Triggering the biocontrol of *Botrytis cinerea* by *Trichoderma harzianum* through inhibition of pathogenicity and virulence related proteins

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**Abstract** This study reports a strain of *Trichoderma harzianum* CCTCC-SBW0162 with potential to enhance biocontrol activity against gray mold pathogen, *Botrytis cinerea*, and with a pivotal role in tomato (*Solanum esculentum*) plant growth enhancement. A total of 254 *Trichoderma* isolates were screened by *in vitro* antagonistic assay. Of these, 10 were selected for greenhouse experiments based on their greater inhibition of *B. cinerea*. The *in vitro* antagonistic assay and greenhouse experiments indicated that *T. harzianum* CCTCC-SBW0162 gave the highest inhibition rate (90.6%) and disease reduction (80.7%). Also, to study the possible mechanism associated with antifungal activity of CCTCC-SBW0162 against *B. cinerea*, molecular docking was used to assess the interactions between CCTCC-SBW0162-derived metabolites, and pathogenicity and virulence related proteins of *B. cinerea*. The molecular docking results indicated that the combination of harzianopyridone, harzianolide and anthraquinone C derived from CCTCC-SBW0162 could synergistically improve antifungal activity against *B. cinerea* through the inhibition/modification of pathogenicity and virulence related proteins. However, this computerized modeling work emphasized the need for further study in the laboratory to confirm the effect *T. harzianum*-derived metabolites against the proteins of *B. cinerea* and their interactions.

**Keywords** anthraquinone, *Botrytis cinerea*, harzianolide, harzianopyridone, molecular docking, *Trichoderma harzianum*

**1 Introduction**

*Solanum esculentum*, although technically a fruit, is one of the most economically important vegetable fruit cultivated worldwide. However, its productivity is substantially decreased by gray mold, foliar diseases, root rots and wilts by several phytopathogens[1–3]. The key phytopathogens belong to the genera *Botrytis*, *Fusarium*, *Macrophomina*, *Phytophthora*, *Rhizoctonia* and *Sclerotinia*[4]. Of these, *Botrytis* is particularly hazardous to the tomato plants, causing gray mold disease[1]. Moreover, continued use of chemical fungicides is hazardous to the environment and can also lead to the resistance of the phytopathogens such as *Botrytis cinerea* and *B. fabae*[5]. Therefore, there is a clear need for an effective biocontrol agent to reduce the impact of *B. cinerea*. In this regard, several biocontrol microorganisms, such as *Bacillus*, *Pseudomonas* and *Trichoderma*, are available to reduce the impact of phytopathogens[6,7]. *Trichoderma* strains have potential to reduce gray mold rot[8–10] and can improve plant growth through production of plant hormones, vitamins, triggering plant immunity and nutrient uptake[11]. *Trichoderma harzianum* has received global attention as an effective biocontrol agent for several plant pathogens[12–15]. *T. harzianum* inhabits various ecological niches, such as soil, rhizosphere, lakes, forest sediments and coastal vegetation soil from agriculture or non-agriculture ecosystems[16]. This fungus also occurs in close association with plants and has been isolated from different substrata of the plants[17].

*B. cinerea* is ranked as the second most important phytopathogen in the world[11], and it causes severe plant diseases that result in significant loss of yield in economically important crop plants including beans, berries, grapes and tomato[18]. However, the underlying mechanism of disease incidence, virulence and pathogenicity of *B. cinerea* is not fully understood. The first and foremost
process of interaction is initiated through contact of the fungal cell wall with the host plant. Hence, the fungal cell wall is essential for the penetration, colonization and infection of the plant tissues[10] and the fungal cell wall proteins (glycoproteins) are involved in the host-pathogen interactions, virulence and pathogenicity[20,21]. The genome of B. cinerea exhibits over 100 putative GPI (glycosylphosphatidylinositol) proteins and these cell wall glycoproteins are involved in pathogenicity, virulence and host interaction of B. cinerea[21,22]. According to recent work, bcpmr1 from the B. cinerea is essential for protein glycosylation, cell wall structure and virulence of B. cinerea. In addition, examination of O-linked glycosylation pathways demonstrated that the PMT genes were crucial for the fungal pathogenicity[23].

