Competitive Index: Mixed Infection-based virulence assays for genetic analysis in *Pseudomonas syringae*-plant interactions

Alberto P. Macho[1,2], José S. Rufián[1], Javier Ruiz-Albert, Carmen R. Beuzón*

Instituto de Hortofruticultura Subtropical y Mediterránea, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Dpto. Biología Celular, Genética y Fisiología, Campus de Teatinos, Málaga

E-29071, Spain

1 These authors contributed equally to this work.

2 Present address: Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences, Shanghai, China.

*For correspondence: cbl@uma.es

Phone: ++ 34-952-131959

Fax: ++ 34-952-132001

Summary

When studying bacterial plant pathogens, the genetic analysis of the contribution of virulence factors to the infection process has traditionally been hindered by their high degree of functional redundancy. In recent years, it has become clear that the use of competitive index in mixed infections provides an accurate and sensitive manner of establishing virulence phenotypes for mutants for which other assays have failed. Such increases in sensitivity and accuracy are due to the direct comparison between the respective growths of the co-inoculated strains within the same infection, each strain replicating as they would in individual infections. Interferences between the co-inoculated strains must be therefore avoided using the appropriate experimental settings. In this chapter, we will present the optimal experimental conditions to achieve maximum sensitivity on virulence assays using the phytopathogenic bacterium *Pseudomonas syringae*, as well as some additional considerations to ensure the correct interpretations of the results.

Keywords

Mixed Infection; Competitive Index; Virulence; Immunity; Resistance; Genetic analysis.

1. Introduction

Bacterial pathogens employ a multitude of virulence factors to colonize plants and cause disease. Due to the functional redundancy described for many virulence factors, the genetic analysis of their individual contribution to virulence can be sometimes hindered by the absence of a relevant phenotype for the respective single mutants. Based on the knowledge generated from the study of animal pathogens, we set up the use of competitive index (CI) assays in mixed infections for the study of bacterial plant pathogens [1]. CI assays are based on the direct comparison between growths of co-inoculated strains within the same plant (Fig. 1). Since one of the strains (e.g. wild-type) is used as an internal control, technical and experimental variations are drastically reduced, providing an accurate and sensitive manner of establishing virulence phenotypes for mutants for which other assays fail [1-5]. CI assays are extremely versatile, allowing their adaptation to analyse virulence, or to characterize defence responses [6-8]. In recent years, CI assays have been also successfully adapted to the analysis of bacterial growth in several plant hosts, for three of the most relevant bacterial plant pathogens: *Pseudomonas syringae, Ralstonia solanacearum* and *Xanthomonas campestris* [1,9,10].

In order to obtain reliable data from CI assays in mixed infections and to avoid misleading interpretations, several considerations need to be taken into account while designing the experiments and analysing the resulting data. An essential requirement to allow for a reliable measurement of virulence of individual strains within mixed infections, is to ensure that each strain replicates as they would in individual infections. This can be achieved under the appropriate experimental settings. Here, we use the well-established model of interaction between *P. syringae* and *Arabidopsis thaliana* to explain the basic procedure to perform CI assays in mixed infections including experimental details and tips to take full advantage of this technique.

2. Materials

2.1 Bacterial growth

1. Lennox Broth (LB) [11]: Modification of Luria-Bertani [12] with NaCl concentration halved. Weight 10 g of Tryptone, 5 g of Yeast Extract and 5 g of NaCl and resuspend into 800 mL of distilled water. Fill up to 1L with distilled water using a measuring cylinder and add 10 g of bacteriological agar. Autoclave at 121ºC for 20 min. Cool down to a temperature about 50ºC and add the appropriate antibiotic (see 2.1.2). Pour about 20 mL of LB agar per 10 cm Petri dish.
2. This protocol is presented for CI assays of kanamycin-resistant *Pto* DC3000 mutant derivatives for which media is supplemented with kanamycin (15 µg/mL). However, examples of the concentrations of different antibiotics we have also used to select *P. syringae* strains are: ampicillin (300 µg/mL for liquid cultures and 500 µg/mL for plates), rifampicin (15 µg/mL), gentamycin (10 µg/mL), spectinomycin (50 µg/mL) (see **Notes 1 and 2**).
3. Magnesium chloride 10 mM: From a 1M stock, add 1 mL to 99 mL of distilled water.
4. All bacterial suspensions must be prepared in sterile conditions.

