Please cite this article as: Imène *et. al.*, (2017). Bioassay of *Xanthomonas albilineans* Attachment on Sugarcane Leaves, *Bio-protocol* 7 (2): e2111. DOI: 10.21769/BioProtoc.2111.

bio-protocol www.bio-protocol.org/e2111 Vol 7, Iss 02, Jan 20, 2017 DOI:10.21769/BioProtoc.2111

Bioassay to Study the Attachment of *Xanthomonas albilineans* on Sugarcane Leaves

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[Abstract] Sugarcane (interspecific hybrids of *Saccharum* species) is an economically important crop that provides 70% of raw table sugar production worldwide and contributes, in some countries, to bioethanol and electricity production. Leaf scald, caused by the bacterial plant pathogen *Xanthomonas albilineans*, is one of the major diseases of sugarcane. Dissemination of *X. albilineans* is mainly ensured by contaminated harvesting tools and infected stalk cuttings. However, some strains of this pathogen are transmitted by aerial means and are able to survive as epiphytes on the sugarcane phyllosphere before entering the leaves and causing disease. Here we present a protocol to estimate the capacity of attachment of *X. albilineans* to sugarcane leaves. Tissue-cultured sugarcane plantlets were immersed in a bacterial suspension of *X. albilineans* and leaf attachment of *X. albilineans* was determined by two methods: leaf imprinting (semi-quantitative method) and leaf washing/homogenization (quantitative method). These methods are important tools for evaluating pathogenicity of strains/mutants of the sugarcane leaf scald pathogen.

Keywords: Attachment, Leaf imprinting, Leaf scald, Pathogenicity, Phyllosphere, Sugarcane, Tissue culture, *Xanthomonas albilineans*

[Background] The mechanisms that govern the interactions between *X. albilineans* and its host plant (the sugarcane) are not well known. Albicidin, a phytotoxin produced by *X. albilineans*, is the only molecular factor which has been demonstrated to play a role in pathogenicity of this pathogen (Birch, 2001). However, pathogenicity of *X. albilineans* doesn't completely depend on albicidin. Albicidin-deficient mutants are still able to colonize efficiently the sugarcane stalk and to produce disease symptoms (Birch, 2001; Rott *et al.*, 2011). Studies using full grown sugarcane are space and time consuming. Bioassays using miniaturized plants (tissue-cultured plants) or detached leaf bioassays can be very useful because they are less space consuming and they allow the study of plant-pathogen interactions in controlled environments. *In vitro* propagation of plants is widely used to rapidly propagate disease-free planting material under controlled conditions (Kumar and Reddy, 2011). Additionally, leaf imprinting has been widely used to study the ecology of bacteria associated with the phyllosphere (Hirano and Upper, 2000; Yadav *et al.*, 2010). However, to our knowledge, these techniques have never been associated to decipher pathogenicity of bacterial plant pathogens. To identify additional pathogenicity factors of *X. albilineans*, especially factors involved in the early phases of infection (epiphytic phase), we developed a new miniaturized bioassay using tissue cultured sugarcane plantlets.

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Attachment of *X. albilineans* to sugarcane leaves under axenic condition was reproduced (Fleites *et al*., 2013; Mensi *et al*., 2016). This bioassay will permit the rapid testing of leaf attachment capacity of wild type and mutant strains of the pathogen causing leaf scald disease, but also of other bacteria colonizing the sugarcane leaf canopy.

