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Biocontrol of *Botrytis cinerea* by chitin-based cultures of *Paenibacillus elgii* HOA73

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Abstract The chitinase-producing bacterium, *Paenibacillus elgii* HOA73, is a biocontrol agent that limits the damage caused to plants by microbial pathogens, insects, and nematodes. However, the mechanisms involved in the biocontrol of plant diseases by HOA73 have not been determined. The objective of this study was to elucidate the role of extracellular chitinase obtained from isolate HOA73 in the control of the fungal pathogen *Botrytis cinerea*, the causative agent of gray mold in tomato. The HOA73 strain grew efficiently in a chitin-containing broth and produced chitin oligomers through chitinase activity; protease, lipase, and Fe-chelating siderophores were also secreted by the bacterium. Cultures containing intact bacteria inhibited *B. cinerea* conidia germination to a greater extent than did the bacterial cells alone or the cell-free culture supernatant. The antifungal activity increased with culture age and was heat-sensitive because of chitinase-mediated production of long-chain chitin oligomers. The biocontrol efficacy of undiluted bacterial cultures against gray mold in tomato was comparable to that of a standard fungicide. This study demonstrated that *P. elgii* HOA73 bacterial cultures grown on chitin-based minimal medium may be an effective formulation for the integrated control of gray mold.

Keywords Chitin-based medium · Chitin oligomer · Extracellular enzymes · Gray mold

Young Cheol Kim and Jin Young Hur contributed equally as first authors.

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Introduction

Gray mold disease caused by the fungal pathogen *Botrytis cinerea* results in serious losses in more than 200 crop species worldwide (Williamson et al. 2007). The pathogen infects through dead tissues or wounds in a process favored by high humidity and low temperature (Eden et al. 1996). In tomato, gray mold occurs on the fruits, leaves, stems, and flower petals remaining on the fruits, as well as in pruning wounds on stems (Eden et al. 1996; O'Neill et al. 1997). The disease is controlled by fungicides, but the improper and continuous application of the "same mode of action" fungicide group can lead to the accumulation of toxic residues, contamination of the environment, and development of resistant pathogens (Fernández-Ortuño et al. 2014; Kim et al. 1995; Myresiotis et al. 2007; Rupp et al. 2017). Consequently, other control approaches are required as alternatives to using fungicides.

Numerous biocontrol agents (BCA) are being developed as alternatives to fungicides; however, under field conditions, most lack the efficacy displayed in the

laboratory (AbuQamar et al. 2016; Junaid et al. 2013). Thus, inconsistency in performance and low effectiveness limit the use of BCA by farmers (Le Mire et al. 2016) and more effective and stable biocontrol approaches are urgently required to meet farmers' demands (Parnell et al. 2016). Recent studies have suggested that next-generation formulations should include metabolites and additives not only to enhance shelf life and efficiency, but also to have an impact on a wider array of targets (Arora and Mishra 2016). Isolates of the Gram-positive bacterium *Paenibacillus elgii* exhibit biocontrol activity against an array of pathogens and nematodes (Kim et al. 2004; Kumar et al. 2015; Nguyen et al. 2013), thereby promoting growth (Das et al. 2010) and inducing systemic resistance in plants (Sang et al. 2014).

Biocontrol is correlated with the lytic enzymes chitinase and gelatinase (Nguyen et al. 2013), as well as with an iron-chelating siderophore (Wen et al. 2011). Several antimicrobial metabolites, including butyl 2,3-dihydroxybenzoate, methyl 2,3-dihydroxybenzoate, and protocatechuic acid, are effective antifungal compounds (Lee et al. 2017; Nguyen et al. 2015a, b). Lipopeptide antibiotics like pelgipeptins (A, B, C, and D) are effective against both bacteria and fungi (Ding et al. 2011b; Qian et al. 2012; Wu et al. 2010), while bacteriocins like elgicins are only antibacterial compounds (AbuQamar et al. 2017; Teng et al. 2012).

The draft genome sequence of *P. elgii* B69 reveals that it produces pelgipeptins and paenibactin, a novel catecholic siderophore, as well as other unknown secondary antimicrobial compounds synthesized by polyketide synthetase (PKS), non-ribosomal peptide synthetases (NRPS), hybrid NRPS-PKS synthetases, and a lantibiotic-synthetic pathway (Ding et al. 2011a).

