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SHORT COMMUNICATION

Silicon, *Clonostachys rosea*, and their interaction for gray mold management in cucumber

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Abstract

Cucumber is one of the most profitable crops cultivated worldwide and the occurrence of gray mold, caused by *Botrytis cinerea*, can dramatically decrease yield and fruit quality. This study investigated the potential of using silicon (Si), *Clonostachys rosea*, and their interaction, to control gray mold and on the potentiation of biochemical mechanisms of host defense. The treatments used in this study were: plants not-supplied with Si and not-receiving *C. rosea* (*Cr*) in the substrate before sowing (-Si -*Cr* plants), plants not-supplied with Si and receiving *C. rosea* (-Si +*Cr* plants), plants supplied with Si and not-receiving *C. rosea* (*Cr*) (+Si-*Cr* plants), and plants supplied with Si and receiving *C. rosea* (+Si+*Cr* plants). The foliar Si concentration significantly increased by 78% for +Si-*Cr* plants and by 98% for +Si +*Cr* plants in comparison to -Si-*Cr* and -Si+*Cr* plants, respectively. Significant increases of 70 and 73% for the incubation period of gray mold occurred for -Si+*Cr* and +Si+*Cr* plants, respectively, in comparison to -Si-*Cr* plants. There were significant reductions of 41 and 51%, respectively, for -Si+*Cr* and +Si+*Cr* plants in comparison to -Si-*Cr* plants for area under gray mold progress curve. The -Si+*Cr* and +Si+*Cr* plants showed high chitinase activity at 5 and 7 days after inoculation (dai) and at 9 hai for +Si-*Cr* plants in comparison to -Si-*Cr* plants. For +Si-*Cr* plants at 3 and 7 dai and for +Si+*Cr* plants at 3 and 5 dai, β-1,3-glucanase activity was significantly higher in comparison to -Si-*Cr* plants. Polyphenoloxidase activity was significantly higher for +Si-*Cr* plants at 5 dai and for -Si+*Cr* plants at 7, 8, and 10 dai in comparison to -Si-*Cr* plants. For -Si+*Cr* plants at 3 and 7 dai, +Si+*Cr* plants at 3 and 8 dai, and +Si-*Cr* plants at 7 and 8 dai, peroxidase activity was significantly higher in comparison to -Si-*Cr* plants. This study brings new insights into the potential of applying *C. rosea* and Si to boost cucumber resistance against gray mold with a bright prospect to be used in cucumber production under greenhouse conditions.

Keywords *Botrytis cinerea* · *Cucumis sativus* · Host defense responses · PR proteins · Phenylpropanoid pathway

Cucumber (*Cucumis sativus* L.) is one of the most profitable crops cultivated worldwide mainly in greenhouses, where it achieves higher yield and the fruits show great quality (Ramírez-Pérez et al. 2018). The importance of cucumber also is linked to its form of human consumption, which could be fresh or industrialized. Gray mold, caused by the necrotrophic fungus *Botrytis cinerea* Pers.:Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel], is the second most common disease in the world and can affect all aerial parts of the cucumber plants (Cota et al. 2009). Gray mold causes plant defoliation, flower death, and necrotic lesions on flowers, fruits, leaves, and stems compromising, therefore, yield and fruit quality (Elad et al. 1998). Fungal infection can also cause significant economic losses during preharvest or postharvest transport to distant markets (Cota et al. 2009). Gray mold control is challenging to achieve because the pathogen is capable of infecting more than 200 plant species (e.g., cucumber, tomato, strawberry, pepper, and kidney bean), has multiple mechanisms of attack, and infect all aerial organs of the cucumber plants (Cota et al. 2009). Resistant cultivars are not

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available to the growers due to the great aggressiveness of *B. cinerea* and its high genetic variability (Elad et al. 1998; Cota et al. 2009). Fungicides spray has been the most method of control adopted by the growers, but there is concern regarding their residues on fruits, the chance to select fungicide-resistant isolates, and the waste resulting from spray drift (Cota et al. 2009; Song et al. 2016).

