

Submission ID #: 72014

Scriptwriter Name: Sri Lakshmi Priya

Project Page Link: <https://review.jove.com/account/file-uploader?src=21964318>

Title: Isolation of Tomato Seed-Associated Microbiota Through Controlled Germination and Comparative Culturing

Authors and Affiliations:

Daniel Silva¹, Joana do Carmo Gomes¹, Kusum Niraula¹, Jesús González-López^{2,3}, Juan Ignacio Vilchez¹

¹Instituto de Tecnologia Química e Biológica (ITQB), NOVA, iPlantMicro Lab.

²Microbiology and Environmental Technologies Section, Water Research Institute, University of Granada

³Department of Microbiology, Faculty of Pharmacy, University of Granada

Corresponding Authors:

Juan Ignacio Vilchez nacho.vilchez@itqb.unl.pt

Email Addresses for All Authors:

Daniel Silva	daniel.silva@itqb.unl.pt
Joana do Carmo Gomes	jgomes@itqb.unl.pt
Kusum Niraula	kniraula@itqb.unl.pt
Jesús González-López	jgl@ugr.es
Juan Ignacio Vilchez	nacho.vilchez@itqb.unl.pt

Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

If **Yes**, please provide the **full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author willing to participate.

Juan Ignacio Vilchez, Assistant Researcher and Group Leader at iPlantMicro Lab

Daniel Silva, MSc student

Joana do Carmo Gomes, MSc student

Current Protocol Length

Number of Steps: 19

Number of Shots: 53

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Answers are slightly edited in accordance with journal guidelines. Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. Dr. Juan Ignacio Vilchez: Our research focuses on developing a culturomic workflow to study tomato seed-associated microbiota during early seedling establishment.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. Daniel Silva: The dry-seed protocols may miss microorganisms that are recoverable only after controlled germination; this protocol consistently recovers them.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. Joana do Carmo Gomes: This protocol allows researchers to compare culturable microorganisms recovered from dry seeds and germinated seedlings.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. Daniel Silva: The key challenge in this protocol is keeping sterilization, germination, and culturing conditions identical across samples.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. Dr. Juan Ignacio Vélchez: Future studies building on this work can test whether enriched isolates improve germination, seedling establishment, or stress tolerance.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

What value do you expect this video article to have for other researchers working in your field?

1.6. **NOTE: No details were provided:** (authors will present their testimonial statements live)

1.6.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

How does the video format of your article help you to share your work with the broader scientific community?

1.7. **NOTE: No details were provided:** (authors will present their testimonial statements live)

1.7.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Surface Sterilization of Tomato Seeds and Sterility Verification

Demonstrator: NOTE: Not provided

- 2.1. To begin, obtain seeds from a single tomato seed lot and allocate them to the appropriate experimental groups [1].
 - 2.1.1. WIDE: Talent sitting with the tomato seeds on the workbench. *Videographer: Please obtain CU shot as well for this shot.*
- 2.2. Transfer 3 to 5 seeds into each sterile 2-milliliter microcentrifuge tube inside a laminar flow hood [1]. Add 1 milliliter of 70 percent ethanol to the tube to fully immerse the seeds [2]. Incubate the tubes for 5 minutes with gentle mixing using a tube rotator at 25 rpm [3]. Using a sterile pipette, remove the ethanol completely [4]. Immediately add 1 milliliter of the 20 percent sodium hypochlorite working solution to fully immerse the seeds [5].
 - 2.2.1. Talent transferring 3 to 5 tomato seeds into sterile 2 milliliter microcentrifuge tubes inside a laminar flow hood.
 - 2.2.2. Talent adding 1 milliliter of 70% ethanol to each microcentrifuge tube to fully immerse the seeds.
 - 2.2.3. Talent placing the microcentrifuge tubes on a tube rotator for incubation.
 - 2.2.4. Talent removing the ethanol completely from each tube using a sterile pipette.
 - 2.2.5. Talent adding 1 milliliter of the 20% sodium hypochlorite working solution to each tube.
- 2.3. Incubate the tubes for 10 minutes with gentle agitation using a tube rotator at 25 rpm [1]. Then, using a sterile pipette, remove the sodium hypochlorite solution completely [2].
 - 2.3.1. Talent placing the microcentrifuge tubes on a tube rotator for incubation.
 - 2.3.2. Talent removing the sodium hypochlorite solution from each tube using a sterile pipette.
- 2.4. Next, add 1 milliliter of sterile distilled water, mix gently for 30 seconds, allow the seeds to settle for 30 seconds [1], and remove the wash solution [2]. Repeat the wash 2 more times and retain the final rinse water from each tube for sterilization efficiency controls [3].