Chitin-based BCA bioformulations have been used to control microbial pathogens and insect pests and to boost plant health (Hidangmayum et al. 2019; Kamil et al. 2018; Kim et al. 2008, 2010, 2017a; Kishore and Pande 2007; Pusztahelyi 2018; Sharp 2013). Formulations containing chitin and its derivatives promote plant growth and induce plant defense responses against diseases (Sharp 2013). Such formulations control soil-borne diseases, such as damping-off and root-knot caused by nematodes (Ha et al. 2014; Rajkumar et al. 2008), and foliar plant diseases (Kim et al. 2008; Kim et al. 2010; Kishore and Pande 2007; Kishore et al. 2005a, b; Seo et al. 2007). Application of microbial cultures grown in a chitin-supplemented medium containing cells, metabolites, and the remaining growth substrates increases the survival and multiplication of chitinolytic bacteria introduced onto plant surfaces (Kishore et al. 2005a) and activates defense-related enzymes in the treated plants (Kishore et al. 2005b). An array of extracellular lytic enzymes, including chitinase, protease, and lipase, as well as antimicrobial compounds including lipopeptides, pyrrolnitrin, and violacein are present in the cell-free supernatants (CFS) obtained from the cultures of Gram-negative chitinolytic biocontrol bacteria (Kamil et al. 2018; Kim et al. 2017a). However, the roles of the individual components of bacterial cultures grown in chitin-based medium in the biocontrol of plant diseases are still largely unknown for both Gram-positive and Gram-negative biocontrol bacteria.

The chitinase-producing strain *P. elgii* HOA73, isolated from field-grown tomato roots in Korea (Neung et al. 2014), controls root-knot nematode and diamondback moth, as well as gray mold and Fusarium wilt (Neung et al. 2014; Nguyen et al. 2013, 2015a, b). Additionally, protocatechuic acid and butyl 2,3-dihydroxybenzoate from *P. elgii* HOA73 are key antifungal compounds against *B. cinerea,* the causal agent of gray mold, as well as wilt in tomato caused by *Fusarium oxysporum* (Nguyen et al. 2015a, b).

In this study, we examined the biocontrol efficacy of *P. elgii* HOA73 grown in a chitin-supplemented minimal medium developed for other Gram-negative, chitin-degrading biocontrol bacteria (Kim et al. 2008, 2017a). To determine the role of extracellular chitinase from *P. elgii* HOA73 cultures, bacterial cultures and chitin oligomers were tested for biocontrol activity by screening them for their ability to inhibit *B. cinerea* spore germination in vitro. The efficacy of each biocontrol formulation was compared with that of a standard fungicide in greenhouse-grown tomatoes. Our study indicated that the *P. elgii* cultures grown in chitin-based medium for over 15 days controlled tomato gray mold under low disease conditions, suggesting that such formulations could be used as an alternative for the integrated control of gray mold in the field.

Materials and methods

Microorganisms and growth conditions

Botrytis cinerea was obtained from the Korean Agricultural Type Culture Collection Center (KACC40574,

Wanju, South Korea). The pathogen was grown on potato dextrose agar (PDA; Difco Inc., Detroit, MI, USA) plates at 25 °C for routine cultivation, maintained on PDA plates at 4 °C, and subcultured every 3 weeks. To prepare *B. cinerea* conidial suspensions, an agar-mycelium plug was inoculated onto PDA and grown for 10 d at 25 °C. The fungal mass was harvested from the PDA plate by suspension in sterile water and filtration through sterile cheesecloth to remove hyphal fragments. The spore concentration was measured on a hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany) under a light microscope (Leica Microsystems, Wetzlar, Germany). The conidial suspension was diluted with sterile water to a final concentration of 1×10^4 conidia mL^{-1} for the in vitro spore germination assay. *Paenibacillus elgii* HOA73 (KACC 19018) was grown routinely on nutrient agar or nutrient broth (NA or NB, Difco) at 28 °C and stored at −80 °C in NB containing 20% glycerol.

Determination of bacterial cells and extracellular protein production

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated. The chitin-based minimal medium used in this study contained 1.5 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 1.5 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, and 1.0 g crude chitin (Sokcho Nanobiotech., Sokcho, South Korea) per liter of distilled water (Kim et al. 2008). In each experiment, 100 μL of bacterial culture (approximately 1×10^8 colony-forming units [cfu] mL^{-1}) grown in NB for 24 h at 28 °C with shaking at 180 rpm was inoculated into 100 mL of the chitin-based minimal broth medium and cultivated at 28 °C with shaking at 180 rpm for a defined number of days. Bacterial cultures (10 mL) were collected at 2- or 5-d intervals to determine cell density, antifungal activity, extracellular enzyme production, and potential to inhibit *B. cinerea* spore germination. The bacterial cell density was determined by plating bacteria onto NA plates containing 50 μg mL^{-1} ampicillin, 50 μg mL^{-1} polymyxin B, and 50 μg mL^{-1} vancomycin to prevent potential contamination. The bacterial colonies on the dilution plates were counted 4 d after incubating at 28 °C and expressed as $\log_{10}$ cfu mL^{-1}.

