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# Citokinin-mediated Suppression of Cucumber Powdery Mildew Disease: 6-benzyladenine Suppresses the Growth of Cucumber Powdery Mildew Fungus, *Sphaerotheca fuliginea*

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**Abstract:** The effect of a cytokinin, 6-benzyladenine (6-benzylaminopurine, BA), on the growth of a powdery mildew fungus was investigated. The growth of *Sphaerotheca fuliginea* on cucumber leaf disks was suppressed by BA treatment and the suppressive effect of BA was dose-responsive. Compared with $H_2O$ treatment, treatment of the leaf disks with 500 μM BA decreased the number of haustoria and total hyphal length per one colony, but not with 5 μM BA. Observation of the hyphal growth using a light microscope showed that BA treatment suppressed the elongation and branching of hyphae by *S. fuliginea*. BA treatment for short period suppressed the formation of conidiophores 96 h post inoculation. Interestingly, the formation of conidiophores was also suppressed even if BA was treated at the later infection processes of *S. fuliginea*. These results suggest that the suppressive effect of BA on the growth of *S. fuliginea* is durable and immediate. On cotyledons inoculated with *S. fuliginea*, BA treatment suppressed the disease symptoms formed by *S. fuliginea* and hence decreased the source of secondary inoculum. These studies might contribute to the development of unique disease management systems to control powdery mildew diseases using plant hormones.

**Key words:** Plant hormones, cytokinin, 6-benzyladenine, plant protection, cucumber, powdery mildew

## INTRODUCTION

*Sphaerotheca fuliginea* is cucumber powdery mildew fungus and causes severe diseases to cucurbit crops worldwide. The breeding program at Cornell University, Michigan State University and others has been proceeded to breed resistant cucumber plants against this powdery mildew (Jahn *et al*., 2002). However, in general, protection and management against cucumber powdery mildew disease depend on the application of several fungicides. There is no doubt that fungicides are effective to control powdery mildew diseases and contributive to prevent the economical loss of commercial crops. On the other hand, agriculturalists have requested the environmentally safe method for plant protection, since most of consumers have required vegetables and commercial crops that have never applied by any chemicals, including fungicides, insecticide and herbicides. Therefore, plant pathologists have been looking for new strategies to control powdery mildew diseases (Bélanger and Labbé, 2002; Salmeron *et al*., 2002).

At present, biotechnologists are able to create novel resistant plants using biotechnological procedures. Susceptible *Arabidopsis* transformed with *RPW8s*, which confer resistance to *Arabidopsis* powdery mildews, acquired broad-spectrum powdery mildew resistance (Xiao *et al*., 2001). Thus, transformation of resistance genes or defense related genes into susceptible plants can effectively give resistance against the infection of powdery mildews to susceptible plants (Salmeron *et al*., 2002). Although these Genetically Modified Organisms (GMOs) are not supported by the general public still, the creation of resistant plants using biotechnological techniques could be one powerful method to protect powdery mildew diseases and also to decrease the application of fungicides. On the other hand, the application of biological agents and reagents is thought to be relatively the safe management of powdery mildew diseases. Several living organisms are candidates of biological agents, because these organisms kill powdery mildew fungi by parasitism and/or antibiosis (Sullivan and White, 2000; Urquhart and Punja, 2002; Verhaar *et al*., 1996). For example, AQ10 (provided by Ecogen Inc., Langhorne, PA) is a commercialized biofungicide to cucurbit powdery mildew disease and a pelleted formulation of conidia of a mycoparasite, *Ampelomyces*

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*quisqualis*. Newly born inocula produced by the powdery mildew on squash leaves were reduced by AQ10 treatment (Shishkoff and McGrath, 2002). However, it appears to be difficult that living organisms are generally utilized as biological fungicides, since these microorganisms require optimum conditions to show high efficiency of parasitism and/or antibiosis (Bélanger *et al*., 1998).