Materials and Reagents

1. Sterile scalpels blades (Swan Morton, catalog numbers: n° 11 and n° 24)
2. Sterilized pipette tips
   200 µl (Thermo Fischer Scientific, Fischer Scientific, catalog number: 02-681-2)
   1,000 µl (Thermo Fischer Scientific, Fischer Scientific, catalog number: 02-681-4)
3. Sterile plastic loops (Greiner Bio One, catalog number: 731171)
4. Falcon 15 ml conical centrifuge tubes (SARSTEDT, catalog number: 62.554.502)
5. Soft tissue (Orapi Hygiène, catalog number: 186)
6. Disposable, sterile splinter removers/tweezers – 11.1 cm. (4 ¼ in.) (TSIC Solution, catalog number: UTIL-1037)
7. 90 x 15 mm Petri dishes (Corning, Gosselin™, catalog number: BP93B-15)
8. 1.5 ml microcentrifuge tube (SARSTEDT, catalog number: 72.690.001)
9. Disposable pellet pestle for 1.5 ml centrifuge tube (Kimble Chase Life Science and Research Products, catalog number: 749521-1500)
10. Sugarcane plantlets (cultivar CP68-1026 susceptible to leaf scald disease of sugarcane)
11. *Xanthomonas albilineans* wild type strains and mutants affected in pathogenicity (grown for 4 to 5 days on Wilbrink medium + appropriate antibiotics); for characteristics of mutants, see Fleites *et al*. (2013) and Mensi *et al.* (2016)
12. Sterile distilled water
13. Tween 20 (Sigma-Aldrich, catalog number: P2287)
14. Sucrose (Merck Millipore, catalog number: 107687)
15. Peptone (BD, Bacto™, catalog number: 211677)
16. Potassium phosphate, dibasic, trihydrate, $K_2HPO_4 \cdot 3H_2O$ (EMD Millipore, Calbiochem®, catalog number: 529567)
17. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (EMD Millipore, catalog number: 105886)
18. Sodium sulfite (Na_2SO_3) (EMD Millipore, catalog number: 106657)
19. Agar (BD, Bacto™, catalog number: 214010)
20. Potassium bromide, KBr (Sigma-Aldrich, catalog number: P0838)
21. Benomyl (Sigma-Aldrich, catalog number: 381586)
22. Cycloheximide (Sigma-Aldrich, catalog number: C1988)
23. Ethanol (Sigma-Aldrich, catalog number: 32294)
    Note: This product has been discontinued.
24. Cephalexin (Sigma-Aldrich, catalog number: C0675000)

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25. Novobiocin (Sigma-Aldrich, catalog number: 1475008)
26. Kasugamycin (Sigma-Aldrich, catalog number: 19408-46-9)
27. Ammonium nitrate (NH_4NO_3) (Sigma-Aldrich, catalog number: A3795)
28. Potassium nitrate (KNO_3) (Sigma-Aldrich, catalog number: P8291)
29. Calcium nitrate tetrahydrate ($Ca(NO_3)_2 \cdot 4H_2O$) (Sigma-Aldrich, catalog number: C2786)
30. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Sigma-Aldrich, catalog number: 63138)
31. Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich, catalog number: P5655)
32. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P5405)
33. Boric acid (H_3BO_3) (Sigma-Aldrich, catalog number: B6768)
34. Manganese(II) sulfate monohydrate ($MnSO_4 \cdot H_2O$) (Sigma-Aldrich, catalog number: M7899)
35. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Sigma-Aldrich, catalog number: Z1001)
36. Potassium iodide (KI) (Sigma-Aldrich, catalog number: P8166)
37. Ammonium molybdate tetrahyddrate ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) (Sigma-Aldrich, catalog number: M1019)
38. Copper(II) nitrate trihydrate ($Cu(NO_3)_2 \cdot 3H_2O$) (Sigma-Aldrich, catalog number: 61194)
39. Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (EMD Millipore, catalog number: 103965)
40. $Na_2EDTA \cdot 2H_2O$ (Sigma-Aldrich, catalog number: E5134)
41. Nicotinic acid (Sigma-Aldrich, catalog number: N4126)
42. Pyridoxol hydrochloride (Sigma-Aldrich, catalog number: P6280)
43. Myo-inositol (Sigma-Aldrich, catalog number: I7508)
44. Thiamine dichloride (Sigma-Aldrich, catalog number: T1270)
45. Phytagel (Sigma-Aldrich, catalog number: P8169)
46. Wilbrink medium (WM) (see Recipes)
47. Wilbrink Selective Davis (WSD) medium (see Recipes)
48. Macronutrients (see Recipes)
49. Micronutrients (see Recipes)
50. Ferric EDTA (see Recipes)
51. Fuji vitamins (see Recipes)
52. Nutritive medium for growth of sugarcane plantlets (see Recipes)

Equipment

1. Growth chamber
2. 200 x 20 mm Pyrex test tubes with cap (Legallais, catalog number: 761224)
3. 200 mm long tweezers with blunt tips (VWR, catalog number: 82027-436)
4. Micropipettes
   20-200 µl (Eppendorf, catalog number: 3120000054)
   100-1,000 µl (Eppendorf, catalog number: 3120000062)
5. Scalpels (Swan Morton, catalog numbers: N° 3G S/S and N° 4G S/S)

