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TECHNICAL ADVANCE

A high-throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana*

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Summary

Measuring the growth of pathogenic bacteria in leaves is a mainstay of plant pathology studies. We have made significant improvements to standard methods that will not only increase the throughput but also reduce the space limitations. Additionally, the method described here is as accurate as the standard method. Briefly, we infected leaves by dipping whole seedlings of *Arabidopsis* into a bacterial solution containing a surfactant. After harvest, the seedlings were then simply shaken in buffer. The resulting bacterial solutions were diluted in microtitre plates and spotted onto agar plates. Colony-forming units were then counted 40 h after plating. Therefore, we have eliminated most of the labour-intensive steps involved in measuring the growth of bacteria in *Arabidopsis*, and describe a method that could be automated. The assay is sensitive enough to detect small differences between pathogens or ecotypes.

Keywords: *Arabidopsis*, resistance genes, *Pseudomonas syringae*, phytopathogenic bacteria, counting bacteria

Introduction

The study of the interactions between plants and their pathogens is a strong and vibrant field in modern biology. Measuring the growth of pathogens *in planta* is critical to evaluate the response of the plant to a known quantity of pathogen. We focus on the interaction between *Arabidopsis thaliana* (*Arabidopsis*) and strains of *Pseudomonas syringae* (Ps, Glazebrook *et al.*, 1997). Four cloned resistance genes (*R*) condition resistance to bacterial strains expressing corresponding, cloned avirulence (*avr*) genes from four strains of *P. syringae* have been characterized (reviewed by Jones, 2001; Nimchuck *et al.*, 2001). Additionally, there are a number of virulent Ps strains that do not express these genes and cause disease on *Arabidopsis*. Thus, this interaction also provides an excellent model for dissecting the virulence mechanisms of *P. syringae*.

Landmark efforts in the early 1990s (Debener *et al.*, 1991; Dong *et al.*, 1991; Whalen *et al.*, 1991) described the interactions between these two organisms. Specifically, Whalen *et al.* (1991) described different procedures to inoculate *Arabidopsis*, namely pressure infiltration, dip, vacuum infiltration, and spray. Pressure infiltration continues to be the most commonly used method for measuring growth of bacteria *in planta*. In this method, leaves of adult, 4–5-week-old, short-day plants are inoculated. After that, a measured fragment of the leaf is detached with a cork borer, ground, and the bacteria serially diluted and plated in a agar plate on the appropriate selective medium (e.g. Morel and Dangl, 1998). For analyses of large numbers of plant–bacterial strain combinations, the amount of labour quickly becomes overwhelming. We revisited the dip inoculation protocol previously described (Whalen *et al.*, 1991; Wanner *et al.*, 1993), in order to facilitate our analyses of bacterial growth in a large number of mutant and transgenic lines of *Arabidopsis*. We optimized this method using small plants (2-week-old, 8 h day) to both increase the number of data points generated per unit of growth space and reduce the amount of labour. We have also developed a new simple method for measuring bacterial growth following dip

Figure 1. Macroscopic symptoms in *Arabidopsis thaliana* 5 days post-inoculation (dpi) with Pst (*avrRpm1*) by dipping.
The left part of the pot contains wild-type Col-0 plants, and the right part the mutant *rpm1-1*. Note that *rpm1-1* is glabrous (Oppenheimer *et al.*, 1991).

inoculation of plants. We believe that these modifications will improve considerably the time and space needed to quantify growth of pathogenic bacteria in *Arabidopsis*.

