Drug Sensitivity Assay of Xanthomonas. citri subsp. citri Using REMA Plate Method

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[Abstract] Resazurin Microtiter Assay (REMA) is a simple, rapid, reliable, sensitive, safe and cost-effective measurement of cell viability. Resazurin detects cell viability by converting from a nonfluorescent dye to the highly red fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth (Palomino et al., 2002). The REMA assay can be used as a fluorogenic oxidation-reduction indicator in a variety of cells, including bacteria, yeast and eukaryotes (Silva et al., 2013).

Materials and Reagents

1. Chemicals: Synthetic esters of gallic acids (Ximenes et al., 2010)
2. Bacterial strain: Wild type Xanthomonas citri subsp. citri strain 306 (Schaad et al., 2005)
3. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
4. Kanamycin (Sigma-Aldrich, catalog number: K4000)
5. Luria-Bertani broth (LB) culture medium
6. Resazurin sodium salt (Sigma-Aldrich, catalog number: R7017)

Equipment

1. 96-well plate, polystyrene, with clear flat bottom wells (Greiner Bio-one, catalog number: 655101)
2. SPECTRAfluor Plus (Tecan) microfluorimeter
3. Multichannel pipetman (Eppendorf)

Procedure

A. Prepare stock solutions of chemicals (dried-powder samples) dissolving in 10% in DMSO (diluted in sterile water).
B. Add 100 µl of water to columns 1 and 12 to avoid evaporation (Table 1).
C. Dilute the stock solutions in LB medium directly in a 96-well plates using a 2-fold scheme (final volume of 100 µl per a well); after serial dilution, the most concentrated sample should have maximum 1% DMSO.

D. Cells were grown in LB medium at 30 °C under rotation (200 rpm) until OD600 0.6 (log phase).

E. Add 10 µl of bacterial inoculum (standardized to 10^5 CFU/well).
   a. Negative control: 1% DMSO dissolved in LB.
   b. Positive control: Kanamycin at 15.6 µg/ml.

Table 1. Example for setup of REMA 96-well assay plate

<table>
<thead>
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<tbody>
<tr>
<td>A</td>
<td>200 µl  H2O</td>
<td>200 µl  drug 1</td>
<td>200 µl  drug 2</td>
<td>200 µl  drug 3</td>
<td>200 µl  drug 4</td>
<td>200 µl  drug 5</td>
<td>200 µl  drug 6</td>
<td>200 µl  drug 7</td>
<td>200 µl  drug 8</td>
<td>200 µl  drug 9</td>
<td>100 µl  negative control</td>
<td>200 µl  H2O</td>
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<tr>
<td>B</td>
<td>200 µl  H2O</td>
<td>100 µl  2A</td>
<td>100 µl  3A</td>
<td>100 µl  4A</td>
<td>100 µl  5A</td>
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<td>100 µl  8A</td>
<td>100 µl  9A</td>
<td>100 µl  10A</td>
<td>100 µl  negative control</td>
<td>200 µl  H2O</td>
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<td>C</td>
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<td>100 µl  9B</td>
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<td>100 µl  negative control</td>
<td>200 µl  H2O</td>
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<td>D</td>
<td>200 µl  H2O</td>
<td>100 µl  2C</td>
<td>100 µl  3C</td>
<td>100 µl  4C</td>
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<td>100 µl  7C</td>
<td>100 µl  8C</td>
<td>100 µl  9C</td>
<td>100 µl  10C</td>
<td>100 µl  negative control</td>
<td>200 µl  H2O</td>
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<td>E</td>
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<td>100 µl  7D</td>
<td>100 µl  8D</td>
<td>100 µl  9D</td>
<td>100 µl  10D</td>
<td>100 µl  positive control</td>
<td>200 µl  H2O</td>
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<td>F</td>
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<td>100 µl  2E</td>
<td>100 µl  3E</td>
<td>100 µl  4E</td>
<td>100 µl  5E</td>
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<td>100 µl  positive control</td>
<td>200 µl  H2O</td>
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F. Incubate the test plates at 30 °C for 6 h.

G. Add 15 µl of a 0.01% (w/v) resazurin solution, and incubate at 30 °C for 2 h.

H. Measure fluorescence at 530 nm (excitation) and 590 nm (emission) using a fluorescence scanning.

I. Percentage of inhibition is defined as:
   \[
   \frac{[\text{average FU negative control} - \text{average FU test}]}{\text{average FU negative control}} \times 100
   \]
   FU: Fluorescence Units
Figure 1. Example for calculation of growth inhibition

Note: Three independent experiments should be conducted, and the data is used to construct plots of chemical concentration versus cell growth inhibition in order to determine the MIC* (Figure 1).

*The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antibiotic able to inhibit the growth of 90% of organisms.

Acknowledgments

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References


