Video Article

High-throughput Siderophore Screening from Environmental Samples: Plant Tissues, Bulk Soils, and Rhizosphere Soils

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Abstract

Siderophores (low-molecular weight metal chelating compounds) are important in various ecological phenomenon ranging from iron (Fe) biogeochemical cycling in soils, to pathogen competition, plant growth promotion, and cross-kingdom signaling. Furthermore, siderophores are also of commercial interest in bioleaching and bioweathering of metal-bearing minerals and ores. A rapid, cost effective, and robust means of quantitatively assessing siderophore production in complex samples is key to identifying important aspects of the ecological ramifications of siderophore activity, including, novel siderophore producing microbes. The method presented here was developed to assess siderophore activity of in-tact microbiome communities, in environmental samples, such as soil or plant tissues. The samples were homogenized and diluted in a modified M9 medium (without Fe), and enrichment cultures were incubated for 3 days. Siderophore production was assessed in samples at 24, 48, and 72 hours (h) using a novel 96-well microplate CAS (Chrome azurol sulphonate)-Fe agar assay, an adaptation of the traditionally tedious and time-consuming colorimetric method of assessing siderophore activity, performed on individual cultivated microbial isolates. We applied our method to 4 different genotypes/Lines of wheat (*Triticum aestivum* L.), including Lewjain, Madsen, and PI561725, and PI561727 commonly grown in the inland Pacific Northwest. Siderophore production was clearly impacted by the genotype of wheat, and in the specific types of plant tissues observed. We successfully used our method to rapidly screen for the influence of plant genotype on siderophore production, a key function in terrestrial and aquatic ecosystems. We produced many technical replicates, yielding very reliable statistical differences in soils and within plant tissues. Importantly, the results show the proposed method can be used to rapidly examine siderophore production in complex samples with a high degree of reliability, in a manner that allows communities to be preserved for later work to identify taxa and functional genes.

Introduction

Siderophores are important biomolecules involved primarily in iron-chelation for bioavailability, but with a wide array of additional purposes in terrestrial and aquatic ecosystems ranging from microbial quorum sensing, signaling to microbial plant-hosts, plant growth promotion, cooperation and competition within complex microbial communities[1,2]. Siderophores can be broadly classified according to their active sites and structural features, creating four basic types: carboxylate, hydroxamate, catecholate, and mixed types[3,4]. Many microorganisms are capable of excreting more than one type of siderophore[5] and in complex communities, a vast majority of organisms biosynthesize the membrane receptors to allow the uptake of an even wider variety of siderophores[1,6]. Recent work indicates that siderophores are particularly important at the community level, and even in inter-kingdom communications and biogeochemical transfers[7,8,9,10,11].

Chrome azurol sulphonate (CAS) has been used for over 30 years as a chelating agent to bind iron (Fe) in such a way that addition of ligands (i.e., siderophores) can result in dissociation of the CAS-Fe complex, creating an easily identifiable color change in the medium[12]. When the CAS is bound with Fe, the dye appears as a royal blue color, and as the CAS-Fe complex dissociates, the medium changes color according to the type of ligand used to scavenge the Fe[13]. The initial, liquid-based medium established by Schwyn and Neilands in 1987, has been modified in many ways to accommodate changing microbial targets[14], growth habits and limitations[15], as well as a variety of metals besides Fe, including aluminum, manganese, cobalt, cadmium nickel, lithium, zinc[16], copper[17], and even arsenic[18].

Many human pathogens, as well as plant growth promoting microorganisms (PGPM) have been identified as siderophore-producing organisms[3,19,20], and important rhizosphere and endophytic PGPM often test positive for siderophore-production[4]. The traditional Fe-based liquid method has been adapted to microtiter testing of isolates in cultivation for siderophore production[21]. However, these techniques fail to recognize the importance of the microbial community as a whole (the microbiome), in cooperation and potential regulation of siderophore production in soils and plant systems[22]. For that reason, we have developed a high-throughput community-level assessment of siderophore production from a given environment, based on the traditional CAS assay, but with replication, ease of measurement, reliability, and repeatability in a microplate assay.