Results and discussion

Inoculation

Seeds are sown in a pot, covered with nylon mesh, and grown under 8 h day conditions (Figure 1). Inoculation is performed 14 days after transfer to the growth chamber. Several factors were tested and found to be critical for reproducibility. First, the inoculum is prepared with bacteria grown on plates with the appropriate antibiotic for 24 h. Bacteria cultivated in liquid media do not generate reproducible results (data not shown). Second, once the bacteria have been resuspended in 10 mM $MgCl_2$, Silwet L-77 is added at a final concentration of 200 μl l^{-1}. This surfactant was also found to enhance the transformation of *Arabidopsis* by *Agrobacterium tumefaciens* (Clough and Bent, 1998). Third, because *P. syringae* can infect the leaf via stomata (Goto, 1992), we kept a transparent lid on the tray of pots after inoculation because stomatal opening is dependent on high humidity. We found that the uniformity of the inoculation was improved if we left the lid closed for 1 h. When high humidity was maintained for longer periods (24 h), the resistance response was reduced (data not shown). With these modifications, *P. syringae* is able to reproducibly infect *Arabidopsis* by dipping.

We used *P. syringae* pathovar tomato, isolate DC3000 (Pst), which is an aggressive pathogen on *Arabidopsis*. We optimized bacterial concentration by using the concentration that did not produce a visual response during an incompatible (plant resistant) interaction and also yielded the highest difference in growth of bacteria between compatible (plant susceptible) and incompatible interactions. In our case, this concentration is OD_{600} = 0.05 (approximately 2.5×10^7 colony-forming units per ml (cfu ml^{-1})). This concentration of bacteria is higher than the inoculum generated in nature when rain runs through an infected leaf (10^5–10^7 cfu ml^{-1}; Goto, 1992). However, the traditional pressure infiltration method typically uses a starting concentration of approximately 10^5 cfu ml^{-1} for growth determinations, and this produces inoculum levels of 10^3–10^4 cfu cm^{-2} of leaf (e.g. Morel and Dangl, 1998). This inoculum corresponds to roughly 2.5×10^2–2.5×10^3 cfu mg^{-1} of leaf (1 cm^2 of a 3-week-old leaf weighs approximately 4 mg, data not shown). This number is very close to the effective inoculum of 10^3–10^4 cfu mg^{-1} we use here.

Figure 1 shows symptoms 5 days post-infection (dpi) for plants that have been dip-inoculated using the method described above with Pst (*avrRpm1*) (Dangl *et al.*, 1992). The plants growing in the left half of the pot are plants of *Arabidopsis* accession Col-0, which express *RPM1* (Grant *et al.*, 1995) and are thus resistant to this particular strain of Pst. At this bacterial concentration, there are no visible lesions in Col-0 and growth of the bacteria is suppressed. We used trypan blue staining to aid in the visualization of the hypersensitive response (HR) in plants. The HR is a programmed cell death process associated with some resistance response (reviewed in Morel and Dangl, 1997). Uninoculated leaves of Col-0 do not stain positively for trypan blue, except along the veins (data not shown). In contrast, leaves of Col-0 inoculated with Pst (*avrRpm1*) show occasional HR foci that stain positively for trypan blue as early as 8 h post-inoculation (hpi).

Plants carrying the *rpm1-1* mutant allele (Grant *et al.*, 1995) are shown in Figure 1, growing in the right half of the pot. In the absence of active *RPM1*, Pst (*avrRpm1*) is able to thrive. The disease symptoms are apparent (Figure 1) within 5 dpi. There is no cell death in *rpm1-1* as determined by trypan blue staining at 8 hpi. After 1 day, micro-colonies of bacteria were visible under epi-fluorescence, but still no cell death was observed. Therefore, the expected responses (determined by pressure infiltration) for different lines of *Arabidopsis* were observed in response to dip inoculation of Pst (*avrRpm1*).

