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Identification of Rhizobacteria that Increase Yield and Plant Tolerance to Angular Leaf Spot Disease in Cucumber

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Abstract

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The biological control of angular leaf spot disease (ALS) of cucumbers (*Cucumis sativus*), caused by *Pseudomonas syringae* pv. *lachrymans* (*Psl*), using promising rhizobacteria (RB) and to compare RB efficacy to that of acibenzolar-*S*-methyl (ASM) was investigated. Effects of ASM and RB isolate *Pseudomonas putida* AA11/1 that was isolated from the healthy cucumber root surface on disease severity and plant growth were evaluated using ALS-susceptible and tolerant cucumber cultivars in a growth chamber and a soilless growing system. ASM and AA11/1 significantly reduced average disease severity of ALS by 69 and 34% in the susceptible cultivar and 92 and 21% in the tolerant cultivar, respectively. ASM treatment significantly reduced *Psl* populations, but AA11/1 did not inhibit *Psl* growth in either cultivar. In the soilless system, disease severity was limited by either ASM or AA11/1, whereas only AA11/1 treatments significantly increased cucumber yield by 68 and 33% in the susceptible and tolerant cultivar, respectively.

Keywords: *Pseudomonas syringae* pv. *lachrymans*; induced tolerance; plant growth promoting rhizobacteria; acibenzolar-*S*-methyl

Angular leaf spot disease (ALS), caused by *Pseudomonas syringae* pv. *lachrymans* (*Psl*), is one of the most common bacterial diseases in cucumbers, and it results in significant yield losses. Several studies have examined the development of resistance to streptomycin and copper in *Psl* and other *P. syringae* pathovars (Yano *et al.* 1978; Scheck *et al.* 1996), and the adverse effects of pesticide usage for disease control on environment and human health have been elucidated in recent years. Therefore, biological control methods for plant diseases have become increasingly important, and the induction of plant resistance is considered a promising tool.

A variety of environmental signals and biological inducers trigger plant defence, including well-known phenomena of induced resistance. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced plant resistance. For instance, ISR is stimulated by plant growth promoting rhizobacteria (PGPR), which directly or indirectly contribute to plant health and development (Saharan & Nehra 2011). Furthermore, ISR is dependent on the phytohormones ethylene and jasmonic acid (Van Loon 2007). On the other hand, SAR, triggered by some chemicals or pathogens, is dependent on the phytohormone salicylic acid, and associated with the accumulation of pathogenesis-related (PR) proteins (Durrant & Dong 2004). SAR disease control levels triggered by acibenzolar-*S*-methyl (ASM) have ranged between virtually no control to 99% in different pathosystems (Walters & Fountaine 2009). This illustrates the potential for impressive levels of disease control with ASM, but the effects were not consistent. Additionally, ASM reduced growth and

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yield of some crop plants. For example, Romero *et al*. (2001) and Hukkanen *et al*. (2008) showed that ASM suppressed the disease, but it also may result in a yield loss.

The purpose of this study was to determine the effects of PGPR and ASM treatments on the severity of ALS in cucumbers, *Psl* population dynamics, and the marketable yield of ALS-sensitive and tolerant cucumber cultivars grown in a soilless system.

MATERIAL AND METHODS

Isolation and identification of rhizobacteria. The rhizobacteria (RB) used in this study were isolated from healthy cucumber roots collected in the western Aegean region of Turkey in 2008–2009. The roots of cucumber plants were washed with tap water and dried with sterile blotting paper. A 1-g sample was taken from the root surface tissue and placed in 100 ml of phosphate buffer. After extraction on a rotary shaker for 30 min at 120 rpm, 10-fold serial dilutions (10^{-1} to 10^{-3}) were made, and 0.1 ml of each dilution was spread on triplicate plates of King's medium B agar (KB) amended with cycloheximide (100 mg/l) and plates were incubated at 24°C for 48 hours. RB colonies, which produced a fluorescent pigment on KB medium, were Gram-negative and which did not induce the hypersensitive reaction on tobacco leaves were selected. RB strains were identified based on the concatenated nucleotide sequences of housekeeping genes *gyrB* and *rpoD,* which were amplified with primer sets UP-1E/AprU and 70F/70R, respectively (Yamamoto *et al.* 2000).