In this study, a cost-effective, high-throughput CAS-Fe assay for detecting siderophore production is presented to assess the enrichment of siderophore production from complex samples (i.e., soil and plant tissue homogenates). Bulk, loosely-bound, and tightly-bound rhizosphere soil (in terms of how the soil was bound to the root) were obtained along with grain, shoot, and root tissues from four distinct wheat (*Triticum aestivum* L.) genotypes: Lewjain, Madsen, PI561725, and PI561727. It was hypothesized that fundamental differences in the wheat genotypes could result in differences in recruitment and selection of siderophore producing communities. Of particular interest is the difference between microbial communities associated with the PI561725 isogenic line, which is aluminum tolerant because it possesses *ALMT1* (Aluminum-activated

Malate Transporter 1), compared with the aluminum sensitive PI561727 isogenic line, which possesses a non-aluminum responsive form of the gene, *almt1*[23,24,25,26]. The chief objective of the study was to develop a straightforward, rapid method of quantitatively assessing siderophore production in siderophore enrichment cultures of complex sample types while preserving the cultures for future work.

Protocol

NOTE: Location of Field Site: Washington State University, Plant Pathology Farm (46°46'38.0"N 117°04'57.4"W). Seeds were sown using a mechanical planter on October 19, 2017. Each wheat genotype was planted in headrows, approximately 1 meter apart to avoid overlapping of root system. Plant and soil samples were collected on August 9, 2018, when plants were ready for harvest. Samples were gathered from three replicates of four wheat genotypes: PI561727, PI561725, Madsen, Lewjain.

1. Preparation of modified M9 medium

1. Use $Na_2PO_4 \cdot 7H_2O$ (12.8 g/200 mL), KH_2PO_4 (0.3 g/200 mL), NaCl (0.5 g/200 mL), and NH_4Cl (1 g/20 mL) reagents to prepare the M9 salt solution.
2. Use 18 g of $MgSO_4 \cdot 7H_2O$ in 100 mL of double deionized water (ddH_2O) to prepare 0.75 M $MgSO_4 \cdot 7H_2O$.
3. Make 1 M of $CaCl_2 \cdot 2H_2O$ by adding 14.7 g of $CaCl_2 \cdot 2H_2O$ with 100 mL of ddH_2O.
4. Prepare the buffer solution by dissolving 6.048 g of PIPES into 156 mL of ddH_2O with stirring. Adjust the pH to 6.8 with 5 M NaOH.
5. Prepare 20% glucose (dextrose monohydrate) by dissolving 20 g of glucose into 100 mL of ddH_2O.
6. Prepare solutions and individually autoclave all but the glucose. Filter sterilize the glucose solution for addition to the M9 media after autoclaving (0.22 µm).
7. Prepare the modified M9 medium by mixing 156 mL of PIPES Buffer solution, 40 mL of M9 salts solution, 20 µL of $CaCl_2 \cdot 2H_2O$ solution, 266 µL of $MgSO_4 \cdot 7H_2O$, and 4 mL of 20% glucose solutions together in the biosafety cabinet, using sterile technique.
8. For preservation of the modified M9, seal the container, cover with aluminum foil to protect from UV, and place at 4 °C.

2. Preparation of CAS-Fe-Agar medium

1. Acid wash all glassware in 100 mM HCl/100 mM HNO_3 for a minimum of 2 h prior to utilization in the CAS assay.
2. Prepare an aluminum baking pan filled with laboratory grade sand and cover it with aluminum foil. Autoclave at 121 °C for 30 min and set aside.
3. Prepare HDTMA (hexadecyltrimethylammonium bromide) by adding 0.0365 g to 20 mL ddH_2O and place the solution at 37 °C to promote solubilization.
4. Prepare 10 mM HCl and generate 1 mM $FeCl_3 \cdot 6H_2O$ using 10 mM HCl as the solvent. Add 0.0302 g of CAS to 25 mL of ddH_2O while gently stirring with a sterile magnetic stir bar. Then add 5 mL of 1 mM $FeCl_3 \cdot 6H_2O$ (in 10 mM HCl) to the 25 mL of CAS solution while continuing to gently stir (the solution turns into dark reddish black color).
5. Slowly add the 20 mL of HDTMA solution while gently stirring, into the Fe-CAS solution (this yields a dark blue solution).
6. Prepare the buffer solution by dissolving 15.12 g of PIPES into 375 mL of ddH_2O, with gentle stirring. Adjust the pH to 6.8 with 5 M NaOH. Add water to bring the volume to 450 mL. Add 5 g of agarose to the solution. Autoclave the PIPES buffer solution and the CAS-Fe solution at 121 °C for 30 min.
7. Carefully add the entirety of the CAS-Fe solution to the entirety of the PIPES Buffer in the biosafety cabinet after each of them is autoclaved.
8. Place the mixed solution in a water bath at 50 °C.
   NOTE: Freshly prepare all the reagents in the CAS-Fe-Agar medium before each assay, as long-term storage at 50 °C results in precipitation of the CAS-Fe complex, and cooling results in solidified medium.
9. Place a sterile reagent boat in the sterile sand in the biosafety cabinet and heat to 50 °C. Transfer the CAS-Fe-Agar to the boat, then quickly aliquot 100 µL to each well in a clear, flat-bottom, sterile 96-well microplate.