- 2.4.1. Talent adding sterile distilled water to the seeds, mixing it, and leaving it undisturbed.
- 2.4.2. Talent removing the wash solution.
- 2.4.3. Talent retaining the final rinse water from each tube.
- 2.5. Transfer the sterilized seeds to sterile Petri dishes and tubes, depending on whether they will be processed immediately as dry seeds or used for controlled germination [1].
 - 2.5.1. Talent transferring the sterilized seeds to sterile Petri dishes and sterile tubes.

NOTE: Insert a chapter title: Verification of Sterilization Efficiency

- 2.6. Prepare 10-fold serial dilutions of the final rinse water by transferring 100 microliters of rinse water into 900 microliters of 0.45 percent sodium chloride and repeating the dilution sequentially up to a 1 in 10,000 dilution [1]. Plate 100 microliters of each dilution onto LB (L-B) agar using the same plating method as for the dry-seed and germinated-seedling homogenates [2]. Then, incubate the plates at 28 degrees Celsius for 24 to 48 hours [3].
 - 2.6.1. Talent transferring 100 microliters of the rinse water into a microcentrifuge tube containing 900 microliters of sterile 0.45% sodium chloride
 - 2.6.2. Talent plating 100 microliters of each dilution onto Luria-Bertani agar plates.
 - 2.6.3. Talent placing the agar plates in an incubator.
- 2.7. After incubation, inspect the plates for colony growth [1] and record the presence or absence of colonies for each dilution [2]. If colonies are present, record the dilution level, the approximate colony number, and the visible colony morphology [3].
 - 2.7.1. Talent inspecting the Luria-Bertani agar plates for colony growth.
 - 2.7.2. Talent recording the presence or absence of colonies for each dilution.
 - 2.7.3. Talent documenting the dilution level, approximate colony number, and visible colony morphology for plates with colony growth.

3. Dry-Seed Processing

Demonstrator: NOTE: Not provided

- 3.1. Transfer pooled sets of 3 to 5 sterilized dry seeds, corresponding to one biological replicate, into sterile 2-milliliter microcentrifuge tubes containing 1-milliliter of sterile 0.45 percent sodium chloride [1]. Using a sterile disposable pestle, homogenize the seeds until the seed tissues are visibly disrupted and no intact seed structures remain [2]. Vortex the homogenate for 30 seconds to uniformly resuspend the microbial cells

[3].

