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Avirulent *Colletotrichum* strain for controlling anthracnose disease in chilli caused by *Colletotrichum capsici*

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Abstract Anthracnose disease caused by *Colletotrichum capsici* (syn. *Colletotrichum truncatum*) is still a major problem in chilli horticulture. Nonpathogenic fungal strains offer an alternative means to control plant disease using green technology. *Colletotrichum siamense* G4211 was isolated and verified as an avirulent strain for chilli seeds, fruits and seedlings. Seeds treated with G4211 showed 99.25% germination. Application of G4211 resulted to produce a strong compatible interaction in chilli with no disease symptoms. Disease severity rating (DSR) was 0.15 on green and 0.1 on red fruits while 1 in seedlings. Whereas the pathogen, *C. capsici* Cc17, showed infection on fruits with DSR 2.5 on green and DSR 4 on red fruits while DSR 5 in seedlings. *C. siamense* G4211 exhibited antagonistic activity against anthracnose disease caused by Cc17. When chilli fruits and seedlings were inoculated with G4211 for 48 h before inoculation with Cc17; the fruits and seedlings showed few symptoms of disease, with DSR lower than 1. β-1, 3-glucanase and chitinase activities accumulated in the leaf of seedlings was high when pre-treated with G4211 followed by Cc17. The experiments indicated that G4211 is shown to be an elicitor of plant response by producing pathogenesis-related proteins such as β-1, 4-glucanase and chitinase. The research findings suggested that avirulent strain of *Colletotrichum siamense* G4211 proved to produce healthy seedlings, possible reduce chemical usage and support an eco-friendly disease management strategy.

Keywords: Avirulent, Biocontrol, Chilli disease, Pathogenic fungi

Introduction

Chilli anthracnose caused by *Colletotrichum* spp. and it is a major disease that negatively impacts to chilli crop yield and quality. The anthracnose can affect all parts of the growing plant: the seeds, seedling, flowers, leaves and fruits. Moreover, the pathogen can occur as a cross infection in different hosts, leading to the disease spreading out in various crops and fruits (Than *et al.*, 2008). The anthracnose is devastated to chilli farmers during pre-harvest and

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post-harvest with losses of production up to 80% (Poonpolgul and Kumphai, 2007). *Colletotrichum acutatum* and *Colletotrichum capsici* are the major causing agents and both are common and widespread in Thailand (Than *et al.*, 2008). Chemical fungicides are effective and popular for controlling anthracnose diseases; however, those should be limited application because chemical fungicides cause serious health and environmental problems, facing obligatory restrictions in many countries. Bio-fungicides using *Pseudomonas*, *Streptomyces* and *Trichoderma* have been reported to control many diseases. An example of commercial products called Mycostop® (Kemira Agro Oy, Helsinki, Finland), which is being marketed for the elimination of soilborne diseases including chilli anthracnose disease (Suwan *et al.*, 2012).

As an alternative biological method to control the anthracnose, avirulent fungal strains are being chosen as a strategy that activates plant innate immunity to induce systemic resistance (ISR), enabling a broad spectrum of plant protection against pathogens including fungi, bacteria and viruses (Pieterse *et al.*, 1996). *Colletotrichum* strains were used as nonpathogenic strains in many plants, such as *Colletotrichum lagenarium* reported to be an elicitor in a plant protection response in cucumbers (Kuć *et al.*, 1975), and *Colletotrichum fragariae* reported to be an avirulent strain against disease in strawberries (Salazar *et al.*, 2007). However, there are no reports of avirulent strains used in chilli. Biocontrol of chilli anthracnose has been reported using plant extracts (Johnny *et al.*, 2011), microbial agents (Raghunandan *et al.*, 2019) and microorganisms - particularly fungal species, i.e *Trichoderma*, *Fusarium*, and *Chaetomium* (Vasanthakumari and Shivanna, 2013; Mukherjee *et al.*, 2014). The mechanisms of antagonistic fungi involved mycoparasitism, antibiosis, competition for nutrients and their ability to induce systemic resistance in the plants. Higher plants have a mechanism to protect themselves against stress conditions such as wounding, salinity, drought, cold, heavy metals, air pollutants, ultraviolet rays and including pathogen attack of fungi, bacteria and viruses (van Loon *et al.*, 1998). Another mechanism involves β-1,3-glucanases and chitinase, which are usually expressed at low concentration in plants; when plants are infected, both enzyme concentrations are increased.

