Research Article

Received: 30 April 2018 Revised: 14 August 2018 Accepted article published: 22 August 2018 Published online in Wiley Online Library: 3 October 2018

(wileyonlinelibrary.com) DOI 10.1002/ps.5181

Systemic resistance inducer acibenzolar-S-methyl (ASM) and its microencapsulated formulations: their long-lasting control efficacy against cucumber diseases and mitigation of phytotoxicity

Hideo Ishii,[a,b*] Masahiro Fujiwara[c] and Kumiko Nishimura[a]

Abstract

BACKGROUND: The development of fungicide resistance by pathogens is a major limiting factor for the control of plant diseases. To combat resistance development, the use of broad-spectrum but nonfungitoxic resistance inducers such as acibenzolar-S-methyl (ASM) is a promising approach because the orchestrated mechanisms underlying systemic acquired resistance induced by ASM are less likely to be overcome easily by pathogens. However, phytotoxicity is the main limiting factor of ASM.

RESULTS: ASM was highly active at inducing systemic resistance against powdery and downy mildews, the two major cucumber diseases. Based on the duration of the control, ASM effectiveness against these diseases was maintained well in plastic greenhouses and the number of sprays could be reduced. Control efficacy after seed treatment with ASM and the applications of microencapsulated ASM was also high against mildews in pots as well as in greenhouse experiments, with no problematic phytotoxicity.

CONCLUSION: The use of ASM is a potential integrated pest management-based tactic to control cucumber powdery and downy mildews because its long-lasting efficacy allows the application of typical fungicides to be reduced. The risk for resistance development in mildew pathogens will also be reduced. ASM seed treatment as well as soil amendment with microencapsulated ASM is effective in lowering the risk for the phytotoxicity of this compound.

Keywords: acibenzolar-S-methyl; cucumber; downy mildew; durability; microcapsule; phytotoxicity; powdery mildew; systemic acquired resistance

1 INTRODUCTION

The use of disease-resistant or -tolerant cultivars is an important part of integrated disease management. New cultivars of cucumber tolerant to major diseases such as powdery mildew [pathogen: *Podosphaera xanthii* (Castagne) U. Braun & N. Shishkoff = *P. fusca* (Fr.) U. Braun & N. Shishkoff], downy mildew [pathogen: *Pseudoperonospora cubensis* (Berkeley et Curtis) Rostowzew] and Corynespora leaf spot [pathogen: *Corynespora cassiicola* (Berk. & Curt.) Wei] are commercially available. However, these cultivars are not grown very widely because yield, quality or other cultural characteristics are not fully acceptable to farmers. As a result, control of cucumber diseases still largely relies on spray applications of standard synthetic fungicides.

Quinone outside inhibitor fungicides such as azoxystrobin and kresoxim-methyl have been commonly used to control major diseases on cucumber since the late 1990s. However, soon after their introduction, resistant strains of powdery mildew, downy mildew and *C. cassiicola* rapidly developed and spread, causing a serious problem for disease control.[1–3] More recently, novel succinate dehydrogenase inhibitors such as boscalid and penthiopyrad have been commercialized, but strains resistant to these fungicides have also appeared quickly in populations of *C. cassiicola* and powdery mildew fungus.[4–7]

Systemic acquired resistance (SAR) is a highly desirable form of resistance that generally provides long-lasting and broad-spectrum resistance in plants.[8] Our research group has been intensively studying the control efficacy and action mechanisms of the systemic disease-resistance inducer acibenzolar-S-methyl (ASM) using cucumber[9–12] and Japanese pear.[13–16] These studies

* *Correspondence to: H Ishii, School of Agriculture, Kibi International University, Minamiawaji, Hyogo 656-0484, Japan. E-mail: hi481204@yahoo.co.jp*

a *National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan*

b *School of Agriculture, Kibi International University, Minamiawaji, Hyogo, Japan*

c *Tohoku Center, National Institute of Advanced Industrial Science and Technology (AIST-Tohoku), Sendai, Miyagi, Japan*

have implicated multiple and complex mechanisms in the expression of ASM-induced SAR on plants, suggesting that these mechanisms will be difficult for fungal or bacterial pathogens to overcome and develop resistance. In fact, probenazole, the first resistance inducer to be commercially introduced, has been widely used in Japan for over 40 years as a major blast control agent on rice with no sign of resistance in the target pathogen *Pyricularia oryzae* Cavara.[17,18] Therefore, the use of ASM is a promising tactic to control diseases in horticultural crops with low risk for resistance development in the target pathogens.

In addition to the suppressive activity of ASM in potted cucumber plants against powdery mildew,[19] excellent SAR-inducing activity of ASM has been documented against two other cucumber diseases, anthracnose and scab caused by *Colletotrichum orbiculare* (Berk. & Mont.) Arx [= *C. lagenarium* (Pass.) Ell. & Halst.] and *Cladosporium cucumerinum* Ell. & Arthur, respectively.[9,20] Thus, ASM may be a resistance inducer with a wide spectrum of disease control capacity. Resistance inducers would be more valuable if their SAR activity were long-lasting under practical growing conditions and conventional fungicide applications can thus be reduced. However, the main limiting factor of resistance inducers, and of ASM in particular, is phytotoxicity such as growth inhibition, chlorosis and necrosis on leaves of treated plants.[21–23] The recent development of an environmentally friendly microcapsule to deliver potential bioinsecticides such as an insect immunosuppressive protein to insect pests[24] may provide a method to increase the shelf life and duration of ASM activity. Microencapsulation of a *Bacillus subtilis* strain has also been reported to be highly effective against Rhizoctonia tomato rot and to have a longer shelf life than that of wettable powders.[25]

The main objectives of this study were to evaluate the control efficacy of (1) ASM against the two major diseases of cucumber, powdery and downy mildews, in pots and in a plastic greenhouse using various methods of treatment and (2) newly formulated silica microcapsules of ASM and examine their contribution to controlling the diseases as well as minimizing the potential phytotoxicity of ASM.