3. Pyoverdine/EDTA standard preparation

1. Pyoverdine preparation
   1. Prepare 800 µM pyoverdine standard (mixture of succinic acid, 2-hydroxy glutaramide, and succinaminde forms of pyoverdine – see **Table of Materials**), in previously prepared modified M9 medium.
   2. Dilute this solution into 400, 200, 100, 50, 25, 12.5, and 6.25 µM solutions.

2. EDTA standard preparation
   1. Add 0.594 g of disodium ethylenediaminetetraacetic acid (EDTA: $C_{10}H_{14}N_2Na_2O_8.2H_2O$), to 500 mL of previously prepared modified M9 medium to prepare 3200 µM EDTA standard.
   2. Dilute this solution into 1600, 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM solutions.

3. Standard curve generation
   1. Add 100 µL of each concentration of pyoverdine and EDTA to separate wells of a 96-well microplate containing 100 µL of CAS-Fe Agar medium. Make duplicate technical replications of each concentration. Also, add blank wells with only M9 (no EDTA or pyoverdine).
   2. Using a microplate reader, measure absorbance (at 420 nm and 665 nm) after 1, 6, and 24 h incubation at 22 °C, and use absorbance measurements to generate standard curves.
      NOTE: For 420 nm measurements, subtract absorbance of blanks from the absorbance of pyoverdine or EDTA containing wells. For 665 nm, subtract absorbance of pyoverdine or EDTA containing wells from absorbance of blanks. Then, $\log_{10}$(µM pyoverdine or

EDTA) is regressed against the absorbance measurements. For ease of interpreting sample results, use absorbance as the x-axis and $\log_{10}$(μM pyoverdine or EDTA) as the y-axis.

4. Collection of environmental samples: soil and plant tissues

1. Wash sampling equipment (shovels and scissors) with 0.22 μm filtered ddH_2O followed by 70% ethanol, and wipe with paper towels before sampling and in between samples to maintain sterile technique and reduce cross-contamination.
2. Excise the plant tissues (grains and shoots) from plants in the field, and place them in a labeled plastic storage bag, leaving enough shoot stubble for ease in uprooting the desired soil and root samples.
3. Excavate a small root ball approximately 15 cm deep and 23 cm wide and place it in a separate, labeled plastic bag for sample preparation in the laboratory environment. This step is similar to the methods of McPherson et al.[27].
4. Place all samples (grains, shoots, bulk soil, and root balls in separate bags) directly on ice and keep at 4 °C until samples are processed for the siderophore production assay.
5. Separate root associated soil samples into bulk, loosely-bound rhizosphere soil, and tightly-bound rhizosphere soil.
    1. Take the root balls out of the bags. Gently shake off soil from the root ball. Shaken off soil, along with the soil left in the bag comprises the “bulk” soil.
    2. Use a rubber mallet to further remove soil from the root ball. This is the loosely-bound rhizosphere soil.
    3. Generate tightly-bound rhizosphere soil by taking roots with the tightly-bound sample and putting them in a centrifuge tube. Add 30 mL of ddH_2O and vortex it for 2-3 minutes. Remove roots to get the tightly-bound rhizosphere soil slurry dilution.

5. Preparation of siderophore enrichment cultures and CAS-Fe siderophore production assay

NOTE: All glassware should be acid washed prior to beginning the assays.