Experiments in planta. The ALS-tolerant Crispina F1 (Cr) (Nunhems Seed Co. Ltd., Haelen, The Netherlands) and ALS-susceptible 22-46 F1 (Rijk Zwaan Co. Ltd., De Lier, The Netherlands) cultivars were selected for experiments from the 18 available cucumber cultivars (Akköprü 2012). The susceptible cultivar 22-46 F1 was used in PGPR screening tests. Fifty-three RB isolates were tested for suppression of ALS in two separate seedling assays in a growth chamber. The candidate RB isolates were applied twice to each plant. For the first application, isolates were grown on KB medium for 48 h, adjusted to a concentration of approximately 10^8 CFU/ml, collected by centrifugation and then suspended in 1.5% carboxymethyl cellulose (CMC). Cucumber seeds were mixed with the bacterial CMC in a sterile beaker. After 30 min, the coated seeds were transferred to sterile blotting paper and maintained at 4°C overnight before sowing on a peat growing medium (Klasmann-Deilmann GmbH, Geeste, Germany). For the second application, 48 h before the pathogen inoculation, each seedling at the second true leaf stage was drenched with 30 ml of an aqueous suspension of the RB isolate at a concentration of 10^8 CFU/ml. At this same time, the leaves of previously non-treated seedlings were sprayed with 0.2 g l-1 ASM (BION-Syngenta Crop Prot. Pty. Ltd., North Ryde, Australia). Sterile distilled water was applied as a negative control.

Seedlings were inoculated with *Psl* strain CFBP 2262 (obtained from CFBP/INRA, Angers, France) by spraying with a 10^7 CFU/ml suspension 48 h after the second application of RB or ASM treatments. Seedlings were maintained in > 80% relative humidity for 48 hours. Thereafter, the seedlings were grown at 24°C with 14 h light/10 h dark and 60% relative humidity in a growth chamber, and 20-ml complete nutrient solution was provided to seedlings on a weekly basis (Gül 2000). The disease severity ratings (0–6 scale) were based on the infected leaf area as follows: 0: no symptoms; 1: 1–3 spots or ≤ 10%, 2: 11–25%, 3: 26–50%, 4: 51–70%, 5: 71–90%, and 6: ≥ 91%. The disease index was calculated using the following formula:

$$\text{Disease severity} = [\Sigma (\text{rating number} \times \text{number of leaves in the rating}) / (\text{total number of leaves} \times \times \text{highest rating})] \times 100$$

The efficacy of the treatment was calculated as the percentage of reduction in disease severity compared to the pathogen-alone treatment. Experiments were conducted according to a completely randomised design with fifteen replicates and repeated twice. The effects of ASM and RB isolate on the growth of cucumber cvs Crispina and 22-46 F1 were evaluated by weighing fresh and dried roots and shoots 14 days after inoculation (dai) with *Psl*.

Monitoring Psl colonisation of seedlings. *Psl* colonisation of cucumber cvs Crispina (ALS-tolerant) and 22-46 F1 (ALS-susceptible), treated with ASM and selected RB strains, was monitored periodically. Seedlings were grown and treated as described above. All leaves of three plants from each treatment were collected at 2 h, 24 h, 4 days, 7 days, and 14 days after *Psl* inoculation. The leaves of each plant were crushed in plastic bags (Bioreba AG, Reinach, Switzerland), and serial dilutions were spread on triplicate plates containing the semi-selective medium for *Psl* called KBZ (KB medium amended with

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boric acid, cephalexin, triphenyltetrazolium chloride, pararosaniline, and cycloheximide), which was prepared according to the protocol of INRA-*PaVe*, Angers, France (Akköprü 2012). Colony counts were converted to colony forming units per gram of fresh weight (CFU/g FW), and colonies on KBZ medium were confirmed as *Psl* using specific "Lac24" primer pairs (F: CGTAACAAATCGTACTAGG, R: ATTCGAGTTCGGAGAAGGTC) as described by Manceau and Brin (2003). A completely randomised experimental design was used for the assay, which was repeated twice.