2.2 Plant growth

1. Soil mixture: 3 parts of potting soil per 1 part of vermiculite, or another mixture suitable for growing Arabidopsis plants.
2. Growth conditions: Arabidopsis can be cultivated in growth chambers or controlled environment rooms under short-day conditions (8h light, 16 h
3. darkness), at 23ºC and 100-150mE $m^{-2}s^{-1}$.

2.3 Inoculation and Grinding

1. Inoculation: 1 mL needleless syringe.
2. Grinding: Cork-borer set (Sigma-Aldrich). Polypropylene Pestles for 1.5 mL microcentrifuge tubes (Sigma-Aldrich).

3. Methods

For simplicity, the co-inoculated strains will be named generically as wild-type and mutant isogenic strain, illustrating a mixed infection assay to detect virulence attenuation of a single knockout mutant strain, carrying a kanamycin resistance cassette that allows selection and differentiation from the wild type. However, CI assays can be used to compare growth between any given two isogenic strains as long as their respective growth can be differentiated *i.e.* the strains display different antibiotic resistance, or one of the strains expresses a fluorescent protein such as the Green Fluorescent Protein (GFP).

3.1 Bacterial growth and preparation of bacterial suspensions

1- Under sterile conditions, streak out the *Pseudomonas syringae* strain from the -80ºC stock culture onto a LB agar medium plate supplemented with the appropriate antibiotics (**see Notes 1 and 2**), and incubate during 2 days at 28ºC.

2- Scrape out bacterial biomass from a fresh Petri dish and resuspend in 10 mM $MgCl_2$. Adjust the OD_{600} to 0.1 by adding 10mM $MgCl_2$ and measure OD_{600} by using a spectrophotometer (**see Note 3**). For most strains, an OD_{600} of 0.1 in a bacterial suspension of *P. syringae* corresponds to approximately $5x10^7$ cfu/mL. For mutants displaying altered colony morphology, this correspondence should be previously established by bacterial counts on plated serial dilutions of bacterial solutions to which the OD_{600} has been measured.

3- Perform steps 1-3 for each strain.

3.2 Preparation of mixed inocula

1- Mix 500 µl of each bacterial suspension into a microcentrifuge tube to obtain 1 mL of mixed concentrated inoculum (**see Note 4**). Perform serial dilutions by adding 100 µl of the mixed inoculum to 900 µL of 10 mM $MgCl_2$ in a sterile microcentrifuge tube and mix by vortexing.

2- Perform the previous step a total of 3 times (final dilution 1:1000), to obtain a bacterial suspension containing approximately $5x10^4$ cfu/mL ($2.5x10^4$ cfu/mL

of each strain) (**see Note 5**). To increase the volume of the final mixed suspension, in order to obtain enough bacterial suspension to inoculate several plants by infiltration (e.g. 10 mL), the last serial dilution can be performed by adding the whole millilitre to 9 mL of 10 mM $MgCl_2$, (see below).

3- Before proceeding to the plant inoculation, collect an aliquot of the mixed diluted inoculum and make 2 additional serial dilutions (1:10 and 1:100). Plate both dilutions in agar plates containing LB and agar plates containing LB supplemented with kanamycin. Incubate these plates at 28ºC, and use the resulting colony counts to calculate the actual strain ratio within the input of each experiment (see below, section 3.6).

3.3 Plant growth

1- Sow *Arabidopsis thaliana* seeds in the soil mixture, cover with a transparent plastic lid and stratify during 2 days at 4ºC in the dark, or stratify them before sowing.