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6. Incubator for microbiology (Memmert, model: B40)
   Note: This product has been discontinued.
7. Benchtop vortex (Scientific Industries, model: Vortex Genie 2)
8. Spectrophotometer (Eppendorf Biophotometer)
9. 250 or 500 ml wide neck Erlenmeyer flasks (Borosilicate glass) (Duran, catalog number: 21 226 36 or 21 226 44)
10. Laminar flow cabinet or sterile hood
11. Autoclave

Software

1. Package R, version 2.14.1 (R Development Core Team)

Procedure

A. Preparation of tissue-cultured sugarcane plantlets
   1. Tissue cultured plantlets of sugarcane cultivar CP68-1026 originating from apex tissue are propagated and maintained in a growth chamber at 28 °C with 12 h light.
   2. Four weeks prior to inoculation, young secondary tillers are transferred into new 200 x 20 mm test tubes containing the nutritive medium for tissue cultured plantlets, as follows:
      a. Using 200 mm long tweezers, remove the tissue cultured-plantlet from the tube and place it on a glass plate under a sterile hood, separate young secondary tillers from the primary tiller with a scalpel and sterile blades.
      b. Using 200 mm long tweezers, place each secondary tiller in a new 200 x 20 mm test tube containing the nutritive medium for sugarcane plantlets. Secondary tillers may vary in size but only tillers that were 150-200 mm long were transferred for future inoculation. All transferred plantlets must have two to four unfolded leaves.
   3. Incubate the test tubes in a growth chamber at 28 °C with 12 h of light for four weeks.

B. Bacterial inoculum preparation
   1. Five days before plant inoculation, streak out each strain of *X. albilineans* to be tested from -80 °C distilled water stocks using a sterilized blade or a pipet tip onto WM agar plates with appropriate antibiotics (for mutant strains) and let the cultures grow for 5 days in an incubator at 28 °C.
   2. For each strain, suspend bacterial colonies from the agar plate with a sterile polypropylene loop in 10 ml of sterile distilled water in a 15 ml Falcon conical centrifuge tubes (to obtain a turbid suspension). Vortex the bacterial suspension manually or gently with a benchtop vortex and then measure its optical density at 600 nm (OD_{600}) using a spectrophotometer.

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3. To prepare the stock suspension, adjust the bacterial suspension with sterile distilled water to obtain an OD_{600} = 0.25-0.30 which corresponds to 10^9 Colony Forming Units (CFU)/ml.
4. To prepare the inoculum at 10^7 CFU/ml, dilute 5 ml of the bacterial suspension calibrated at 10^9 CFU/ml in 495 ml of sterile distilled water in a 500 ml Erlenmeyer flask.

C. Sugarcane plantlets inoculation by immersion

1. For inoculation, use plantlets with two to four fully unfolded leaves (Figure 1).

Figure 1. Sugarcane plantlet exhibiting three fully expanded leaves in a test tube

2. Use sterile tweezers to immerse the leaves of the tissue-cultured plantlets into the bacterial suspension adjusted at 10^7 CFU/ml for 30-40 sec (Figure 2). During immersion of leaves, make gentle circular movements of the Erlenmeyer flask in order to optimize the contact of the bacterial suspension with the sugarcane leaf surfaces.

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Figure 2. Immersion of leaves of a sugarcane plantlet for 30-40 sec in a *X. albilineans* suspension adjusted at 10^7 CFU/ml

3. After immersion, remove excess of bacterial suspension by dragging the leaves across the edge of the wall of the Erlenmeyer flask: no visible droplet should be seen on the leaf surfaces. Then, replace the inoculated plantlets in the same test tubes containing the same nutritive medium (using sterile tweezers).
4. Keep inoculated plantlets in a growth chamber at 28 °C with 12 h of light for 14 days.
5. Inoculate at least six (for leaf imprinting assay, cf. D.1) to 15 (for leaf washing and tissue homogenization assay, cf. D.2) sugarcane plantlets per strain of *X. albilineans* and distribute them randomly in the growth chamber.
6. Inoculate control plantlets with only sterile distilled water.

D. Determination of *X. albilineans* leaf attachment

Leaf attachment of *X. albilineans* can be determined using two methods: 1. imprints of sugarcane leaves on selective medium (semi-quantitative method) and 2. leaf washing and tissue homogenization (quantitative method).