In this work, an avirulent strain of *Colletotrichum siamense* was isolated and investigated for its ability to control anthracnose disease caused by *C. capsici* (syn. *C. truncatum*) and to evaluate the ability of the avirulent strain to induce enzyme activity of the plant. Finally, the effect on disease incidence on chilli seeds, fruits and seedlings was determined.

Materials and methods

Isolation of Colletotrichum spp.

Chilli leaves and fruits were collected in order to isolate fungi from chilli producing fields in four districts of Roi-Et province of Thailand. Pieces of tissue were surface sterilized with sodium hypochlorite (3%) for 5 min, rinsed with sterile distilled water, and then placed on potato dextrose agar (PDA) and incubated at 28 ℃. After 5 days of inoculation, growing mycelial tips were subcultured on PDA medium. Single spore isolation was done on water agar (2% Bacto agar) (Ho and Ko, 1997). A single germinating spore was subcultured on PDA for 5 days at 28 ℃. The isolates were transferred to PDA slants and kept at 4 ℃ for preservation and further use.

Spore preparation

Sterile distilled water containing Tween 80 (0.1%) was added to fungal isolates grown on PDA slant for 7 days at 28 ℃. The conidial suspension was counted and adjusted to a final concentration of 1×10^6 conidia/ml. For fungal pathogen, *C. capsici* (syn. *C. truncatum*) isolate Cc17 derived from Krumkhuntot *et al.* (2017), was multiplied on PDA using single spore to obtain pure culture and maintained on PDA slants at 4 ℃ for stock culture. The spore suspension was prepared the same as with the other fungal isolates.

Planting material

Chilli seeds used were “Jinda Chilli” (*Capsicum annuum* L.) from Chia Tai Co., Ltd. The seeds were surface sterilized by soaking in 3% sodium hypochlorite for 3 min and washed with sterile water twice. Then, the seeds were placed in a peat at a depth of 2 cm, in plastic pots which were sterilized by autoclave and 70% alcohol. The pots were moistened with sterilized water and maintained in a greenhouse for 30 days. Healthy plants containing three to four young leaves were used as seedlings in further experiments.

Pathogenicity tests for screening of avirulent strains

Pathogenicity on fruits

Ten green and red chilli fruits were surface-sterilized by soaking in 3% sodium hypochlorite for 5 min then, washed in sterilized water and air dried. Each fruit was wounded with sterile needle and then inoculated with 20 µl conidial suspension of the isolated fungi or sterilized distilled water, as a

control. The wounded and inoculated fruits were placed in sterile-plastic box (20x35x18 cm) with plastic-mesh-screens and incubated at 28 ℃. Paper towels were placed under plastic mesh and were filled with 100 ml of sterile distilled water to maintain relative humidity in 100%. The plastic boxes were covered with lids throughout the incubation period. The lesions on chilli fruits were evaluated at 7 days after inoculation (DAI), and longitudinal lesion diameter was measured using a vernier caliper. Disease severity rating (DSR) was expressed as the sum of longitudinal lesion diameters relative to the total length of the fruit inoculated. The severity of the presented symptoms was assessed according to the method of Jetiyanon *et al.* (2003) using the following scale 0 = fruit was healthy, 1 = 10% of fruit area was infected, 2 = 25% fruit area infected, 3 = 50% of fruit area infected, 4 = 75% of fruit area infected and 5 = 100% of fruit area infected.

Pathogenicity on seeds

Sterile seeds were tested for pathogenicity of isolated fungi with standard blotter plate technique (Elwakil and Ghoneem, 2002). The seeds were soaked in conidial suspension (1×10^6 conidia/ml) of the lisolated fungi or sterile distilled water for 1 h and placed on a sterile towel within blotter plate in four replicates of one hundred seeds (25 seeds per plate), and incubated at 28 ℃ under alternating cycles of 12/12 h of light and darkness for 14 DAI. The seeds were observed for germination rate (germinated seeds x 100/total seeds).