Biocontrol and growth promotion assays under soilless growth conditions. Seedlings were treated with ASM, RB strains, and *Psl* as described above and were subsequently transferred to a greenhouse 3 dai with *Psl*. The seedlings were transplanted in plastic pots (16 l) filled with cocopeat bricks (Tartes Tarım Ind. Trade Co. Ltd., Izmir, Turkey), and two plants were placed in each pot. An experiment including 6 treatments (RB, ASM, and controls) was set up according to randomised blocks with four replicates and each plot had four plants. A complete nutrient solution was applied to cucumber plants using a drip irrigation system (Gül *et al.* 2000, 2013). Disease severity was measured with the 0–6 scale described above at 4, 7, 14, 21, 28, and 35 dai with *Psl*. Cucumber fruits were picked when the expected length was reached and weighed to determine marketable yield. The cumulative yield was evaluated at the end of the experiment, at 70 dai with *Psl*.

In vitro tests with RB strain AA11/1. The selected RB strain AA11/1 used in the plant experiments was analysed for siderophore production using the blue chromeazurol-*S* (CAS) agar method (Schwyn & Neilands 1987), quantitative estimation of indole-3-acetic acid (IAA) (Asghar *et al.* 2002), and the ability to solubilise tricalcium phosphate in NBRIB agar medium (Nautiyal 1999). The methods of Bakker and Schippers (1987) were used to detect the production of hydrogen cyanide. Tests for antibiosis between the strain AA11/1 and *Psl* were conducted in KB medium and KB supplemented with Fe^{3+} (Jetiyanon & Kloepper 2002).

Statistical analysis. The experimental variants for the two cultivars were as follows: (1) negative control plants (NC); (2) *Psl*-inoculated (PC); (3) RB-treated; (4) ASM-treated; (5) RB-treated + *Psl*-inoculated; (6) ASM-treated + *Psl* inoculated. The data were analysed using SPSS v17.0 statistical software. Significant differences between treatments were determined using Duncan's multiple range test with a significance level of $P \leq 0.05$. *Psl* population data were log-transformed prior to analyses.

RESULTS

Cucumber cultivars and traits of selected RB strains. The tolerant Crispina F1 (Cr) and susceptible 22-46 F1 cultivars were selected from the 18 available cucumber cultivars based on respective reactions to *Psl* (Akköprü 2012). RB strain AA11/1 was selected for in-depth experiments among the 53 RB strains that were isolated from healthy cucumber roots and screened for the suppression of ALS on the suscep-

