Assays to Assess Virulence of *Xanthomonas axonopodis* pv. *manihotis* on Cassava

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[Abstract] Cassava (*Manihot esculenta*) is a root crop that provides calories for people living in more than 100 tropical and subtropical countries and serves as a raw material for processing into starch and biofuels as well as feed for livestock (Howeler *et al*., 2013). *Xanthomonas axonopodis* pv. *manihotis (Xam)*, the causal agent of cassava bacterial blight (CBB), can cause extensive crop damage [reviewed in Lopez *et al.* (2012), Lozano (1986)]. Bacterial movement, growth in planta and the ability to cause disease symptoms are all important measures of bacterial fitness and plant susceptibility to CBB. Here we present a protocol for visualizing the movement of *Xam* within the plant. We also provide a detailed method of assaying bacterial growth in the cassava leaf midvein, and bacterial growth and disease symptom development in the leaf apoplast. These methods will be important tools for determining *Xam* strain pathogenicity and for developing cassava varieties that are resistant to CBB.

Materials and Reagents

1. Cassava plants, cultivar TMS60444 (see Note 1)
2. *Xam* strains (grown 48 h on NYGA plates + antibiotic selection)
3. $MgCl_2$
4. Peptone (Thermo Fisher Scientific, catalog number: BP1420-500)
5. Yeast extract (Thermo Fisher Scientific, catalog number: BP1422-500)
6. Glycerine
7. Agar (Thermo Fisher Scientific, catalog number: BP1423-500)
8. NYGA (see Recipes)

Equipment

1. 28 °C incubator
2. 5 ¾" glass disposable Pasteur pipet (Thermo Fisher Scientific, catalog number: 13-678-20B), with the tip broken and filed to make a smooth 2 mm diameter tip (Figure 3A)
3. 2 in. x 4 in. cardboard covered with Parafilm
4. Mini-Beadbeater-96 (Biospec Products, catalog number: 1001)

5. 3 mm glass beads (Thermo Fisher Scientific, catalog number: 11-312A)
6. 100 x 15 mm petri dishes
7. Spectrophotometer (Pharmacia Biotech Ultrospec 3000)
8. Approximately 0.6 cm^2 cork borer
9. 1.7 ml Posi-Click tubes (Denville, catalog number: C-2170)
10. Single edge razor blade (Garvey Products Inc., catalog number: 40475)
11. 1 ml needleless syringe (BD, catalog number: 309659)
12. 96 well plate reader for measuring luminescence (PerkinElmer, catalog number: 2104-0010)
13. 96 well OptiPlate, black (PerkinElmer, catalog number: 6005270)
14. Dark room, x-ray imaging cassettes with film, ring stand

Procedure

Part I. Visualization of *Xam* movement

A. Preparation of bioluminescent bacteria

1. Make and transform competent *Xam* cells [as in Do Amaral *et al*. (2005)] with an expression vector containing the *lux* operon genes *luxCDABEG* from *Vibrio fischeri* (pLAFR-*lux,* unpublished, see acknowledgements).
2. Successful transformants may be screened for luciferase expression using an EnVision microplate reader and the US LUM 96 default protocol before inoculation into cassava.
   a. Grow transformed *Xam* colonies at 28 °C for 2 days on NYGA plates containing the appropriate antibiotics (Rifampicin 100 µg/ml, Tetracycline 10 µg/ml).
   b. Resuspend *Xam* in 10 mM $MgCl_2$ at OD_{600} = 0.6.
   c. Add 100 µl to each well of a 96 well OptiPlate, including negative controls (10 mM $MgCl_2$ alone, untransformed *Xam*) as well as a positive control (*E. coli* with pLAFR-*lux*).
   d. Run the US LUM 96 protocol and read the output for each well. Look for samples with values equal to or greater than the positive control.

B. Inoculations

1. Two days prior to the inoculation, streak out the *Xam* strains to be used from a glycerol stock onto NYGA plates containing the appropriate antibiotics and place at 28 °C for 2 days.
2. Prepare an inoculum from this plate by suspending the bacteria at OD_{600} = 0.01 in 1 ml 10 mM $MgCl_2$ (see Note 2).
3. On the abaxial side of a leaf, create 2-3 close, shallow nicks with the tip of a razor blade, avoiding major veins (Figure 1A).