Finding alternative methods for gray mold management is necessary to avoid the yield losses caused by this disease nowadays. The intensities of important root and foliar diseases on crops of economic importance have been decreased with the supply of silicon (Si) (Debona et al. 2017). The increased resistance of plants supplied with Si against pathogens has been associated with a physical barrier that prevents or slows fungal penetration; this physical barrier is the result of an increase in the density of the long and short silicate cells in the leaf epidermis or from a thick silica layer below cuticle as reported for the rice-*P. oryzae* pathosystem (Debona et al. 2017). High concentrations of phenolics, lignin, and phytoalexins; an increase in the activities of defense enzymes such as chitinases (CHI), β-1,3-glucanases (GLU), polyphenoloxidase (PPO), peroxidases (POX), and phenylalanine ammonia-lyase (PAL) as well as the rapid transcription of genes related to host resistance are the mechanisms of host resistance potentiated by Si (Rodrigues et al. 2005; Debona et al. 2017).

Biological control has been used to control gray mold on some crops (Cota et al. 2008; Saraiva et al. 2014a, b). The fungus *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Siefert & Gams (formerly *Gliocladium roseum* Bainier) (Cr) has excellent potential to reduce *B. cinerea* growth and sporulation through hyperparasitism and competition for nutrients (Chatterton et al. 2008; Saraiva et al. 2014a,b). The fungus *C. rosea* is widely spread through the agricultural regions, can colonize all plant tissues (alive, senescing, and dead tissues), is endophytic in many plant species, and effective against pathogens of different lifestyles infecting many crops (Saraiva et al. 2014a, b).

The present study aimed to investigate the potential of Si, *C. rosea*, and their interaction, on gray mold control in cucumber plants and the possible potentiation of biochemical mechanisms of host defense.

Cucumber seeds (cultivar "Caipira") were surface sterilized in 1% (v/v) NaOCl for 5 min, rinsed in sterilized water for 10 min, and sowed at the rate of six seeds per plastic pot filled with 2 kg of substrate [1:1:1 mixture of pine bark, peat, and expanded vermiculite; available Si (extracted with 0.01 M $CaCl_2$) of 17 mg/dm^3]. One seedling was kept per pot. Plants were growth in greenhouse (temperature of 25 ± 5 °C and relative humidity of 65 ± 5%) and fertilized with nutrition solution (100 ml/plant) composed of: 40 mM KNO_3, 10 mM $NH_4H_2PO_4$, 10 mM $MgSO_4.7H_2O$, 15 mM $Ca(NO_3).4H_2O$, 2.4 mM $ZnSO_4.7H_2O$, 3 mM H_3BO_3, 10 mM K_2SO_4, 3.3 mM CH_4N_2O, and 7.5 mM $NH_4H_2SO_4$ (Dallagnol et al. 2012) weekly. The isolate NCR 61/F of *C. rosea* was used in this study because of its exceptional ability to establish on the leaves of different hosts and suppress *B. cinerea* sporulation (Nobre et al. 2005). The *C. rosea* was grown on Petri dishes containing potato-dextrose-agar (PDA) medium. The dishes were incubated in a growth chamber at 25 °C with a 12 h light/12 h dark photoperiod for 15 days. After this period, conidia were carefully removed from the dishes with a soft bristle brush using sterile water. The conidial suspension was calibrated with a hemacytometer to obtain a concentration of 1 × 10^7 conidia/mL. Conidia suspension of *C. rosea* was applied to each pot as a drench (20 mL/pot) 24 h before sowing. Pots receiving sterile water served as the control treatment. Plants were irrigated daily with 100 mL of a potassium silicate solution prepared using deionized water with a concentration of 2 mM Si/L (0.32 mL of K_2SiO_3/L; FertiSil®, 13% K_2O, 26.59% SiO_2, and 12.2% soluble Si; PQ Corporation, São Paulo, Brazil). The pH was adjusted to 5.5 using HCl 1 M. In order to normalize the amount of potassium supplied to the plants receiving potassium silicate solution with the plants from control treatment, they were irrigated daily with 100 mL of a solution of KCl at the concentration of 1.23 mM K (Sigma-Aldrich, São Paulo, Brazil) with pH adjusted to 5.5 using HCl 1 M (Dallagnol et al. 2012). The isolate UFV-DFP *Bc*1 of *B. cinerea* was used to inoculate the plants. The fungus was grown in Petri dishes containing PDA medium. The dishes were incubated in a growth chamber at 20 °C with a 12 h light/12 h dark photoperiod for 12 days. After this period, conidia were carefully removed from the dishes with a soft bristle brush using sterile water. The conidial suspension was calibrated with a hemacytometer to obtain a concentration of 1 × 10^6 conidia/ml. The conidial suspension was sprayed on the leaves of plants (at 38 days after emergence) with the aid of an atomizer (Paasche Airbrush Co., Chicago). After inoculation, plants were kept in a moist chamber at 18 °C for 48 h with 12 h light/12 h dark photoperiod and relative humidity of 90 ± 5%. Thereafter, plants were transferred to a greenhouse (temperature of 25 ± 3 °C and relative humidity of 80 ± 5%) until the end of the experiments. The incubation period (IP) of gray mold was determined by examining the five leaves per plant, from base to apex, for the appearance of lesions every 6 h after inoculation (hai). At 4, 6, 8, 10, 12, 14, and 16 days after inoculation, leaves were collected, scanned at 300 dpi resolution, and the images were processed using the QUANT software to obtain severity values (Resende et al. 2009). The area under gray mold progress curve (AUGMPC) for each leaf was computed using the trapezoidal integration of the gray mold progress curve over time (Shaner and Finney 1977).