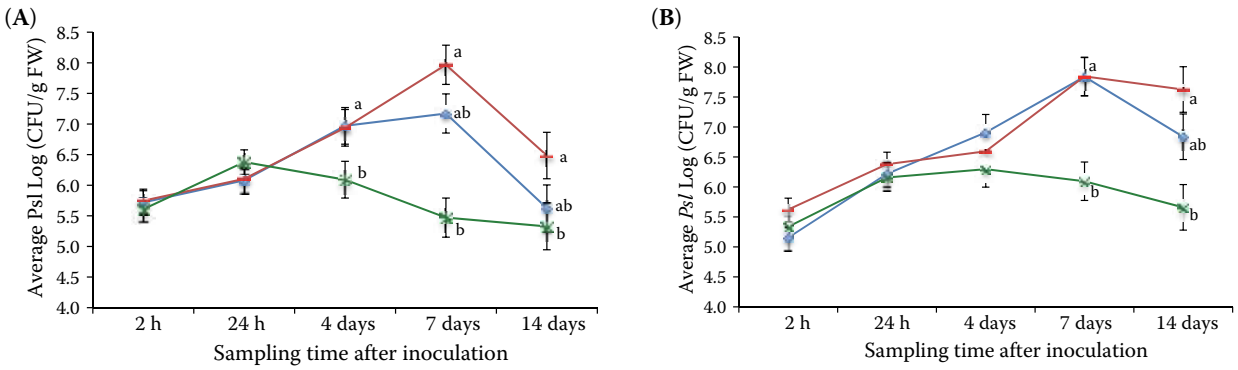

Figure 1. Influence of acibenzolar-*S*-methyl (AMS) and RB strain *Pseudomonas putida* A11/1 on the population dynamics of *Pseudomonas syringae* pv. *lachrymans* (*Psl*) in leaves of cucumber seedlings. The angular leaf spot-tolerant cultivar of cucumber Crispina (**A**) and the susceptible cultivar 22-46 (**B**) were treated with water (blue), RB strain AA11/1 (red), or ASM (green) and inoculated with *Psl* CFBP 2262. Mean *Psl* population sizes were estimated from crushed foliar tissue samples over time. Mean values followed by the same letter at the same time point are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$ significance level ($N \geq 15$)

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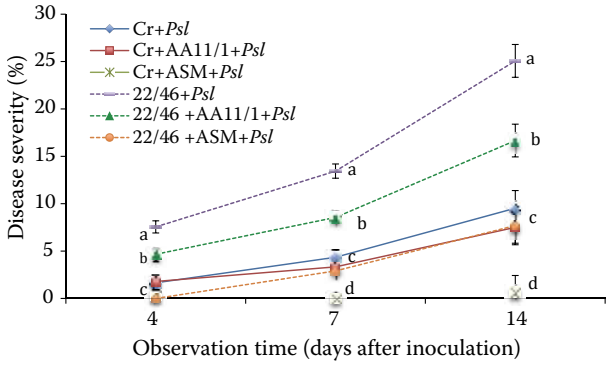

Figure 2. Severity of angular leaf spot (ALS) on cucumber seedlings, inoculated with *Pseudomonas syringae* pv. *lachrymans* (*Psl*), and maintained in a growth chamber. Seedlings of the cultivar Crispina (Cr, ALS-tolerant) and 22-46 (22/46, ALS-susceptible) were treated with water, RB strain All/1, or acibenzolar-*S*-methyl, and inoculated with *Psl* CFBP 2262. Disease severity was monitored over time. Mean values followed by the same letter in a day are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$ significance level ($N \geq 15$)

tible cultivar 22-46 F1 in the seedling assay (data not shown). RB strain AA11/1 reduced the average disease severity of ALS by 32.9%. After the *in planta* screening, the selected isolate AA11/1 was tested to determine its characteristics *in vitro*, and was found to produce high quantities of IAA (60 µg/ml), large zones (15 mm diameter) on CAS agar associated with siderophores and zones of inhibition of *Psl* on KB medium (4.75 mm). However, the phosphatase and hydrogen cyanide production of AA11/1 was not detectable.

RB strain AA11/1 was identified as *Pseudomonas putida* (MF083943, MF083944) according to the sequence analysis of *rpoD* and *gyrB*.

Growth chamber tests. Among the treatments, the first changes in *Psl* populations on both cultivars were observed after the fourth day. ASM significantly reduced the growth of *Psl*, but AA11/1 did not affect the growth of *Psl* on either cucumber cultivar on the seventh day (Figure 1). On the 14th day, AA11/1 did not have any effect on *Psl* populations in the susceptible cultivar, but the populations were significantly lower on the tolerant cultivar Cr treated with AA11/1 and ASM (Figure 1). On the other hand, disease symptom formation was reduced by ASM and AA11/1 on both cultivars (Figure 2). The most successful treatment for disease severity suppression was the ASM application (Figure2), and the efficacy rates of ASM were 69 and 92% in susceptible and tolerant cultivar, respectively. AA11/1 suppressed disease severity on the 14th day in susceptible cultivar by 34% (Figure 2), even though *Psl* populations were not reduced compared to the control (Figure 1).

