figure 7. dSynCom impacts the beta diversity of the sorghum microbiome in the field. (A) Schematic diagram of the field trial design. Sorghum plants cultivar BTx642 were treated once (seeds) or twice (seeds and 4-week old plants) with mock or dSynCom. Two irrigation treatments were applied, a drought stress, no irrigation, for four weeks after the second inoculation (5 wap – 9 wap), or irrigation periodically. Microbiome profiling was analyzed at the end of drought stress (9 wap) and the plant phenotyping was recorded also at 9 wap and after three months of the restored irrigation at 25 wap. (B) Beta diversity analysis of the sorghum microbiome of the field trial. Constrained Analysis of Principal Coordinate (CAP) ordination plot of the microbial community in the sorghum (B,C) rhizosphere and (D-E) roots sampled under (B,D) normal irrigation (blue colors) and (C,E) drought stress (brown colors) inoculated twice with mock or dSynCom.

weight) under watered conditions for the dSynCom treated plants compared to the mock treatments. These results indicate a mutual benefit between the plant and the microbial community.

To explore how the dSynCom performs in a native context, we conducted experiments in the field through inoculation of the dSynCom onto seeds and young growing seedlings. In this experiment, sorghum seeds were first subjected to either a preincubation with the dSynCom treatment or a mock inoculation prior to sowing into the field at the Kearney Agricultural Research and Extension Center. A second round of inoculation (dSynCom or mock) was performed on a select group of 4-week old seedlings in each plot to explore the impact of alternative methods of SynCom deployment. Subsequently, half of the plants in all inoculation treatment types were subjected to a period of drought stress, during which no water was applied for four consecutive weeks (5 - 9 weeks after planting, wap). The other half of the plants remained regularly irrigated throughout the experiment (**Figure 7A**). At the end of the drought treatment (9 wap), we collected root tissue and biomass phenotypes from all treatment groups and performed 16S rRNA (V3-V4) community profiling of root and rhizosphere using Illumina MiSeq sequencing (9 wap). In accordance with previous studies on the sorghum microbiome [8], microbial diversity significantly differed between roots and rhizosphere (**Figure 7B-E**), showing a lower Shannon diversity in roots, and also showed differences according to watering treatment with overall reduced diversity in droughted samples compared with watered treatments. It was

