

Quantification of Root Colonizing Bacteria

Maged M. Saad^{1,*}, Axel de Zélicourt^{1,2}, Eleonora Rolli², Lukas Synek¹ and Heribert Hirt^{1,*}

¹Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia; ²Institut of Plant Sciences Paris-Saclay (IPS2), UMR 9213/UMR1403, CNRS, INRA, Université Paris-Sud, Université d'Evry, Université Paris-Diderot, Sorbonne Paris-Cité, Bâtiment 630, 91405 Orsay, France

*For correspondence: Maged.saad@kaust.edu.sa; Heribert.hirt@kaust.edu.sa

[Abstract] Here we describe a simple method to quantify the number of viable bacteria (e.g., *Enterobacter* sp. SA187) that colonize the root system of *Arabidopsis thaliana*.

Keywords: *Arabidopsis thaliana*, Colony forming unit, Endophytes

[Background] Colonization of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant. *Enterobacter* sp. SA187 is an endophytic bacterium that has been isolated from root nodules of the indigenous desert plant *Indigofera argentea* (Andrés-Barrao *et al.*, 2017; Lafi *et al.*, 2017). SA187 promotes the growth of the model plant *Arabidopsis thaliana* under diverse abiotic stresses such as salinity, drought or high temperature, demonstrating an important potential for application as Plant Growth Promoting Bacteria (PGPR) to improve abiotic resistance and yield of crops in arid lands. SA187 can colonize both roots and shoots of *A. thaliana* on ½ MS agar plates or in soil (de Zélicourt *et al.*, 2018). To follow the fate of inoculant strain SA187 on the non-host plant *A. thaliana*, we applied a routinely cultivation-dependent method. This protocol has been successfully used to mounter the bacterial number colonized both root and shoot of the *A. thaliana* plant under different abiotic stresses (de Zélicourt *et al.*, 2018).

Materials and Reagents

1. Mechanical pipette tips
2. 1.5, 2 ml Eppendorf tube
3. Razor Blades (LabSupplyOutlaws, catalog number: 12-640)
4. Nunc™ 96 Microtiter Plates (Thermo Fisher Scientific, Thermo Fisher™, catalog number: 262162)
5. Disposable Tissue Grinder Pestle (Corning, Axygen®, catalog number: PES-15-B-SI)
6. TissueLyser II .Gentech Biosciences (GENTECH BIOSCIENCES, catalog number: 85300)
7. Lab Parafilm M (Labdirect, catalog number: PM992)
8. 100 mm Petri dish (Corning, Falcon®, catalog number: 351029)
9. Square Petri Dish with Grid (SIMPORT, catalog number: D210-16)
10. Stainless Steel Beads, 2.3 mm diameter (Bio Spec Products, catalog number: 11079123ss)

11. Seeds of *Arabidopsis* (*Arabidopsis thaliana*)
12. Murashige and Skoog Basal Salt Mixture (MS) (Sigma-Aldrich, catalog number: M5524)
13. LB Broth with agar (Lennox) (Sigma-Aldrich, catalog number: L2897)
14. Magnesium chloride anhydrous, $\geq 98\%$ (Sigma-Aldrich, catalog number: M8266)
15. Sodium chloride BioXtra, $\geq 99.5\%$ (Sigma-Aldrich, catalog number: S7653)
16. Potassium hydroxide (Sigma-Aldrich, catalog number: 484016)
17. Silwet[®] L-77 (PhytoTechnology Laboratories, catalog number: S7777)

Equipment

1. Tweezers with Sharp Tip (Labdirect, catalog number: 560.002.115)
2. Top centrifuge (Eppendorf)
3. Vortex (VWR digital Vortex Mixer)
4. 28 °C shaker-incubator
5. Growth chamber (Percival Scientific or similar types. Set the growth conditions to 22 °C, 16 h light/8 h dark cycles)
6. TissueLyser II[®] (QIAGEN)
7. Autoclave (TOMY SEIKO, model: LSX-500 or similar types)
8. Mechanical pipettes (Gilson, catalog numbers: F123601 (P200) and F123602 (P1000))
9. Analytical balance (0.1 mg resolution)

Procedure

A. Prepare the bacterial culture

1. Revive the SA187 bacterial strains (or any other strain) on LB Agar media (or equivalent media) and incubate at 28 °C for 24 h.
2. Inoculate 10 ml LB media with a single colony on a shaker incubator with 220 rpm at 28 °C for 16 h (overnight) (pre-culture).
3. Take 100 μ l of the O.N. culture to start a new fresh 2 ml culture and let the bacteria grow for 2-3 h.
4. Prepare and autoclave $\frac{1}{2}$ MS with 0.9% agar adjusted to pH 5.8 with 1 M KOH. Allow the medium to cool down to reach 37-40 °C before pouring the plates.