1. Soil sample preparation (for each of the three soil sample types)
    1. Homogenize each soil sample within the sample bag, by mixing and turning the soil as much as possible without opening the bag.
    NOTE: This helps reduce natural soil spatial variability and aligns with normal soil sampling procedures. Other methods may be utilized to homogenize environmental samples, as appropriate, and depending on the experimental design.
    2. After each sample has been thoroughly mixed, aliquot and suspend 2.0 g of each soil in 20 mL of modified M9 medium in a sterile 50 mL centrifuge tube with a sterile foam plug to allow aeration.
    3. For tightly-bound rhizosphere samples, add 2 mL of the rhizosphere soil slurry to 20 mL of modified M9 medium in a sterile 50 mL centrifuge tube with a sterile foam plug to allow aeration.

2. Tissue sample (root, shoot and grain) preparation
    1. Surface sterilize the sample with 70% ethanol. Macerate 2.0 g of fresh tissue in 20 mL of modified M9 medium using a blender on high for 30 seconds. Transfer the sample to a sterile 50 mL centrifuge tube with a sterile foam plug to allow aeration.

3. Enrichment of Siderophore Production Through Fe Limitation
    1. Incubate 50 mL centrifuge tubes at room temperature and shake at 160 rpm.

6. CAS-Fe agar assays for detection of siderophore production in environmental samples

1. At 24, 48, and 72 h after initiating the enrichment culture, remove 1 mL subsamples from the enrichment tubes using sterile technique and centrifuge at 10,000 x g for 1 min in 2 mL centrifuge tubes to pelletize the cells.
2. Collect the separated supernatant. Using sterile technique, add 100 μL of the supernatant to 100 μL solution of CAS-Fe-Agar in duplicate or triplicate in the microplate. Also add 100 μL of sterile M9 medium (as blanks). Then incubate the plate at 28 °C.
3. Suspend the remaining supernatant and pellet for each sample (not added to microtiter plate) into its own, sterile 2 mL centrifuge tube. Add 400 μL of sterile glycerol into each sample-supernatant tube and resuspend the pellet to create glycerol stocks. Freeze the stock at -80 °C for later analyses.
   NOTE: This step can be modified to generate glycerol stocks according to any preferred in-house protocol.
4. Measure absorbance at 6, 24, 48, and 72 h, at 420 nm wavelength.
5. Use standard curves generated from pyoverdine or EDTA to interpret sample absorbance measurements in terms of pyoverdine equivalents.
   NOTE: Pyoverdine was determined to be a superior standard compared with EDTA (vide infra), so EDTA was not used to interpret results in the current study.

Representative Results

A pyoverdine mixture biosynthesized by *Pseudomonas fluorescens* was used as a standard to interpret and quantify absorbance (at 420 nm) of samples in terms of pyoverdine equivalents in µM. **Figure 1** shows the relationship between absorbance (420 nm) and starting concentration of pyoverdine (Log_{10} molarity in µM). EDTA did not provide an adequate standard because samples exhibited greater absorbance measurements than were attainable with pyoverdine, and the R^2 was lower (**Figure 2**). While initial work using the CAS-Fe assay as a method of siderophore detection measured absorbance at 630 nm, in a related study using a very similar method (CAS-Fe-Agar was mixed 1:1 with modified M9 to generate a 200 µL column in the microplate), it was observed that the peak absorbance was at 665 nm, but that 420 nm was more reproducible in terms of changes in absorbance induced by samples (**Figure 3**).

Siderophore production was observed in enrichment cultures of all tissue types after 72 h of Fe-deficit enrichment and siderophore activity appeared to stabilize after 48 h of incubation (**Supplementary Figure 1**). Thus, siderophore activity of the 72 h enrichment was assessed at 48 h incubation to determine the influence of genotype and sample type on siderophore isolation (**Figure 4**). Siderophore activity in bulk soil samples was relatively low and did not exhibit differences between the wheat genotype from which the bulk soil was sampled (**Figure 4A**). Enrichments of loosely bound soil isolated from the PI561725 genotype exhibited greater siderophore production compared with loosely bound soil from Madsen and PI561727, but not Lewjain (**Figure 4B**). Siderophore production in enrichments from tightly bound soil was not heavily influenced by genotype (**Figure 4C**).

Enrichment cultures of grain tissue yielded relatively low siderophore production regardless of genotype (**Figure 4D**). Enrichments of Lewjain shoot tissue had significantly lower siderophore production than the other genotypes, and PI561725 shoot tissue cultures resulted in more variable siderophore production (**Figure 4E**). Siderophore activity was more than 200% greater in in root tissue enrichment cultures of PI561725 compared with all other genotypes (**Figure 4F**).

