

Bacterial Growth Inhibition Assay for *Xanthomonas oryzae* pv. *oryzae* or *Escherichia coli* K12 Grown together with Plant Leaf Extracts

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[Abstract] We performed a growth inhibition assay to test antibacterial compounds in leaf extracts from transgenic rice plants. The assay is based on over-night co-incubation of a defined concentration of colony forming units (cfu) of the respective bacteria together with aqueous extracts of ground leaf tissue.

Keywords: Crop plant, Rice, pathogen, Bacteria, Antimicrobial, Jacalin, Lectin, Dirigent

[Background] Defense of plants against harmful organisms can be specific against particular pathogen species or groups of pathogens. Aiming at increasing pathogen resistance of crop plants, breeding for resistance against particular diseases can be useful but the ultimate goal is to implement broad-spectrum disease resistance. The rice protein OsJAC1 is a modular protein consisting of a jacalin-related lectin domain predicted to bind to sugar residues and a dirigent domain that might act during coupling of monolignols. This fusion protein is specific to *Poaceae* and represents a novel type of resistance protein. The protocol described here was used to assess the antimicrobial capabilities of leaf extracts from transgenic rice plants overexpressing the *OsJAC1* cDNA (Weidenbach *et al.*, 2016). For this assay, the bacterial lab strain *Escherichia coli* K12 and the bacterial rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO86, causing bacterial blight, were used.

Materials and Reagents

1. Test tubes for bacterial liquid cultures
2. Pipettes and sterile tips
3. Inoculation loops
4. Safe lock reaction tubes (1.5 and 2.0 ml) (e.g., Eppendorf tubes)
5. Sterile toothpicks
6. 0.2 µm syringe filter (Sartorius, catalog number: 16532)
7. *E. coli* K12 (standard lab strain)
8. *Xanthomonas oryzae* pv. *oryzae* PXO86 (received from C.M. Vera Cruz, IRRI, Manila, Philippines)
9. Rice plants, approx. 2-3 weeks old (see Weidenbach *et al.*, 2016)
10. Sterilized tap water
11. Liquid nitrogen
12. Peptone (Duchefa Biochemie, catalog number: P1328)

13. Sodium chloride (NaCl) (Carl Roth, catalog number: 3957)
14. Yeast extract (Duchefa Biochemie, catalog number: Y1333)
15. Agar-agar (Carl Roth, catalog number: 5210)
16. Sucrose (Carl Roth, catalog number: 4621)
17. Trisodium phosphate (Na_3PO_4) (Carl Roth, catalog number: 8613)
18. Calcium nitrate [$\text{Ca}(\text{NO}_3)_2$] (Carl Roth, catalog number: P740)
19. Ferrous sulphate (FeSO_4) (Carl Roth, catalog number: P015)
20. HCl (Carl Roth, catalog number: 2607)
21. LB-medium (see Recipes)
22. Modified Wakimoto medium (see Recipes)
23. Ferrous sulphate (FeSO_4) stock solution (see Recipes)

Equipment

1. Laminar flow hood
2. 28 °C Thermo constant incubator
3. 37 °C Thermo constant incubator
4. 37 °C Thermo shaking incubator
5. -80 °C low temperature freezer
6. Spectrophotometer and respective cuvettes
7. Mortar and pestle (e.g., Carl Roth, catalog numbers: 1568 and 3831)
8. Autoclave
9. Benchtop centrifuge (cooled at 4 °C)

Procedure

Notes:

- a. All work with bacterial cultures is carried out under sterile conditions using a laminar flow hood. Culture medium for *E. coli* is LB-medium and incubation temperature is 37 °C. For *X. oryzae* pv. *oryzae* (Xoo) modified Wakimoto medium is used and bacteria are incubated at 28 °C.
 - b. Streak bacterial cells from glycerol stocks (see Note 1) stored at -80 °C on appropriate media agar plates and incubate overnight for *E. coli* (on LB-medium agar plates; incubation at 37 °C) or until colonies get visible for Xoo (after 2-3 days at 28 °C on modified-Wakimoto medium agar plates).
1. Day 1
 - a. *E. coli*:
 - i. Inoculate 3 ml liquid LB-medium in a test tube with a single colony picked with a sterile toothpick or an inoculation loop from the overnight incubation plate.
 - ii. Shake culture at 210 rpm and 37 °C.