2 MATERIALS AND METHODS

2.1 Chemicals

ASM (*S*-methylbenzo[1, 2, 3]thiadiazole-7-carbothiate) was supplied courtesy of Syngenta AG, Basel, Switzerland as a 50% (a.i.) water-dispersible granular (WG) or 2% granular (G) formulation. As a reference fungicide, commercial formulations of chlorothalonil (TPN; 40% flowable) were used. Sodium silicate (water glass No. 3) and NH_4HCO_3 were purchased from Kishida Chemical Co., Ltd (Osaka, Japan). Other reagents for microcapsule preparation were obtained from Wako Pure Chemical Corporation (Osaka).

2.2 Preparation of silica microcapsules and encapsulation of ASM

Silica microcapsules were prepared by a reported procedure[26] as follows: *n*-hexane solution (72 mL) with Tween 80 (1.0 g) and Span 80 (0.50 g) was mixed using a homogenizer at 8200 rpm. To this solution, a solution (36 mL) of deionized water and sodium silicate (29.9 g, 144 mmol) as an inner water solution was added to form a W/O emulsion. After emulsifying for 1 min at 8200 rpm, this mixture was quickly poured into an outer water solution as noted in Table 1 (252 mL). After mixing this resulting solution for 2 h, the solid was filtered, washed with fresh deionized water three times and with a sufficient amount of methanol. Finally, the solid was dried at 100 °C for 12 h. The porosity of the silica shell of the microcapsules was analyzed by nitrogen adsorption–desorption isotherms measured using a Belsorp Mini instrument (Bel Japan). Specific surface area was determined by the Brunauer–Emmett–Teller method. Barrett–Joyner–Hallender estimation was used to analyze pore volume and peak pore diameter using adsorption branches. Porosity data for silica microcapsules before ASM encapsulation are summarized in Table 1.

ASM was encapsulated into silica microcapsules by impregnating the silica microcapsules (10 times for ASM by mass) with a toluene or acetone solution of ASM. Optical microscope images were obtained using Nikon Model Eclipse E600 POL apparatus (Fig. 1) and scanning electron microscopy (SEM) images using a JEOL JSM-5200 SEM (Fig. 2). The contents of ASM in silica microcapsules were determined by thermogravimetric analysis (TGA) using a Shimadzu TGA-50 apparatus. All samples were held in a platinum sample holder and were heated under air at a rate of 5 °C/min. The mass decreases from 150 to 800 °C were used to estimate ASM content in microcapsules.

2.3 Plants and pathogens

Cucumber cvs. Shin Suyo Tsukemidori (Tohoku Co., Ltd, Tochigi, Japan), Natsubayashi, Natsusuzumi, V-road and Kyoushizuku (Takii & Co., Ltd, Kyoto, Japan), and Pilot 2 (Tokiwa Co., Ltd, Saitama, Japan) were grown in 0.5 L pots with soil at 25 °C in a phytotron under natural light conditions and used for pot and/or plastic greenhouse experiments.

The stock isolates of cucumber powdery and downy mildews were collected from Ibaraki Prefecture and maintained in the laboratory. For successive generations of the powdery mildew fungus, every 10 days, a cotton swab with conidia was rubbed on healthy detached young leaves of cucumber (cv. Shin Suyo Tsukemidori) on a moist filter paper in plastic Petri dishes, which were then

Table 1. Porosity data for silica microcapsules used to encapsulate ASM

Sample encapsulating ASM	Outer water solution (concentration)	Specific surface area(m^2/g)	Pore volume(mL/g)	Peak pore diameter(nm)
eASM-1	NH_4HCO_3 (2 M)	436	0.909	12.12
eASM-2	NH_4Cl (2 M)	211	0.250	2.97
eASM-3	$(NH_4)_2SO_4$ (2 M)	476	0.535	9.23
eASM-4	NH_4Cl (3 M)	461	0.215	1.99
eASM-5	NH_4Cl (3 M)	515	0.231	1.72
eASM-6	NH_4Cl (4 M)	600	0.338	1.72

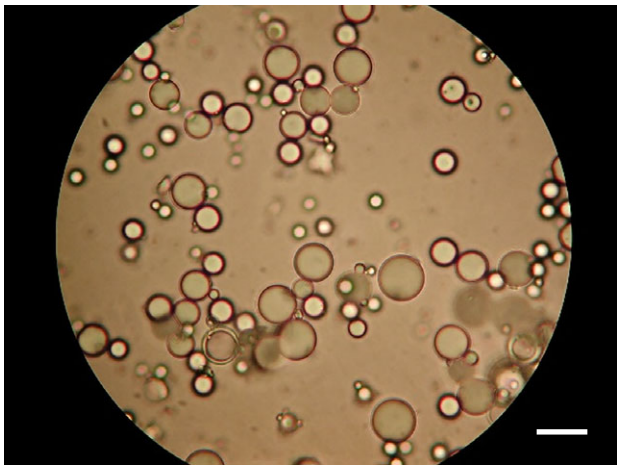

Figure 1. Silica microcapsule formulation of ASM (light micrograph, bar = 20 μm).

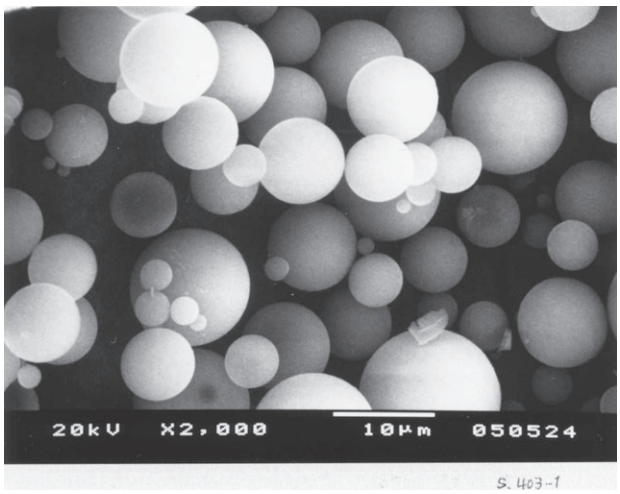

Figure 2. Silica microcapsule formulation of ASM (scanning electron micrograph).

incubated at 20 °C under a 12 h photoperiod using fluorescent light. Zoosporangial suspensions of downy mildew pathogen were dropped onto detached cucumber leaves periodically and maintained in the laboratory using similar conditions.