At the defined days, the culture fluid was centrifuged at 15000×*g* for 10 min at 4 °C and the CFS was filtered aseptically through a 0.2-μm pore size Minisart® Syringe Filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Each CFS was stored at −20 °C and subsequently used to determine extracellular enzyme activity, siderophore production, and chitin oligomer composition. The protein concentration of the CFS was quantified using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard according to the manufacturer's instructions. All experiments were performed in three independent trials, with three replicates per trial.

Extracellular enzyme and siderophore production in a CFS fraction

The CFS of *P. elgii* HOA73 grown in the chitin-based minimal medium was used to determine the production of extracellular enzymes and siderophores. Chitinase activity was determined with 4-methylumbelliferyl β-D-*N,N′*-diacetylchitobioside [4-MU(GlcNAc)$_2$] as previously described (Kim et al. 2017b). Protease activity was quantified using the Pierce Fluorescent Protease Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and lipase activity was determined using a routine turbidimetric assay with Tween 20 as the substrate (von Tigerstrom and Stelmaschuk 1989). Siderophore production was measured using the chrome azurol S (CAS) assay solution, as previously described (Alexander and Zuberer 1991). The CFS samples or non-inoculated chitin-based minimal medium as control were added to the reaction solutions in 96-well microplates (Thermo Fisher Scientific) and incubated for 30 min at 37 °C. Fluorescence was measured using a BioTek® FLX-800™ (BioTek, Winooski, VT, USA) with excitation at 360 nm and emission at 440 nm for chitinase, and excitation at 485 nm and emission at 538 nm for protease. The absorbance of the samples was measured at 500 nm for lipase and at 630 nm for the siderophores, using a BioTek uQuant microplate reader (BioTek).

For the chitinase enzyme assay, the amount of 4-methylumbelliferone (4-MU) released from the reaction mixture was estimated using 2–125 μM 4-MU as a standard, with one unit representing one micromole of the liberated 4-MU per minute per milliliter of the CFS. For protease activity, the detected values were compared with those of the proteases released by trypsin digestion (0.25–2.5 μg mL^{-1}); one unit was equal to the amount of trypsin proteolysis per minute (1 μg mL^{-1} min^{-1}). For the siderophores, peak activity was estimated using 1.57

to 100 μM deferoxamine mesylate as a standard, and one unit was expressed as one micromole of the liberated deferoxamine mesylate per minute per milliliter of CFS. After subtracting the absorbance value of the blank from that of a sample, the relative activity of each sample was determined and expressed as the relative percentage of the maximum detected value. Each experiment was repeated twice, with three replicates per experiment.

Determination of chitin oligomers

The chitin oligomers [G1 (GlcNAc) to G6 $(GlcNAc)_6$] were analyzed by thin-layer chromatography. Each CFS (0.5 mL) collected on different growth days, and a mixture of 5 μg of mono to hexasaccharides as standards (Qingdo BZ Oligo Biotech Co., Qingdo, China), were spotted onto a silica gel 60 F254 plate (Merck Co., Berlin, Germany), and developed with a solvent system of 1-butanol: methanol: 20% ammonium hydroxide: water (25:20:9:6, *v*/v). Chitin oligomers were visualized by heating the plate at 130 °C for 5 min after spraying it with an aniline/diphenylamine solution (a mixture of 2 mL aniline, 2 g diphenylamine, 100 mL acetone, and 15 mL of 85% phosphoric acid) (Itoh et al. 2014).

Conidial germination inhibition assay

Inhibition of conidial germination was evaluated at different dilutions of bacterial cultures, CFS, CFS boiled for 10 min at 100 °C, and bacterial cell suspensions of *P. elgii* HOA73 grown in the chitin-based minimal medium. Bacterial cells grown in chitin minimal medium for 10 d at 28 °C with shaking at 180 rpm were centrifuged and then washed once with sterile distilled water to remove soluble compounds. The cells were suspended in sterile water and adjusted to a concentration of approximately 1×10^8 cfu mL^{-1} to mimic the cell density of a bacterial culture. Authentic chitin oligomers (Sigma-Aldrich Co.) were diluted to between 5 and 500 μg mL^{-1} with sterile distilled water.

An equal volume (10 μL) of the prepared sample, conidial suspension (1×10^4 conidia mL^{-1}), and potato dextrose broth (PDB) were added to the wells of a 96-well microplate and incubated at 25 °C. Sterile water was used as a control. After 12 h, conidial germination was evaluated using an inverted microscope (Leica). The experiment was repeated twice, with three replicates per experiment and at least 100 conidial spores per sample. The percentage inhibition of conidial germination was calculated using the following formula: % Inhibition = (inhibition rate in the control – inhibition rate with treatment)/inhibition rate in the control × 100. When a spore formed a germ tube twice its length, it was counted as a germinated spore.