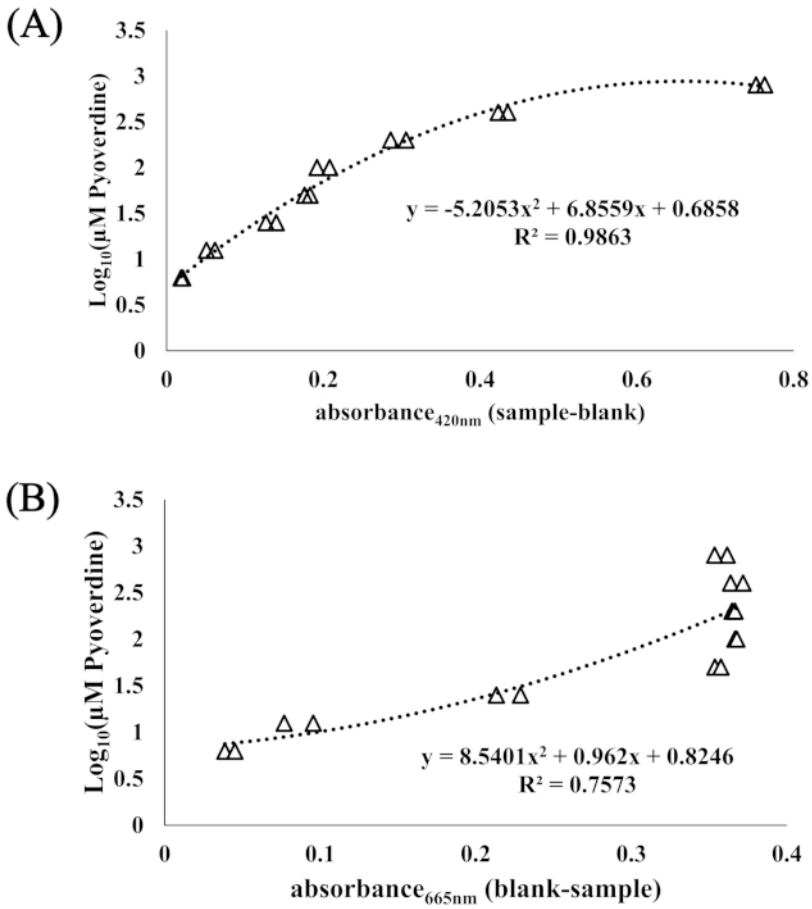


Figure 1. Absorbance at 420 nm and 655 nm regressed against the log10 concentration of pyoverdine. (**A**) Absorbance at 420 nm regressed against the log_{10} concentration of pyoverdine in µM. A polynomial curve was fit to obtain an explanatory equation for interpreting absorbance in terms of pyoverdine equivalents. (**B**) Absorbance at 665 nm regressed against the Log_{10} concentration of pyoverdine in µM. R^2 is the square of the Pearson correlation coefficient, and the equation explains the fitted curve. Points are duplicates of absorbance measurements at 800, 400, 200, 100, 50, 25, 12.5, and 6.25 µM pyoverdine after 6 h incubation at 28 °C. Please click here to view a larger version of this figure.