2.4 Inoculation in pot experiments

The SAR treatment of cucumber plants was performed with ASM as described by Ishii *et al.*[20] Briefly, the first true leaves of plants at the three-leaf stage were dipped for about 5 s in aqueous ASM suspensions at 20 mg L^{-1}, 50 mg L^{-1} a.i. or in distilled water (DW) as a control. Three hours after treatment, whole plants (ASM- or DW-treated first true leaves and untreated upper leaves) were sprayed with conidial suspensions of *P. xanthii* or zoosporangial suspensions of *P. cubensis* (about 1 to 2.5 × 10^5 conidia mL^{-1} DW each for the two pathogens). After inoculation, plants were kept in a dew chamber for 24 h at 20 °C for *P. cubensis* in the dark then transferred to a phytotron at 25 °C with natural light. The plants inoculated with *P. xanthii* were incubated at 25 °C under a 12 h photoperiod using fluorescent light. Each treatment consisted of three replicate plants. Seven days after inoculation, development of downy mildew was assessed. Powdery mildew disease was assessed 10 days after inoculation. Disease severity (DS) was calculated based on the degree of lesion development according to the methods of Ishii *et al.*,[1] and represented as the mean and 95% confidence interval (95% CI) to determine significance.

2.5 Resistance-inducing activity after seed soaking in ASM

In a preliminary experiment, cucumber seeds (cv. Shin Suyo Tsukemidori) were soaked for various times (1, 2, and 3 days) at room temperature (approximately 25 °C) in suspensions of 10, 20, and 50 mg L^{-1} a.i. ASM or DW as a control with gentle stirring in a glass beaker, air-dried, sown into pots of soil and placed at 25 °C in a phytotron. Eighteen days after the start of seed soaking, plant height was measured. Significant suppression of plant growth was visually evident when seeds were soaked for 2 or 3 days, irrespective of the concentration of ASM or DW. In contrast, plant height did not differ after the 1-day soak in ASM at 0, 10, 20, and 50 mg L^{-1} a.i. (data not shown.). Therefore, a 1-day soak in ASM at 50 mg L^{-1} was chosen to test control against two pathogens in subsequent experiments.

2.6 Efficacy of microencapsulated ASM formulations in pot experiments

Soil was amended with either of the granule or the microencapsulated formulations of ASM at the time of seed sowing, and *P. xanthii* was inoculated onto the plants 23 days (Exp. 1) or 28 days (Exp. 2) later (2- to 2.5-leaf-stage). Inoculation with *P. cubensis* was done 29 days (Exp. 3) after sowing (3- to 3.5-leaf stage).

2.7 Plastic greenhouse experiments with natural infection

The experiments were conducted at the National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki, Japan. Potted cucumber plants that had been grown at 25 °C in a phytotron were transplanted in a plastic greenhouse and maintained according to the recommended agricultural practice. The control efficacy of ASM and its silica microcapsule formulations against powdery and downy mildew diseases was assessed using the methods of Ishii *et al.*,[1] and data were expressed as the mean with 95% CI.

(1) Four four-leaf-stage plants (cv. V-road, tolerant to powdery mildew, downy mildew, and viral diseases; each of two replicates in double rows) were transplanted on May 24. Either 100 mg L^{-1} ASM or 400 mg L^{-1} TPN (reference fungicide) were sprayed four times at 2-week intervals using a hand sprayer starting on May 25 until July 6. In one plot, ASM was sprayed twice instead of four times (on June 8 and July 6). Two weeks after the last spray, powdery mildew on whole leaves of plants was assessed.

(2) Susceptible cv. Pilot 2 and cv. Natsubayashi tolerant to powdery and downy mildews were transplanted on August 25 and sprayed with 100 mg L^{-1} ASM or 400 mg L^{-1} TPN four times at 2-week intervals between August 30 and October 11. Downy mildew was assessed on October 18.

(3) (a) Cvs. Natsubayashi and Kyoushizuku (tolerant to Corynespora leaf spot) were transplanted on May 23 into soil amended only with the microencapsulated formulation of ASM eASM-2 at 250 mg (12.5 to 25 mg a.i.). In another plot, soils were amended with 0.5 g of ASM 50 WG (0.25 g a.i.) in the same way. Plants in these two plots were then sprayed with 100 mg L^{-1} a.i. of ASM on June 11 and June 25. In two other plots, plants were sprayed with 100 mg L^{-1} a.i. of ASM or the reference fungicide TPN (400 mg L^{-1} a.i.) at 2-week intervals starting on May 28 until June 25 (three

sprays). Two weeks after the last spray, powdery and downy mildews were assessed.

(b) Plants of cvs. Natsubayashi and Kyoushizuku were transplanted on August 30. Six treatments were included in the test: (1) 100 mg L^{-1} a.i. ASM was sprayed three times after transplanting every 2 weeks starting on September 4, (2) seeds were soaked with 50 mg L^{-1} a.i. ASM at room temperature for 24 h then grown in 0.5 L soil at 25 °C in a phytotron before transplanting, (3) plants were grown in 0.5 L soil amended with 5 mg a.i. ASM immediately before sowing and transplanted, (4) 0.375 g a.i. ASM was added to soil before transplanting, (5) a mixture of the two microencapsulated formulations of ASM, 0.25 g of each (14.2 mg and 12.9 mg a.i. for eASM-4 and eASM-5, respectively) was added to the soil before transplanting. In treatments 2, 3, 4, and 5, transplanted plants were also sprayed with 100 mg L^{-1} a.i. ASM on September 18 and on October 2. In the two reference plots, (6) TPN was sprayed at 400 mg L^{-1} a.i. six times every 7 days starting on September 4, and (7) no treatments were applied before or after transplanting. The disease severity of powdery and downy mildews was assessed 7 days after the last spray with TPN.