A total of 21 leaf discs (1 cm^2 in size) were obtained from the first leaf of each plant per replication of each treatment at 72, 120, 168, 192, and 240 hai and also from the leaves of non-inoculated plants (control treatment) to determine the electrolyte

leakage (EL) according to Lima et al. (2002). The EL values were used to calculate the area under EL progress curve (AUELPC) for each sample using the trapezoidal integration of EL progress curve over time (Shaner and Finney 1977).

The second, third, and fourth leaves of each plant per replication of each treatment were collected at 0, 72, 120, 168, 192, and 240 hai to determine the activities of the defense enzymes CHI, GLU, PPO, and POX. Leaf samples were kept in liquid nitrogen during sampling and stored at −80 °C until analysis. A total of 100 mg of leaf tissue was macerated with liquid nitrogen in a mortar with the addition of polyvinylpyrrolidone 1% (wt/vol) to obtain a fine powder. The powder was homogenized in 2 mL of 50 mM sodium phosphate (pH 6.5) containing 1 mM phenylmethylsulfonylfluoride and 0.1 mM acid etilenodiaminotetracetic. The homogenized material was centrifuged at 20,000 *g* for 25 min at 4 °C and the supernatant was used to determine the enzymes activities following the procedures described by Rios et al. (2014), which were expressed on the basis of protein quantified according to Bradford (1976).

Foliar Si concentration was determined on 0.1 g of dried and alkali-digested tissue using the colorimetric analysis (Resende et al. 2009).

A 2 × 2 × 2 factorial experiment, consisting of two Si doses (0 and 2 mM Si, referred to -Si and +Si plants), non-application or application of *C. rosea* (referred to -*Cr* and +*Cr* plants), and non-inoculated or inoculated plants with *B. cinerea*, was arranged in a completely randomized design with six replications. Each experimental unit consisted of a plastic pot containing one plant. The experiment was repeated once. Data from the foliar Si concentration, AUGMPC, IP, AUELPC, and the activities of CHI, GLU, PPO, and POX from the two experiments were analyzed using the MIXED procedure of the SAS software (Release 8.02 Level 02 M0 for Windows, SAS Institute, Inc. 1989, Cary, NC, USA) to determine if data from the two experiments could be combined (Moore and Dixon 2015). Data were submitted to analysis of variance and treatment means were compared by Tukey's test ($P \leq 0.05$) using the SAS software.