4. Use a 1 ml needleless syringe to inoculate the bacterial suspension (OD_{600} = 0.01) into the leaf via the nicks to a total area of approximately 0.25 cm^2. Using a finger on the free hand, push the leaf against the syringe while infiltrating, applying enough pressure to encourage the inoculum to enter the leaf (Figure 1B). Alternatively, a toothpick dipped in the *Xam* (pLAFR-*lux*) inoculum may be used to inoculate by directly puncturing the leaf through the midvein or apoplast, though results may vary more than with apoplast infiltrations.

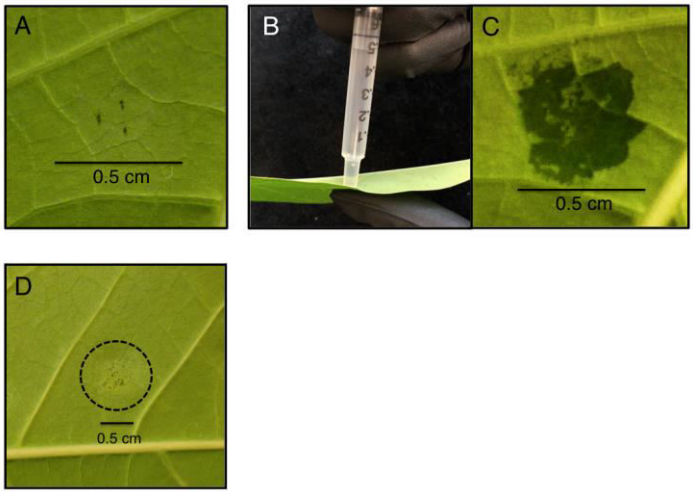

Figure 1. Apoplast inoculations and sample processing. A. Cassava leaf with 3 shallow abaxial nicks made by a razor blade. B. Demonstration of leaf infiltration using needleless syringe. C. Picture showing bacterial suspension inoculated into the leaf apoplast with a needleless syringe. D. Diagram showing sampled area of inoculated leaf apoplast for growth assay 6 days post inoculation (dpi).

C. Visualization

1. After 5-6 days of growth at ambient temperature, the bacteria may be visualized by exposing the leaf directly to x-ray film (Figure 2). For a one-time visualization, leaves may be removed and imaged in a standard film cassette for about 1 h. For continued tracking of bacterial movement in the same leaf, place the plant in a dark room and use binder clips to sandwich the leaf and a sheet of x-ray film between two pieces of cardboard. Keep the leaf elevated using a ring stand with a platform. After 1 h, take off the clips, image the film, and then return the plant to its growth room intact for subsequent imaging.

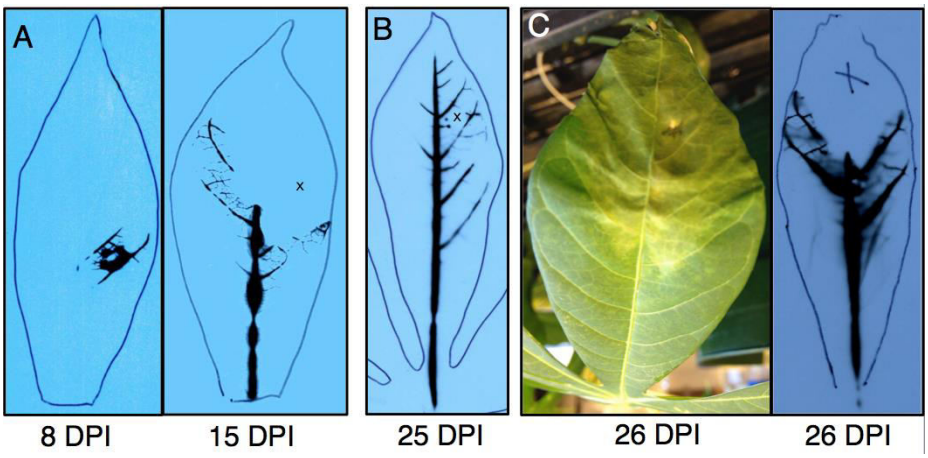

Figure 2. Visualization of *Xam* vascular movement in cassava by a luciferase reporter construct after inoculation into the apoplast. A. Examples of syringe infiltrated leaves (OD_{600} = 0.01) showing *Xam* entry into nearby veins and into the midvein. B. A toothpick inoculated leaf (OD_{600} = 0.01). C. A diseased leaf inoculated via toothpick puncture and its *Xam* profile. Signal loss is often observed at the site of inoculation most likely due to leaf tissue wilting. (X = inoculation point; DPI = days post inoculation).