(4) Young plants of cvs. Pilot 2 and Natsubayashi were transplanted on May 15 for spray treatment with ASM or TPN between May 19 and June 23 as follows: (1) 100 mg L^{-1} a.i. ASM every 2 weeks, three times total, (2) 100 mg L^{-1} a.i. ASM mixed with TPN 400 mg L^{-1} a.i. every 2 weeks, three times total, (3) TPN at 400 mg L^{-1} a.i., every week, six times total, and (4) no treatment. Downy mildew development was assessed 7 days after the last spray application of TPN.

(5) Young plants of cvs. Natsubayashi and Kyoushizuku were transplanted to soil on May 15 for use in three experimental plots that were amended with ASM just before transplanting as follows: (1) 0.75 g of ASM (50% WG), (2) 0.5 g of microencapsulated formulation eASM-6 and (3) 0.75 g of eASM-6. Subsequently, for all treatments, 100 mg L^{-1} a.i. ASM was sprayed every 2 weeks, three times total, starting on May 26. As a reference, in treatment (4) TPN was sprayed at 400 mg L^{-1} a.i. every week, six times total, between May 19 and June 23, and (5) untreated control was also included. Downy mildew was assessed 7 days after the last TPN spray.

3 RESULTS

3.1 SAR activity of ASM against powdery mildew disease on potted cucumber plants

When three cucumber cvs. Natsubayashi, Natsusuzumi and Shin Suyo Tsukemidori, differing in their susceptibility to powdery mildew disease, were used, 96.7% to 100% powdery mildew control was obtained by ASM both on treated first true leaves and untreated upper leaves. Seven days after SAR treatment and inoculation, powdery mildew was completely controlled by ASM at 20 mg L^{-1} and 50 mg L^{-1} on cvs. Natsubayashi and Natsusuzumi, both tolerant to powdery mildew. On Shin Suyo Tsukemidori, 20 mg L^{-1} ASM gave 97.1% control and 50 mg L^{-1} gave 96.7% control. In this experiment, DS on DW-treated control plants was 36.7 ± 17.4 on Natsubayashi, 31.1 ± 4.3 on Natsusuzumi and 75.6 ± 6.5 on Shin Suyo Tsukemidori, clearly showing the powdery mildew tolerance of the first two cultivars.

3.2 Resistance-inducing activity after seed soaking in ASM

(1) About 1 month after seed soaking, plants were inoculated with *P. xanthii* and disease was evaluated. The tests were conducted twice. Treatment at 50 mg L^{-1} ASM resulted in 83.1% ± 12.6% control under high disease pressure (DS 66.8 ± 37.7 on DW-treated plants) with no sign of phytotoxicity or growth detriment. In the subsequent experiment, 100% and 63.4% control, respectively, was recorded at 50 mg L^{-1} when inoculated 47 days or 61 days after treatment (DS 55.6 ± 8.8 and 65.6 ± 8.7 on DW-treated plants).

(2) Following inoculation with downy mildew pathogen 1 month after seed soaking, ASM applied at 50 mg L^{-1} gave 74.5% ± 8.2% mean control against the disease in two experiments performed under severe disease pressure (69.2 ± 17.9 lesions/leaf on DW-treated plants).

3.3 Efficacy of microencapsulated ASM formulations in pot experiments

(1) Experiment 1: Under heavy powdery mildew pressure (75.0 ± 3.3 lesions/leaf on DW-treated plants), 16 mg (0.8 to 1.6 mg a.i.) or 8 mg (0.4 to 0.8 mg a.i.) eASM-2 achieved moderate control (57.7% and 44.4%, respectively) and suppressed mycelial growth after penetration and subsequent conidiation of the pathogen in infected cucumber tissues with no phytotoxicity. Very high efficacy (97.7% control) was gained by treatment with 10 mg of ASM 50 WG (5 mg a.i.); however, slight chlorosis appeared on leaf margins and blades of the cucumber plants. After treatment with 40 mg and 20 mg of ASM 50 WG (20 mg and 10 mg a.i., respectively), plants either died or growth was severely inhibited at the time of inoculation.

(2) Experiment 2: Soil amendment with the microencapsulated formulation eASM-2 at 32, 16 and 8 mg (1.6 to 3.2, 0.8 to 1.6 and 0.4 to 0.8 mg a.i., respectively) suppressed the development of powdery mildew by 96.6%, 65.5% and 31.1%, respectively, under heavy disease pressure (64.5 ± 4.4 lesions/leaf on DW-treated control plants). Furthermore, complete suppression of disease was achieved with ASM 50 WG at 40, 20 and 10 mg (20, 10 and 5 mg a.i.), but the leaves became chlorotic, phytotoxicity was especially severe with 20 mg and 40 mg of ASM 50 WG. Thus, eASM-2 has a high potential to effectively control powdery mildew and does not cause chemical injury.

(3) Experiment 3: Soil amendment with 40 mg of ASM 50 WG (20 mg a.i.) completely controlled downy mildew but caused very severe phytotoxicity: growth stopped and plants died. Treatment with 20 or 10 mg (10 or 5 mg a.i.) still caused growth inhibition, leaf mosaic and dwarfing, but 8 mg, 16 mg and 32 mg of the microencapsulated formulation eASM-2 (0.4 to 0.8 mg, 0.8 to 1.6 and 1.6 to 3.2 a.i., respectively) caused no phytotoxicity.

These results from pot experiments encouraged us to do greenhouse experiments to confirm the control efficacy of ASM and its microencapsulated formulations in practice.

3.4 Plastic greenhouse experiments with natural infection

(1) Under a moderate level of powdery mildew incidence (38.7% ± 18.2% of leaves diseased and 18.7 ± 9.3 lesions/leaf on untreated controls), four sprays of ASM at 100 mg L^{-1} provided slightly superior efficacy (74.7% ± 5.9% control) over TPN applied at 400 mg L^{-1} (61.6% ± 17.2% control). Two spray applications of ASM at 100 mg L^{-1} at 4-week intervals were also effective (79.6% ± 4.0% control) on powdery mildew, indicating durable efficacy of ASM applications (Fig. 3).