Evaluation of biocontrol properties in greenhouse tomatoes

To test the biocontrol efficacy of the *P. elgii* HOA73 culture and CFS grown in the chitin-based broth against tomato gray mold, a field trial was conducted on semi-forced tomato in a commercial greenhouse located at Kwangyang-si, Chonnam Province, South Korea, in 2015–2016. The greenhouse, with an area of 660 m^2, contained 3000 SeoKwang tomato seedlings (Seminis Vegetable Seeds Inc., Seoul, Korea) that were transplanted on November 1, 2015. The cultivation practices recommended by the Rural Development Administration, Wanju, Korea, were followed.

A seed inoculum of HOA73 culture (500 mL) grown in NB broth for 24 h was transferred to 500 L of sterile chitin medium in a large fermenter (1000 L) (Heuksalim, Chungbuk, Korea) and cultured for 15 days at 28 ± 2 °C with stirring. Bacterial suspensions were prepared with the same volume of sterile water after harvesting of the bacterial cells by centrifugation as described above. The chemical fungicide fludioxonil (suspension concentrate, 20% active ingredient, Syngenta Korea, Seoul, Korea) was used as a positive control at the standard dose recommended by the manufacturer. The tomato plants were sprayed till run-off with the non-diluted culture containing intact cells at 1×10^8 cfu mL^{-1}, a bacterial cell suspension (1×10^8 cfu mL^{-1}), 2000-fold diluted fludioxonil, or tap water as control, using a power sprayer (Maruyama, Tokyo, Japan). The foliar treatments were applied three times at 10-day intervals. The 45-m^2 experimental plots were arranged in a randomized complete block design with five replicates per treatment. The biocontrol efficacies of the treatments were assessed by evaluating the marketable fruit yield. The incidence of natural gray mold was determined by counting the number of diseased fruits in 20 plants per treatment 10 d after the final treatment.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA, $P < 0.05$) using SPSS (version 23, SPSS Inc., Chicago, IL, USA) and if the F test was significant, the differences in each measurement were further evaluated with Dunnett's test ($P < 0.05$). The significance of the effect of heating on the CFS of *P. elgii* HOA73 was evaluated by Student's t test ($P < 0.05$). Regression analysis was used to analyze a putative correlation between the inhibition of conidial germination in bacterial cultures and the growth period of *P. elgii* HOA73. Spearman's correlation coefficient (R^2-value) and its corresponding P value were calculated.

Results

Growth and extracellular enzyme production of *P. elgii* in chitin-based minimal medium

Cell growth in the chitin-based minimal medium (1×10^8 cfu mL^{-1}) reached the maximum density 2 days after inoculation (DAI), and was maintained at a high cell density up to 20 DAI (Supplementary Fig. 1A). Cell growth was not correlated with extracellular protein levels, which appeared at 7 DAI (Supplementary Fig. 1A). Extracellular chitinase, protease, and lipase activity, as well as siderophore secretion, were recorded during *P. elgii* HOA73 cultivation in the chitin-based minimal medium, reaching their maxima between 5 and 8 DAI (Supplementary Fig. 1B).

Comparison of the antifungal activities of *P. elgii* cultures

Regression analysis of the data showed that inhibition of conidial germination was related to culture age (Fig. 1). The values varied with the dilution of the cultures or their supernatants, namely, the 25-fold diluted bacterial culture ($y = -16.85 + 18.84x$, $R^2 = 0.849$, $P < 0.001$), 15-fold diluted bacterial culture ($y = -1.53 + 22.51x$, $R^2 = 0.794$, $P < 0.001$), 7.5-fold diluted cell-free supernatant ($y = -6.84 + 19.23x$, $R^2 = 0.847$, $P < 0.001$), and 5-fold diluted cell-free supernatant ($y = -7.43 + 23.02x$, $R^2 = 0.861$, $P < 0.001$).

Spore germination was inhibited 100% with 20-day bacterial cultures. The inhibition of spore germination by the 5-fold diluted CFS from 5- and 10-day cultures was significantly reduced by boiling. No effect of boiling was observed for the undiluted CFS from 15- or 20-day cultures (Fig. 2). Complete inhibition of *B. cinerea* spore germination was observed with the <5-fold diluted *P. elgii* bacterial cultures, <5-fold diluted CFS, <2-fold diluted boiled CFS, and undiluted bacterial cells (Fig. 1 and Supplementary Fig. 2). This result indicated that CFS application effectively inhibited the germination of *B. cinerea* spores.