The IP of gray mold significantly increased by 70 and 73%, respectively, for the -Si +*Cr* and +Si +*Cr* plants in comparison to -Si -*Cr* plants (Table 1). The AUGMPC was significantly reduced by 41 and 51%, respectively, for -Si +*Cr* and +Si +*Cr* plants in comparison to -Si -*Cr* plants (Table 1). For non-inoculated plants, the foliar Si concentration significantly increased by 81% for +Si -*Cr* plants and by 74% for +Si +*Cr* plants in comparison, respectively, to -Si -*Cr* and -Si +*Cr* plants (Fig. 1a). For inoculated plants, the foliar Si concentration significantly increased by 78% for +Si -*Cr* plants and by 98% for +Si +*Cr* plants in comparison, respectively, to -Si -*Cr* and –Si +*Cr* plants (Fig. 1a). There was no significant difference among treatments for AUELPC from non-inoculated plants (Fig. 1b). For inoculated plants, the AUELPC was significantly lower by 26% for –Si +*Cr* plants and by 22% for +Si +*Cr* plants in comparison to -Si -*Cr* plants (Fig. 1a). The AUELPC for inoculated plants was also significantly lower by 29% for -Si +*Cr* plants and by 25% for +Si +*Cr* plants in comparison to +Si -*Cr* plants (Fig. 1a). The CHI activity was significantly higher for –Si +*Cr* and +Si +*Cr* plants at 5 and 7 dai and for +Si -*Cr* plants at 8 hai in comparison to -Si -*Cr* plants (Fig. 2a). The GLU activity was significantly higher for +Si -*Cr* plants at 3 and 7 dai and for +Si +*Cr* plants at 3 and 5 dai in comparison to -Si -*Cr* plants (Fig. 2b). The PPO activity was significantly higher for +Si -*Cr* plants at 5 dai and for -Si +*Cr* plants at 7, 8, 10 dai in comparison to -Si -*Cr* plants (Fig. 2c). The POX activity was significantly higher for –Si +*Cr* plants at 3 and 7 dai, for +Si +*Cr* plants at 3 and 8 dai, and for +Si -*Cr* plants at 7 and 8 dai in comparison to -Si -*Cr* plants (Fig. 2d).

Table 1 Incubation period (IP) and area under gray mold progress curve (AUGMPC) for cucumber plants non-supplied (-Si) or supplied (+Si) with silicon (Si) and non-treated (-*Cr*) or treated (+*Cr*) with *Clonostachys rosea* (*Cr*) and inoculated with *Botrytis cinerea*

Treatments	IP (hours)	AUGMPC
-Si -*Cr*	90 b	2207 a
-Si +*Cr*	153 a	1314 b
+Si -*Cr*	141 ab	2015 a
+Si +*Cr*	156 a	1089 b

Means of the treatments, in each column, followed by different letters are significantly different ($P \leq 0.05$) according to Tukey's test. Data are from two pooled experiments. $n = 12$

Many studies investigated the potential of Si (Rodrigues et al. 2005; Dallagnol et al. 2012; Araujo et al. 2016; Debona et al. 2017) and *C. rosea* (Cota et al. 2008, 2009; Saraiva et al. 2014a, b) to decrease the intensities of diseases affecting several profitable crops, including cucumber. However, this is the first study, to the best of the authors' knowledge, to present some biochemical evidence regarding gray mold control using Si, *C. rosea*, and their interaction. The use of *C. rosea* and its combination with Si increased the time for the appearance of disease symptoms on the leaves and lowered disease development. Even though the foliar Si concentration increased for Si-supplied plants, regardless of the presence of *C. rosea*, the IP and AUDPC were not affected. The AUELPC was lower for plants treated with *C. rosea* and also for those supplied with Si and treated with *C. rosea* indicating lower cellular damage caused by *B. cinerea* infection and corroborating with the reduced disease symptoms on those plants. Lower EE values on melon and rice leaves infected by *Podosphaera xanthii* and *Pyricularia oryzae*, respectively, were linked with lower disease severities (Dallagnol et al. 2011, 2012; Debona et al. 2012).

Interestingly, the activities of the defense enzymes studied seemed to be high for Si-supplied plants during the infection process of *B. cinerea* and may have contributed somehow to