Part II. Assaying *Xam* growth and symptom development

A. Culture growth

Grow *Xam* strains on NYGA plates + antibiotic selection for 40-48 h in a 28 °C incubator.

B. Preparation of inoculum

1. Resuspend plated bacteria in 1 ml of 10 mM $MgCl_2$ at approximately OD_{600} = 1.0 for each strain. Dilute with 10 mM $MgCl_2$ to OD_{600} = 0.2 for leaf midvein growth assays, OD_{600} = 0.00005 (first to OD_{600} = 0.01, then to final OD) for apoplast growth assays, or OD_{600} = 0.01 for apoplast symptom assays.
2. Select and label cassava leaves to inoculate (see Note 3). Leaves should be labeled with a marker to indicate the inoculated bacterial strain, timepoint, and replicate number (see Note 4).

C. Inoculation

Leaf midvein growth assay

1. Hold the leaf on a piece of Parafilm-covered cardboard, adaxial side up.
2. Dip the 2 mm diameter Pasteur pipet tip in 10 mM $MgCl_2$ and then use it to punch a clean hole through the midvein approximately 4-5 cm in from the leaf tip. Make sure

that a film of 10 mM $MgCl_2$ stays in the hole to prevent air from entering the midvein as this will block uptake of the bacterial suspension (Figure 3A-B).

3. Immediately pipette a 5 µl drop of OD_{600} = 0.2 bacterial suspension on the hole (Figure 3C). Move on to the next inoculation point and repeat. Let the drops dry completely without being disturbed. This can take up to 30 min.
4. When all inoculations are complete and dry, begin tissue extraction for Day 0.

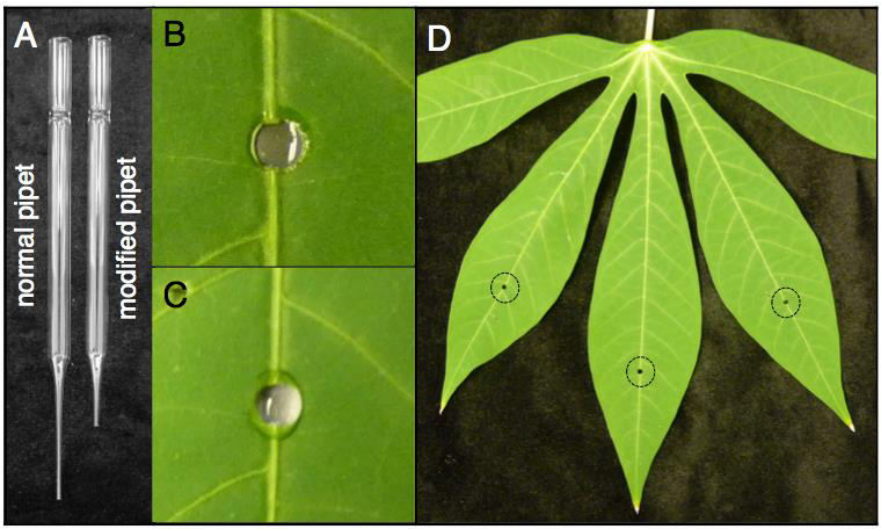

Figure 3. Midvein growth assay inoculations and sample processing. A. A glass Pasteur pipet is modified to make an approximately 2 mm hole in the leaf midvein. B. Midvein inoculation point showing film of 10 mM $MgCl_2$ after puncture. C. Midvein inoculation point with 5 µl drop of bacterial suspension. D. A cassava leaf with midvein inoculations on the middle 3 leaflets. A 0.6 cm^2 disc including the inoculation point is taken at days 0 and 6 (dashed lines).