Determination of chitin oligomers and their effect on spore germination

The thin-layer chromatography (TLC) analysis of *P. elgii* HOA73 CFS showed the release of chitin oligomers of various lengths at 10 days; chitin oligomer production was the greatest at 20 days (Fig. 3a).

Treatments with authentic pentameric and hexameric chitin oligomers completely inhibited spore germination when applied in the range of 50 to 500 μg mL^{-1}. Application of chitin dimers and tetramers at the highest concentration (500 μg mL^{-1}) resulted in 70–80% inhibition of spore germination (Fig. 3b). This indicated that inhibition of spore germination was dependent on the length of the chitin oligomers.

Control efficacy of *P. elgii* culture against tomato gray mold in the field

Gray mold was first observed in the tomatoes in the commercial field on February 15, 2016, with the seedlings having been transplanted in November 2015. At this early developmental stage, three foliar sprays of the 10-day-fermented undiluted culture containing intact cells, applied at 10-d intervals, significantly reduced the incidence of tomato gray mold ($P < 0.05$) to a level similar to that observed with application of the fungicide. The biocontrol efficacy of the bacterial suspension (1×10^8 cfu mL^{-1}) was relatively less effective (Fig. 4).

Discussion

A bioformulation based on the use of cultures of intact *P. elgii* HOA73 grown in a chitin-based minimal medium resulted in effective biocontrol of *B. cinerea* in vitro and in tomato field trials. These results augment the findings of studies in which the application of cultures of intact chitinase-producing Gram-negative bacterial

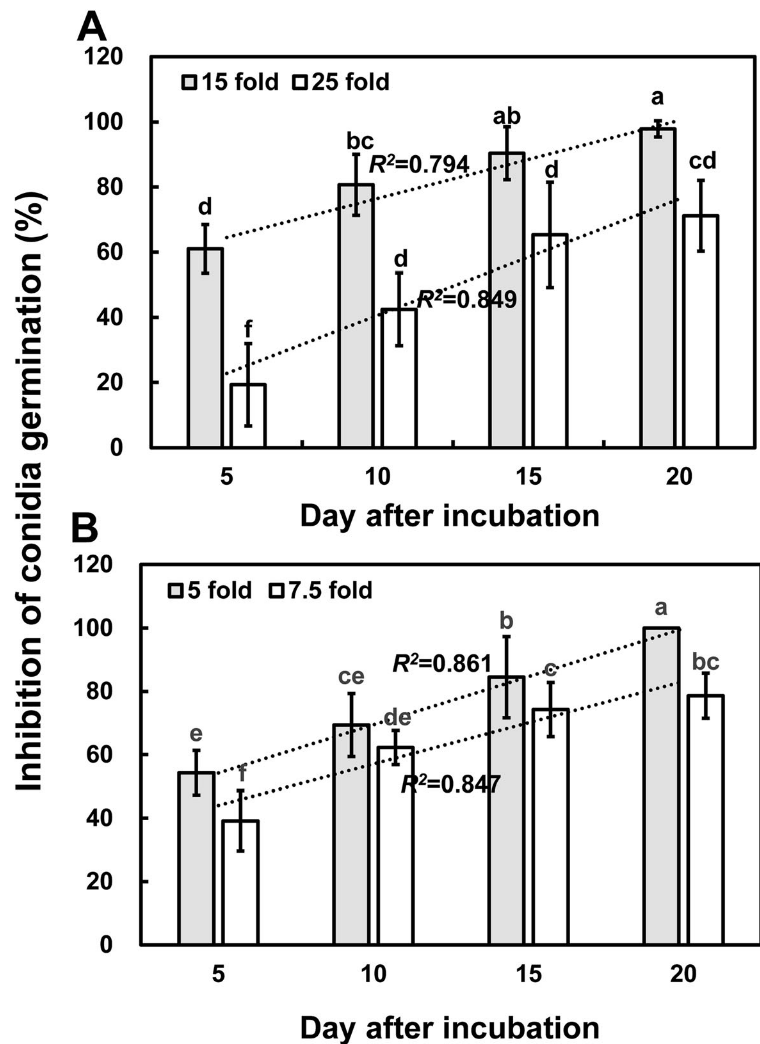

Fig. 1 Inhibition of *Botrytis cinerea* spore germination by *P. elgii* HOA73. Cultures of intact cells (**a**) and cell-free supernatants (**b**) of *P. elgii* HOA73 grown in a chitin-based minimal medium were used at defined dilutions. The experiment was repeated twice, with three replicates per experiment and at least 100 conidial spores for each sample. Different letters indicate significant differences between treatments based on Dunnett's test at $P < 0.05$. Spearman's correlation coefficients (R^2-value) were obtained by performing a regression analysis between the inhibition of conidial germination by bacterial cultures and the growth period of *P. elgii* HOA73