1. Determination of capacity of *X. albilineans* leaf attachment by leaf imprinting
   a. Fourteen days after immersion, remove the inoculated sugarcane plantlets (one by one) from the test tubes and put them on a sterile soft tissue (Figure 3). Use a scalpel with a sterile blade to remove leaves (remove and imprint only non-withered leaves).

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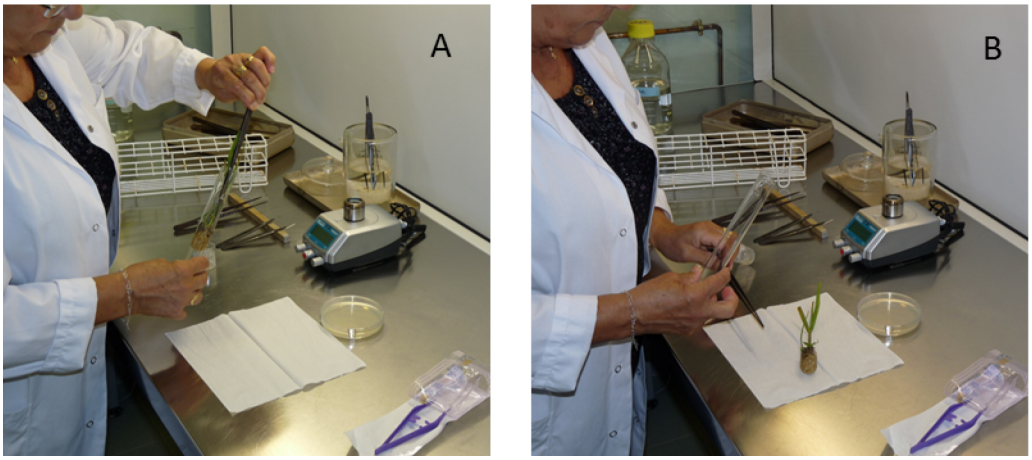

Figure 3. Removal of the sugarcane plantlet from the test tube in an aseptic work area (laminar flow cabinet). A. Remove the inoculated sugarcane plantlet from the test tube using long sterile tweezers, 14 days after immersion and B. put it on a sterile soft tissue.

b. Using disposable sterile tweezers, gently place detached leaves on WSD medium in 90 x 15 mm plates and apply a soft pressure with the tweezers to imprint the lower and then the upper leaf surface on the agar medium (Figure 4). Leaves for imprinting may vary in length but must have a green color. If the length of the leaf exceeds the plate diameter, cut the leaf into two or three fragments before performing the imprint. The quality of the imprinted leaf area can be observed by trans-illumination using day or artificial light.

Figure 4. Imprint the inoculated sugarcane leaf (upper and lower surfaces) on WSD medium using disposable sterile tweezers

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c. Place agar plates in an incubator at 28 °C for 5 days.
d. Five days after incubation, examine plates for presence or absence of *X. albilineans* colonies in the imprint area. Density of *X. albilineans* colonies growing in the imprint area can vary between absences of colonies to confluent growth of bacteria in the imprint area (Figure 5).