We routinely used this method to evaluate different *Arabidopsis* mutants that show qualitative differences in resistance against Pst (*avrRpm1*). We were able to successfully discriminate between several levels of resistance, such as those exhibited by strong and weak loss of function alleles of *RPM1* (Tornero and Dangl, unpublished results). Nevertheless, once we had found qualitative differences with this method, we found ourselves switch-

ing to hand inoculation of older plants in order to generate quantitative data. As the standard method requires 4–5-week-old plants, this represented a loss of time and resources. In our system, the plants are too small to punch leaf discs of known size, so we decided to express the number of bacteria per fresh weight of the plant. Three seedlings per replicate were removed from pots containing inoculated plants. Care was taken to avoid taking soil or roots. These seedlings were placed in pre-weighed 1.5 ml microfuge tubes containing 200 μl of $MgCl_2$. The tubes were weighed again to determine the amount of plant tissue for each sample. Data were collected in quadruplicate. Thus, each data point is the mean and standard error of four independent measurements and required 12 seedlings. The tubes were then processed with a grinder (Dual Range Stirrer with a custom-made stainless steel tip, Caframo Ltd, Ontario, Canada) as described previously (e.g. Morel and Dangl, 1998). With these changes in the method, we are able to use less space in the short-day chambers (a limitation in most laboratories) and less time. Thus, in our method we use a tray (28 × 54 cm) holding up to 20 pots (8.9 cm diameter) for 3 weeks. This allows the analysis of 20 plant lines with one pathogen. With pressure infiltration, we used the same surface for 5 weeks growing 36 plants (9 × 4 plant matrix), which resulted in data for nine plant lines (using four individuals per analysis). The improvement is considerable, from 1.8 lines per week/unit surface in the hand inoculation, to 6.7 lines per week/unit surface in the dip inoculation. There is also an important saving in hands-on time. Thus, hand inoculation of the same nine plant lines takes between 20 and 30 min, while dip inoculation of the same growing surface (20 pots) takes between 5 and 10 min. Therefore, the time required for hand inoculation is 2.2–3.3 min $line^{-1}$ versus 0.25–0.5 min $line^{-1}$ for dip inoculation, a significant improvement. However, the most important benefit is obtained in time required for sample handling. As the samples are shaken for 1 h, this time is used for preparation of the dilutions in the next step. With 20 lines, our estimation of the time needed for grinding the tissue is 3 h of hands-on work.

We took 1 h after inoculation as time zero, as this is the time when we open the lid. The intention of this measurement is to assess the uniformity of the inoculation. In our case, the differences in phenotypes between control incompatible and compatible interactions are obvious at 3 and 5 dpi (Figure 1). However, we prefer not to measure beyond day 3, as by 4 dpi, some of the leaves in the compatible interactions are completely desiccated but still carry viable bacteria. This is a source of error when plants are picked, as we express growth per weight, so we decided to avoid it. If an extended time course is needed, a low initial inoculum (as low as $OD_{600} = 5 \times 10^4$ cfu mL^{-1}) is recommended.

Bacterial extraction from infected leaves

We also designed a method to rapidly extract bacterial cells from infected leaves, further reducing the amount of labour required to process samples. The same number of seedlings per sample as above was used. In order to improve the reproducibility, we increased the volume of the buffer to 1 ml per tube, and added 0.2% v/v Silwet L-77. Up to 96 microfuge tubes were then placed into a single 2 l flask and shaken for 1 h at 28°C, 18.7 g (250 rpm). Shaking for longer periods of time (3 h) did not result in an observable increase in the number of bacterial cells recovered from leaves of *Arabidopsis* (data not shown). Washing leaves to remove any bacteria possibly growing on the surfaces prior to shaking also did not affect the number of cells recovered.

Titration of bacteria and plating

After bacterial extraction, we used the dilution procedure already established to measure the number of cfu per ml following pressure infiltration. Thus, we prepared a microtitre plate by adding 180 μl of 10 mM $MgCl_2$ to each well. Aliquots (20 μl) of bacterial solution were removed from each tube and independently added (undiluted from the tube) to the wells in the first column of the microtitre plate. We then used a multi-channel pipette to make serial 10-fold dilutions in wells corresponding to columns 2–6. Drops (2 μl) from each well were spotted onto a single 150 mm agar plate carrying the appropriate antibiotics. Drop sizes larger than 2 μl often mixed with one another. Thus, one agar plate is sufficient for measuring 96 dilutions of bacteria. The number of colony-forming units is then counted as described below.