In general, *Psl* inoculation negatively affected plant growth in both cultivars (Table 1), and ASM treat-

Table 1. Effects of treatments on plant growth parameters of potted plants of cucumber cultivars Crispina (ALS-tolerant) and 22-46 (ALS-susceptible) at 14 dai with *Pseudomonas syringae* pv. *lachrymans* (*Psl*)

	Treatment	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)
cv. Crispina	NC	$8.83^{a} \pm 0.54$	$0.62^{a} \pm 0.05$	$1.4^{a} \pm 0.11$	$0.059^{a} \pm 0.005$
	Psl (PC)	$5.25^{b} \pm 0.54$	$0.36^{b} \pm 0.05$	$0.91^{b} \pm 0.11$	$0.032^{b} \pm 0.005$
	A11/1	$7.93^{a} \pm 0.54$	$0.60^{a} \pm 0.05$	$1.48^{a} \pm 0.11$	$0.056^{a} \pm 0.005$
	ASM	$7.85^{a} \pm 0.54$	$0.68^{a} \pm 0.05$	$1.2^{ab} \pm 0.11$	$0.052^{a} \pm 0.005$
	AA11/1+*Psl*	$5.03^{b} \pm 0.54$	$0.36^{b} \pm 0.05$	$0.93^{b} \pm 0.11$	$0.032^{b} \pm 0.005$
	ASM+*Psl*	$4.39^{b} \pm 0.54$	$0.29^{b} \pm 0.05$	$0.56^{c} \pm 0.11$	$0.021^{b} \pm 0.005$
cv. 22/46	NC	$7.27^{ab} \pm 0.60$	$0.48^{b} \pm 0.04$	$0.61^{bc} \pm 0.60$	$0.034^{ab} \pm 0.004$
	Psl (PC)	$6.46^{b} \pm 0.60$	$0.50^{b} \pm 0.04$	$0.51^{c} \pm 0.60$	$0.03^{ab} \pm 0.004$
	AA11/1	$8.41^{a} \pm 0.60$	$0.53^{b} \pm 0.04$	$0.74^{b} \pm 0.60$	$0.036^{a} \pm 0.004$
	ASM	$8.14^{ab} \pm 0.60$	$0.55^{b} \pm 0.04$	$0.45^{c} \pm 0.60$	$0.027^{ab} \pm 0.004$
	AA11/1+*Psl*	$7.79^{ab} \pm 0.60$	$0.56^{b} \pm 0.04$	$0.53^{c} \pm 0.60$	$0.032^{ab} \pm 0.004$
	ASM+*Psl*	$6.56^{ab} \pm 0.60$	$0.45^{b} \pm 0.04$	$0.52^{c} \pm 0.60$	$0.023^{b} \pm 0.004$

NC – water-treated, non-inoculated (negative control); PC – water-treated, pathogen-inoculated (positive control); mean values followed by the same letter in a cultivar column are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$ significance level ($N \geq 15$)

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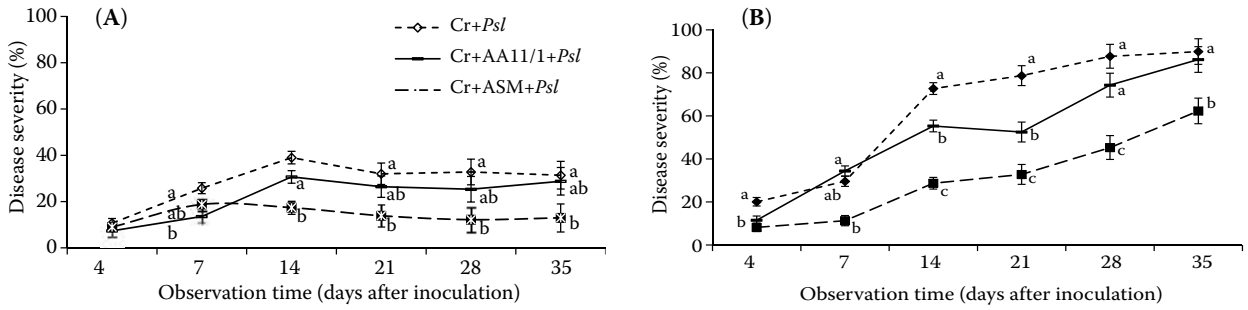