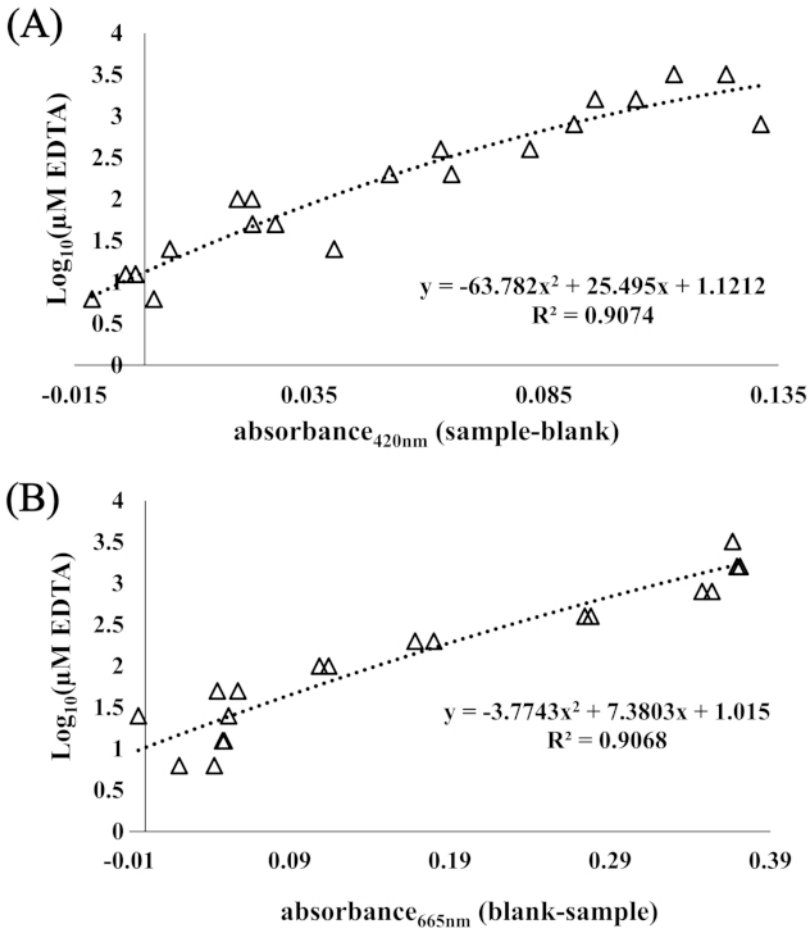


Figure 2. Absorbance at 420 nm and 655 nm regressed against the log10 concentration of EDTA. (**A**) Absorbance at 420 nm regressed against the $\log_{10}$ concentration of EDTA in μM. A polynomial curve was fit to obtain an explanatory equation for interpreting absorbance in terms of pyoverdine equivalents. (**B**) Absorbance at 665 nm regressed against the Log_{10} concentration of EDTA in μM. R^2 is the square of the Pearson correlation coefficient, and the equation explains the fitted curve. Points are duplicates of absorbance measurements at 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, and 6.25 μM EDTA after 6 h incubation at 28 °C, and error bars . Please click here to view a larger version of this figure.

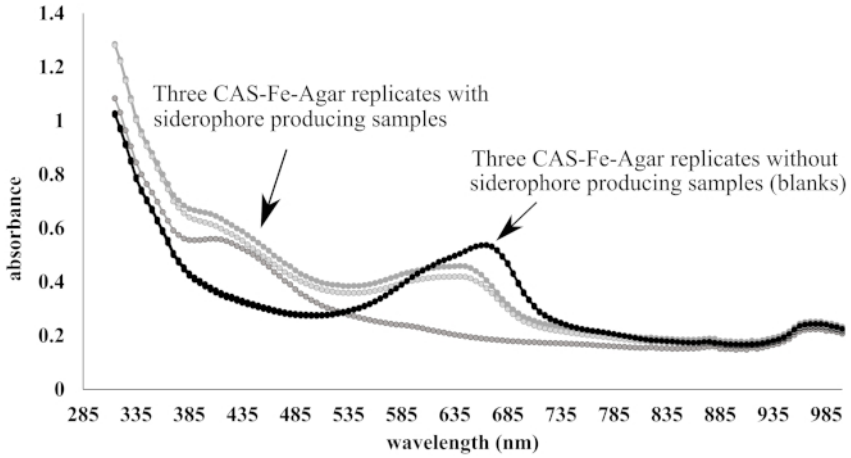


Figure 3. Absorbance scans from 315−1000 nm of microplate wells containing 200 µL columns of 1:1 CAS-Fe-Agar and modified M9 or M9 medium with siderophore producing samples. The plate was incubated at 28 °C for 72 hours before measuring absorbance in a microplate reader. Absorbance scans show the three blanks containing no sample (black lines) yielded tightly clustered curves with a peak at 665 nm. Absorbance scans show the three blanks containing siderophore producing samples (gray lines) yielded curves with more variability, but with more consistent absorbance at 420 nm compared with 665 nm. Please click here to view a larger version of this figure.