We have also used an alternative method to spotting 2 μl drops onto plates. A 96-pronged stamp can also be used to spot a small aliquot of each bacterial dilution onto the plates in a single motion. The weight of the stamp is enough to make a reproducible impression in the agar without breaking the surface. Between different microtitre plates, the stamp is briefly dipped in ethanol and then blotted three times onto absorbent paper. No cross-contamination results using this treatment (data not shown). The agar plates are then incubated at 25°C for 40 h and the number of colony-forming units is counted. We can reliably count dilutions that have between 1 and 20 per drop. The total number of bacteria (cfu/mg fresh weight) is calculated by multiply the number of colonies by a constant '*k*' (see Experimental procedures) adjusting the dilution, and dividing by the weight of the tissue as described in Experimental procedures.

Figure 2 indicates that recovery of bacterial cells at 3 dpi by shaking is as effective as that obtained by grinding leaf tissue. The data presented on the left show the growth of

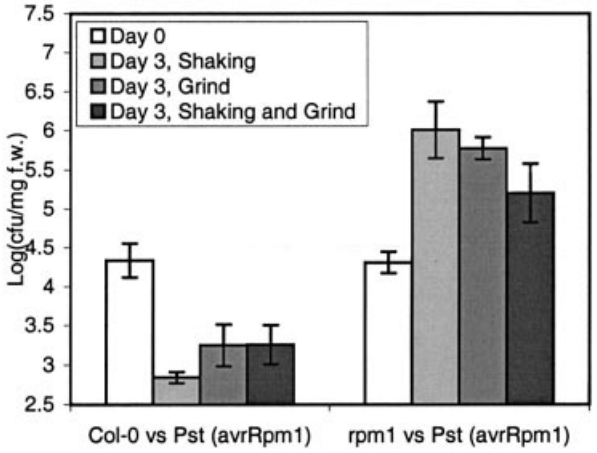

Figure 2. Comparison of different methods of extracting bacteria from *Arabidopsis*.
Plants of Col-0 (left group of columns) or *rpm1-1* (right group of columns) were inoculated by dipping with Pst (*avrRpm1*). At day 0 (white columns) and day 3 (light grey columns), bacteria were extracted from the plants by shaking. After the buffer had been removed, the tissue was blotted and ground with a grinder in order to quantify the remaining bacteria (black columns). Independent samples with no previous treatment were also ground (dark grey columns).

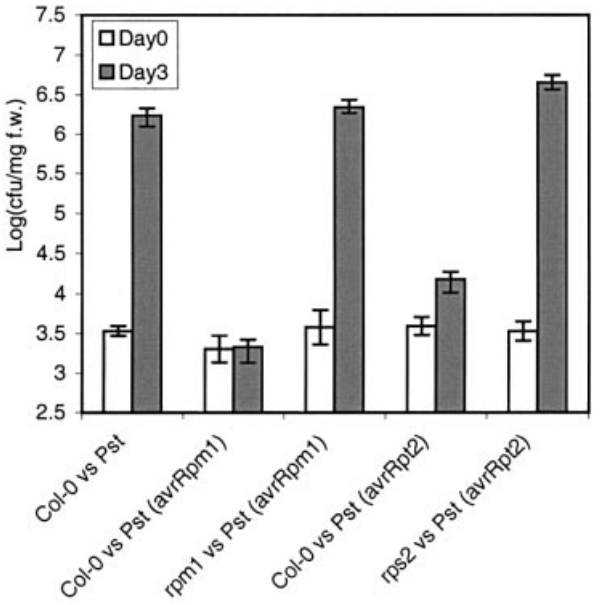

Figure 3. Comparison of different pathogens in the same plants.
Plants of Col-0, *rpm1-1* or *rps2-101c* were inoculated by dipping with Pst, Pst (*avrRpm1*) or Pst (*avrRpt2*). At day 0 (white columns) and day 3 (light grey columns), bacteria were extracted from the plants by shaking.