Figure 3. Effects of treatments with A11/1 or ASM on disease severity of ALS on two cucumber cultivars grown in a soilless system. The angular leaf spot-tolerant cultivar Crispina (**A**) and the susceptible cultivar 22-46 (**B**) were treated with water (diamond), RB strain AAll/1 (dash), or ASM (asterisk), and inoculated with *Psl* CFBP 2262. Disease severity was monitored over time. Mean values followed by the same letter in a day are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$ significance level ($N = 12$)

ments on both cultivars significantly decreased the root fresh weight compared to the negative control. Moreover, ASM + *Psl* treatments significantly decreased root fresh weight in the tolerant cultivar Crispina and root dry weight in the susceptible cultivar 22-46. On the other hand, AA11/1 increased root fresh and dry weights compared to NC in susceptible cultivar, and the AA11/1 + *Psl* treatment increased shoot fresh weights compared to the only *Psl* inoculated positive control in susceptible cultivar (Table 1).

Soilless growing system experiments. Regarding the tolerant cultivar, the disease increased on the 14th day, and did not increase thereafter. However, in the susceptible cultivar, disease severity increased until the end of the experiment (Figure 3). On the 21st day, AA11/1 limited ALS severity ratings up to 33 and 17% in susceptible and tolerant cultivar, respectively. However, the suppressive effect of AA11/1 on ALS gradually decreased in susceptible cultivar, likely because of the high *Psl* disease pressure. ASM decreased the disease severity at rates of 59 and 31% in tolerant and susceptible cultivar, respectively, until the end of the experiment (Figure 3). On the other hand, some phytotoxicity formations were observed, including necrotic spots, growth deficiency, and leaf curl of ASM-treated plants (data not shown) that were not observed with the other treatments. Under the high disease pressure, treatment with ASM or AA11/1 did not significantly increase the marketable yield of susceptible and tolerant cultivars (Table 2). However in the absence of disease pressure, the RB strain AA11/1 significantly increased the total marketable yield both in tolerant and susceptible cultivar at rates of 68 and 33%, respectively (Table 2).

DISCUSSION

Bacterial characteristics, such as lipopolysaccharides, or the production of salicylic acid, siderophores, IAA, HNC, or antibiotics that are known to stimulate plant growth and induce resistance (Van Loon 2007; Pieterse *et al.* 2014) are frequently used as criteria to select new PGPR candidates *in vitro*. Interestingly, Meziane *et al.* (2005) reported that lipopolysaccharides, flagella, and siderophores of *P. putida* WCS358 triggered ISR in *Arabidopsis*, but a mutant strain lacking those elicitors also triggered ISR. Consequently, because assays for putative ISR traits *in vitro* may not be predictive of *in planta* efficacy, we screened our candidate RB strains directly on seedlings for the ability to decrease disease severity. After the *in planta* screening, the selected isolate AA11/1 was tested to determine its characteristics *in vitro*, and was found to produce large zones associated with siderophores and high quantities of IAA.

Our RB strain AA11/1 significantly increased the total marketable yield both in tolerant and susceptible cultivars at rates of 68 and 33%, respectively, in the absence of disease pressure. The IAA production of this isolate may be a factor that underlies increased plant growth parameters and marketable yield of the cucumber cultivars tested (Tables 1 and 2). Similar results were reported by Gül *et al.* (2013), who showed highly significant relationships between IAA production of PGPR, as well as increased cucumber fruit number and weight. Additionally, some researchers proposed that the IAA production ability might be used as a marker to select candidate PGPR strains (Khalid *et al.* 2004).