strains was successful in controlling plant diseases and root-knot nematodes under field conditions (Pusztahelyi 2018; Sid Ahmed et al. 2003; Singh et al. 2013; Zarei et al. 2011). The results of this study suggest there is synergism between different components of the bacterial cultures. Complete inhibition of *B. cinerea* spore germination was observed in vitro with 15-fold diluted bacterial cultures, 5-fold diluted CFS, and a suspension of 1×10^7 mL^{-1} bacterial cells. This result corroborates previous findings that bacterial cells and their products contribute to biocontrol efficacy (Kim et al. 2017a).

We documented the presence of secreted chitinase, protease, lipase, and siderophores in the supernatants. Previous studies indicated that extracellular bacterial chitinases inhibit pathogenic fungi, including *F. oxysporum* and *Cladosporium* spp. (Kim et al. 2017a, b; Singh et al. 2013). Proteases also act against *Rhizoctonia solani, Aspergillus niger, Magnaporthe oryzae,* and *Fusarium* spp. (Al-Askar et al. 2015; Cui et al. 2012; Singh and Chhatpar 2011; Yen et al. 2006). Iron chelation by siderophores has been implicated in enhancing plant growth and controlling *A. niger, F. oxysporum,* and *Gaeumannomyces graminis* (Ahmed and Holmstrom 2014; Bharucha et al. 2013; Saha et al. 2016). However, the effectiveness of each of these factors and their interactions have still not been investigated. In a previous study, a chitinase purified from the HOA73 strain inhibited the germination of *B. cinerea* conidia, but only at a high concentration (Kim et al. 2017b). A novel finding of these studies was that inhibition of germination was differentially sensitive to heat depending on the age of the culture; the loss of activity

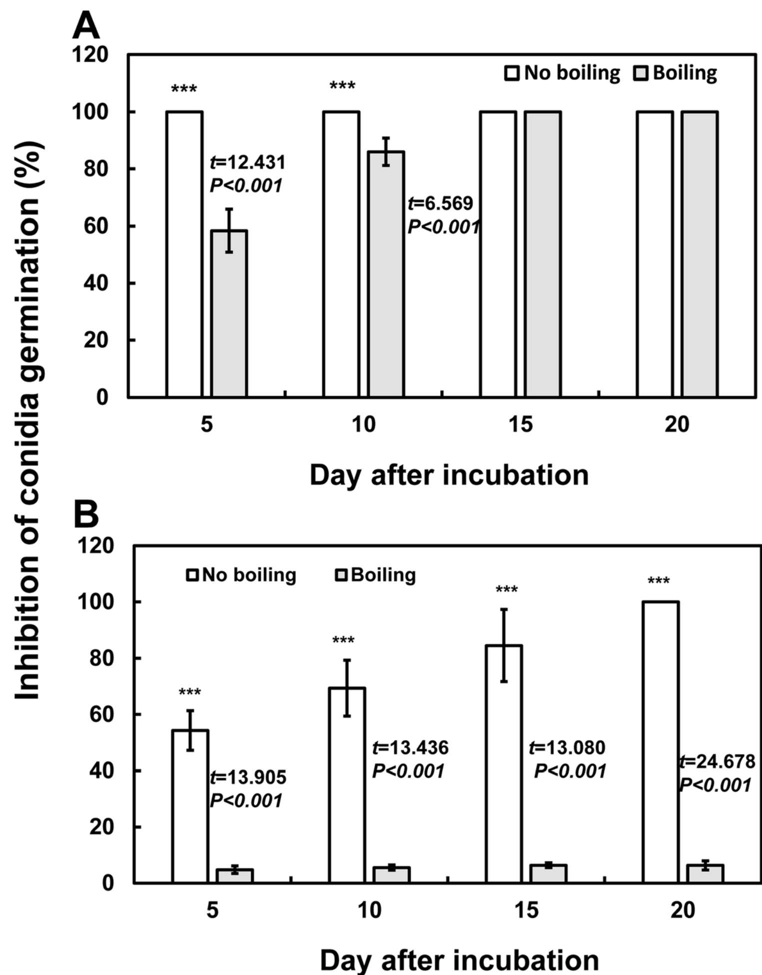

Fig. 2 Effect of heating the *P. elgii* HOA73 cell-free supernatant on the inhibition of *B. cinerea* spore germination. The cell-free supernatants of *P. elgii* HOA73 were obtained from chitin medium at defined days after inoculation, and half the samples were heated at 100 °C for 10 min. Undiluted cell-free supernatants (**a**) and cell-free supernatants diluted fivefold with sterile water (**b**) were used to determine the inhibition of conidial germination of *B. cinerea* in 96-well microplates. Sterile water was used as a control. The experiment was repeated twice, with three replicates per experiment and at least 100 conidial spores for each sample. The *P*- and *t*-values of the Student's *t* test are provided, and *** indicates differences between samples based on the Student's *t* test at $P < 0.01$

in early cultures could be related to reduced chitinase enzyme activity as a result of boiling (Cui et al. 2012; Kim et al. 2017b; Wang et al. 2002; Yen et al. 2006).

