**22 October 2018**

**Authors’ Response to Review and Revisions**

The authors sincerely appreciate the considerable time and efforts taken for a thorough review and constructive input by the reviewers and editor. We have incorporated all the input and provided line-by-line responses to each concern below. Below you will find each comment individually addressed in the order each was received, with the reviewer comment in plain text, and the response in bold, blue font, directly below or beside the comment. Thank you for this opportunity to revise and resubmit for publication with *The Journal of Visualized Experiments*.

**Editorial Comments:**  
  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**The authors have done so and feel the manuscript is much improved.**

2. Keywords: Please provide at least 6 keywords or phrases.

**The authors have added keywords.**

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Milli-Q, Waring, Lab companion, etc.

**The authors have removed all instances of commercial language and replaced with generic terms with reference to the Table of Materials.**

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

**The authors have revised to ensure conformation to the JoVE format and apologize for the previous mistakes.**

5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

**The authors have revised the instructions to be in the imperative tense, and moved those relevant statements to the discussion section.**

6. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

**The authors have added more detail throughout, as well as the appropriate references.**

7. Line 120: Please specify the samples (grains, shoots, roots, or soil samples).

**The authors have reorganized and revised the manuscript so that Lines 220 through 297 all specify the different sample types and how each should be handled.**

8. Lines 122-123: Please describe the criteria for separating the soil samples.

**The authors have reorganized and revised the manuscript so that Lines 220 through 297 all specify the different sample types and how each should be handled.**

9. Line 130: How is the soil sample homogenized? Are loosely-bound and tightly-bound rhizosphere soil both homogenized?

**Lines 241 through 263 now give a better description of the process of handling these types of samples. Lines 257-262 now also describes how to homogenize a soil sample and that each type of environmental sample can be handled slightly differently, but that is beyond the scope of this particular article.**

10. Line 133: Do tissue samples refer to grains and shoots?

**The authors have reorganized and revised the manuscript so that Lines 268 through 272 now give more specifics on the types of tissues.**

11. Line 153: Do you mean measuring absorbance of pyoverdine standards at different time periods?

**The authors have reorganized and revised the manuscript so that the preparation of standards and curves and presented in Lines 188 through 218, and have clarified the time points for standards measurements.**

12. Lines 174-175: How about the rest of the supernatant? Is it discarded or left with the pellet?

**The authors have rewritten this step with better description of the process in Lines 290-293.**

13. Line 230: Please describe the specific actions being performed here.

**The authors have rewritten the manuscript in such a way that the actions previously described as “Lewis et al 2018” are described throughout the protocol, primarily Lines 278 -300.**

14. Please include single-line spaces between all paragraphs, headings, steps, etc.

**Done**

15. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please note that shorter steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

**Done**

16. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Done.**

17. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Done**

18. Figure 2: Please define error bars in the figure legend.

**The figure has been revised so that there are no longer error bars in Figure 2, but all figure legends have been elaborated on.**

19. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique  
20. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

**The authors have revised and rewritten the discussion according to the guidelines provided and the new Discussion (with all the above items addressed) may be found in Lines 373 though 426.**

**Reviewer #1:**  
1. The manuscript titled is not suitable with content because there are many reports which revealed wheat plant can able to sequestrates siderophore by itself. On this experiment they assess siderophore from tissue of the wheat plant. My opinion on that this siderophore may not be produce from microorganism and need this further confirmation.

**The authors gratefully acknowledge the reviewer’s extent of knowledge in the area and have accordingly altered the manuscript title to address this concern and broaden the scope of the assay. The new title now reads, “High-throughput siderophore screening from environmental samples: plant tissues, bulk soils, and rhizosphere soils.”**  
2. The word use in the title should not be repeated in the key words.  
**The authors have revised the list of keyword to a more appropriate set. (Lines 25-26)**

3. My recommendation the author need to isolate the microorganisms from the plant tissue separately and then to test their efficacy alone and along with plant tissues to underpin the actual consequence.

**The authors acknowledge this as a very useful follow-up study to the protocol as given. However, the objective of the current manuscript is to simply describe the protocol for detecting siderophores in environmental samples in a high-throughput, economical and robust manner. Because we also describe how to generate glycerol stocks, future researchers can use this method to isolate cells and communities capable of siderophore production and then perform a wide array of assays and further tests in the future.**

Minor Concerns: Some minor revision will be required.  
**The authors feel the concerns of this reviewer were thoroughly addressed.**

**Reviewer #2:**  
Major Concerns:  
The manuscript would be greatly improved if the protocol, representative results, and discussion section were restructured, as indicated below. I'm not sure any of these items are major, but collectively indicate that the manuscript needs major revision.

Introduction section: This section is generally fine, and does a nice job of introducing the protocol. However, much of it is redundant (for example: Microbial siderophores are well recognized recently as important biomolecules.... could be streamlined to say: Siderophores are important biomolecules involved....). The introduction also talks about enrichment cultures, but does not explain what exactly is being enriched.

**The authors have revised and streamlined the lines 65-68, mentioned by the reviewer to:**

**“Siderophores are important biomolecules involved primarily in iron-chelation for bioavailability, but with a wide array of additional purposes in terrestrial ecosystems ranging from microbial quorum sensing, signaling to microbial plant-hosts, plant growth promotion, cooperation and competition within complex microbial communities1,2.”**

Also, throughout the manuscript, the authors claim that the improved protocol is cost-effective and rapid but provide no evidence or reasoning for that claim.