Leaf apoplast growth and symptom assays

1. On the abaxial side of a leaf, create 2-3 close, shallow nicks with the tip of a razor blade, avoiding major veins (Figure 1A).
5. Use a 1 ml needleless syringe to inoculate the bacterial suspension (OD_{600} = 0.00005 for growth assays and OD_{600} = 0.01 for symptom assays) into the leaf via the nicks to a total area of approximately 0.25 cm^2. To do this, use a finger on the free hand, push the leaf against the syringe while infiltrating, applying enough pressure to encourage the inoculum to enter the leaf (Figure 1B).
2. For symptom assays, observe each site daily and keep track of the appearance and severity of water soaking symptoms, often observed by 6 days post inoculation (Figure 4).

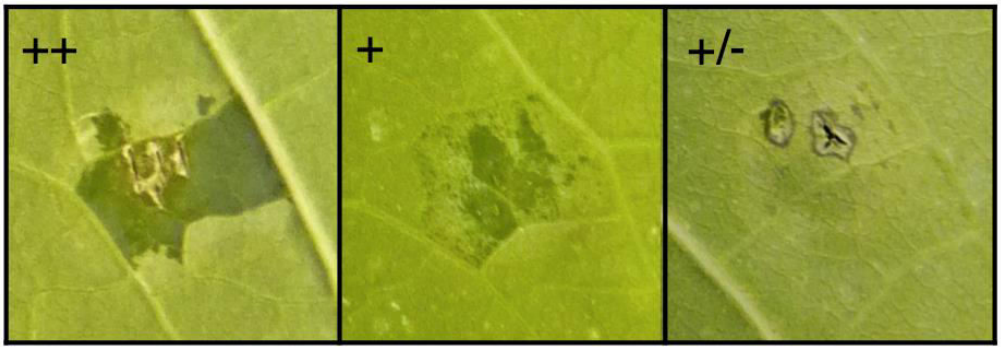

Figure 4. Representative examples of *Xam* water soaking symptoms. ++ indicates high levels of water soaking, + indicates medium water soaking, +/- indicates little or no water soaking.

D. Tissue extraction for growth assays

Leaf midvein growth assay (see Note 5)

1. Using a cork borer, take an approximately 0.6 cm^2 round leaf punch with the inoculation point exactly in the middle (Figure 3D). Place the punch in a Posi-Click tube containing a 3 mm glass bead and 200 µl 10 mM $MgCl_2$.
2. Pulverize the tissue using a bead beater (2 min., 36 oscillations/second). Bring each sample to a volume of 1 ml by adding 800 µl 10 mM $MgCl_2$.

Leaf apoplast growth assay

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E. Dilutions and plating

1. Make serial dilutions of the 1 ml sample of pulverized tissue in 10 mM $MgCl_2$ and plate countable colony forming units (CFUs) (Figure 5A).

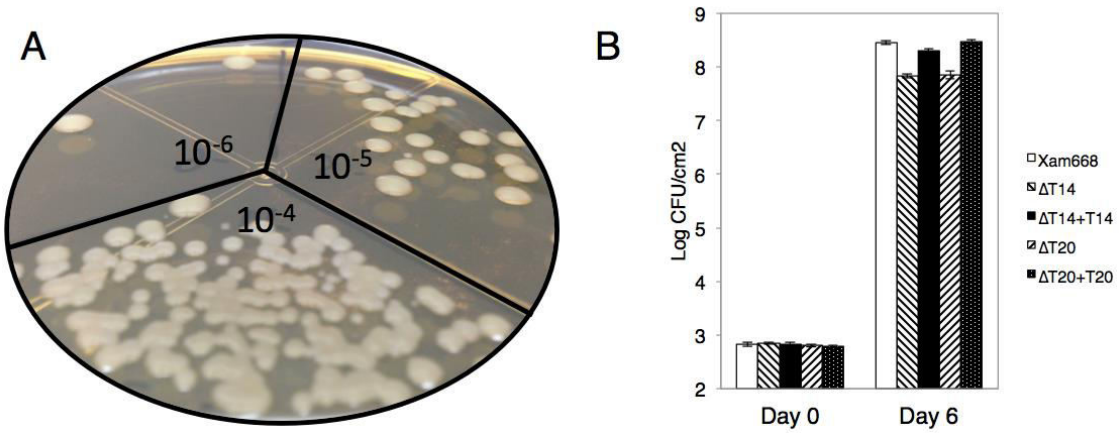

Figure 5. Plating and counting Colony Forming Units (CFUs). A. Diagram of plated samples showing serial dilutions. B. Chart showing representative results of an apoplast growth assay [previously published in Cohn *et al.* (2014)].