Several workers have studied the pathogenesis and virulence of B. cinerea[24–30]. One of the findings indicated that bcpmr1 encoded a P-type Ca2+/Mn2+-ATPase involved in protein glycosylation, cell wall structure and virulence of B. cinerea[30]. The identification of the function of the monocarboxylate is, however, reported from the mammalian metabolism rather than fungi. The presence of this monocarboxylate transporter BcMctA has been reported as being essential for B. cinerea pathogenicity[28]. Our study aimed to investigate the effects of T. harzianum and its metabolites on plant growth and enhanced biocontrol of B. cinerea through the modification/inhibition of the pathogenicity and virulence related proteins (bcpmr1 encoding a P-type Ca2+/Mn2+-ATPase, BcMctA) of B. cinerea.

### 2 Materials and methods

#### 2.1 Microorganisms

Trichoderma strains were isolated from a coastal wetland ecosystem[31], grown on modified potato dextrose agar (PDAm)[32] and preserved in 20% glycerol at –80°C. A culture of B. cinerea isolated from infected tomato leaves was obtained from the Center of Trichoderma Culture Collection of Shanghai Jiao Tong University (CCTCCSJ), China.

#### 2.2 In vitro screening

In vitro antagonism of 254 Trichoderma isolates (T1-T254) against B. cinerea was tested by the dual culture method, as described by Dennis and Webster[33]. Percentage of mycelial growth was calculated by measuring the B. cinerea growth competing with Trichoderma isolates on PDAm in Petri dishes (9 cm).

#### 2.3 Trichoderma enhanced plant growth

Seeds of tomato were surface-sterilized by the method of Huang et al.[34], and germinated on sterile wet-paper at room temperature for 4 d. The pre-germinated tomato seedlings were planted in a greenhouse in pots containing the natural agriculture soil, sterilized at 180°C for 6 h to remove other microbes, insects and weeds. The conidial suspension of Trichoderma and B. cinerea were prepared according to the method of Vinale et al.[35], and Nelson and Powelson[36]. The microbial inoculation of Trichoderma pathogens on tomato seedlings was applied by spraying[37]. A 5 mL conidia suspension of Trichoderma (2.6 × 10⁶ conidia per mL) and/or a 5 mL conidia suspension of B. cinerea (5 × 10⁴ conidia per mL) were sprayed on tomato seedlings per treatment.

To analyze Trichoderma induced plant growth regulation, 12 treatments were applied: CK1, uninoculated; CK2, seedlings sprayed with B. cinerea; T1, seedlings sprayed with Trichoderma atroviride CCTCC-RW0008 and B. cinerea; T2, seedlings sprayed with Trichoderma asperellum CCTCC-RW0011 and B. cinerea; T3, seedlings sprayed with T. harzianum CCTCC-RW0006 and B. cinerea; T4, seedlings sprayed with T. harzianum CCTCC-SBW0162 and B. cinerea; T5, seedlings sprayed with T. atroviride CCTCC-RW0008 and B. cinerea; T6, seedlings sprayed with T. atroviride CCTCC-SBW0138 and B. cinerea; T7, seedlings sprayed with T. aureoviride CCTCC-SBW0122 and B. cinerea; T8, seedlings sprayed with T. atroviride CCTCC-SBW0074 and B. cinerea; T9, seedlings sprayed with T. atroviride CCTCC-SBW0068 and B. cinerea; and T10, seedlings sprayed with T. atroviride CCTCC-SBW0073 and B. cinerea. One month after microbial inoculation, the Trichoderma induced growth regulating indicators of tomato, including shoot length, root length, shoot biomass, root biomass and total biomass, were measured. Botrytis disease reduction was evaluated using the modified formula of Saravanakumar et al.[31]. Each treatment had three replicates in a randomized design.