Plant hormones are closely related to plant resistances and candidates for biological reagents. Endogenous ethylene is one of signaling molecules for the induction of disease resistance as well as expression of salicylic acid and jasmonic acid (Dong, 1998; Enyedi *et al*., 1992; Pieterse and van Loon, 1999). Tobacco plants transformed with *rgp1*, a gene encoding a Ras-related small GTP binding protein, induced endogenous cytokinins, zeatin and zeatin ribose (Sano *et al*., 1994). This induction of endogenous cytokinins leaded to the expression of acidic pathogenesis-related proteins and the acquirement of resistance against tobacco mosaic virus. Therefore, exogenous application of plant hormones might act as inducers of plant resistance and be utilized as biological reagents to control plant diseases. In the present study, we used a cytokinin, 6-benzyladenine (6-benzylaminopurine) as a biological reagent to control powdery mildew diseases. Our results demonstrated that the treatment of cucumber plants with 6-benzyladenine suppressed the formation of haustoria, secondary hyphae, conidiophores and colonies by cucumber powdery mildew.

## MATERIALS AND METHODS

**Test fungus:** *Sphaerotheca fuliginea* was tested throughout. This fungus was maintained on cucumbers (*Cucumis sativum* L. cv. Shinhokusei 1) grown at 25°C in a greenhouse.

**Treatment of *S. fuliginea*-inoculated cucumber cotyledons with 6-benzyladenine**: 6-benzyladenine (6-benzylaminopurine, BA) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). BA was prepared to the indicated concentrations with 1% ethanol every experiment. Leaf disks (9 mm in diameter) were prepared from cotyledons of 8 days cucumber seedlings using a cork borer (Cohen, 1993). Newly born conidia of *S. fuliginea* were inoculated on the abaxial surfaces of leaf disks by a paint brush. The leaf disks were settled on filter papers saturated with various concentrations of BA and incubated in a growth chamber at 25°C and 60% relative humidity under light (150 $\mu mol^{-2}s^{-1}m^{-2}/\mu A$, 12 h light/day). Abaxial surfaces of detached cotyledons were inoculated with *S. fuliginea* and treated with BA through petioles. Adaxial surfaces of intact cotyledons of 8 days seedlings were also inoculated with *S. fuliginea.* After incubation for 24 h, 5 mL of 500 μM BA per one seedling was sprayed to the cotyledons using an atomizer. The detached cotyledons and the seedlings were incubated in the growth chamber for the indicated days. $H_2O$ and 1% ethanol were used as experimental controls.

**Observations of the infection processes of *S. fuliginea* on BA-treated cucumber cotyledons:** After incubation of *S. fuliginea* on cucumber cotyledons for the indicated period, abaxial epidermal layers were pealed off and mounted on a glass slide. Observation of the infection processes of *S. fuliginea on* the epidermis was done using a light microscope. This is a superior procedure to observe the infection processes of powdery mildews without displacing germlings attached loosely on host cells. The number of haustoria and conidiophores per one colony was counted under a microscope and total length of hyphae per one colony was measured using a micrometer. Furthermore, rates of germination, appressoria formation, the formation of primary haustoria and the formation of secondary hyphae were calculated using the following formulae:

Rate of germination (%) = (number of germinated conidia/number of total conidia)×100

Rate of appressoria formation (%) = (number of conidia with matured appressoria/number of germinated conidia)×100

Rate of the formation of primary haustoria = (number of conidia with primary haustoria/number of conidia with matured appressoria)×100

Rate of the formation of secondary hyphae = (number of conidia with secondary hyphae/number of conidia with primary haustoria)×100

**Statistics:** Results were presented as means±standard errors. Statistical analysis was performed using student's t-test.

## RESULTS

**Suppressive effect of BA treatment on the growth of *S. fuliginea*:** BA treatment suppressed the growth of *S. fuliginea* on cucumber cotyledons. The formation of haustoria and the elongation of hyphae by *S. fuliginea* on the leaf disks were suppressed 60 h post inoculation with dose-dependent manner (Fig. 1), although treatment of 1% ethanol, used as an experimental control, showed the slight suppression of *S. fuliginea* growth compared with