Heat stability of secreted antimicrobial compounds may also be important for biocontrol (Guo et al. 2012; Réblová 2012; Wu et al. 2010). Biocontrol *P. elgii* strains produce various antimicrobial compounds, including siderophores, butyl 2,3-dihydroxybenzoate, methyl 2,3-dihydroxybenzoate, protocatechuic acid (Lee et al. 2017; Nguyen et al. 2015a, b), and lipopeptides like pelgipeptins and paenibactin (Ding et al. 2011b; Qian et al. 2012; Wu et al. 2010). When cultured in the chitin-based minimal medium, protocatechuic acid, which is active against *B. cinerea,* and butyl 2,3-dihydroxybenzoate, effective against *F. oxysporum* were identified in HOA73 (Nguyen et al. 2015a, b). We are currently investigating the levels of antimicrobial compounds in *P. elgii* HOA73 CFS when this isolate is grown in the chitin-based minimal medium, as well as the minimal inhibition concentrations of the compounds to determine their importance in biocontrol activity.

The chitin and chitin oligomers generated during growth may also contribute to the suppression of tomato gray mold. Chitin was shown to support growth and chitinase production in other beneficial chitinolytic microbes (Sharp 2013). In the tomato, chitosan effectively inhibited postharvest fungal diseases like black rot caused by *Alternaria alternata*, gray mold caused by *B. cinerea*, and blue mold caused by *Penicillium expansum* (Liu et al. 2007; Reddy et al. 2000). Chitosan affects cell membrane functions by disturbing the electrostatic balance of the negatively-charged phospholipids (Palma-Guerrero et al. 2010). After entering the cell by disrupting the fungal cell membrane, chitosan inhibits nucleic acid and protein synthesis (Verlee et al. 2017). Here, we found that authentic pentameric and hexameric chitin oligomers inhibit *B. cinerea* spore germination, in agreement with that observed in studies on *Fusarium solani* (Kendra and