#### 2.4 Molecular interaction

Molecular interaction of T. harzianum-derived secondary metabolites and pathogenicity related proteins from B. cinerea were assessed with a computer-based molecular docking program. The presence of the known T. harzianum metabolites, including T22azaphilone, harzianopyridine, harzianolide, 1-hydroxy-3-methyl-anthraquinone and anthraquinone C[38], in CCTCC-SBW0162 was confirmed by preliminary biochemical experiments (data not shown) and subsequent detailed chemical characterization of compounds. Therefore, these compounds were used for the molecular interaction study. The ligand structures (metabolites) were obtained from PubChem (NCBI-PubChem Compound), and the ligand was prepared by using the ACD/ChemSketch

Earlier reports showed that bcpmr1 encodes a P-type Ca2+/Mn2+-ATPase, and BcMctA from B. cinerea is
involved in pathogenicity and virulence\textsuperscript{[29,30]}. A BLAST analysis indicated that \textit{BcMctA} is identical to MFS monocarboxylate transporter (CCD50452), and the \textit{bcpmr1} is identical to Ca\textsuperscript{2+}/Mn\textsuperscript{2+}-transporting P-type ATPase PMR1 (NP_011348) and hypothetical protein SS1G_09885 (EDN94018). Therefore, in the present study, \textit{bcpmr1} was considered identical to PMR1 (NP_011348) and SS1G_09885 (EDN94018), and \textit{BcMctA} was identical to MFS (CCD50452)\textsuperscript{[29,30]}. Hence, available protein sequences of \textit{bcpmr1} and \textit{BcMctA} for molecular interaction with \textit{T. harzianum} metabolites were used, after retrieving the protein sequences from NCBI protein database and the protein structure were predicted using SWISS-MODEL. Molecular docking was analyzed with ArgusLab 4.0.1, and interactions of the protein and ligand were visualized with BIOVIA Discovery Studio 2016 (Accelrys Software Inc., San Diego, CA, USA).

### 3 Results and discussion

#### 3.1 In vitro antagonism

\textit{In vitro} antagonistic experimental results indicated that mycelial growth of \textit{B. cinerea} was significantly inhibited by \textit{Trichoderma} isolates in dual culture. The percentage inhibition ranged from 1.56\% to 90.6\% with \textit{T. harzianum} CCTCC-SBW0162 exhibiting the highest inhibition of \textit{B. cinerea} (Table S1). Out of 254 \textit{Trichoderma} isolates tested, the top 10 were selected for greenhouse experiments to assessing the \textit{Trichoderma} induced enhanced growth in tomato (Fig. 1) based their high percentage of inhibition of \textit{Trichoderma}. The selected isolates were \textit{T. harzianum} RW0006 (81.3\%), \textit{T. harzianum} SBW0162 (90.6\%), \textit{T. atroviride} SBW0138 (76.6\%), \textit{T. aureoviride} SBW0122 (79.7\%), \textit{T. atroviride} SBW0074 (81.3\%), \textit{T. atroviride} SBW0068 (85.9\%), \textit{T. atroviride} SBW0008 (78.1\%), \textit{T. asperellum} RW0011 (84.4\%) and \textit{T. atroviride} RW0008 (75.0\%).

#### 3.2 Plant growth enhancement induced by \textit{Trichoderma}

The effects of \textit{Trichoderma} on reduction of \textit{B. cinerea} and enhancement of tomato growth under greenhouse conditions are shown in Table 1 and Fig. S1. Average shoot length was significantly affected by the treatments and ranged from 18.6±2.5 to 41.4±2.6 cm. Shoot length was increased significantly by about 1.22 times in T4 compared
Table 1  Antagonistic effect of *Trichoderma* against *B. cinerea* on growth factors of tomato seedlings