- 3.1.1. Talent transferring pooled sets of 3 to 5 sterilized dry seeds into sterile 2 milliliter microcentrifuge tubes containing 1 milliliter of 0.45% sodium chloride.
- 3.1.2. Talent homogenizing the seeds using a sterile disposable pestle.
- 3.1.3. Talent vortexing the homogenate.
- 3.2. Prepare 10-fold serial dilutions of the homogenate in sterile 0.45% sodium chloride by transferring 100 microliters of homogenate into 900 microliters of sodium chloride and repeating the dilution sequentially up to 1 in 1,000,000 dilution [1]. Plate 100 microliters of the selected dilutions onto LB agar as previously demonstrated [2]. Incubate the plates at 28 degrees Celsius for 24 to 72 hours [3].
 - 3.2.1. Talent transferring 100 microliters of the homogenate into a microcentrifuge tube containing 900 microliters of sterile 0.45% sodium chloride
 - 3.2.2. Talent spread plating 100 microliters of the selected dilutions onto LB agar plates.
 - 3.2.3. Talent placing the agar plates in an incubator.
- 3.3. Select the plates corresponding to the dilution range that yields isolated and countable colonies for downstream colony-forming unit or CFU (C-F-U) estimation and isolate recovery [1]. Record the total colony count, visible colony morphotypes, and any differences in growth pattern among replicates [2].
 - 3.3.1. Talent selecting the agar plates with isolated and countable colonies.
 - 3.3.2. Talent recording the total colony count, visible colony morphotypes, and differences in growth pattern among replicates.

NOTE: Insert the chapter title: Axenic Germination of Sterilized Seeds

- 3.4. Place the sterilized seeds on Murashige and Skoog or MS (M-S) agar plates [1]. Space the seeds evenly to avoid contact between emerging seedlings [2]. Seal the plates with laboratory sealing film or an equivalent material that reduces external contamination while allowing normal germination [3]. Incubate the seeds at 25 degrees Celsius in the dark in a horizontal position [4].
 - 3.4.1. Talent placing the sterilized seeds onto a sterile Murashige and Skoog agar plate.
 - 3.4.2. Close-up of the seeds spaced evenly on the agar surface.
 - 3.4.3. Talent sealing the agar plate with laboratory sealing film.
 - 3.4.4. Talent placing the wrapped agar plate horizontally in the incubator.
- 3.5. Allow the seeds to germinate under sterile conditions until early radicle establishment is clearly visible [1]. Inspect the plates regularly for signs of contamination, abnormal germination, or excessive condensation [2].

- 3.5.1. Close-up of germinated seeds showing clearly visible early radicle emergence on the agar plate.
- 3.5.2. Talent inspecting the agar plate for contamination, abnormal germination, and excessive condensation.

4. Isolation of Bacteria from Germinated Seedlings

Demonstrator: NOTE: Not provided

- 4.1. Using sterile forceps, collect the germinated seedlings inside a laminar flow hood [1]. Transfer pooled material from 3 to 5 germinated seedlings, corresponding to one biological replicate, into sterile 2-milliliter microcentrifuge tubes [2]. Add 1 milliliter of sterile 0.45% sodium chloride to each tube [3]. Using a sterile disposable pestle, homogenize the pooled seedling material thoroughly, including both the emerging root and the associated residual seed tissue, until the tissues are visibly disrupted [4]. Vortex the homogenate for 30 seconds to uniformly resuspend the microbial cells [5].
 - 4.1.1. Talent collecting germinated seedlings using sterile forceps inside a laminar flow hood.
 - 4.1.2. Talent transferring pooled material from 3 to 5 germinated seedlings into a sterile 2 milliliter microcentrifuge tube.
 - 4.1.3. Talent adding 1 milliliter of sterile 0.45% sodium chloride to the microcentrifuge tube.
 - 4.1.4. Talent homogenizing the pooled seedling material with a sterile disposable pestle until the tissues are visibly disrupted.
 - 4.1.5. Talent vortexing the homogenate.
- 4.2. Prepare 10-fold serial dilutions of the homogenate by transferring 100 microliters of homogenate into 900 microliters of sterile 0.45% sodium chloride and repeating the dilution sequentially up to a 1 in 10,000 dilution [1]. Plate 100 microliters of the selected dilutions onto LB agar using sector drop plating [2]. Incubate the plates at 28 degrees Celsius for 24 to 72 hours [3]. Record the total colony count, visible colony morphotypes, and differences relative to the dry-seed samples [4].
 - 4.2.1. Talent transferring 100 microliters of the homogenate into a microcentrifuge tube containing 900 microliters of sterile 0.45% sodium chloride
 - 4.2.2. Close-up shot of dispensing 100 microliters of each selected dilution as separate drops onto the designated sectors of an LB agar plate.
 - 4.2.3. Talent placing the agar plates in an incubator.
 - 4.2.4. Talent examining the incubated agar plates and recording the total colony count, visible colony morphotypes, and differences relative to the dry-seed samples.