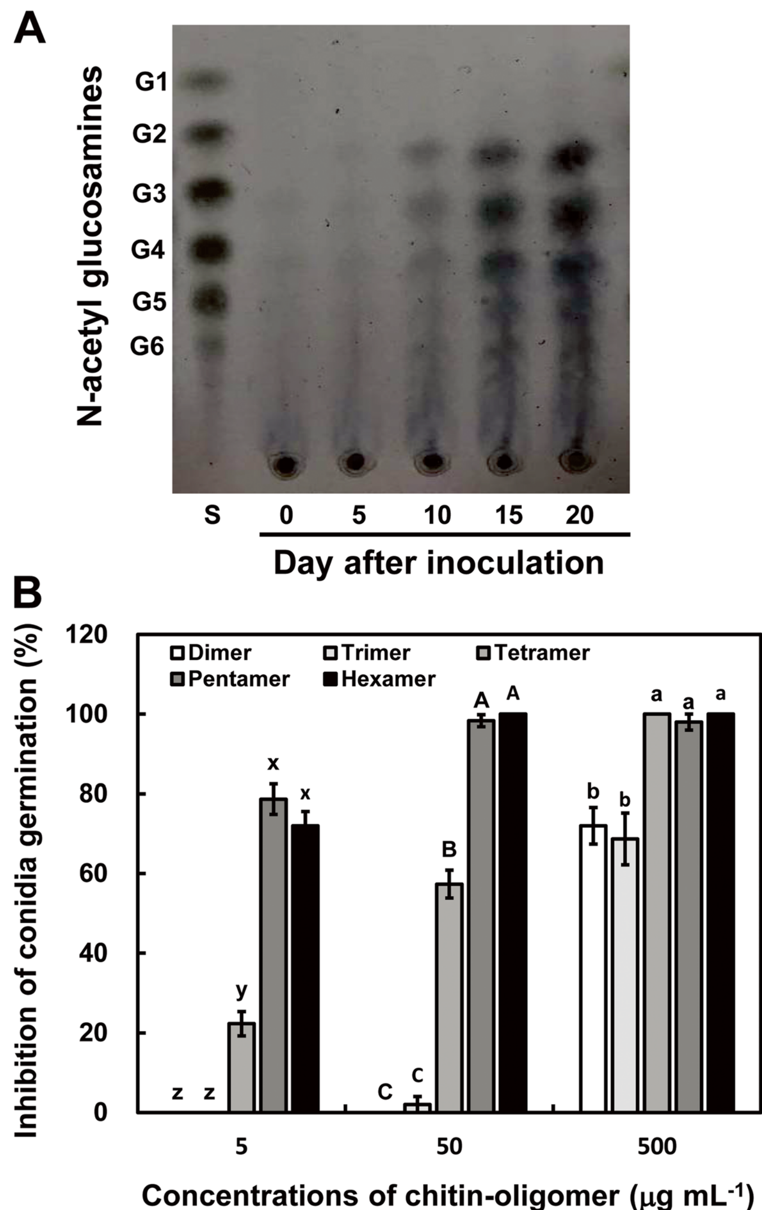

Fig. 3 Chitin oligomer production in the cell-free supernatant of *P. elgii* HOA73 (**a**) and antifungal activity of authentic chitin oligomers on *B. cinerea* spore germination (**b**). The cell-free supernatants of *P. elgii* HOA73 grown in chitin-based minimal medium were collected on different growth days and, together with a mixture of authentic chitin oligomers as standards (S), were spotted onto a silica gel plate and developed to detect chitin oligomers. The image of the TLC analysis is typical of three independent experiments with similar results. **b** Authentic chitin oligomers [G1 (GlcNAc) to G6 $(GlcNAc)_6$] at defined concentrations between 5 and 500 μg mL^{-1} were used to determine inhibition of *B. cinerea* spore germination. The experiment was repeated twice, with three replicates per experiment and at least 100 conidial spores for each sample. Different letters indicate a significant difference between treatments based on Dunnett's test at $P < 0.05$

Hadwiger 1984). In addition, chitin oligomers elicit defense responses in a wide range of both monocot and dicot plants (Felix et al. 1993; Shibuya and Minami 2001), showing that the use of a chitin growth substrate has several advantages for plant health.

We documented the effective biocontrol of tomato gray mold using a *P. elgii* HOA73 formulation under commercial field conditions. Application of the *P. elgii* HOA73 formulation at the start of gray mold development engendered effective control comparable to that of a standard fungicide, indicating that application of *P. elgii* HOA73 formulations in the field could be effective in preventing gray mold development.

In summary, the present study demonstrated the synergistic effects of heat-labile and heat-stable extracellular products in the inhibition of *B. cinerea* spore germination and biocontrol of gray mold in tomato. The *B. cinerea* bacterium is cultured effectively by large-scale fermentation on a cost-effective medium (Kim et al. 2008, 2017a). Taken together, our results show that *P. elgii* HOA73 cultures grown in a chitin-based minimal medium could be applied as an eco-friendly fungicide.

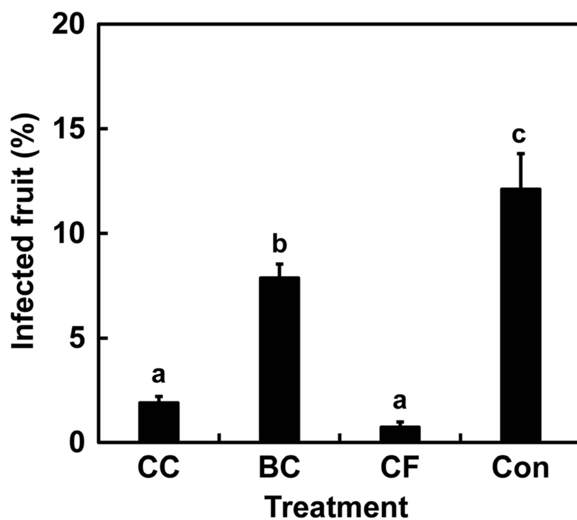

Fig. 4 Control efficacy of undiluted bacterial culture (CC), bacterial suspension (BC), 2000-fold diluted chemical fungicide (CF), and tap water (Con) against the development of gray mold in tomato in a commercial greenhouse in 2016. The bacterial culture was cultivated in 500 L sterile chitin medium in a large fermenter with agitation. Disease incidence (A) was determined by counting the number of diseased fruits in 20 plants per treatment 10 d after the final treatment. Experimental plots (45 m^2) were arranged in a randomized complete block design with five replicates per treatment. Values followed by the same letter within each column are not significantly different at $P < 0.05$ according to Dunnett's test

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Compliance with ethical standards The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

Competing interests The authors declare that they have no competing interests.

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