| Treatment | Average shoot length/cm | Average root length/cm | Average shoot biomass/g | Average root biomass/g | Total biomass/g | Disease reduction/% |
|-----------|-------------------------|------------------------|-------------------------|------------------------|----------------|------------------|
| CK1       | 30.2±1.2 (0.62)         | 5.0±1.2 (0.56)         | 1.0±0.02 (4.0)          | 0.4±0.08 (1.0)         | 1.4±0.3 (2.50) | 56.7             |
| CK2       | 18.6±2.5                | 3.2±0.8                | 0.2±0.01                | 0.2±0.09               | 0.4±0.1        | 31.7             |
| T1        | 32.0±2.1 (0.72)         | 3.6±0.1 (0.13)         | 0.9±0.03 (3.5)          | 0.3±0.05 (0.5)         | 1.1±0.6 (0.75) | 50.0             |
| T2        | 29.7±1.5 (0.59)         | 4.2±0.6 (0.31)         | 0.2±0.02 (0.0)          | 0.2±0.06 (0.0)         | 0.4±0.8 (0.00) | 40.0             |
| T3        | 37.8±3.2 (1.03)         | 22.1±2.2 (5.90)        | 2.6±0.01 (12.0)         | 0.5±0.06 (1.5)         | 3.1±0.4 (6.75) | 40.0             |
| T4        | 41.4±2.6 (1.22)         | 11.5±0.4 (2.59)        | 3.8±0.03 (18.0)         | 1.6±0.04 (7.0)         | 5.4±0.5 (12.50) | 80.7             |
| T5        | 24.4±1.2 (0.31)         | 8.6±0.6 (1.68)         | 1.8±0.06 (8.0)          | 0.6±0.06 (2.0)         | 2.4±0.6 (5.00) | 33.3             |
| T6        | 32.4±0.6 (0.74)         | 6.5±1.2 (1.03)         | 3.4±0.06 (16.0)         | 0.2±0.04 (0.0)         | 3.6±0.2 (8.00) | 56.7             |
| T7        | 29.5±1.8 (0.58)         | 12.2±1.4 (3.00)        | 2.7±0.04 (2.5)          | 0.3±0.06 (0.5)         | 2.9±0.1 (1.50) | 46.7             |
| T8        | 20.0±1.0 (0.07)         | 3.5±0.6 (0.09)         | 1.2±0.06 (5.0)          | 0.5±0.01 (1.5)         | 1.7±0.2 (6.25) | 33.3             |
| T9        | 37.7±2.4 (1.02)         | 4.5±1.8 (0.40)         | 2.4±0.04 (11.0)         | 0.4±0.02 (1.0)         | 2.8±0.6 (6.00) | 60.0             |
| T10       | 32.6±1.6 (0.75)         | 7.0±1.6 (1.18)         | 1.9±0.09 (8.5)          | 1.1±0.03 (4.5)         | 3.0±0.1 (6.50) | 56.7             |

Note: The values shown are means±SE (df = 60 seedlings per treatment) and one way ANOVA followed by multiple comparison using Duncan’s test. The values in parentheses are the relative increase in treatment compared to negative control inoculated with *B. cinerea* and without *Trichoderma* treatment. CK1, uninoculated sterile soil; CK2, soil inoculated with *B. cinerea*; T1–T10, inoculated with different *Trichoderma* strains and *B. cinerea* as detailed in the materials and methods.

to the CK2. Average root length varied with the treatments and it increased significantly 5.9 times in T3 when compared to CK2. Average shoot biomass (18 times; *P* < 0.05), average root biomass (7 times; *P* < 0.05) and total biomass (12.5 times; *P* < 0.05) increased significantly in T4 compared to CK1. Disease reduction was significant between the treatments (*P* < 0.05) with greatest reduction (80.7%) in T4 compared to (31.7%) in CK2. Thus, treatment T4 showed that *T. harzianum* CCTCC-SBW0162 significantly reduce gray mold and improved tomato growth, which is consistent with other reports that *Trichoderma* spp. promote plant growth[39].

