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 OD_{600} 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

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 µl H_2O	200 µl drug 1	200 µl drug 2	200 µl drug 3	200 µl drug 4	200 µl drug 5	200 µl drug 6	200 µl drug 7	200 µl drug 8	200 µl drug 9	100 µl negative control	200 µl H_2O
B	200 µl H_2O	100 µl 2A	100 µl 3A	100 µl 4A	100 µl 5A	100 µl 6A	100 µl 7A	100 µl 8A	100 µl 9A	100 µl 10A	100 µl negative control	200 µl H_2O
C	200 µl H_2O	100 µl 2B	100 µl 3B	100 µl 4B	100 µl 5B	100 µl 6B	100 µl 7B	100 µl 8B	100 µl 9B	100 µl 10B	100 µl negative control	200 µl H_2O
D	200 µl H_2O	100 µl 2C	100 µl 3C	100 µl 4C	100 µl 5C	100 µl 6C	100 µl 7C	100 µl 8C	100 µl 9C	100 µl 10C	100 µl negative control	200 µl H_2O
E	200 µl H_2O	100 µl 2D	100 µl 3D	100 µl 4D	100 µl 5D	100 µl 6D	100 µl 7D	100 µl 8D	100 µl 9D	100 µl 10D	100 µl positive control	200 µl H_2O
F	200 µl H_2O	100 µl 2E	100 µl 3E	100 µl 4E	100 µl 5E	100 µl 6E	100 µl 7E	100 µl 8E	100 µl 9E	100 µl 10E	100 µl positive control	200 µl H_2O
G	200 µl H_2O	100 µl 2F	100 µl 3F	100 µl 4F	100 µl 5F	100 µl 6F	100 µl 7F	100 µl 8F	100 µl 9F	100 µl 10F	100 µl positive control	200 µl H_2O
H	200 µl H_2O	100 µl 2G	100 µl 3G	100 µl 4G	100 µl 5G	100 µl 6G	100 µl 7G	100 µl 8G	100 µl 9G	100 µl 10G	100 µl positive control	200 µl H_2O

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:

   [(average FU negative control) - (average FU test)]/(average FU negative control) x 100

   FU: Fluorescence Units

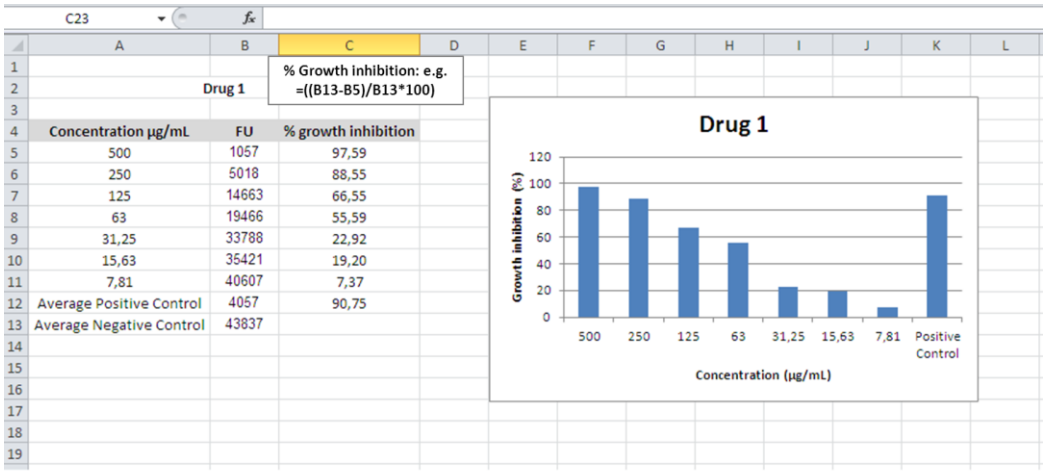

Figure 1. Example for calculation of growth inhibition

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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alfalfae (ex Riker and Jones, 1935) dye 1978 as *X. alfalfae* subsp. *alfalfae* (ex Riker *et al.*, 1935) sp. nov. nom. rev.; and "var. fuscans" of *X. campestris* pv. *phaseoli* (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. *Syst Appl Microbiol* 28(6): 494-518.

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