2. Day 2

a. *E. coli*:

- i. Harvest bacteria from overnight culture by centrifugation and re-suspend pellet in sterilized tap water.
- ii. Determine the OD₆₀₀ using a spectrophotometer. Using the measured OD₆₀₀ value dilute to a calculated OD₆₀₀ of 1.25×10^{-6} (equivalent to approximately 1×10^3 cells ml⁻¹ when assuming that OD₆₀₀ = 1.0 is equivalent to approximately 8×10^8 cells ml⁻¹ for *E. coli*) in sterilized tap water.

b. *Xoo*:

- i. Scrape off bacteria from modified Wakimoto-medium plates (see Note 2) using a sterile inoculation loop, re-suspend the bacteria in sterilized tap water and measure OD₆₀₀ in the linear absorption range of the spectrophotometer.
- ii. Dilute to approximately OD₆₀₀ 1.25×10^{-6} in sterilized tap water (see above).

c. Plant leaf extracts:

- i. Snap freeze 0.3 g leaf material from transgenic or wild-type rice plants in liquid nitrogen and grind to a fine powder using a mortar and pestle.
- ii. Transfer the frozen powder to a 1.5 ml safe lock reaction tube and mix with 1 ml sterilized tap water.
- iii. After centrifugation at 16,000 x g at 4 °C in a cooled benchtop centrifuge, the supernatant is transferred to a fresh safe lock reaction tube and mixed 1:2 (v/v) with the bacterial suspension (100 µl supernatant + 100 µl diluted bacterial suspension). Controls consist of bacterial suspension and leaf extracts from wild-type plants.
- iv. The mixtures (100 µl) are plated in triplicates on agar plates of the appropriate media. Plates with *E. coli* are incubated overnight at 37 °C. *Xoo* plates are incubated at 22 °C for 4-5 days (see Note 3).

3. Day 3

Count the number of cfu on *E. coli* plates.

4. Day 6-7

Count the number of cfu on *Xoo* plates.

Data analysis

Calculate mean value and standard deviation of the number of cfu on the triplicate plates. The number of cfu of the wild-type sample is set to 100%. Test for significant reduction of bacterial growth in sample compared to control by applying Student's *t*-test.

Notes

1. Glycerol stocks are prepared by adding 750 µl of bacterial culture to 250 µl of sterile glycerol in a safe lock reaction tube; mix well and snap freeze in liquid nitrogen. Store at -80 °C.
2. Use freshly streaked out plates (~3 days) to ensure vitality of Xoo.
3. Incubation for a longer time at lower temperature ensures better growth of well separated colonies for easier counting.

Recipes

1. LB-medium
10.0 g·L⁻¹ peptone
10.0 g·L⁻¹ sodium chloride (NaCl)
5.0 g·L⁻¹ yeast extract
15 g·L⁻¹ agar-agar
Dissolve components in distilled water and sterilize by autoclaving
2. Modified Wakimoto medium
20 g·L⁻¹ sucrose
5.0 g·L⁻¹ peptone
1.9 g·L⁻¹ trisodium phosphate (Na₃PO₄)
0.5 g·L⁻¹ calcium nitrate [Ca(NO₃)₂]
15 g·L⁻¹ agar-agar
Dissolve components in distilled water and sterilize by autoclaving
Afterwards add 1 ml L⁻¹ sterile FeSO₄ stock solution (see below)
3. Ferrous sulphate (FeSO₄) stock solution for modified Wakimoto medium
0.5 g·L⁻¹ ferrous sulphate (FeSO₄)
3.0 ml 2 N hydrogen chloride (HCl)
10 ml distilled water
Sterilize stock solution by using a 0.2 µm syringe filter and store at room temperature in darkness

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References

1. Weidenbach, D., Esch, L., Moller, C., Hensel, G., Kumlehn, J., Hofle, C., Huckelhoven, R. and Schaffrath, U. (2016). [Polarized defense against fungal pathogens is mediated by the jacalin-related lectin domain of modular poaceae-specific proteins.](#) *Mol Plant* 9(4): 514-527.