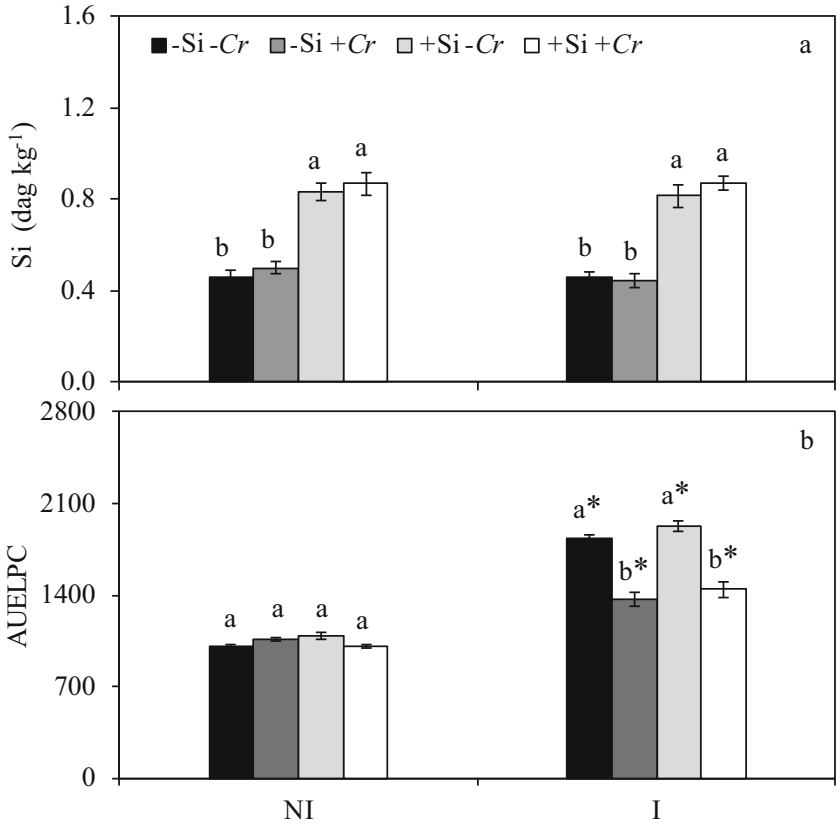

Fig. 1 Foliar silicon (Si) concentration (**a**) and area under the electrolyte leakage progress curve (AUELPC) (**b**) for cucumber plants non-supplied (-Si) or supplied (+Si) with Si, non-treated (-*Cr*) or treated (+*Cr*) with *Clonostachys rosea* (*Cr*), and non-inoculated (NI) or inoculated (I) with *Botrytis cinerea*. Means for the -Si -*Cr*, -Si+*Cr*, +Si -*Cr*, and + Si +*Cr* treatments followed by different letters within each NI and I treatment and means between NI and I treatments for each -Si -*Cr*, -Si +*Cr*, +Si -*Cr*, and + Si +*Cr* treatment followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. Data are from two pooled experiments. $n = 12$