(2) Downy mildew was severe on untreated control plants of both cultivars (lesions/leaf: Pilot 2, 65.0 ± 11.3; Natsubayashi, 55.5 ± 13.8). Under such high disease pressure, the reference fungicide TPN failed to control downy mildew (9.4% ± 22.1% and 11.4% ± 65.0% on Pilot 2 and Natsubayashi, respectively). In contrast, ASM still achieved moderate activity (Pilot 2, 47.4% ± 9.4%;

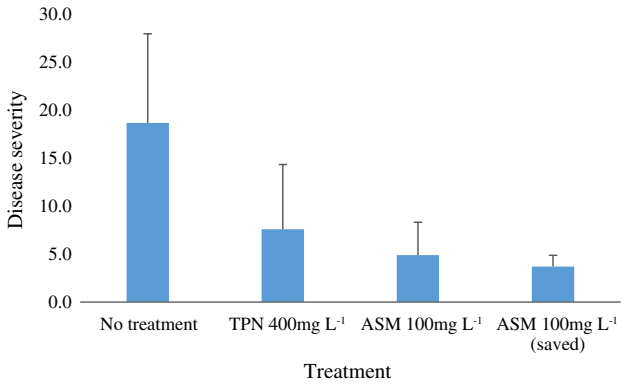

Figure 3. Control efficacy of ASM on cucumber powdery mildew in a plastic greenhouse. DS in each plot is shown by the mean and 95% CI.

Natsubayashi, 55.7% ± 42.8%), further demonstrating the durable efficacy of ASM.

(3) (a) For powdery mildew, severity was low on untreated Natsubayashi (DS 23.5 ± 13.3), but efficacy of TPN was low (28.9% control) because the longevity of this protectant fungicide was shorter than the 2-week spray interval used in this trial. On the contrary, spray application of ASM (58.7% control), soil amendment with either ASM (44.3% control) or eASM-2 (59.6% control) followed by the subsequent ASM sprays all yielded a higher level of control than did TPN. Interestingly, however, the severity of powdery mildew was greater on Kyoushizuku (DS on untreated plants: 64.9 ± 6.96) than on Natsubayashi, and neither TPN (6.2% control), ASM (17.6% and 16.6% control with spray applications and soil amendment, respectively) nor eASM-2 (23.9% control with soil amendment) treatments exhibited high control efficacy against powdery mildew on this Corynespora leaf spot-tolerant cultivar.

For downy mildew, the efficacy of TPN was extremely low on both cultivars even under low disease pressure. On Natsubayashi (DS 23.6 ± 0.9 on untreated plants), the efficacy of the ASM soil amendment (62.3% control) or spray application (48.3% control) was superior to TPN sprays (34.3% control), but the soil amendment with eASM-2 followed by supplemental ASM spray applications was less effective (39.4% control). Similar to the case for powdery mildew, none of the treatments with ASM (11.1%, 22.8%,and −6.9% control after spray applications of ASM, soil amendment with ASM and soil amendment with eASM-2, respectively) or TPN sprays (30.5% control) were very effective against downy mildew on Kyoushizuku (DS 37.7 ± 32.0 on unsprayed plots).

(b) The results are shown in Table 2. On the powdery mildew-tolerant cv. Natsubayashi, treatments 1–4 ([1] three sprays of ASM after transplanting, [2] seed soaking in ASM plus two sprays after transplanting, [3] ASM soil amendment at sowing plus two sprays after transplanting, and [4] two ASM sprays after ASM soil amendment at transplanting) provided 100% control of powdery mildew under low to moderate disease pressure (DS 18.8 ± 36.9 in untreated plots). Treatment [5] (soil amendment with microencapsulated ASM formulations at transplanting plus two ASM sprays) also gave high control activity of 87.2%. However, treatment [6] with TPN only gave moderate control (50.3%) even though it was sprayed every 7 days (six times total).

On the Corynespora leaf spot-tolerant cv. Kyoushizuku, powdery mildew was severe (DS 68.4 ± 5.6) in untreated plots (Table 2). On this cultivar, the efficacy of ASM was surprisingly low after treatment [1] (30.7% control; three sprays after transplanting) and treatments [2–5] described above (55.4%, 56.8%, 58.3% and 45.9% control, respectively), but control was higher than after six sprays of the reference fungicide TPN (29.2% control). Growth of plants was inhibited for both cultivars only after treatment [3] with ASM added to the soil at sowing, followed by growth in the phytotron, transplanting and two ASM sprays.

For downy mildew, DS was 42.0 ± 3.9 in the [7] untreated plots of cv. Natsubayashi (Table 3). All the ASM treatments [1–5] yielded a moderate control against this disease (44.2%, 40.0%, 43.3%, 41.0%, and 43.6% control, respectively), only slightly inferior to the reference TPN (51.3% control). On cv. Kyoushizuku, downy mildew was more severe in the [7] untreated plots (DS 59.8 ± 10.7) than on Natsubayashi. Furthermore, the efficacy of ASM was much lower, 19.1%, 8.7%, 29.8%, 32.9%, and 5.3% control in the treatments [1–5] than that of TPN (53.1% control).

(4) Under moderate downy mildew severity (DS in unsprayed control plots: 26.4 ± 5.8 on Pilot 2 and 28.6 ± 2.8 on Natsubayashi, respectively), three sprays of ASM alone (control 72.9% and 86.2% on Pilot 2 and Natsubayashi, respectively) gave better control than six sprays of the reference fungicide TPN applied every week (control 60.0% and 66.5% on these two cultivars, respectively) (Table 4). The highest control efficacy (80.5% and 93.9% on these cultivars, respectively) was obtained with three sprays of ASM mixed with TPN at 2-week intervals.

(5) On cv. Natsubayashi, severity of downy mildew was moderate (DS 37.9 ± 6.0) on untreated plants (Table 5). Treatment [1], ASM in soil plus three sprays gave the best control (83.4%). Treatments [2] eASM-6 and [3] eASM-6 in soil plus three sprays gave 64.3% and 61.6% control, respectively, equivalent to the 64.9% control by treatment [4], six TPN sprays representing durable activity of ASM and its microencapsulated formulations compared with an ordinary fungicide.