Leaf midvein growth assay

a. Inoculation point (day 0): Make 1 ml 10^{-2} and 10^{-4} dilutions in microcentrifuge tubes (see Note 6). Plate fractions of these dilutions to make final dilutions of 10^{-4}, 10^{-5}, and 10^{-6} (see Note 7).

b. Inoculation point (day 6): Make 1 ml 10^{-4} and 10^{-6} dilutions in microcentrifuge tubes. Plate fractions of these dilutions to make final dilutions of 10^{-5}, 10^{-6}, and 10^{-7} (see Note 8).

Leaf apoplast growth assay

a. Inoculation site (day 0): Plate 100 µl of the pulverized tissue sample for a 10^{-1} dilution.

b. Inoculation site (day 6): Make 1 ml 10^{-2} and 10^{-4} dilutions in microcentrifuge tubes. Plate fractions of these dilutions to make final dilutions of 10^{-4}, 10^{-5}, and 10^{-6}.

3. Place plates in a 28 °C incubator for 4 days.
4. Count colonies and correct for dilutions. For example, 5 colonies on the 10^{-6} dilution plate translates to 5,000,000 CFUs present in the original sample.
5. Convert to $\log_{10}$ CFUs per portion of leaf sampled and plot (Figure 5B).

Notes

1. Plants should be 2-3 months old. Our plants are propagated through cuttings (Note 9) and grown in a greenhouse room with no artificial lighting, mist (schedule: 15 min on/15 min off between 6:00 am and 6:00 pm) and an average temperature of 27 °C.
2. An OD_{600} of 0.1 is approximately 10^8 colony forming units (CFU) per ml.

3. Careful leaf selection is very important to cut down on technical variation. Only the first 2 fully unfolded leaves should be used. It is best to use leaflets that are similar in size.
4. We typically do 6 replicates per strain per timepoint. An average experiment will have 6 strains to be tested at days 0 and 6, so with 2 timepoints and 6 replicates per strain, that makes 72 inoculation points.
5. In addition to assaying midvein growth at the inoculation point, one can also measure growth in the proximal midvein section, though this technique is more technically challenging and leaf choice becomes very important in order to cut down on sample variation (see Note 3). To measure bacterial growth in the proximal midvein section, use a razor blade to cut out the 3 cm of leaf midvein directly above the leaf punch encompassing the inoculation point at day 6. Place the midvein section in a Posi-Click tube containing a 3 mm glass bead and 200 µl 10 mM $MgCl_2$. Pulverize the tissue using a bead beater (2 min, 36 oscillations/second). Bring each sample to a volume of 1 ml by adding 800 µl 10 mM $MgCl_2$. Make 1 ml 10^{-2} and 10^{-4} dilutions in microcentrifuge tubes (see Note 6). Plate fractions of these dilutions to make final dilutions of 10^{-4}, 10^{-5}, and 10^{-6} (see Note 7).
6. For example, to make a 10^{-2} dilution, add 10 µl of the pulverized tissue suspension to 990 µl of 10 mM $MgCl_2$.
7. For example, to plate a 10^{-6} dilution, put 10 µl of a 10^{-4} dilution on a plate and spread; to plate a 10^{-7} dilution, put 100 µl of a 10^{-6} dilution on a plate and spread.
8. These dilutions are guidelines as the actual dilutions will depend on the virulence of the *Xam* strains that you are testing. The dilutions should be determined based on what will allow you to clearly count colonies after plating.
9. To propagate plants through cuttings, cut all the leaves off of a plant that is woody along at least half of the stem (6-12 months old). Cut the stem into sections, making sure to include 3-4 nodes per section. Place each section into fertilized soil, taking care to maintain up/down orientation of the stem section and making sure that at least one node is below the soil surface, and at least one node is above the soil surface. Provide the cutting plenty of water. New leaves should begin growing from the top node after about a week.

Recipes

1. NYGA (1 L)
   5 g peptone
   3 g yeast extract
   20 ml 100% glycerine
   1.5 g agar
   a. Combine peptone, yeast extract, and glycerine, then bring to 1 L with water, mixing on low heat.

b. Bring pH to 7.0 using 1 M NaOH.
c. Mix in agar.
d. Autoclave 30 min.

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