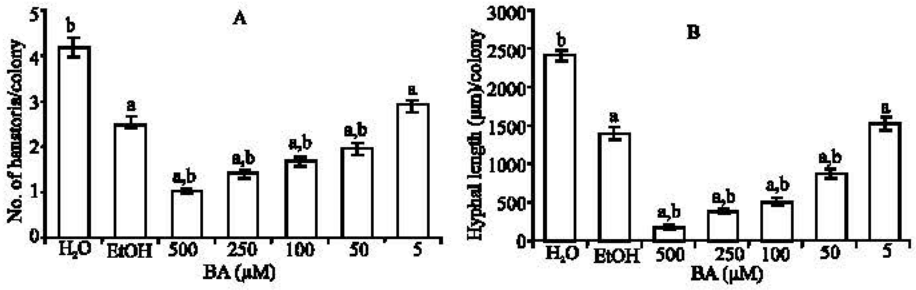



Fig. 1: Suppressive effect of BA treatment on the growth of *S. fuliginea*. Leaf disks inoculated with *S. fuliginea* were treated with various concentrations of BA, $H_2O$ or 1% ethanol (EtOH). After incubation for 60 h, the number of haustoria (A) and total hyphal length (B) per one colony were investigated. Bar, standard error (n = 10). [a] $p < 0.01$ (compared with $H_2O$). [b] $p<0.01$ (compared with EtOH)

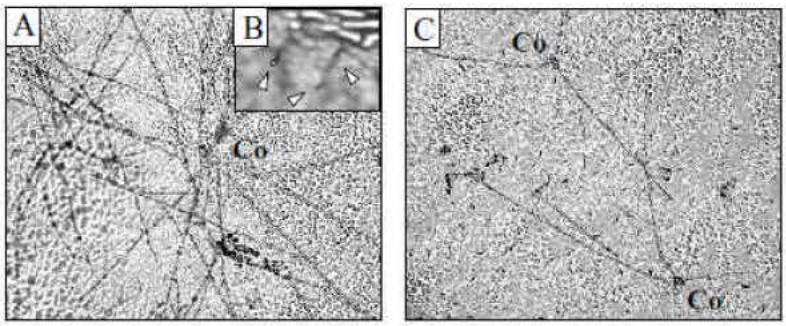



Fig. 2: Colonies of *S. fuliginea* on BA-treated leaf disks. (A) Colonies on $H_2O$-treated leaf disks. The extensive elongation of hyphae was visible 96 h post inoculation. (B) Conidiophores (arrowheads) formed on the colony in Fig. 6A. (C) Colonies on BA-treated leaf disks. The elongation and branching of hyphae was suppressed by 500 μM BA 96 h post inoculation. Co, conidium

$H_2O$ treatment (another experimental control). Treatment of 500 μM BA decreased the number of haustoria and hyphal length per one colony by 70 and 90%, respectively, compared with $H_2O$ treatment. The suppressive effect of 500 μM BA treatment on *S. fuliginea* growth could be also observed compared with ethanol treatment. Most of *S. fuliginea* on the leaf disks, which were treated with 500 μM BA, could not form secondary haustoria 60 h post inoculation (Fig. 1A). *S. fuliginea* formed spoke-like colonies and conidiophores on cucumber cotyledons 96 h post inoculation (Fig. 2A and B). BA treatment suppressed the elongation and branching of hyphae and hence the formation of conidiophores (Fig. 2C). Taken together, these results suggested that BA treatment effectively suppressed the growth of *S. fuliginea* on cucumber cotyledons.

**No effect of BA treatment on the early infection processes of *S. fuliginea*:** To determine the effect of BA on the early infection processes of *S. fuliginea*, observations were performed every 4 h after inoculation and BA treatment. Germination and appressoria formation were not suppressed by treating *S. fuliginea*-inoculated leaf disks with 500 μM BA (Fig. 3A, B). BA also didn't suppress the formation of primary haustoria 24 h post inoculation (Fig. 3C). On the other hand, BA treatment suppressed the formation of secondary hyphae by 50% 36 h post inoculation compared with $H_2O$ and ethanol treatment (Fig. 3C). These results suggested that the suppressive effect of BA might act on the formation and elongation of secondary hyphae by *S. fuliginea*, but not on germination and the formation of appressoria and primary haustoria at the early infection processes of *S. fuliginea*.