3.3 Generation of protein and metabolite structures

The predicted protein structures of *bcpmr1* [PMR1 (NP_011348) and SS1G_09885 (EDN94018)] and *BeMctA* [MFS (CCD50452)] are shown in Fig. 2. The structure of *T. harzianum* metabolites (ligand) such as T22azaphilone, harzianopryridone, harzianolide, 1-hydroxy-3-methyl-anthraquinone, and 1, 8-dihydroxy-3-methyl-anthraquinone are shown in Fig. 3.

3.4 Molecular interaction studies

A total of three target proteins were tested for interactions with *T. harzianum*-derived compounds and the results indicated that they can have a significant inhibitory effect against pathogenicity and virulence related proteins of *B. cinerea* (Table 2). Secondary metabolites produced from *T. harzianum* are known to inhibit the pathogens such as *Gaeumannomyces graminis, Pythium ultimum* and *Rhi zoctonia solani* in vitro[38]. Similarly the present work indicated that the *T. harzianum*-derived metabolites can inhibit the growth of *B. cinerea* through the inhibition/modification of pathogenicity and virulence proteins. The antifungal effect of *T. harzianum*-derived metabolites significantly varied between different types of phytopathogens[35]. Similarly, the present study indicated that among the five tested compounds anthraquinone C can provide the greatest inhibition of *bcpmr1* (<43.91 and −49.47 kJ·mol⁻¹ for PMR1 and SS1G, respectively) and *BeMctA* (<57.71 kJ·mol⁻¹) than the other compounds tested for docking energy.
3.5 Molecular interaction of Bcpmr (PMR1 and SS1G_09885) with T. harzianum-derived metabolites

The examination of five candidate compounds of T. harzianum for molecular interaction indicated that they all had significant ability to inhibit and/or modify the pathogenicity and virulence related proteins Bcpmr (PMR1) of B. cinerea with strong docking scores from $-38.22$ to $-43.91$ kJ·mol$^{-1}$ (Table 2). Among the compounds, anthraquinone C showed the highest docking score of $-43.91$ kJ·mol$^{-1}$ with a strong interaction with hydrogen residues such as Arg182 and Asp703. Other residues were observed in the binding pockets such as Glu75, Gly241, Gly263, Ile76, Leu148 Phe262, Phe266, Thr702, Val704, Val682 and (Fig. 4). This interaction strongly indicated the potential of anthraquinone C to modify targeted protein structures and such modification could change protein function.

The results indicated that all the tested compounds showed significant potential interactions with the SS1G_09885; and among the tested compounds anthraquinone C had the highest docking score of $-49.47$ kJ·mol$^{-1}$ with strong interactions with hydrophobic residues, such as Met447, Gln444 and Met520, and other residues, such as Glu524, Ile826, Ile830, Leu834, Leu495, Leu503, Leu443, Pro521, Thr441, Val523, Phe827, and Val499 (Fig. 5). This demonstrates that the fungal metabolites could have a significant ability to interact with and interfere with the functioning of Bcpmr-encoded protein.

3.6 Molecular interaction of BcMctA (MFS) with Trichoderma harzianum-derived compounds

BcMctA is significantly involved in the pathogenicity and virulence of B. cinerea and it was found that among the tested compounds, anthraquinone C could interact significantly with the BcMctA protein with a docking score of $-57.71$ kJ·mol$^{-1}$ and strong interaction with protein residues such as Leu111, Phe114, Ser110, Leu404, Phe401 and Phe117 (Fig. 6). All the docking scores of T. harzianum-derived compounds with pathogenicity and virulence related proteins in B. cinerea indicated the potential of T. harzianum to inhibit B. cinerea by targeting this protein. Several researchers have reported a potential reduction of protein function as evidenced by negative docking scores from the computational method using ArgusLab$^{35,40–43}$ and the present results suggest that

![Fig. 3](structure.png) Structure of metabolites (ligand) of T. harzianum. (a) T22azaphilone; (b) harzianopyridone; (c) harzianolide; (d) 1-hydroxy-3-methyl-anthraquinone; (e) 1,8-dihydroxy-3-methyl-anthraquinone.