5. Colony Counting and Isolate Recovery

Demonstrator: NOTE: Not provided

5.1. Select the dilution plates that yield countable and well-separated colonies [1]. Use plates containing 30 to 300 colonies for CFU estimation [2].

5.1.1. Talent examining the dilution series and selecting plates with well-separated colonies.

5.1.2. Close-up of the selected agar plate showing approximately 30 to 300 well-separated colonies.

5.2. Count the colonies on the selected plates [1]. If more than one plate from the same dilution is used, calculate the mean colony count for that dilution [2]. Calculate the CFU per biological replicate [3-TXT].

5.2.1. Close-up of the selected agar plate as the talent counts the colonies using a colony marker or counter.

5.2.2. Close-up of the selected replicate agar plates as the talent compares the colony counts and calculates the mean colony count.

5.2.3. Close-up of the selected agar plates while the colony-forming unit calculation is illustrated. **TXT: CFU per replicate = (number of colonies × dilution factor)/plated volume (mL)**

5.3. Record the colony morphology using standardized descriptors, including size, shape, color, edge, elevation, and opacity [1]. Select colonies for downstream isolation to minimize sampling bias [2]. Select all distinguishable morphotypes when colony numbers are low, [3] or randomly select multiple colonies across plates when colony numbers are high [4].

5.3.1. Talent recording the size, shape, color, edge, elevation, and opacity of the colony.

5.3.2. Talent marking colonies selected for downstream isolation on the underside of the agar plate.

5.3.3. Close-up of an agar plate with a few colonies as the talent marking each visibly distinct colony morphotype on the underside of the plate.

5.3.4. Close-up of multiple agar plates with numerous colonies as the talent marking colonies distributed across different regions of the plates for random selection

5.4. Restreak the selected colonies onto fresh LB agar plates to obtain pure cultures [1]. Repeat the restreaking until each isolate shows a uniform colony morphology on the plate [2].

5.4.1. Shot of restreaking a selected colony onto a fresh Luria-Bertani agar plate.

5.4.2. Close-up of a pure culture plate showing uniform colony morphology after repeated restreaking.

5.5. Identify representative bacterial isolates by 16S ribosomal RNA gene sequencing [1].

5.5.1. Shot of purified genomic DNA samples, polymerase chain reaction tubes, and a thermal cycler prepared for 16S ribosomal RNA gene amplification.

Results

6. Results

- 6.1. Processing of dry, non-germinated seeds yielded lower culturable recovery [1] than germinated seedlings, as shown by the absolute CFU distribution [2].
 - 6.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the stacked bar “Dry seed”*
 - 6.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the stacked bar “Germinated seed”.*
- 6.2. Germination also changed the relative composition of recovered culturable taxa, with several microorganisms increasing after germination [1].
 - 6.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight both the stacked bars.*
- 6.3. Species-level analysis showed selective enrichment after germination, including increases in *Stutzerimonas stutzeri*, *Priestia megaterium*, and *Paenibacillus amylolyticus* [1].
 - 6.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight the bars for Stutzerimonas stutzeri, Priestia megaterium, and Paenibacillus amylolyticus.*
- 6.4. Representative plates illustrated colony recovery, serial dilution, and purification of isolates with distinct morphologies [1].
 - 6.4.1. LAB MEDIA: Figure 4.