Pst (*avrRpm1*) on Col-0, and the data on the right show growth of the same bacteria on *rpm1-1*. For the day 0 data (white columns), the number of bacterial cells was determined in each genotype of plant at 1 hpi by the method described here. These results indicate that the inoculations were uniform between genotypes of plants. The remaining columns indicate that, by 3 dpi, Pst (*avrRpm1*) can successfully grow in *rpm1-1* plants, but not in Col-0. The decrease in the number of colony-forming units at 3 dpi, relative to 0 dpi, in Col-0 is most likely due to the increase in leaf mass in the ratio cfu mg^{-1}. More importantly, the data clearly show that the recovery of bacterial cells from *rpm1-1* plants by shaking (column 2) or grinding (column 3) is comparable. Column 4 represents the bacteria released from *rpm1-1* plants that were first shaken for 1 h, the tissue briefly blotted, and then ground using a grinder. Although it appears that a significant number of bacterial cells remain in the plant, this represents only approximately 10% of the total number of bacterial cells recovered by shaking alone. Therefore, shaking of plants to recover bacterial cells from plants is as efficient as grinding the leaf tissue, but requires significantly less labour.

Figure 3 shows that the method presented here is sensitive enough to measure small differences in the growth of different strains of Pst on *Arabidopsis*. Pst (*avrRpm1*) and Pst (*avrRpt2*) (Innes *et al.*, 1993) grow to different levels on susceptible near-isogenic lines of *Arabidopsis* that carry mutations in the respective *R* genes, *RPM1* and *RPS2* (Bent *et al.*, 1994; Grant *et al.*, 1995; Mindrinos *et al.*, 1994). In previous studies, growth of the bacterial strains was measured following pressure infiltration. We used the method described here to determine whether we could observe the same patterns of growth on these *Arabidopsis* lines. As seen in Figure 3, we detect the same differences in growth of the different strains of Pst on the different lines of *Arabidopsis*, i.e. Col-0 plants exhibit a more effective resistance to Pst (*avrRpm1*) than to Pst (*avrRpt2*). We realize that our results are somehow different to the ones reported previously. Thus, Ritter and Dangl (1996) reported a difference in growth of Pst (*avrRpm1*) in Col-0 of more than 10-fold between days 0 and 3. We speculate that dip inoculation, which reflects natural infection more accurately, is more sensitive to small differences between pathogens or plants. This idea was first proposed by Wanner *et al.* (1993). Also, Mittal and Davis (1995) reported no difference in growth of bacteria with and without the phytotoxin Coronatine in *Arabidopsis* when the bacteria were hand-infiltrated. However, the authors reported strong differences when the same bacteria were dip-inoculated. In our hands, the symptoms observed with the dip method are more pronounced that in the hand inoculation method. For instance, with Pst (*avrRpm1*) no macroscopic symptoms were observed in Col-0 at day 5 by dip inoculation (Figure 1), as opposed to hand inoculation where mild disease symptoms are frequent (data not shown). Conversely, the symptoms observed in the *rpm1* mutant inoculated with Pst

(*avrRpm1*) (Figure 1) are more severe than the equivalent hand inoculation (data not shown).

An independent repetition was performed. It is important to mention several aspects of the repetitions. Data from independent experiments should not be pooled together. While genotype-dependent growth differences are maintained, a Student's *t* test (with $\alpha = 0.05$) recognized parallel data from independent experiments as statistically different in 3 out of 10 pair wise comparisons. This phenomenon was observed in several systems, including data from tissue culture experiments and hand inoculations of bacteria in *Arabidopsis* (data not shown). Incidentally, the same *t* test does not recognize differences in bacterial levels at day 0 within an independent experiment. Conversely, differences between days 0 and 3 in each case are catalogued as significant, with the exception of the incompatible interaction between Pst (*avrRpm1*) and Col-0 in both experiments, and the interaction between Pst (*avrRpt2*) and Col-0 in the second repetition. Most importantly, the differences between Col-0 and the respective *r* gene mutants were statistically significant when challenged with the same strain of bacteria. We also used the dip inoculation method to recapitulate results observed in the interactions between Pst (*avrB*) and *RPM1* (Grant *et al.*, 1995; Tamaki *et al.*, 1991) Pst (*avrPphB*) and *RPS5* (Mansfield *et al.*, 1994; Warren *et al.*, 1998) and Pst (*avrRps4*) and *RPS4* (Gassmann and Staskawicz, 1999; Hinsch and Staskawicz, 1996) (data not shown), as well as the interactions between different Pst isolates and mutant lines *lsd1*, *pad4*, *eds1*, *nim1*, *nahG* and some double mutant combinations (Rusterucci *et al.*, 2001; Aviv *et al.*, 2001).