**The authors have added language to support the point about cost in the conclusion:**

**Lines 412-418: “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 dishes30, which inherently take more time and money to prepare than a microplate.”**

Protocol: There are several confusing points in this section.  
Section 1) How long were the plants grown? Were they harvested after maturation?

**The authors have addressed concerns in Lines 122-123 for the representative data: “****Plant and soil samples were collected on August 9, 2018, when plants were ready for harvest.”**

**However environmental sample collection can be varied according to experimental design and plant physiological stage can also be a variable at the experimenter’s discretion.**

Section 2) What exactly is "loosely bound, tightly bound, and bulk soil"? Please provide your working definition.

**The authors have described how the samples were collected and operationally defined in Section 5 on sample collection and preparation, Lines 220-249.**

Section 3) Is it possible to put the recipes for the media first? I find it distracting that I haven't read the recipes before the protocol is described. Ideally I would like to see section 7 as section 3 or 4.

**The authors have reorganized and rewritten the protocol section to be less confusing, so that now the experimental design and site is described first. After that, the preparation of each medium is given, followed by standard curve generation, sample prep, and sample assessment.**

Section 3.4) I do not see how you are enriching siderophore producers. Can you explain?

**Siderophore production was enriched through Fe-deficit limitation. This is now emphasized in the heading for step 6.4.**

**Line 274: “6.4. Enrichment of Siderophore Production Through Fe Limitation”**

Section 3.5) The method indicates that 3 technical replicates are measured (presumably on a plate reader), but only one point on the graph is shown for each dilution. Also, I recommend that three stock solutions and separate dilutions prepared, instead of measuring replicates of a singular dilution. There is no mention at all in the entire protocol of how the reading are measured. Is this a plate reader? Why are you measuring at 420 nm? Most other siderophore methods read at 630 nm...why the difference?

**The authors discovered in preliminary work, the peak absorbance in CAS-Fe medium at 665 nm. We also observed absorbance at 420 nm to be a sensitive and more accurate estimate of changes in the color of CAS-Fe in the presence of samples. In order to address this preliminary work and the rationale for the wavelength choice, we have now added the data and figures for the standard curves generated using absorbance at 420 nm (far better than those examining 665 nm). These points can be gleaned from examining Figures 1 and 3.**

Section 3.7) Was the glucose and other non-autoclaved components filter sterilized?

**These solutions were filter sterilized and the manuscript has been adjusted to reflect this (Lines 141-142).**

Section 3.7.2.4) Here and throughout the manuscript, you reference a "hood". DO you mean a laminar flow hood? Can you just say using sterile technique?

**The language was changed to “biosafety cabinet” and “sterile technique” where appropriate.**

Section 3.7.2.5 remove "hot" and just say "was placed in a 50 degrees C water bath"

**The authors have corrected this as suggested throughout.**

Section 3.7.3) What is described in Lewis et al??

**The authors have removed this reference. We are currently publishing a paper using a similar method with the same M9 formula. But as it is still in the revision process and we have fully described the solution, we felt it was appropriate to remove it from the manuscript and more thoroughly describe the preparation of the Fe-limited M9 medium.**

Representative results: There is no need to place quote marks around "pyoverdine equivalents". Why was pyoverdine chosen over other known siderophores?

The authors have removed the quotation marks, added some information on pyoverdines in Lines 303, and the following was added regarding the selection of pyoverdine:

Lines 384-385: “The results also show that pyoverdine equivalents (pyoverdines are important siderophores in terms of the environment28 and medicine29) provide a good method of quantitatively assessing siderophore production.”  
  
Figures and Table legends:  
  
Figure 1) Please add error bars or indicate that the points shown are from a single experiment.

**The authors have revised the figure so that each replicate is now shown as a point and a new standard curve was generated. The new equation explaining the fitted curve was also generated so the data were all reanalyzed to reflect this. You will see there is basically no change in the results because the new formula is very similar to the old formula. We have also now shown the absorbance measurements at 665nm which we found to the the peak of CAS-Fe absorbance in another study (even though the convention is to measure at 630 nm).**

Figure 2 and Figure s1) These descriptions are pretty confusing. "Figure 2. Pyoverdine equivalents of 72 h enrichment cultures associated with bulk (A), loosely bound (B), and tightly bound (C) (in terms of roots) soil and tissue homogenates of wheat grain  
(D), shoots (E), roots (F). Genotypes/lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after 48 h of incubation. Asterisks  
269 represent significance at alpha = 0.008 (after Bonferroni correction)." would be less confusing if written "Figure 2. Pyoverdine equivalents of 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 were calculated after 72 h of enrichment. Genotypes/lines are Lew = Lewjain, Mad = Madsen, 725 = PI561725, and 727 = PI561727. Siderophore production was assessed after 48 h of incubation with Chrome azurol S. Asterisks  
269 represent significance at alpha = 0.008 (after Bonferroni correction)."

**The authors have altered figure descriptions to address concerns and they have been updated as such (Figure 1 is now Figure 4):**

“**Figure 4.** 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.”

**“Figure S1**. 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 24, 48, and 72 h of incubation with Chrome azurol S. 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.”

According to the author instructions, the discussion should be focused on:  
\* Critical steps in the protocol  
\* Modifications and troubleshooting of the method  
\* Limitations of the method  
\* The significance of the method with respect to existing/alternative methods  
\* Future applications or directions of the method  
  
I'm not sure that the discussion addresses any of these points. Please rewrite and leave out any discussion of the representative results, as instructed.

**The discussion section has been re-written to address all these concerns as per our response to the editor above and reflected in Lines 373-426.**