1. Protease
Pronunciation link: <https://www.merriam-webster.com/dictionary/protease>
IPA: /'proʊ.ti.ɛɪs/
Phonetic Spelling: proh-tee-ays
2. Substrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/substrate>
IPA: /'sʌb.streɪt/
Phonetic Spelling: sub-strayt
3. Potyviral
Pronunciation link: No confirmed link found
IPA: No verified IPA available
Phonetic Spelling: poh-tee-vy-ruhl
4. Synthetic biology
Pronunciation link: <https://www.merriam-webster.com/dictionary/synthetic>
IPA: /sɪn'theɪ.tɪk baɪ'ɔː.lə.dʒi/
Phonetic Spelling: sin-thet-ik bye-ol-uh-jee
5. Therapeutic
Pronunciation link: <https://www.merriam-webster.com/dictionary/therapeutic>
IPA: /ˌθer.ə'piː.ju.tɪk/
Phonetic Spelling: thair-uh-pyoo-tik
6. High-throughput
Pronunciation link: <https://www.merriam-webster.com/dictionary/high-throughput>
IPA: /ˌhaɪ'θruː.pʊt/
Phonetic Spelling: hy-throo-put
7. Variants
Pronunciation link: <https://www.merriam-webster.com/dictionary/variant>
IPA: /'ver.i.ənt/
Phonetic Spelling: vair-ee-uhnt
8. Specificities
Pronunciation link: <https://www.merriam-webster.com/dictionary/specificity>
IPA: /ˌspes.ə'fɪs.ə.ti/
Phonetic Spelling: spess-uh-fis-uh-tee
9. Biochemically
Pronunciation link: <https://www.merriam-webster.com/dictionary/biochemical>
IPA: /ˌbaɪ.əʊ'kem.i.kli/
Phonetic Spelling: bye-oh-kem-ik-lee
10. Catalytic

Pronunciation link: <https://www.merriam-webster.com/dictionary/catalytic>

IPA: /ˌkæt.əˈlɪt.ɪk/

Phonetic Spelling: kat·uh·lit·ik

11. Fluorescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>

IPA: /flʊˈres.əns/

Phonetic Spelling: floor·ess·uhns

12. Electroporation

Pronunciation link: <https://www.merriam-webster.com/dictionary/electroporation>

IPA: /ɪˌlek.troʊ.pəˈreɪ.ʃən/

Phonetic Spelling: ih·lek·troh·puh·ray·shuhn

13. Reagents

Pronunciation link: <https://www.merriam-webster.com/dictionary/reagent>

IPA: /riˈeɪ.dʒənt/

Phonetic Spelling: ree·ay·juhnt

14. EBY100

Pronunciation link: No confirmed link found

IPA: No verified IPA available

Phonetic Spelling: ee·bee·why·one·zero·zero

15. Glycerol

Pronunciation link: <https://www.merriam-webster.com/dictionary/glycerol>

IPA: /ˈɡlɪs.əˌrɔːl/

Phonetic Spelling: glis·uh·rawl

16. Erlenmeyer

Pronunciation link: <https://www.merriam-webster.com/dictionary/Erlenmeyer%20flask>

IPA: /ˈɜː.lənˌmaɪ.ə/

Phonetic Spelling: er·len·my·er

17. Micropipette

Pronunciation link: <https://www.merriam-webster.com/dictionary/micropipette>

IPA: /ˌmaɪ.kroʊ.paɪˈpet/

Phonetic Spelling: my·kroh·pie·pet

18. Conical

Pronunciation link: <https://www.merriam-webster.com/dictionary/conical>

IPA: /ˈkɔː.nɪ.kəl/

Phonetic Spelling: kon·ih·kuhl

19. Biosafety

Pronunciation link: <https://www.merriam-webster.com/dictionary/biosafety>

IPA: /ˌbaɪ.əʊˈseɪf.ti/

Phonetic Spelling: bye·oh·sayf·tee

20. Programmable

Pronunciation link: <https://www.merriam-webster.com/dictionary/programmable>

IPA: /proʊˈgræm.ə.bəl/

Phonetic Spelling: proh·gram·uh·buhl