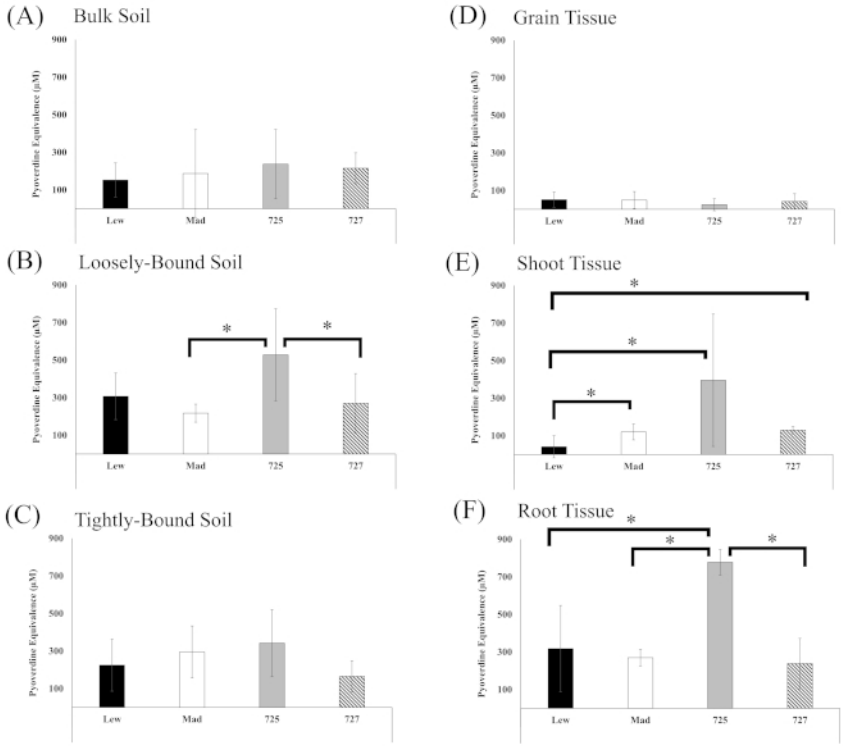
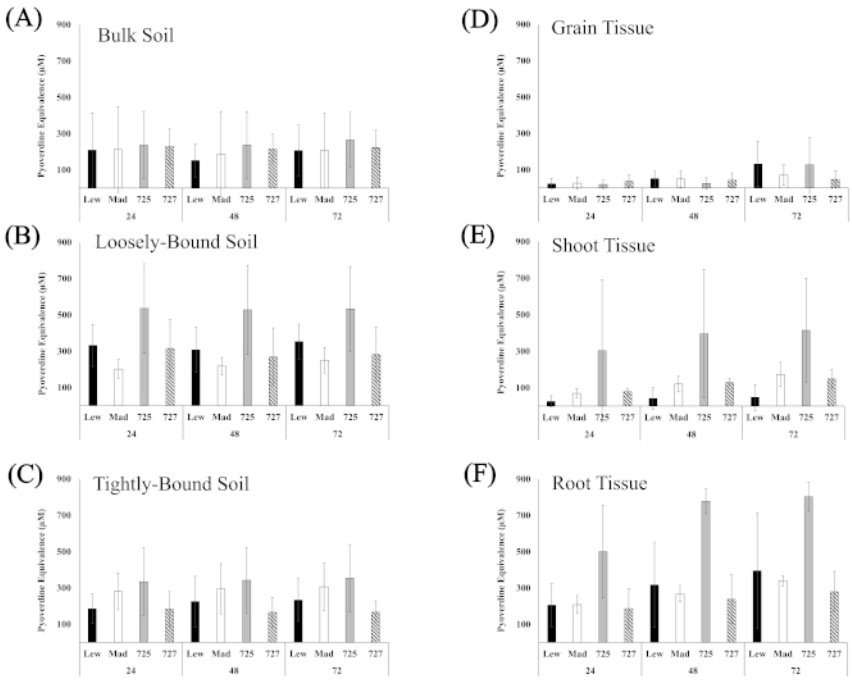


Figure 4. Pyoverdine equivalents of siderophore enrichment cultures. Pyoverdine equivalents of siderophore enrichment cultures associated with (**A**) bulk (**B**) loosely bound, and (**C**) tightly bound soil, and in tissue homogenates of wheat (**D**) grain (**E**) shoots, and (**F**) roots. Siderophore enrichment cultures were incubated for 72 h before transferring subsamples to a microplate and incubating at 28 °C. Siderophore production was assessed after 48 h of incubation with Chrome azurol S. Genotypes/Lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Asterisks represent significance at *alpha* = 0.008 (after Bonferroni correction). Bars are standard deviation. Please click here to view a larger version of this figure.