Table 2 Analysis of interactions between Trichoderma-derived compounds and pathogenicity related protein bcpmr1 of B. cinerea

| S. No. | PubChem CID | T. harzianum derived Compound Name | Mol. Formula | Mol. Wt/(g·mol$^{-1}$) | Docking Score/(kJ·mol$^{-1}$) |
|--------|-------------|-----------------------------------|-------------|------------------------|-----------------------------|
|        |             | Bcpmr PMR1                        |             |                        |                             |
|        |             | SS1G                             |             |                        |                             |
|        |             | MFS                              |             |                        |                             |
| 1       | 76326344    | T22azaphilone                     | C$_{18}$H$_{16}$O$_{6}$ | 330.336                | $-38.56$ $-42.57$ $-42.87$ |
| 2       | 54697782    | Harzianopyridone                  | C$_{14}$H$_{10}$NO$_{5}$ | 281.308                | $-40.36$ $-35.34$ $-38.39$ |
| 3       | 15719532    | Harzianolide                      | C$_{13}$H$_{12}$O$_{3}$ | 222.284                | $-40.27$ $-43.87$ $-45.17$ |
| 4       | 164982      | 1-hydroxy-3-methyl-anthraquinone  | C$_{13}$H$_{12}$O$_{4}$ | 238.242                | $-38.22$ $-41.65$ $-48.13$ |
| 5       | 641293      | anthraquinone C                   | C$_{20}$H$_{17}$ClO$_{4}$ | 356.802                | $-43.91$ $-49.47$ $-57.71$ |

Fig. 4 Interaction of Bcpmr with T. harzianum-derived compounds and pathogenicity related protein Bcpmr1 of B. cinerea

![Fig. 5](interaction.png) Interaction of BcMctA with T. harzianum-derived compounds and virulence related protein BcMctA of B. cinerea.
anthraquinone C could interact significantly with the BcMctA protein and inhibit its functioning. Harzianopyridone, harzianolide and anthraquinone C are known to reduce the gray mold disease in tomato caused by B. cinerea\textsuperscript{[29,35]}. The negative docking scores for these three compounds (Table 2) indicated that they could work synergistically against B. cinerea and inhibit its growth through targeting the pathogenicity and virulence related proteins. These molecular docking results indicate that some secondary metabolites from Trichoderma spp., could potentially interact strongly with phytopathogens. However further detailed laboratory and greenhouse studies are required to confirm this.

4 Conclusions

This work reports a potent Trichoderma strain with potentially useful biocontrol activity against the gray mold pathogen, B. cinerea, investigated by in vitro and
greenhouse experiments. Significant potential molecular interactions between T. harzianum-derived metabolites and pathogenicity, virulence related proteins of B. cinerea indicated that the inhibition of the B. cinerea may not triggered by single metabolite, but is likely to be a synergistic effect of multiple metabolites from T. harzianum. Notably, the negative docking score for anthraquinone C indicated it could have the greatest ability to inhibit B. cinerea, and the combination of harzianopyridone, harzianolide and anthraquinone C may also increase the potential biocontrol activity of T. harzianum against B. cinerea. Taken together, these findings provide important new information about the molecular interactions of metabolites and pathogenicity-related virulence proteins. This contrasts with established methods which are generally based on trial and error testing, with a low probability of success in the laboratory experiments[44]. Although this study needs to be confirmed by further study of the interaction of T. harzianum metabolites with B. cinerea proteins in a microbiology laboratory, this approach should lead to a greater likelihood of success.

Supplementary materials The online version of this article at https://doi.org/10.15302/J-FASE-2018214 contains supplementary materials (Table S1; Fig. S1).

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Compliance with ethics guidelines Kandasamy Saravanakumar, Zhixiang Lu, Hai Xia, Meng Wang, Jianan Sun, Shaoqing Wang, Qiang-qiang Wang, Yaqian Li, and Jie Chen declare that they have no conflicts of interest or financial conflicts to disclose.

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