Table 2. DS and control (%) after treatment with ASM, microencapsulated formulations of ASM and the reference fungicide TPN against cucumber powdery mildew in a plastic greenhouse

	cv. Natsubayashi		cv. Kyoushizuku	
Treatment	DS	Control (%)	DS	Control (%)
(1) ASM sprays after transplanting	0 ± 0.0	100	47.4 ± 14.8	30.7
(2) ASM seed soaking plus sprays after transplanting	0 ± 0.0	100	30.5 ± 7.0	55.4
(3) ASM soil amendment at sowing plus sprays after transplanting	0 ± 0.0	100	29.6 ± 4.0	56.8
(4) ASM soil amendment at transplanting plus sprays thereafter	0 ± 0.0	100	28.5 ± 12.6	58.3
(5) Microencapsulated ASM soil amendment at transplanting plus sprays thereafter	2.4 ± 4.7	87.2	37.0 ± 27.1	45.9
(6) TPN (reference fungicide) sprays only	9.4 ± 15.2	50.3	48.4 ± 11.7	29.2
(7) No treatment	18.8 ± 36.9	–	68.4 ± 5.6	–

Table 3. DS and control (%) after various ASM treatments against cucumber downy mildew in a plastic greenhouse

Treatment	cv. Natsubayashi		cv. Kyoushizuku	
	DS	Control (%)	DS	Control (%)
(1) ASM sprays after transplanting	23.5 ± 0.1	44.2	48.4 ± 3.4	19.1
(2) ASM seed soaking plus sprays after transplanting	25.2 ± 5.3	40.0	54.6 ± 9.1	8.7
(3) ASM soil amendment at sowing plus sprays after transplanting	23.8 ± 10.3	43.3	42.0 ± 7.6	29.8
(4) ASM soil amendment at transplanting plus sprays thereafter	24.8 ± 5.1	41.0	40.1 ± 16.9	32.9
(5) Microencapsulated ASM soil amendment at transplanting plus sprays thereafter	23.7 ± 8.0	43.6	56.6 ± 15.3	5.3
(6) TPN (reference fungicide) sprays only	20.5 ± 9.5	51.3	28.0 ± 3.7	53.1
(7) No treatment	42.0 ± 3.9	–	59.8 ± 10.7	–

Table 4. DS and control (%) of ASM sprays alone or mixed with reference fungicide TPN against cucumber downy mildew in a plastic greenhouse experiment

Treatment	cv. Pilot 2		cv. Natsubayashi	
	DS	Control (%)	DS	Control (%)
(1) ASM	7.2 ± 4.0	72.9	4.0 ± 1.3	86.2
(2) ASM + TPN	5.2 ± 0.3	80.5	1.8 ± 0.3	93.9
(3) TPN	10.6 ± 7.4	60.0	9.6 ± 3.2	66.5
(4) No treatment	26.4 ± 5.8	–	28.6 ± 2.8	–

Interestingly, however, for ASM, the results on cvs. Natsubayashi and Kyoushizuku differed distinctly despite downy mildew being similarly moderate (DS 37.9 ± 6.0 and 40.1 ± 18.7, respectively) on untreated control plants (Table 5). On Kyoushizuku, control by TPN was lower than on Natsubayashi but still at 48.9%. In contrast, none of the other ASM treatments gave any control.

4 DISCUSSION

In the present laboratory experiments, we confirmed high ASM activity against powdery and downy mildews of cucumber, similar to our previous reports on SAR activity induced by ASM against scab and anthracnose diseases on cucumber.[9,20] Desirable resistance inducers need to be active against a wide spectrum of diseases, and ASM fulfills this requirement. The duration of the control on crops also needs to be long-lasting. Such a high level of SAR-inducing activity and long-lasting efficacy were found against powdery and downy mildews after various ASM treatments in plastic greenhouse experiments in this study.

Seed treatment with ASM reduces downy mildew incidence in maize.[27] Cashew seedlings that emerged from seeds that had been soaked in 0.44 mM ASM showed no symptoms of anthracnose caused by *C. gloeosporioides* (Penzig) Penzig & Saccardo.[28] Interestingly, transgenerationally induced resistance and priming against pathogens were reported to be triggered by ASM.[29] However, cucumber seeds soaked in high concentrations of ASM had delayed germination, and a longer soaking time negatively affected seedling vigor, as shown in the present study. Nevertheless, seed treatment with resistance inducers would be attractive if the high control efficacy is long-lasting and there is no unacceptable phytotoxicity so that fewer fungicide applications are needed at early stages of crop cultivation. In fact, probenazole, a resistance inducer for blast and bacterial leaf blight diseases on rice, is currently widely used not only as a foliar spray in paddy fields but also at sowing or transplanting.

Soaking cucumber seeds in ASM was effective for suppressing powdery and downy mildews in our pot experiments. Seed soaking at 50 mg L^{-1} ASM for 24 h under ambient temperature completely suppressed powdery mildew on the disease-tolerant cv. Natsubayashi when treated plants were grown in a pot and inoculated with the pathogen 1.5 months after seed soaking.

However, the seed treatment with ASM was not effective for cultivars Hisaki III and Kyoushizuku, both tolerant to Corynespora leaf spot, even at 100 mg L^{-1} (Ishii H, unpublished). Early reports also indicated that cultivar-dependent differences were evident in the expression of induced resistance in soybean.[30] Relating to this phenomenon, a combination of ASM, β-aminobutyric acid (BABA) and *cis*-jasmonate provided field control of the biotrophic pathogen *Blumeria graminis* (DC) Speer f. sp. *hordei* Marchal and the hemibiotroph *Rhynchosporium secalis* (Oudem.) J.J.Davis, but increased infection by *Ramularia collo-cygni* B.Sutton & J.M.Waller (Rcc) on barley.[31] Thus, trade-offs seem to be associated with a combination of resistance elicitors.