Our method can also be used to detect small differences in growth of bacteria that cannot be detected using the pressure infiltration method. No major differences in growth of Pst (*avrRpm1*) on different *RPM1*-expressing ecotypes of *Arabidopsis* have been reported. However, using the dip inoculation method, we observed a several fold higher growth of Pst (*avrRpm1*) in plants of Landsberga *erecta* relative to Col-0 (Figure 4; first four columns). Our data suggest that the cotyledons of Landsberga *erecta* were considerably more susceptible than the rest of the plant (Figure 4; columns 5 and 6). This suggests a novel developmental control of *RPM1* function in La-*er*. Note that the number of cotyledons per mg is higher than the number of true leaves per mg, explaining the observation that the bacterial titre in cotyledons is higher than in the total plant. We used trypan blue staining of infected cotyledons of Landsberga *erecta* at 5 dpi and observed the presence of microcolonies. No microcolonies were observed in infected leaves of Col-0.

To summarize, this method is fast, reliable, and can unveil small differences between pathogens and ecotypes of *Arabidopsis*. There are disadvantages of this method,

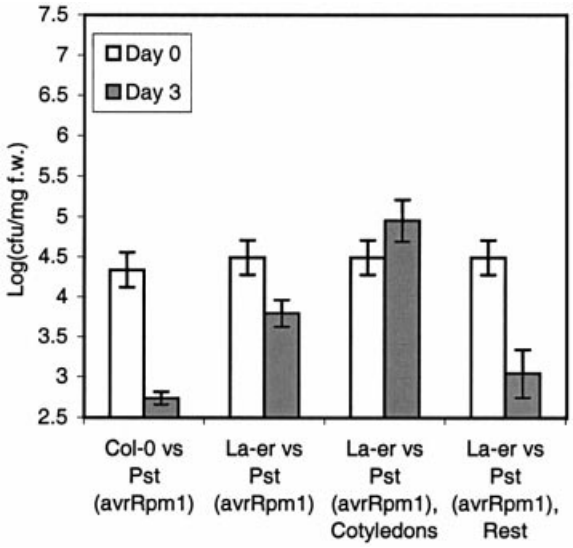

Figure 4. Comparison of different ecotypes infected with the same pathogen.
Plants of Col-0 and Landsberga *erecta* (La-*er*) were inoculated by dipping with Pst (*avrRpm1*) and the bacteria extracted by shaking. At day 3, plants of La-*er* were either processed as whole seedlings (La-*er* vs Pst (*avrRpm1*)) or each plant was divided into cotyledons (La-*er* vs Pst (*avrRpm1*), cotyledons) and the rest of the plant (La-*er* vs Pst (*avrRpm1*), rest). Note that the day 0 data for La-*er* are from the whole seedling in all cases.

mainly the fact that the plant cannot be recovered, as opposed to the detachment and grinding of a single leaf. This aspect may be limiting on occasions were few plants are available. However, we believe that the enormous saving of space, time of growth until experimentation, hands-on time, and the reduction in the fatigue of the researcher overcomes these problems.