**Durable effect of BA treatment on the growth of *S. fuliginea*:** Leaf disks inoculated with *S. fuliginea* were treated with 500 μM BA, $H_2O$ or ethanol for 0, 6, 12 or 24 h followed by washing the leaf disks with $H_2O$ to exclude the remnant of BA on the leaf disks. The washed

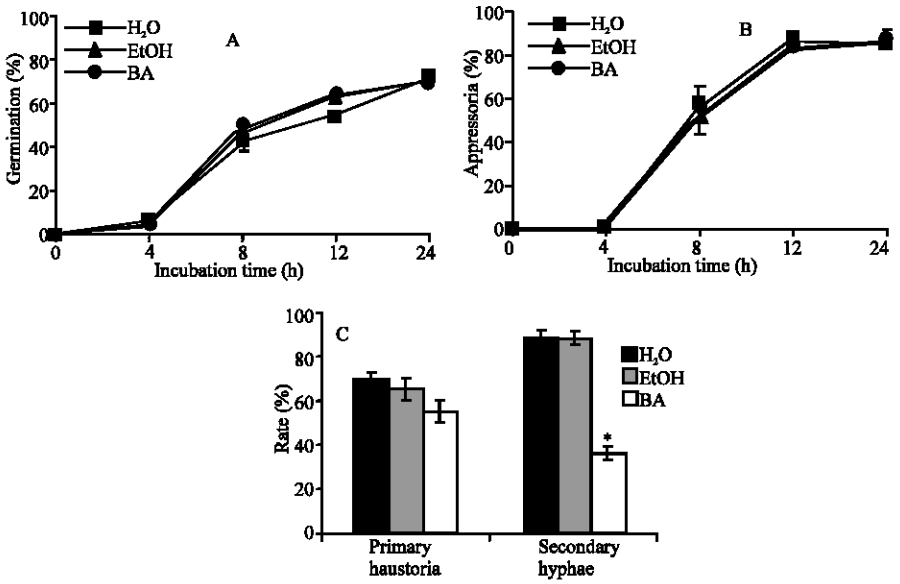



Fig. 3: Effect of BA treatment on the early stage of the infection processes by *S. fuliginea*. Leaf disks inoculated with *S. fuliginea* were treated with 500 μM BA. Treatment of $H_2O$ and 1% ethanol (EtOH) was also performed as controls. The percentage of germination (A) and appressoria formation (B) were calculated every 4 h after inoculation as described in Materials and methods. (C) The formation of primary haustoria and secondary hyphae was observed 24 and 36 h post inoculation, respectively. Bar, standard error (n = 50). * $p<0.01$ (compared with $H_2O$ and EtOH)

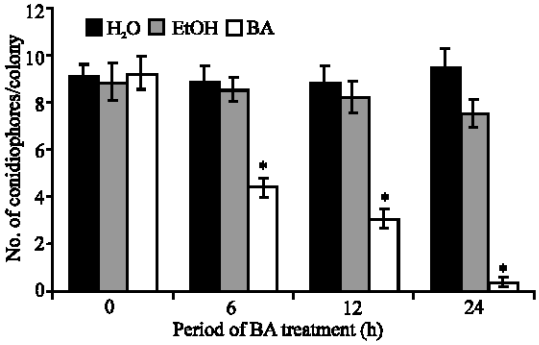



Fig. 4: Durable effect of BA treatment on the growth of *S. fuliginea*. Leaf disks inoculated with *S. fuliginea* were treated with 500 μM BA for 0, 6, 12 or 24 h. The disks were washed off with $H_2O$ to exclude the remnant of BA and further incubated with $H_2O$. Number of conidiophores per one colony was counted 96 h post inoculation. As controls, $H_2O$ or 1% ethanol (EtOH) were treated. Bar, standard error (n = 10). * $p<0.01$ (compared with $H_2O$ and EtOH)

leaf disks were further incubated with $H_2O$ until 96 h post inoculation. Compared with $H_2O$ and ethanol treatment, BA treatment for short period drastically suppressed the formation of conidiophores (Fig. 4). The suppressive effect was observed even if leaf disks were treated with BA only for 6 h. As shown in Fig. 4, extending the period of BA treatment resulted in the enhancement of the suppressive effect. This result suggested that BA treatment for short period was enough to suppress the growth of *S. fuliginea* and that the suppressive effect of BA might be durable.

**Immediate effect of BA treatment on the growth of *S. fuliginea*:** To evaluate whether the suppressive effect of BA on the growth of *S. fuliginea* appears immediately after BA treatment, leaf disks inoculated with *S. fuliginea* were firstly incubated with $H_2O$ for the indicated period followed by further incubation with 500 μM BA. The formation of conidiophores was never observed 96 h post inoculation when BA treatment was started within 24 h post inoculation (Fig. 5). Even if BA treatment was started from 60 h post inoculation, when *S. fuliginea* already formed spoke-like colonies, the formation of conidiophores was drastically suppressed by BA treatment compared with $H_2O$ and ethanol treatment. This result suggested that the suppressive effect of BA on the fungal growth might be immediate.