In the present study, silica microencapsulated formulations of ASM were produced for the first time and tested for enhancing the longevity of disease control and for lowering the potential risk of plant injury caused by ASM. In pot experiments, the microcapsule eASM-2, added to soil at sowing, highly suppressed anthracnose with no phytoxicity (Ishii H, unpublished). The amount of active ingredient in eASM-2 required for high efficacy was nearly equal to or lower than in the reference granule formulations of ASM. Moreover, the efficacy of eASM-2 or the granule formulations of ASM was durable, lasting for 7 weeks after treatment, indicating potential use as a soil amendment. Furthermore, soil amendment with eASM-2 was highly effective for suppressing powdery mildew in pot experiments, suggesting that ASM and its microencapsulated formulations have the potential to control multiple diseases in the field.

In plastic greenhouse experiments to evaluate the potential of ASM and its microencapsulated formulations for practical use in the future, sprays of ASM effectively controlled cucumber powdery mildew when used with a disease-tolerant cultivar such as V-road. ASM spray applications also suppressed downy mildew under severe disease pressure when the reference fungicide TPN failed. Control was higher on cv. Natsubayashi, which is tolerant to powdery and downy mildews, than on susceptible cultivars, suggesting the usefulness of using ASM with disease-tolerant cultivars. An ASM soil amendment at transplanting and after sprays with ASM achieved highly acceptable efficacy against downy mildew on disease-tolerant cultivars. Control provided by the soil amendment with eASM-6 was equivalent to that with TPN when sprays were supplemented with ASM. In contrast, such high control efficacies

Table 5. DS and control (%) of cucumber downy mildew after various ASM treatments in a plastic greenhouse

Treatment	cv. Natsubayashi		cv. Kyoushizuku	
	DS	Control (%)	DS	Control (%)
(1) ASM soil amendment at transplanting plus sprays after transplanting	6.3 ± 3.3	83.4	32.7 ± 9.8	18.4
(2) eASM-6 (0.5 g) soil amendment at transplanting plus sprays after transplanting[a]	13.5	64.3	40.8	−1.9
(3) eASM-6 (0.75 g) soil amendment at transplanting plus sprays after transplanting	14.6 ± 20.5	61.6	43.6 ± 7.7	−8.9
(4) TPN sprays only	13.3 ± 2.4	64.9	20.5 ± 18.1	48.9
(5) No treatment	37.9 ± 6.0	–	40.1 ± 18.7	–

[a] Three plants in only one row were used in this plot.

were never recorded on the cucumber cultivar tolerant to Corynespora leaf spot.

The long-lasting efficacy of ASM against powdery and downy mildews was confirmed during 4 years of plastic greenhouse experiments. At first, sprays of ASM controlled these two diseases better than TPN and reduced the number of sprays required for cucumber cultivars that were susceptible or tolerant to powdery and downy mildews. Soil amendment with ASM or the microcapsule eASM-2 in addition to supplemental sprays of ASM provided good control against mildews on a tolerant cultivar.

Complete control of powdery mildew on the mildew-tolerant cucumber cultivar was achieved after a reduction in the number of sprays of either ASM alone or supplemental sprays of ASM on plants pretreated with ASM by seed soaking, soil amendment at the time of sowing or transplanting. Soil amendment with a mixture of the two microencapsulated formulations of ASM (eASM-4 and eASM-5) at transplanting and the two supplemental spray applications of ASM also gave high control efficacy. Considering all the results, pretreatment of cucumber with ASM or its microencapsulated formulations provided durable control against powdery mildew in the plastic greenhouse although long-lasting durability of the latter formulations has been demonstrated insufficiently yet. Chemicals such as ASM to induce systemic plant resistance should thus be effectively incorporated with host resistance into integrated pest management (IPM) programmes to combat cucumber diseases and thus reduce fungicide use. Recently, a formulation of ASM (Actigard® 50WG, Syngenta) has been submitted to the Japanese authorities for registration to control bacterial diseases in cabbage and Chinese cabbage (Syngenta, personal communication). In this case, drench treatment with ASM is recommended before transplanting from a seedling box.

As described recently,[32] ASM did not influence the diversity of nontarget fungi on cucumber leaves in a field test although ordinary chemical fungicides such as mancozeb and kresoxim-methyl inhibited the establishment of phylloplane fungi. The isolation frequency of those fungi after ASM treatment did not differ significantly from that on untreated cucumber leaves and is a desirable ecofriendly feature to farmers in addition to the practical high efficacy of disease control. Methods and timing of treatment may still need to be optimized, but introduction of ASM and its microencapsulated formulations into commercial vegetable growing is expected to reduce fungicide use and lower the risk for pathogen resistance development.

ACKNOWLEDGEMENTS

The authors are grateful to the field staff and students of the National Institute for Agro-Environmental Sciences for their assistance in field experiments. The authors also thank Mrs Kumi Shiokawa (AIST-Kansai) for her support in microcapsule preparation.