Supplementary Figure 1. Pyoverdine equivalents over time. Pyoverdine equivalents of siderophore enrichment cultures assessed after 24, 48, and 72 h of incubation with Chrome azurol S. Sderophore enrichment cultures associated with (**A**) bulk (**B**) loosely bound, and (**C**) tightly bound soil, and in tissue homogenates of wheat (**D**) grain (**E**) shoots, and (**F**) roots. Siderophore enrichment cultures were incubated for 72 h before transferring subsamples to a microplate and incubating at 28 °C. and subsampled to assess siderophore production at each timepoint. Genotypes/Lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after, 24, 48, and 72 h of incubation. Bars are standard deviation. Please click here to view a larger version of this figure.

Discussion

The primary result of this work is the production of a new methodology that can be used to rapidly enrich for siderophore producing microbes while quantitatively measuring siderophore production/activity in the environmental sample. The methodology is quick, simple, and cost-effective, and the results show how it can be used to detect siderophore activity from complex and novel sample types (e.g., soil and plant tissue). The protocol also results in the production of glycerol stocks of the enrichment cultures, which can easily be taken through time to accommodate studies of shifts in microbial community structure and function during Fe deficiency through DNA or RNA based techniques. Those interested in examining the kinetics of siderophore activity in ecological studies could also likely benefit from this method. The results also show that pyoverdine equivalents (pyoverdines are important siderophores in terms of the environment[28] and medicine[29]) provide a good method of quantitatively assessing siderophore production. An important finding is that absorbance measurements at 665 nm are inadequate for determining siderophore activity compared with those observed at 420 nm (**Figure 1**). Of particular importance was the finding that absorbance at 665 nm clustered across a broad range of pyoverdine concentrations (Pyoverdine μM = 50-800 μM, $\log_{10}$(μM pyoverdine) = 0.18-0.76), suggesting a detection ceiling at this wavelength (**Figure 1B**). It should be noted that while pyoverdine was a superior standard compared with EDTA, it is also costly, so it is suggested that preliminary work is performed with EDTA or other cost-effective chelators to ensure the methodology has been mastered before generating pyoverdine standards.

There are several critical steps throughout the protocol that require close attention. Firstly, it is important to maintain metal-free glassware and other wares wherever possible when working with metals, particularly those necessary in low concentrations, like Fe. Secondly, because cultures were enriched for siderophore production through Fe limitation, it is important to maintain aseptic conditions throughout the workflow to reduce the influence of environmental contaminants. Lastly, preparation of the CAS-Fe-agar requires careful attention to detail and should be prepared as closely to described as possible. For instance, if the CAS-Fe-agar solution is kept warm but is not used quickly, the CAS-Fe will precipitate. Additionally, it is essential to keep the CAS-Fe-Agar warm during transfer to the microplate. This was achieved by using heated, sterile sand and quickly transferring the medium to the microplate in a biosafety cabinet.

One limitation of the methodology is that because some plants also produce siderophores (phytosiderophores); these can contribute to measured siderophore activity in enrichment cultures of plant tissue homogenates. Also, there was relatively high variability in the results from field replicates, suggesting more replication could be beneficial in future studies. Another limitation of the technique is that while the microplate method is high-throughput, the sample gathering and preparation are time-consuming. Still, because a single microplate can be used for 96 samples (including standards), the time and cost inputs are much lower compared with existing techniques. This is primarily because other existing methods rely on performing the CAS-Fe assay in Petri dishes[30], which are inherently less time and cost efficient to prepare than a microplate. Additionally, because solubilized CAS-Fe complexes are prone to precipitation[12], the proposed method using CAS-Fe-Agar medium is superior to liquid-based methods, which have also be adapted to the 96-well format.

In terms of the reported findings, given that the primary difference between the PI561725 and PI561727 is the presence of *ALMT1* vs.*almt1*, respectively, the results suggest the presence of *ALMT1* likely results in the selection of microbial communities in both the plant and the soil which have a greater potential for siderophore production, as assessed *via* enrichment cultures. Future work should further investigate the phenomenon using a larger number of replicates, particularly to clarify if the presence of *ALMT1* specifically selects for enhanced siderophore activity.

Disclosures

The authors have no conflicts of interest to disclose.

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