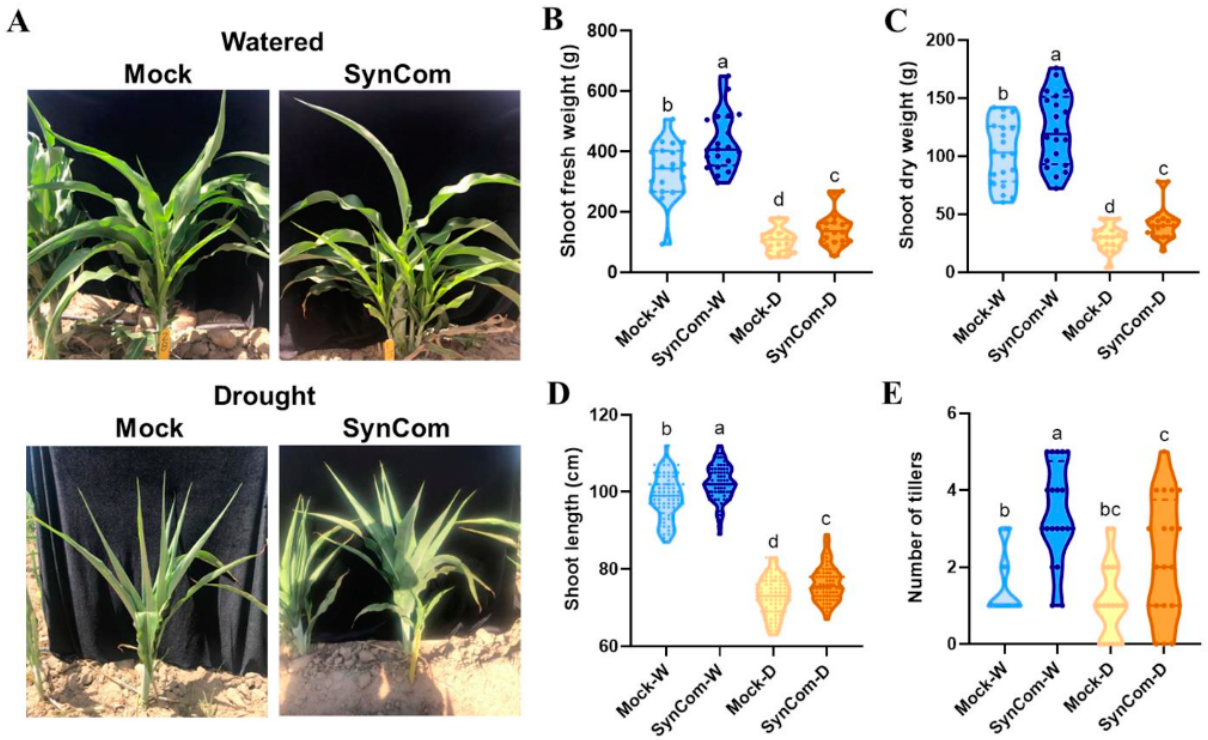


Figure 8. dSynCom enhances the shoot biomass of sorghum plants in the field at nine weeks after planting. (A) Representative pictures of watered or drought stressed plants inoculated twice (seeds and 4-week old plants) with mock or dSynCom. B) Shoot fresh weight. C) Shoot dry weight. D) Shoot length. E) Tiller number. W=normal irrigation (blue colors); D=drought stress (brown colors). Letters above represent statistical differences among treatments by Brown-Forsythe and Welch ANOVA one-way, Holm-Šídák post hoc test $P < 0.05$ for A-B), ANOVA one-way, Tukey post hoc test $P < 0.05$ for D), and Kruskal-Wallis test, Dunn's Test of multiple comparisons $P < 0.05$ for E). A total number of 80 plants were harvested, 20 plants per treatment.

also noted that the dSynCom inoculation significantly impacted alpha diversity of watered root samples (under seed inoculation), increasing the Shannon diversity by approximately 2%, from 5 to 5.15 in mock- and dSynCom-treated plants, respectively (no effect was observed in the rest of the treatments).

To explore the impact of each experimental factor on specific taxonomic fractions of the sorghum microbiome, we analyzed the data at the phylum-level and genus-level using relative abundance plots. In agreement with previous studies of root microbiome response to drought stress [8], we observed an enrichment of Gram-positive bacteria in drought-stressed samples together with a depletion of Gram-negative bacteria. More specifically, the phylum Actinobacteria and the genus *Streptomyces* were strongly abundant in both roots and rhizosphere of drought stressed samples, while the phyla Proteobacteria and Bacteroidetes were less abundant in both sample types.

While we did not observe dSynCom persistence within the rhizosphere environment, positive impacts on plant phenotype were observed in the field following dSynCom treatment. As was observed previously in the lab-based *in planta* experiments, the shoot fresh weight, dry weight, and shoot length were all impacted. Specifically, each of these phenotypes was enhanced with the dSynCom treatment (both seed and 4-week old seedling inoculations) compared to mock-treated plants under both water and drought treatments (**Figure 8A-D**). We also observed a positive impact on the root phenotypes of watered plants that had been inoculated twice with the dSynCom, with significant effects in eight of the 36 features analyzed with a RhizoVision Crown system [9]. For root phenotypes, no significant effect was observed in either the shoot or root phenotypes on those plants that were inoculated only onto seeds. Interestingly, the number of tillers increased for both plants inoculated onto seeds or inoculated twice (seeds and seedlings) with the dSynCom under normal irrigation (**Figure 8E**), which could partially explain overall increases in above ground yield and biomass. Finally, after a period of three months of restored irrigation (25 wap), the shoot fresh weight and dry weight were measured for all treatment groups; we observed increases in both phenotypes for plants inoculated onto seeds or twice with dSynCom under the watered treatment, although no significant effect was observed on droughted plants at this stage of development. Additionally, we noted that dSynCom treatment also positively impacted seed yield for plants under the normal irrigation treatment. Altogether, these results demonstrate that treatment with the dSynCom is effective in increasing biomass and yield-related traits in the field as well as in the laboratory.

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