**Effect of BA treatment on the disease symptoms of *S. fuliginea*:** The effect of BA treatment on the disease symptoms formed by *S. fuliginea* was investigated. White

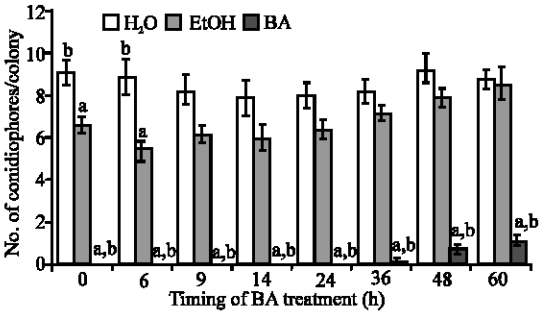



Fig. 5: Immediate effect of BA treatment on the growth of *S. fuliginea*. Leaf disks inoculated with *S. fuliginea* were treated with $H_2O$ for the indicated period before 500 μM BA, $H_2O$ or 1% ethanol (EtOH) were treated. Number of conidiophores per one colony was counted 96 h post inoculation. Bar, standard error (n = 10). [a]p< 0.01 (compared with $H_2O$). [b] p<0.01 (compared with EtOH)

disease symptoms, which are characteristic of powdery mildew diseases, could be seen on leaf disks treated with $H_2O$ and ethanol 10 days post inoculation (Fig. 6A, $H_2O$ and EtOH). Abundant sporulation was observed on these leaf disks using a light microscopy (data not shown). On the contrary, disease symptoms could not be visible on BA-treated leaf disks with naked eyes (Fig. 6A, BA), although a little sporulation was confirmed by the observation of the leaf disks using a light microscope (data not shown). Similarly, disease symptoms formed by *S. fuliginea* were also observed on detached cotyledon treated with $H_2O$, but never on the cotyledons treated with BA (Fig. 6B). Cucumber seedlings having fully expanded cotyledons were inoculated with *S. fuliginea* and incubated for 24 h before 500 μM BA was treated. As shown in Fig. 6C, the disease symptoms were suppressed on the BA-treated seedlings 12 days post inoculation compared with $H_2O$-treated seedlings. Taken together, these results suggested that BA suppressed the disease symptoms of *S. fuliginea* and hence decreased the source of secondary inoculum.

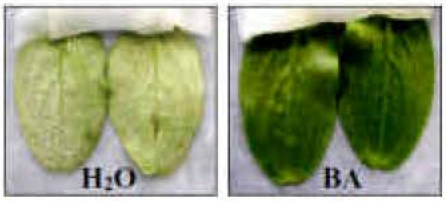
## DISCUSSION

In the present study, to develop the management systems for protecting powdery mildew diseases without fungicides, we evaluated whether exogenous application of a plant hormone can suppress powdery mildew disease in cucumber-*Sphaerotheca fuliginea* interaction. Consequently, a cytokinin, 6-benzyladenine (BA) suppressed the growth of cucumber powdery mildew fungus. The suppressive effects could be observed in the formation of secondary haustoria and secondary hyphae and in the elongation and branching of hyphae above all (Fig. 1-3).