B. Bacterialized vertical plate culture of *Arabidopsis* seedlings

1. In a 50 ml Falcon tube mix 50 ml of warm $\frac{1}{2}$ MS medium with 0.1 ml of a bacterial suspension ($OD_{600} = 0.21$) to obtain 10^7 bacteria, dispense the media in square Petri dishes (Figure 1) and let the agar solidify. For control plates (no bacteria) add 0.1 ml of liquid LB.

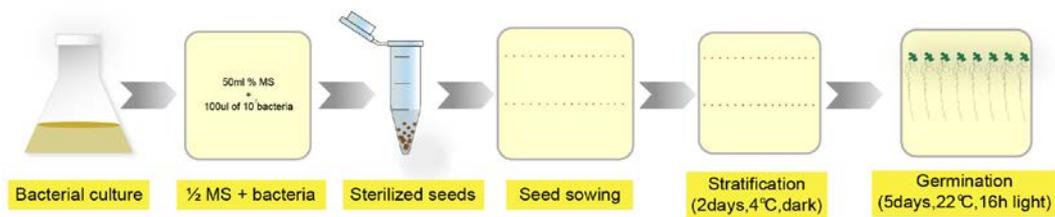


Figure 1. Schematic illustration of different steps for preparing the bacterIALIZED vertical plates of *Arabidopsis* seedlings

2. Sterilize *Arabidopsis* seeds following a standard protocol (de Zélicourt *et al.*, 2018) or similar methods described elsewhere and sow the sterilized seeds. Keep the plates and seeds at 4 °C in the dark for 2 days.
3. Transfer the plates to a growth chamber and keep them vertically for 5 days in long day conditions: 16 h-light /8-h dark cycle at constant 22 °C.
4. Transfer the colonized seedlings to ½ MS plates with or without 100 mM NaCl and incubate for 10 days or as desired.

C. Bacterial Quantification: Colony Forming Units

1. With forceps and razor blades, separate root and shoot and combine three roots and/or three shoots in one 1.5 ml tube, record the fresh weight.
2. Physically grind using the plastic cone grinder first without MgCl₂ and then add 500 µl 10 mM MgCl₂ supplemented with 0.02% Silwet L-77 and vortex vigorously (Figure 2).

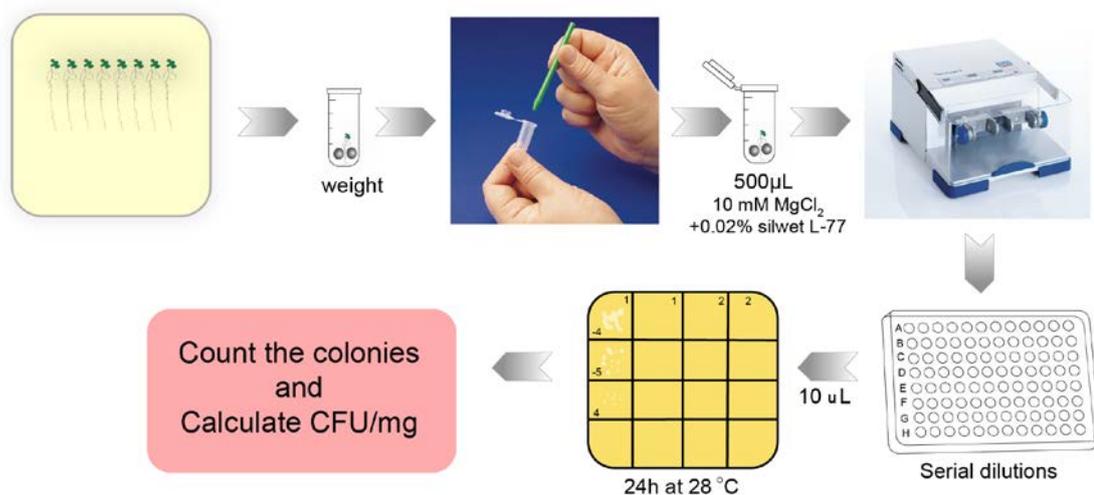


Figure 2. Schematic illustration of different steps for the quantification of bacteria in *Arabidopsis* plants

3. Use TissueLyserII with Stainless Steel Beads to homogenize the plant material.
4. Proceed with serial dilutions by removing 10 µl and add to 90 µl of sterile distilled H₂O in 96 microtiter plates.

5. Spot 10- μ l samples (3 replicates) from each dilution on LB agar plates. Allow spotted samples to dry, cover the plates, and wrap them with Parafilm.
6. Incubate the plates at 28 °C for 24 h or until the colonies are clearly visible. Count colonies from each dilution, record the colony forming units (CFU), and calculate CFU/mg plant material.

Note: All work shall be done under sterile conditions.

Data analysis

Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor. The number of colonies per ml reported should reflect the precision of the method and should not include more than two significant figures.

The CFU/ml can be calculated using the following formula:

$$\text{CFU/ml} = (\text{number of colonies} \times \text{dilution factor}) / \text{volume of culture plate}$$

CFU value could also be divided by the root/shoot fresh weight to get CFU/mg plant material.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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