Experimental procedures

Plant lines

The mutants *rpm1-1* (Grant *et al.*, 1995) and *rps2-101c* (Yu *et al.*, 1993) have been described previously. Note that *rpm1-1* was isolated in a glabrous background (Oppenheimer *et al.*, 1991) as an independent marker. The plants are sown in pots (8.9 cm diameter) covered with a mesh (bridal veil, purchased in a local fabric store, with the threads separated 1 mm), and the mesh is secured with a rubber band. Plants are grown under a short-day regimen, as described previously (Ritter and Dangl, 1996). A small number of plants are sown, in order to avoid crowding. In practice, we thin the plants at day 10 (after transfer to the growth conditions) until 50–100 plants are left. The inoculation is done at day 14.

Bacterial strains, growth and inoculation

Pseudomonas syringae pv. tomato DC3000 (Pst) derivatives containing pVSP61 (empty vector, no *avr* gene) or *avrRpm1* or *avrRpt2* (in the vector pVSP61) were used and maintained as

described previously (Ritter and Dangl, 1996). Approximately 24 h prior to inoculation, a generous sample of the bacteria growing on a plate was plated onto a fresh plate of KB medium (King *et al.*, 1954) with the appropriate antibiotics. The inoculum was then distributed using a spreader in order to obtain a lawn of bacteria. Once the bacteria have grown for 24 h at 25°C, 10 ml of 10 mM $MgCl_2$ is added to the plate. Ten minutes later, the bacterial suspension is washed out of the plates with a pipette, the OD_{600} is measured, samples adjusted to OD_{600} = 0.05, and Silwet L-77 (OSi Specialties Inc., Danbury, Connecticut, USA) is added to a final concentration of 200 μl l^{-1}.

The pot is then turned upside-down and submerged in the bacterial solution for 10 sec. In order to ensure that all the plants are dipped, the pot is submerged approximately 3 cm above the soil. We prepare aliquots of 1 l per 20 pots (8.9 cm of diameter each), discarding the remaining. We inoculate plants at the beginning of the short day to ensure reproducibility. Once the inoculation has been completed, the plants are moved to the short-day chamber with a transparent lid covering the plants.

Bacterial counting

One hour after the inoculation, the lid is removed and the samples for day zero are taken. Briefly, three whole seedlings, without roots, are placed into a pre-weighed 1.5 ml tube containing 10 mM $MgCl_2$ and 0.2% v/v Silwet L-77 and the weight is recorded. Four tubes are prepared for each data point. The tubes are then introduced in a 2 l flask, and this flask is shaken at 28°C, 18.7 g (250 rpm), for 1 h. After this time, 20 μl from each tube are added to the wells of a microtitre plate containing 180 μl of 10 mM $MgCl_2$, and serial 10-fold dilutions are prepared with a multichannel pipette. The bacteria are spotted onto a 150 mm Petri plate of KB containing the appropriate antibiotics with the help of a 96-pronged stamp, and the plate is incubated at 25°C. Forty hours later, the number of colonies are counted. We count the dilution that gives us between 1 and 20 colonies. The number of cfu mg^{-1} fresh weight is determined by the formula:

$$\text{cfu mg}^{-1}\,\text{FW} = k\,(N \times 10^{d-1})/(\text{weight of the tissue})$$

where N is the number of colonies counted in the dilution number d, and k is a constant, calculated as follows. One colony in the first dilution would indicate that the concentration in the first well was 1 cfu/0.6 μl, as this is the volume of liquid delivered by the stamp (determined empirically, data not shown). As this is from a 10-fold dilution, it is equivalent to 10 cfu/0.6 μl from the original tube. Assuming that the volume of the plants is negligible in comparison to the 1000 μl of buffer, this equals 16 667 cfu per tube. Therefore, the constant k is 16 667. Each data point is represented as the mean and standard error of the decimal logarithm of four repeats. Independent experiments were performed on different days to ensure that the method is robust.

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Supplementary Material

The following material is available from http://www.blackwell-science.com/products/journals/suppmat/TPJ/TPJ1136/TPJ1136sm.htm

Appendix 1

Inoculation by dipping.

Appendix 2

Growth curves (dipping).

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