REFERENCES

1 Ishii H, Fraaije BA, Sugiyama T, Noguchi K, Nishimura K, Takeda T *et al.*, Occurrence and molecular characterization of strobilurin resistance in cucumber powdery mildew and downy mildew. *Phytopathology* **91**:1166–1171 (2001).
2 Date H, Kataoka E, Tanina K, Sasaki S, Inoue K, Nasu H *et al.*, Sensitivity of *Corynespora cassiicola*, causal agent of Corynespora leaf spot of cucumber, to thiophanate-methyl, diethofencarb and azoxystrobin (in Japanese with English abstr). *Jpn J Phytopathol* **70**:10–13 (2004).
3 Ishii H, Yano K, Date H, Furuta A, Sagehashi Y, Yamaguchi T *et al.*, Molecular characterization and diagnosis of QoI resistance in cucumber and eggplant pathogens. *Phytopathology* **97**:1458–1466 (2007).
4 Miyamoto T, Ishii H, Seko T, Kobori S and Tomita Y, Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. *Plant Pathol* **58**:1144–1151 (2009).
5 Miyamoto T, Ishii H, Stammler G, Koch A, Ogawara T, Tomita Y *et al.*, Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. *Plant Pathol* **59**:873–881 (2010).
6 Miyamoto T, Ishii H and Tomita Y, Occurrence of boscalid resistance in cucumber powdery mildew disease in Japan and the molecular characterization of iron-sulfur protein of succinate dehydrogenase of the causal fungus. *J Gen Plant Pathol* **76**:261–267 (2010).
7 Ishii H, Miyamoto T, Ushio S and Kakishima M, Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. *Pest Manag Sci* **67**:474–482 (2011).
8 Kachroo A and Robin G, Systemic signaling during plant defense. *Curr Opin Plant Biol* **16**:527–533 (2013).
9 Narusaka Y, Narusaka M, Horio T and Ishii H, Induction of disease resistance in cucumber by acibenzolar-S-methyl and expression of resistance-related genes. *Ann Phytopathol Soc Jpn* **65**:116–122 (1999).
10 Cools HJ and Ishii H, Pre-treatment of cucumber plants with acibenzolar-S-methyl systemically primes a phenylalanine ammonia lyase gene (*PAL1*) for enhanced expression upon attack with a pathogenic fungus. *Physiol Mol Plant Pathol* **61**:273–280 (2002).
11 Deepak SA, Ishii H and Park P, Acibenzolar-S-methyl primes cell wall strengthening genes and reactive oxygen species forming/scavenging enzymes in cucumber after fungal pathogen attack. *Physiol Mol Plant Pathol* **69**:52–61 (2006).
12 Lin TC and Ishii H, Accumulation of H_2O_2 in xylem fluids of cucumber stems during ASM-induced systemic acquired resistance (SAR) involves increased LOX activity and transient accumulation of shikimic acid. *Eur J Plant Pathol* **125**:119–130 (2009).
13 Ishii H, Watanabe H and Tanabe K, *Venturia nashicola*: pathological specialization on pears and control trial with resistance inducers. *Acta Hort* **587**:613–621 (2002).
14 Faize M, Faize L, Koike N, Ishizaka M and Ishii H, Acibenzolar-S-methyl-induced resistance to Japanese pear scab is associated with potentiation of multiple defense responses. *Phytopathology* **94**:604–612 (2004).
15 Jiang S, Park P and Ishii H, Ultrastructural study on acibenzolar-S-methyl-induced scab resistance in epidermal

pectin layers of Japanese pear leaves. *Phytopathology* **98**:585–591 (2008).
16 Faize M, Faize L and Ishii H, Gene expression during acibenzolar-S-methyl-induced priming for potentiated responses to *Venturia nashicola* in Japanese pear. *J Phytopathol* **157**:137–144 (2009).
17 Iwata M, Probenazole – a plant defence activator. *Pest Outlook* **12**:28–31 (2001).
18 Ishii H, Fungicide resistance in rice, in *Modern Fungicides and Antifungal Compounds VI*, ed. by Dehne HW, Deising HB, Gisi U, Kuck KH, Russell PE and Lyr H. DPG Selbstverlag, Braunschweig, Germany, pp. 35–40 (2011).
19 Lin TC, Ishizaka M and Ishii H, Acibenzolar-S-methyl-induced systemic resistance against anthracnose and powdery mildew diseases on cucumber plants without accumulation of phytoalexins. *J Phytopathol* **157**:40–50 (2009).
20 Ishii H, Tomita Y, Horio T, Narusaka Y, Nakazawa Y, Nishimura K *et al.*, Induced resistance of acibenzolar-S-methyl (CGA 245704) to cucumber and Japanese pear diseases. *Eur J Plant Pathol* **105**:77–85 (1999).
21 Obradvic A, Jones JB, Momol MT, Olson SM, Jackson LE, Balogh B *et al.*, Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. *Plant Dis* **89**:712–716 (2005).
22 Elmer WH, Effects of acibenzolar-*S*-methyl on the suppression of Fusarium wilt of cyclamen. *Crop Protect* **25**:671–676 (2006).
23 Yamashita Y, Development of systemic acquired resistance activator acibenzolar-S-methyl, in *Pursuit of the Essence of Plant Pathogenesis*, ed. by Takamatsu S, Kobayashi K, Yoshioka H and Toyoda K. Mie University Co-operative, Tsu, Japan, pp. 172–174 (2004).
24 Richards EH, Wontner-Smith T, Bradish H, Dani MP and Cotterill JV, Novel control methods for insect pests: development of a microencapsulation procedure for proteinaceous bioactives intended for oral delivery. *Pest Manag Sci* **71**:1238–1246 (2015).
25 Ma X, Wang X, Cheng J, Nie X, Yu X, Zhao Y *et al.*, Microencapsulation of *Bacillus subtilis* B99-2 and its biocontrol efficiency against *Rhizoctonia solani* in tomato. *Biol Control* **90**:34–41 (2015).
26 Fujiwara M, Shiokawa K, Tanaka Y and Nakahara Y, Preparation and formation mechanism of silica microcapsules (hollow sphere) by water/oil/water interfacial reaction. *Chem Mater* **16**:5420–5426 (2004).
27 Morris SW, Vernooij B, Titatarn S, Starrett M, Thomas S, Wiltse CC *et al.*, Induced resistance responses in maize. *Mol Plant Microbe Interact* **11**:643–658 (1998).
28 Lopez AMQ and Lucas JA, Effects of plant defence activators on anthracnose disease of cashew. *Eur J Plant Pathol* **108**:409–420 (2002).
29 Veloso J, García T, Bernal A and Díaz J, New bricks on the wall of induced resistance: salicylic acid receptors and transgenerational priming. *Eur J Plant Pathol* **138**:685–693 (2014).
30 Dann E, Diers B, Byrum J and Hammerschmidt R, Effect of treating soybean with 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) on seed yields and the level of disease caused by *Sclerotinia sclerotiorum* in field and greenhouse studies. *Eur J Plant Pathol* **104**:271–278 (1998).
31 Walkers DR, Havis ND, Sablou C and Walsh DJ, Possible trade-off associated with the use of a combination of resistance elicitors. *Physiol Mol Plant Pathol* **75**:188–192 (2011).
32 Chung W-H, Deepak SA and Ishii H, Effect of acibenzolar-S-methyl, strobilurins and other fungicide treatments on phylloplane fungi of crops. *J Gen Plant Pathol* **79**:128–135 (2013).