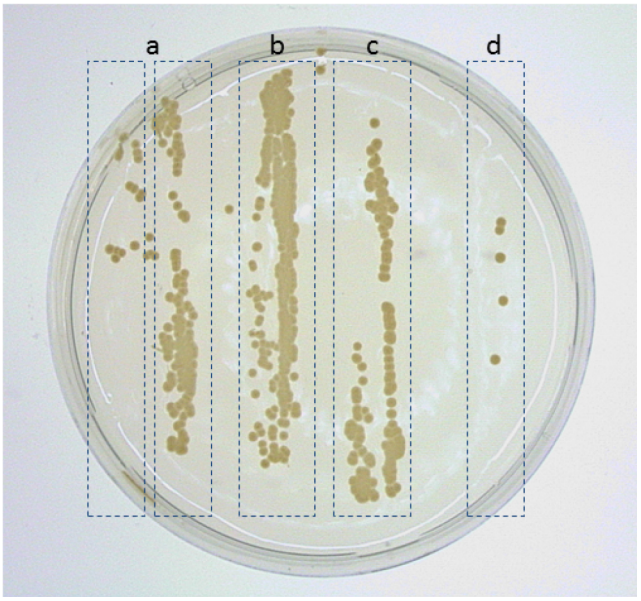

Figure 5. Growth of colonies of *X. albilineans* in imprints of sugarcane leaves on WSD medium, 14 days after immersion of leaves in a bacterial suspension adjusted at 10^7 CFU/ml. a. Imprint of the upper surface of leaf n1; b. Imprint of the lower surface of leaf n1; c. Imprint of the upper surface of leaf n2; d. Imprint of the lower surface of leaf n2. Leaf n1 is the first leaf from the top of the sugarcane plantlet that is completely unfolded. Leaf n2 is the leaf immediately below leaf n1 (Reprinted from Fleites *et al*., 2013).

e. To estimate Extent of Leaf Attachment (ELA) of *X. albilineans*, give a score ranging from 0 to 6 to each leaf imprint (lower and upper surfaces) using the following scale (Figure 6):
0 = 0 to 5 five colonies in the leaf imprint,
1 = 6 to 50 colonies in the leaf imprint,
2 = more than 50 colonies and no confluent growth,
3 = confluent growth of bacteria in less than 10% of the leaf imprint,
4 = confluent growth of bacteria in 10% to 40% of the leaf imprint,
5 = confluent growth of bacteria in 41% to 80% of the leaf imprint,
6 = confluent growth of bacteria in 81% to 100% of the imprint.

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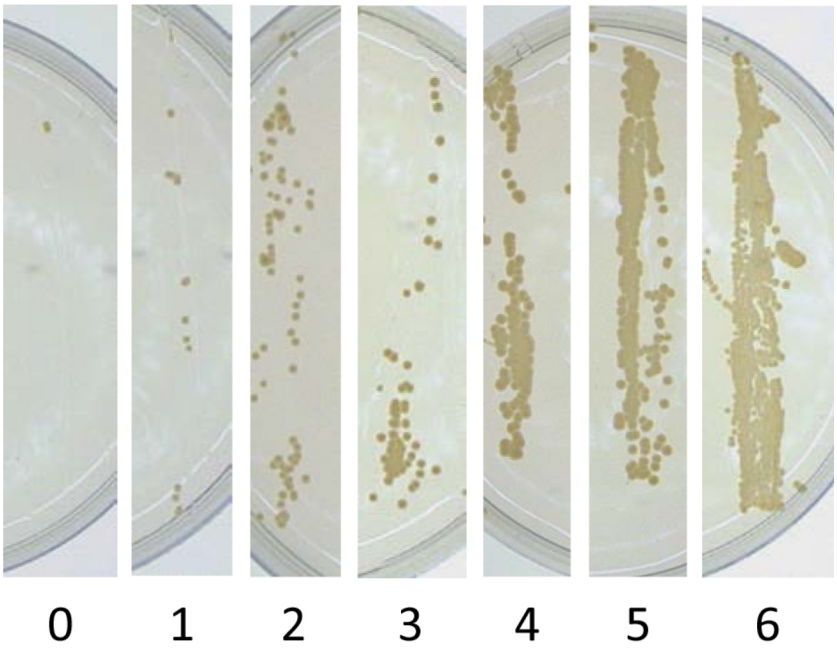

Figure 6. Score used to estimate Extent of Leaf Attachment (ELA). 0 = 0 to 5 five colonies in the leaf imprint, 1 = 6 to 50 colonies in the leaf imprint, 2 = more than 50 colonies and no confluent growth, 3 = confluent growth of bacteria in less than 10% of the leaf imprint, 4 = confluent growth of bacteria in 10% to 40% of the leaf imprint, 5 = confluent growth of bacteria in 41% to 80% of the leaf imprint, 6 = confluent growth of bacteria in 81% to 100% of the leaf imprint (Reprinted from Mensi *et al*., 2016).

f. Calculate ELA for each inoculated plantlet as follows:
$$ELA = 100\ ([1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5 + 6 \times N_6]/[6 \times N_T])$$
Where,
N_i is the number of leaf imprints with score i (i is the individual scale),
N_T is the total number of leaf imprints per plantlet.

g. Calculate average ELA for each bacterial strain or mutant tested using all ELA values obtained for the six inoculated plantlets.