Researchers have observed different effects of ASM in several hosts, including reports of no effect or nega-

https://doi.org/10.17221/41/2017-PPS

Table 2. Effects of the treatments on total marketable yield of cucumber cultivars Crispina (Cr, ALS-tolerant) and 22-46 (22/46, ALS-susceptible) in soilless culture

Treatment		Fruit/plant (g)
cv. Crispina (P = 0.000 SD: ± 37.33)	Cr (NC)	276^{b}
	Cr+*Psl* (PC)	20^{c}
	Cr+ AA11/1	463^{a}
	Cr+ASM	297^{b}
	Cr+AA11/1+*Psl*	81^{c}
	Cr+ASM+*Psl*	119^{c}
cv. 22/46 (P = 0.000 SD: ± 55.19)	22/46 (NC)	598^{B}
	22/46+*Psl* (PC)	0^{C}
	22/46 +AA11/1	794^{A}
	22/46 +ASM	633^{B}
	22/46+AA11/1+*Psl*	0^{C}
	22/46+ASM+*Psl*	0^{C}

NC – water-treated, non-inoculated (negative control); PC – water-treated, pathogen-inoculated (positive control); mean values followed by the same letter are not significantly different according to Duncan's Multiple Range test at $P \leq 0.05$ significance level (N = 10)

tive effects on plants and yield (Romero *et al*. 2001; Hukkanen *el al*. 2008; Mandal *et al.* 2008). In this study, ASM reduced some plant growth parameters in both cultivars (Table 1). Although ASM did not affect marketable yield in the absence of disease pressure (Table 2), we observed some symptoms of phytotoxicity on plants grown in soilless cultures. On the other hand, ASM was the most successful treatment for controlling the disease based on AA11/1 and cultivar effects. The results also indicated that the effects of ASM were likely associated with the suppression of *Psl* growth. Louws *et al.* (2001) and Buonaurio *et al.* (2002) obtained similar results, in that the disease severity in different pathosystems was reduced due to the suppression of pathogen population sizes. In the current study, this effect was observed following a single dose of ASM, but the suppression of disease gradually decreased over time in the susceptible cultivar (Figure 3B). Therefore, ASM must be regularly applied as recommended, and the use of ASM in an integrated pest management approach may be beneficial for disease control. However, cultivar features and dosage should be considered, because increasing the dose might give rise to yield loss or phytotoxicity.

Although A11/1 suppressed ALS in tolerant and susceptible cucumber cultivars, it did not inhibit *Psl* populations on seedlings. Doss and Hevisi (1981) and Block *et al.* (2005) also found that population sizes of pathogenic bacteria were not affected despite decreased symptom formation on plants. This phenomenon is referred to as "systemic acquired tolerance" (SAT) rather than SAR (Block *et al.* 2005; Hammerschmidt 2009). Furthermore, Block *et al.* (2005) proposed that SAT could be related to SAR, and it could be associated with the stimulation level. Van Loon (2007) suggested that tolerance in ISR could have resulted from physiological factors and plant ethylene hormones, and Mecey *et al*. (2011) concluded that genetic and physiological activation of symptom formation could be considered independently of the pathogen population development. Therefore, the effects of AA11/1 on ALS should be evaluated as tolerance within the ISR phenomenon.

In conclusion, although a single application of ASM had negative effects on plants, it significantly decreased the *Psl* population and associated disease severity. Our RB strain *P. putida* AA11/1 significantly increased marketable yields of both cultivars. Although AA11/1 did not decrease disease severity to the same level as ASM, it limited the disease severity by increasing plant tolerance without decreasing *Psl* populations. It was clearly observed in this work that AA11/1 increased the total marketable yield without any disease pressure. The application of AA11/1 combined with other control methods could be beneficial, resulting in increased yield and decreased pesticide and fertiliser input.

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