2- Then move to a controlled environment room to grow at 23ºC under short-day conditions, keeping the lid on. Check every 2 days and water the plants slightly if needed, to ensure that the soil is humid, while carefully avoiding excess water in the tray.

3- Two weeks after sowing the seeds, transfer Arabidopsis seedlings into individual pots or tray wells and cover again with a transparent plastic lid during one week to minimise stress after the transplanting process.

4- Four-to-five week-old healthy-looking plants are suitable for bacterial inoculations by infiltration (**see Note 6**).

3.4 Infiltration of plant leaves with mixed infections

1- Separate 3-5 Arabidopsis plants for each mixed infection (**see Note 7**). Using a permanent marker pen, mark 3 fully expanded rosette leaves

2- Using a needleless syringe, infiltrate the diluted mixed inoculum into the abaxial face of the marked leaves (avoid contact with the central vein), carefully pressing the syringe plunger until the area around the syringe grows darker as the suspension fully enters the leaf (**see note 8**). Infiltrate the three marked leaves per plant and return the plants to the controlled environment room.

3.5 Extraction and dilution of bacteria from plant samples

1- Samples can be taken 2-4 days after inoculation, depending on the purpose of the assay, or the nature of the specific mutant strain tested.
2- Three 10 mm-diameter leaf discs, one from each infiltrated leaf, should be taken from the centre of the leaf using a sterile cork-borer. Place it into a sterile tube containing 1 mL of 10mM $MgCl_2$: this constitutes an independent replicate, containing a bacterial mixed infection from one independent plant. Repeat this procedure with each one of the inoculated plants.
3- Grind and homogenise the plant samples into 10 mM Mg_2Cl by mechanical disruption using sterile material (**see note 9**).
4- Make serial dilutions and plate them in agar plates containing LB and agar plates containing LB supplemented with kanamycin. All plates must contain cycloheximide (2 µg/mL) to avoid fungal growth (**see note 10**).
5- Incubate the plates at 28ºC during 2 days and count bacterial colonies (**see note 11**).

3.6 CI calculation

1- The number of colony-forming units (cfu) of the mutant strain will be obtained from those growing on the kanamycin plates, while the number of wild-type bacteria can be obtained by subtracting the number of mutant bacteria from the total number of bacteria counted on the LB plate.
2- The CI is defined as the mutant-to-wild type ratio within the output sample divided by the mutant-to-wild type ratio within the input (inoculum) [13,14] (Figure 1). The input ratio should be close to 1 (**see note 12**).
3- A CI value will be calculated for each independent replicate, and used to generate the mean CI value and the standard error of the mean. A CI of 1 indicates that both wild-type and mutant strains have similar virulence (measuring virulence as bacterial replication inside the host). A CI lower than 1 indicates that the mutant strain has attenuated virulence compared to the wild-type strain, and vice versa.
4- To determine statistically significant virulence attenuation, use a 2-tailed Student's t-test and the null hypothesis: mean index is not significantly different from 1.0 (P value <0.05). Different CI values can also be compared to determine significant differences in attenuation between different mutant strains, or to determine if a given strain grows differently (CI) in different plant genotypes (**see note 13**). In these cases, the statistical analysis used is One Way ANOVA and Holm-Sidak test for multiple comparison, or One Way

ANOVA on Ranks and Tukey test for multiple comparison when Equal Variance Test failed ($P<0.05$).

4. Notes

1- When using strains with multiple antibiotic resistances (e.g. multiple mutation or carrying plasmids), it is advisable to grow all bacteria in the presence of the corresponding antibiotics, to ensure the maintenance of the plasmids, and/or to avoid chromosomal rearrangements that might lead to the loss of chromosomal antibiotic resistances.

2- Laboratories often use antibiotic concentrations above the minimum inhibitory concentration (MIC) corresponding to their particular strains and antibiotic. Using an antibiotic concentration higher than the MIC will still work to select colonies, but will underestimate the real numbers of cfu of a bacterial suspension. Since CI assays are very sensitive, this could lead to misleading results. If uncertain about this issue, plate the serial dilutions of the kanamycin-resistant strain (or any other antibiotic-resistant strain to be used for CI assays) in both LB with and without the antibiotic at the concentration used in the laboratory. If the number of cfu calculated from both types of plate match, then the antibiotic concentration is appropriate. If they do not match, gradually reduce the antibiotic concentration until they do, while including the appropriate negative controls to guarantee correct selection against antibiotic-sensitive derivates.