2. Determination of *X. albilineans* leaf attachment by leaf washing and tissue homogenization.
For this bioassay, 15 sugarcane plantlets were inoculated per strain. At each sampling time (1 h, 7 and 14 days), five plantlets and two leaves per plantlet were individually used to determine attachment capacity of *X. albilineans*. The experiment is repeated independently at least two times. Leaf washing is performed to detach and quantify bacteria that are located on the leaf surface, whereas leaf homogenization is performed to isolate and quantify bacteria from protected areas (such as stomatal chambers) or from inside the leaf.

a. Leaf washing

i. At 1 h, 7 and 14 days after immersion of sugarcane leaves in the bacterial suspension, use sterile tweezers to remove plantlets from the test tubes, put them on sterile soft

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tissue (Figure 3) and cut two leaves from each plantlet (using a scalpel with a sterile blade). Collect only inoculated and non-withered leaves.

ii. Cut a 7 cm long fragment from each leaf (Figure 7A) and immerse each fragment individually in a 1.5 ml micro-centrifuge tube containing 1 ml of sterile distilled water with 0.005% Tween 20 (Figure 7B). Note that each leaf fragment is analyzed separately.

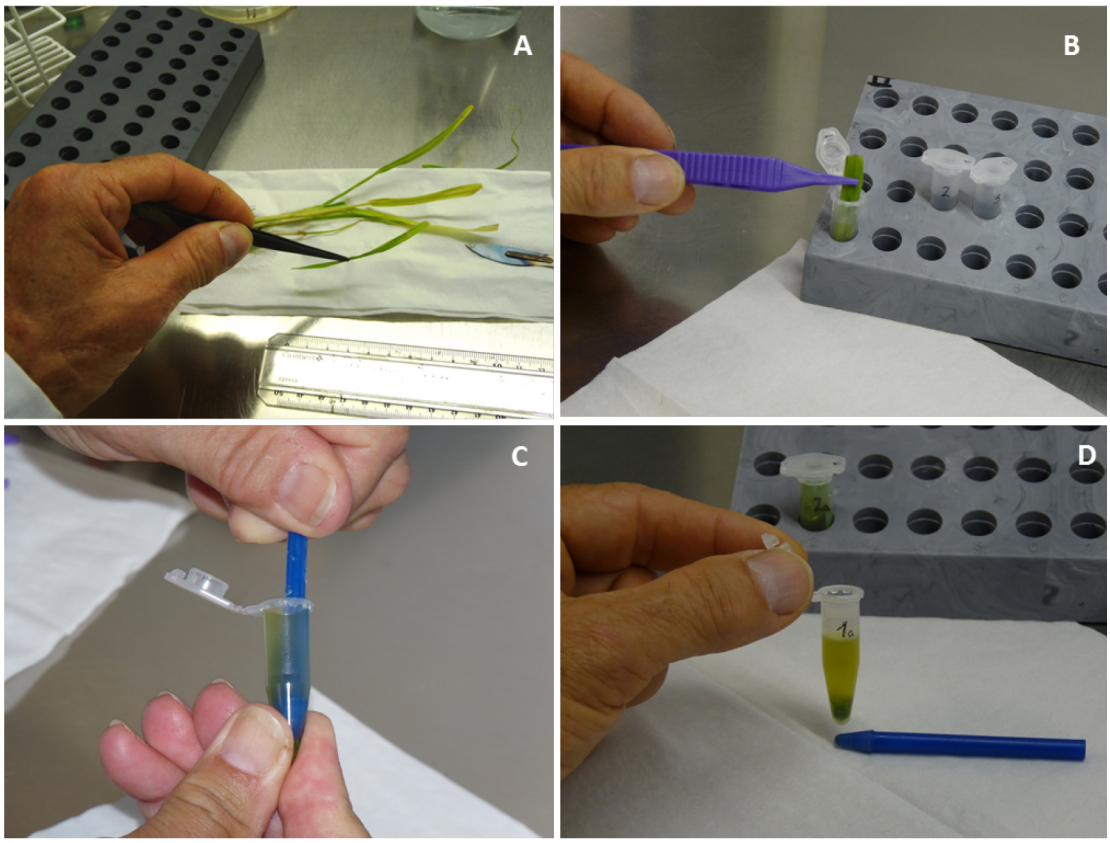

Figure 7. Steps describing leaf washing and tissue homogenization. A. Cut a 7 cm long fragment from two leaves of each inoculated plantlet. B. For leaf washing, immerse each leaf fragment individually in a 1.5 ml micro-centrifuge tube containing 1 ml of sterile distilled water and 0.005% Tween 20. C. For tissue homogenization, use a pestle made for micro-centrifuge tubes and homogenize the leaf fragment in the same tube, each tube containing 820 µl of wash water after removal of 180 µl for bacterial counts. D. Homogenized leaf tissue.