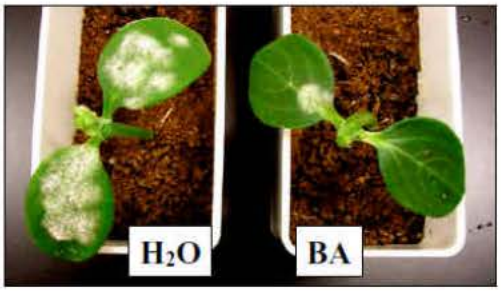
BA promotes the activation of cell division (Riou-Khamlichi *et al*., 1999), the formation and development of plant tissues (Auer and Cohen, 1993; Zhang and Hasenstein, 1999) and the induction of programmed cell death (Carmi *et al*., 2003) by activating or inactivating protein synthesis in plant cells. In our experimental system, we have found that the treatment of cucumber cotyledons with BA induced the expression of the gene encoding rubisco large subunit and altered the metabolism of chloroplasts (data not shown). Therefore, it is plausible that BA might affect the growth of *S. fuliginea* through the alternation of host cells' metabolism. In the interaction of tangelo Nova fruits (Citrus hybrid)-*Phytophthora citrophthora*, BA treatment increased the level of polymethoxyflavones in this fruits and induced the resistance against *P. citrophthora* (Ortuno *et al*., 2002). The induction of antifungal second metabolites in cucumber cells by BA treatment might confer the suppression of the *S. fuliginea* growth, since flavonoid compounds showed antifungal activity *in vitro* and *in vivo* against broad range of fungi. This possibility might be partially supported by evidence that a flavonoid compound rhamnetin, that is a phytoalexin of cucumber, was accumulated by the infection of cucumber powdery mildew fungus (Fawe *et al*., 1998). Moreover, BA stimulated the production of endogenous ethylene in *Arabidopsis thaliana* seedlings (Cary *et al*., 1995). Because ethylene is an inducer molecule of plant resistance, BA treatment might induce ethylene-mediated resistance as well as the induction of antifungal second metabolites. In our experimental system, the occurrence of hypersensitive cell death and the generation of free radicals, which are mediated by ethylene and resistant responses against powdery mildews (Hückelhoven and Kogel, 1998; Hückelhoven *et al*., 1999), were never detected in BA-treated cucumber cells infected by *S. fuliginea*, although a nonpathogen, *Blumeria graminis* f. sp. *tritici*, wheat powdery mildew fungus, induced the hypersensitive cell death in cucumber cells without BA treatment (data not shown). The mechanism(s) to suppress the growth of *S. fuliginea* by a cytokinin BA, remains to be identified. Further studies should be necessary to determine what happens in cucumber cells by BA treatment. In addition, we may not exclude the possibility that BA directly affects *S. fuliginea*.

In conclusion, we introduced that the treatment of cucumber plants with 6-benzyladenine suppressed the


(a)




(b)




(c)

Fig. 6: Effect of BA treatment on the disease symptoms of *S. fuliginea* on cucumber cotyledons. (A) Disease symptoms on leaf disks. Leaf disks inoculated with *S. fuliginea* were treated with 500 μM BA, $H_2O$ or 1% ethanol (EtOH). Observation was performed 10 days post inoculation. (B) Disease symptoms on detached cotyledons. Detached cotyledons inoculated with *S. fuliginea* were treated with 500 μM BA or $H_2O$ through petioles. Observation was performed 8 days post inoculation. (C) Disease symptoms on intact seedlings. Fully expanded cotyledons on 8 days seedlings were inoculated with *S. fuliginea*. After incubation for 24 h, 500 μM BA or $H_2O$ was sprayed on the *S. fuliginea*-inoculated cotyledons and the seedlings were further incubated in a greenhouse. Observation was performed 12 days post inoculation. To take photographs of disease symptoms formed on cotyledons, first and second leaves were removed

growth of cucumber powdery mildew fungus. Our final goal is to establish the management systems to control several powdery mildew diseases by the application of plant hormones. BA treatment has some advantages to control powdery mildew diseases. Firstly, BA treatment through petioles was effective against *S. fuliginea* infection as well as foliar spraying, since the fungal growth was suppressed on detached cotyledons treated with BA through petioles (Fig. 6B). This result raises speculation that BA might be available in horticultural crops by addition of BA into hydroponic solutions. Secondly, the suppressive effect of BA on the growth of *S. fuliginea* was durable and immediate (Fig. 4, 5). This is a superior advantage to control powdery mildew diseases efficiently, since powdery mildew fungi generate and spread next progenies fast. Actually, BA treatment decreased the source of second inoculum (Fig. 6). On the contrary, a few disadvantages might be arisen when BA

is used as a biological reagent in the field. Inasmuch as BA affects the metabolism of plant cells, BA treatment might affect the growth of cucumber plants. Although the growth of seedlings treated with BA appeared to be as same as that of seedlings treated with $H_2O$ during the present study (Fig. 6C), whether BA treatment loses the yield and/or alter the flavor of cucumber fruits remains unclear. If BA does, the suppressive effect of BA on the growth of *S. fuliginea* might be meaningless. Further studies are now under way to understand the mechanism that BA suppresses the growth of *S. fuliginea* and to establish the application of BA without any damages to cucumber fruits.

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