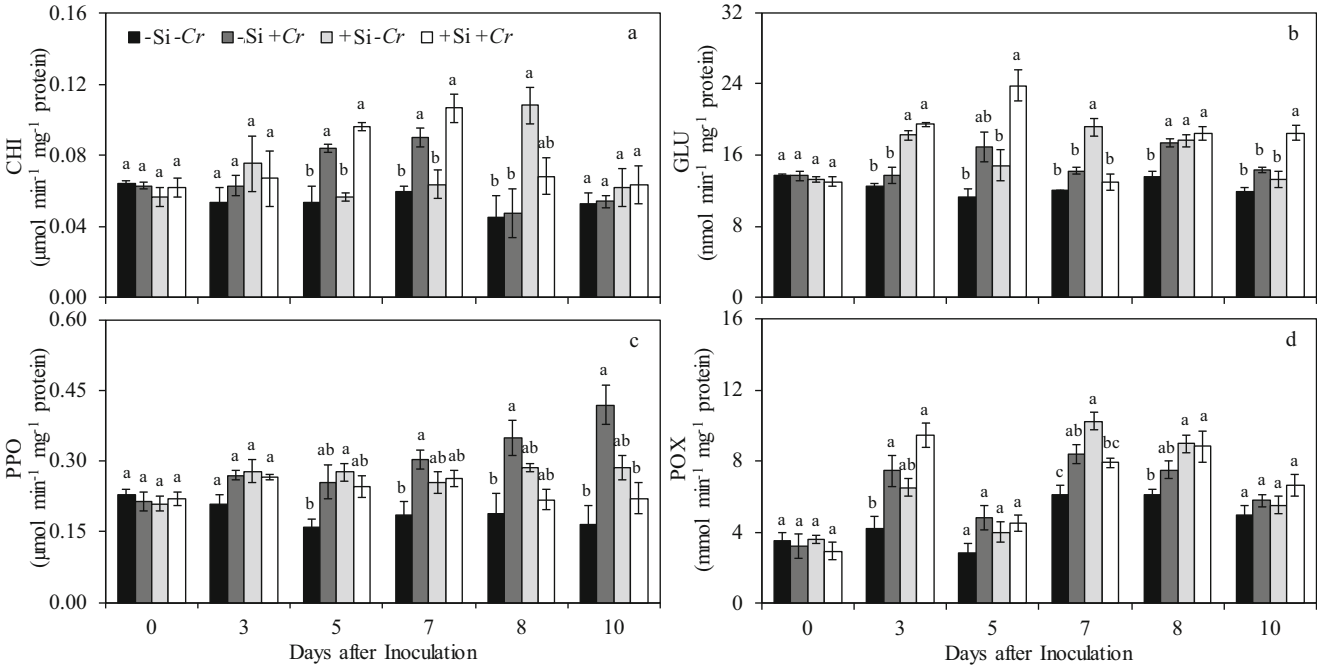

Fig. 2 Activities of chitinase (CHI) (**a**), β-1,3-glucanase (GLU) (**b**), polyphenoloxidase (PPO) (**c**), and peroxidase (POX) (**d**) in the leaves of cucumber plants non-supplied (-Si) or supplied (+Si) with silicon (Si) and non-treated (-*Cr*) or treated (+*Cr*) with *Clonostachys rosea* (*Cr*) and inoculated with *Botrytis cinerea*. Means for the -Si -*Cr*, -Si+*Cr*, +Si -*Cr*, and+Si +*Cr* treatments followed by the different letter, within each evaluation time, are significantly different ($P \leq 0.05$) according to F test. Bars represent the standard error of the means. Data are from two pooled experiments. $n = 12$

impede *B. cinerea* colonization on leaf tissues. The Si-mediated host resistance against pathogens infection has been attributed to the potentiation of the phenylpropanoid pathway or the formation of a physical barrier below the cuticle that delays fungal penetration and lesions expansion (Rodrigues et al. 2005; Dallagnol et al. 2012; Araujo et al. 2016; Debona et al. 2017). Chérif et al. (1994) and Liang et al. (2005) reported that Si potentiated the resistance of cucumber plants against damping-off and powdery mildew through an increase in the activities of PPO and POX. The CHI and GLU catalyze the hydrolysis of chitin and β-1,3-glucan, respectively, in the fungal cell wall and may release elicitors to activate defense responses (Ben-Shalom et al. 2003; Chérif et al. 1994; Liang et al. 2005). Many studies showed evidence that Si was involved in increasing cucumber resistance to foliar and root diseases by enhancing the production of PR-proteins and antimicrobial compounds (Chérif et al. 1994; Liang et al. 2005).

Cucumber plants treated with *C. rosea* exhibited high CHI, GLU, PPO, and POX activities as the infection process of *B. cinerea* took place explaining, therefore, the longer IP for gray mold and the reduced disease symptoms. Even though the potential of using *C. rosea* in agriculture is very well documented, the host defense mechanisms stimulated by this fungus on plants need to be better elucidated (Saraiva et al. 2014a, b). Mouekouba et al. (2014) and Gong et al. (2017) reported high activities of some PR-proteins in the leaves and fruits of tomato plants sprayed with *C. rosea* that resulted in lower disease severity caused by *B. cinerea*. Chitosan and chitin oligomers induced higher CHI and POX activities in cucumber plants infected by *B. cinerea* (Ben-Shalom et al. 2003). The enzymes POX and PPO are involved in the polymerization of phenolics that increase tissues lignification and reducing the cellular damage caused by pathogens infection (Dallagnol et al. 2012; Debona et al. 2012). It is plausible to postulate that high activities of CHI, GLU, PPO, and POX on the leaves of plants treated with *C. rosae* played an important role to increase cucumber resistance against gray mold.

The results of the present study bring new insights of the potential of applying *C. rosea* and Si to boost cucumber resistance against gray mold with a bright prospect to be used in cucumber production under greenhouse conditions.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest in this work. All forms of financial support are acknowledged in the contribution. This work does not involve any human participants or animals. All authors have offered their consent for the submission.

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