iii. Vortex the tubes vigorously for 10 sec to wash leaf fragments.

iv. Use 100 µl of each wash water to make 10 and 100 fold dilutions.

v. Gently drop three or four times 20 µl of each undiluted, 10 and 100-fold diluted wash water on WSD plates.

vi. Place the agar plates in an incubator at 28 °C for 6 days (Figure 8).

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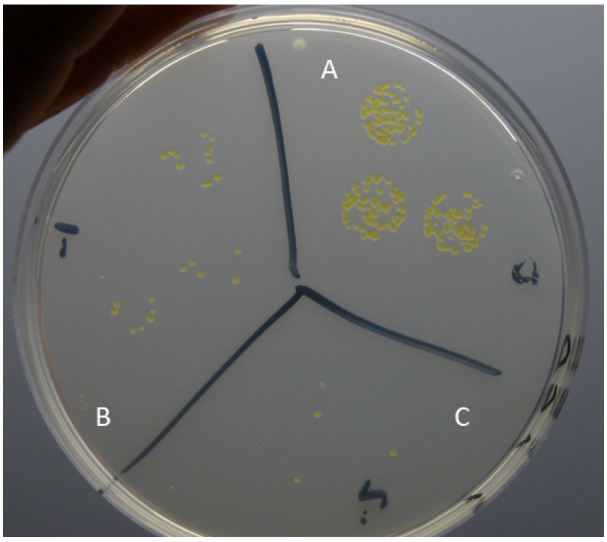

Figure 8. Growth of *X. albilineans* colonies in drops of undiluted (A), 10 (B) and 100 times (C) diluted wash water or homogenized tissue, 6 days after deposition on WSD plates

b. Leaf homogenization after leaf washing
   i. Using a pestle made for 1.5 ml micro-centrifuge tubes, homogenize each leaf fragment in the remaining wash water (820 µl after removal of 180 µl in A. for determination of bacterial populations) for 5 min (Figures 7C and 7D).
   ii. Use 100 µl of each homogenized tissue suspension to make 10 and 100 fold dilutions.
   iii. Gently drop three or four times 20-µl of undiluted, 10 and 100 times diluted homogenized tissue on WSD plates. Place the agar plates in the incubator at 28 °C for 6 days.

c. Determination of bacterial population densities
   i. Count colonies for each dilution and each of the three-four replications after 6 days of growth; calculate the average number of colonies and correct for dilutions. For example, 15 colonies on the 10^{-1} dilution plate correspond to 7,500 CFU present in the leaf section. Convert to $\log_{10}$ CFU per leaf section for the wash water or homogenized tissue.
   ii. For wash water and for homogenized tissue, calculate average $\log_{10}$ CFU for each bacterial strain or mutant tested using all values obtained for the six inoculated plantlets.

Data analysis

1. Data were analyzed using the statistical software package R, version 2.14.1 (R Development Core Team) as described by Mensi *et al*. (2016). Extent of Leaf Attachment (ELA) values is the combination of means of three independent experiments with six replicates (plantlets) per strain or mutant in each experiment. Note that two to four leaves were imprinted per plantlet. Although there can be significant variability between means of ELA between plantlets, all data are used for statistical analysis.

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2. For the determination of leaf attachment by leaf washing and leaf homogenization, data are from 20 leaves and combined from two separate experiments of five plantlets each (two leaves sampled per inoculated sugarcane plantlet and five plantlets used at each sampling time). Individual population data (CFU/ml) are transformed using the log10 function ($y = \log_{10}[(CFU/ml) + 1]$) before calculation of population means. When appropriate (few nil data), individual log transformed data can be used for mean comparison by variance analysis.
3. Data obtained by leaf imprinting (semi-quantitative method) and leaf washing (quantitative method) are correlated and yield similar information as they are both linked to bacterial populations present on the leaf surface. In contrast, data obtained by leaf homogenization are linked to bacteria that cannot be easily detached from the leaf surface and that are located in protected leaf areas or inside the leaf. Leaf imprinting is recommended for rapid and easy screening of bacterial strains for leaf attachment capacity. Leaf washing/homogenization is recommended to obtain more precise and quantitative data, and additional information regarding location of bacteria on or in the leaf.