3- It is essential not to overgrow bacteria in the plate and to avoid 4ºC storage, otherwise the accumulation of extracellular components may alter the OD_{600}.

4- When preparing the inoculum, it is important to mix both bacterial suspensions before making serial dilutions. This way, potential inaccuracies in the dilution process will affect equally to both strains.

5- This inoculum concentration has been optimized and tested to avoid growth interference (complementation of growth or dominant negative effects) between *Pto* DC3000 strains with different pathogenic capabilities (pathogenic *versus* non-pathogenic or virulent *versus* avirulent, respectively) in Arabidopsis. Additional information about inoculum concentrations optimized for other strains or hosts, or even different pathogens, have also been reported [1,9,10]. If using a different pathosystem, it is important to run a test of your inoculum concentration, checking individual growth *versus* growth within the mixed infection, of a non-pathogenic (e.g. a type III-defective mutant) and an avirulent strain (e.g. a strain derivative expressing an effector triggering HR in that host),

6- Results can be misleading when using plants that do not have the appropriate age or that are affected by different stresses. Try to avoid plants that have unexpected morphological phenotypes or that seem affected by contaminating pathogens. Plant growth and watering conditions may need to be set up in advance.

7- Although 3 plants per mixed infection are normally sufficient for the analysis of some mutants, increased accuracy and reproducibility can be achieved by using more replicates.

8- The infiltration technique should be practised in advance until mastered appropriately, using spare plants and water, to minimise mechanical damage. An Arabidopsis leave that has been appropriately infiltrated should not be distinguished from a non-infiltrated leave 1-2 h after infiltration. Mechanical damage can distort the result of the experiment.

9- Different methods for mechanical disruption can be used depending on lab equipment and personal preferences. Commercial pestles, which adapt to 1.5 mL microcentrifuge tubes can be used: when using such tubes, the diameter of the leaf discs and the volume of 10 mM Mg_2Cl can be reduced to facilitate homogenisation. Pestles can also be adapted to drill stands for medium-throughput processing. A combination of 96 deep-well plates with metal beads and a tissue homogenizer can also be used. In all cases, homogenisation should be as thorough as possible, while carefully keeping the sample from heating through mechanical friction (particularly when using drill stands).

10- Several serial dilutions should be plated to ensure that sufficient number of colonies of both strains will be available for counting. This can be set up in advance and optimised if the same strains will be used in subsequent experiments. We recommend to prepare each dilution step by adding 100 µL of bacterial suspension into 900 µL of 10 mM $MgCl_2$ and to plate 100 µL of each dilution into a 9 cm Petri dish. The use of multi-well plates and multi-channel pipettes to make dilutions can speed up the process, but might introduce additional variation, and is therefore not recommended.

11- To maximise the accuracy of the assay, only the plated dilution that contains a number between 50-500 bacterial colonies should be considered for each strain. Lower colony numbers may be subject to stochastic variation, therefore not reflecting the actual bacterial numbers in the sample obtained from the plant.

12- For a reliable calculation of the CI value, the input ratio should be as close as possible to 1. Input ratios lower than 0.8 or higher than 1.3 may be the cause of misleading results and should not be used.

13- When applying CI assays to analyse the role of a plant genotype in the differential growth of the query bacterial strain, it is necessary to consider carefully the actual bacterial cfu numbers obtained for each strain within the mixed infection in each of the plant genotypes assayed. This is aimed to ensure that the plant genotype is actually affecting the growth of the tested strain, and that the CI output is not misleading as a consequence of an altered growth behaviour of the co-inoculated wild type strain. The possible outputs for CI assays of plant genotypes, illustrated with examples that we have encountered throughout our work, are detailed in Ref 8.