Notes

1. Bacterial suspension preparation, plantlet sugarcane immersion, and foliar imprints are performed under a laminar flow cabinet (sterile conditions).
2. The bacterial suspension used to inoculate sugarcane plantlets is prepared freshly (just before the inoculation procedure). The bacterial suspension is prepared with only a few single colonies to obtain the desired bacterial concentration. The number of colonies used will depend on the size of the colonies. The bacterial strains stored in the freezer at minus 80 °C are each issued from a single colony.
3. Choose carefully the sugarcane leaves for imprinting or washing; we choose only green and non-withered leaves.
4. The area of confluent growth was estimated visually using a template with 10% leaf area increments.
5. Sugarcane cultivar CP68-2016 was used herein, but other cultivars susceptible to sugarcane leaf scald can also be tested.

   Non-specific adhering was not observed with the bacterial strains used herein. However, it can be a good idea to identify and include a bacterial species not adapted to sugarcane as a negative control.

Recipes

1. Wilbrink medium (WM) (1 L)

   10 g sucrose

   5 g peptone

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0.50 g $K_2HPO_4 \cdot 3H_2O$
0.25 g $MgSO_4 \cdot 7H_2O$
0.05 g Na_2SO_3
15 g agar
Add distilled water to make up 1 L
Adjust pH to 6.8-7.0
Autoclave 20 min at 121 °C

2. Wilbrink Selective Davis (WSD) medium (1L)
   Same composition as WM medium but supplemented with:
   5 g KBr and 0.004 g Benomyl (fungicide) before heat sterilization
   After autoclaving, add:
   0.1 g cycloheximide dissolved in 500 µl ethanol
   0.025 g cephalexin dissolved in 500 µl sterile distilled water
   0.03 g novobiocin dissolved in 500 µl ethanol
   0.05 g kasugamycin dissolved in 500 µl sterile distilled water
3. Macronutrients (1 L)
   10 g NH_4NO_3
   10 g KNO_3
   5 g $Ca(NO_3)_2 \cdot 4H_2O$
   715 mg $MgSO_4 \cdot 7H_2O$
   3 g KH_2PO_4
   650 mg KCl
   Add distilled water to make up to 1 L
4. Micronutrients (100 ml)
   160 mg H_3BO_3
   1,400 mg $MnSO_4 \cdot H_2O$
   380 mg $ZnSO_4 \cdot 7H_2O$
   75 mg KI
   10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
   35 mg $Cu(NO_3)_2 \cdot 3H_2O$
   Add distilled water to make up to 100 ml
   Solution to be stored at -20 °C in 1.5 ml micro-centrifuge tubes
5. Ferric EDTA
   2.78 g $FeSO_4 \cdot 7H_2O$
   3.73 g $Na_2EDTA \cdot 2H_2O$
   500 ml distilled water
   Dissolve Na_2EDTA and $FeSO_4$ separately and add $FeSO_4$ to Na_2EDTA by mild heating of the solution

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6. Fuji vitamins
   50 mg nicotinic acid (in boiling water)
   10 mg pyridoxol hydrochloride
   10 g myo-inositol
   10 mg thiamine dichloride (to be added last)
   Add distilled water to make up to100 ml
   Solution to be stored at -20 °C in 1.5 ml micro-centrifuge tubes
7. Nutritive medium for growth of sugarcane plantlets (1 L) (Adapted from Chatenet *et al*., 2001)
   100 ml macronutrients
   1 ml micronutrients
   5 ml Ferric EDTA
   2 ml Fuji vitamins
   8 g agar or 4 g Phytagel
   40 g sucrose
   890 ml distilled water
   pH 5.8
   Autoclave for 20 min at 121 °C

Acknowledgments

We thank Marie-Josée Darroussat for excellent technical assistance. Imène Mensi was supported by a PhD fellowship from Cirad. The work is supported by the USDA National Institute of Food and Agriculture [project Hatch/Rott FLA-BGL-005404]. This protocol was adapted from work published by Fleites *et al*. (2013) and Mensi *et al.* (2016).

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