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Figure legend

Figure 1. **Determination and analysis of a competitive Index.** A mix inoculum containing an equal bacterial number of wild type and mutant stains is infiltrated into plant leaves. Bacteria are recovered from plant leaves at the appropriate time point, *i.e.* days post inoculation (dpi), and plated into LB and LB supplemented with antibiotics, to differentiate between the co-inoculated strains. The CI is defined as the mutant-to-wt output ratio divided by the mutant-to-wt input ratio. A CI not significantly different from 1.0 indicates that growth of the mutant strain is not significantly different from growth of the wild type, and therefore the mutation has not effect on virulence. A CI significantly different from 1.0 indicates that growth of the mutant strain is significantly lower than growth of the wild type, and therefore the mutation causes virulence attenuation.

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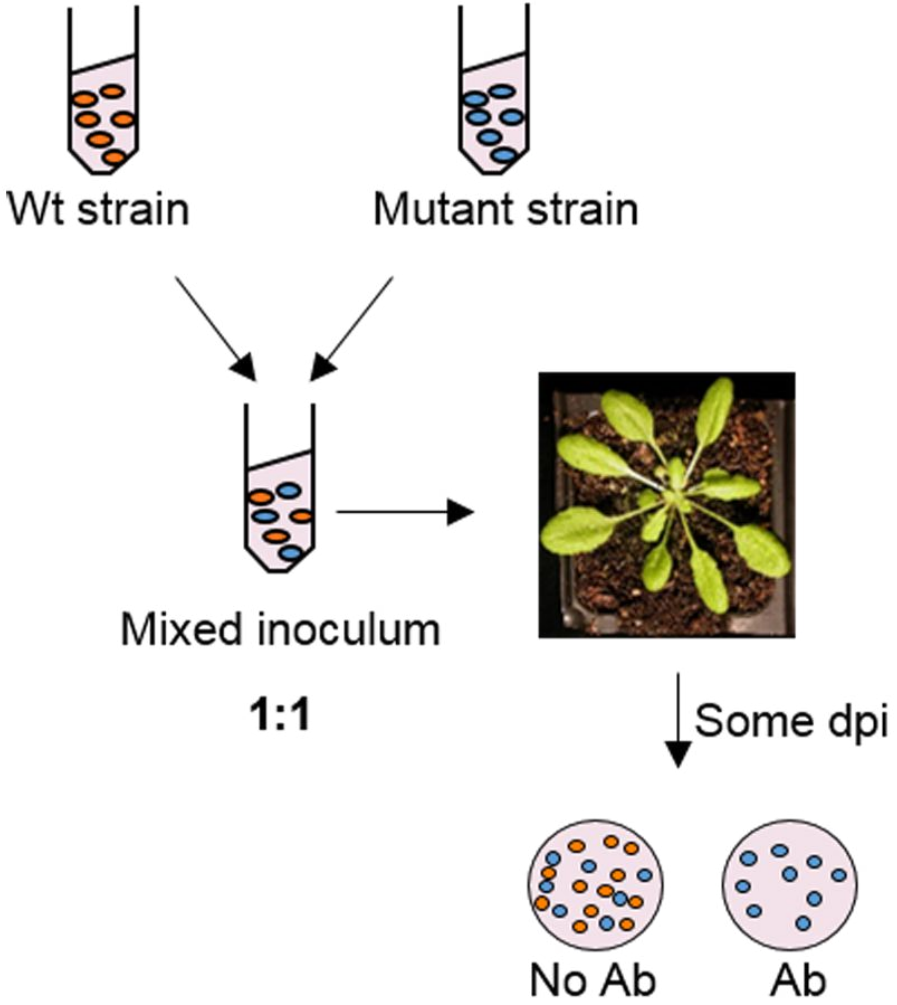
Mixed Infections



Competitive Index (CI)

$$CI = \frac{\text{cfu mutant /cfu wt (Output)}}{\text{cfu mutant / cfu wt (Input)}}$$