Pathogenicity on seedlings

Five seedlings were sprayed with conidial suspension of isolated fungi. For a negative control, the seedlings were sprayed with sterile distilled water. Each inoculated plant was covered with moist plastic bags for 48 h at room temperature. Afterwards, the bags were removed and the plants were observed for disease symptoms at 5, 14, 30 and 50 DAI. Disease severity rating (DSR) was assessed according to the method of Delp and Milholland (1980) using the following scale: 1= healthy petiole without lesions; 2= petiole with lesions < 3 mm; 3= petiole with lesions from 3 to 10 mm; 4= petiole with lesions from 10 to 20 mm and girdling of petiole; 5= entirely necrotic petiole and dead plant.

Effect of avirulent strain against anthracnose disease

Postharvest test

Ten green and red chilli fruits were tested in each treatment. Double inoculation was performed on each fruit by firstly inoculating with 20 µl conidia suspension of avirulent strains for 24 h, and then by second inoculation with 20 µl conidia suspension of the virulent strain *C. capsici* Cc17. Sterile

distilled water was used as a negative control and only virulent strain was used as a positive control. The experiments were performed as pathogenicity tests on fruits as described above. The anthracnose symptoms on chilli fruits were evaluated at 7 DAI and expressed as DSR.

Seedling test

Five seedlings prepared as described above were sprayed with the conidial suspension of avirulent strains for 48 h prior to spraying with conidia suspension of virulent strain *C. capsici* Cc17. As a positive control, seedlings were inoculated with the virulent strain Cc17 and inoculated with sterile distilled water as a negative control. After each inoculation, plants were covered with plastic bags at room temperature for 48 h, after which the bag was removed. DSR was evaluated on 30 DAI and assessed according to the method of Delp and Milholland (1980).

Effect of avirulent strains on plant response

As the seedling test experiment, the leaves of controls and inoculated treatments were taken after inoculation at 72 h and 120 h. One gram of leaves was homogenized in a pre-chilled mortar containing 120 µl of 0.05 M of potassium acetate buffer (pH 5.5). The homogenate was filtered through pre-moistened two-layered cheesecloth and the filtrate was centrifuged at 10,000 g for 20 min at 4 ℃. The supernatant was collected and enzyme activity was determined.

Assay of β-1,3-glucanase

The reaction mixture consisted of 1 ml of the supernatant from leaves and 1 ml of 1% laminarin in 0.05 M potassium acetate (pH 5.0) as a substrate, and incubated for 30 min at 37 ℃. After incubation, the released glucose was further assayed using the DNS method (Miller, 1959). The β-1,3-glucanase activity was calculated using an equation of the glucose standard curve. 1 unit of enzyme was defined as the amount of enzyme produced reducing sugar in 1 µmole/ml per 1 min.

Assay of chitinase

Chitinase activity and the reaction of chitin breakdown was determined by measuring the release of N-acetyl-D-glucosamine (NAG). The reaction mixture consisted of 1 ml of the supernatant from leaves and 1 ml colloidal chitin soluble (1%) in 0.05 M potassium acetate (pH 5.0) as a substrate. The mixture was incubated for 60 min at 37 ℃. After incubation, the reaction was stopped with 50 µl of 1 M HCl and centrifuged at 10,000 g for 10 min at 4 ℃.

The supernatant was determined for its absorbance at 585 nm. The chitinase activity was calculated using an equation of the NAG standard curve. 1 unit of enzyme was defined as the amount of enzyme released NAG in 1 μmole/ml per 1 min.

Identification of avirulent strains

Five-day-old mycelia of avirulent isolates were cut with a cork borer and transferred to PDA at the center of a petri dishes. The plates were incubated at 28 ℃ for 7 days. Then, the characteristics of colony were observed. The shape, size, and color of conidia were examined under the microscope. The morphological characteristics were identified using Sutton's key (Sutton, 1992). Appressoria were determined using slide culture technique and microscope observation. For molecular identification, DNA of avirulent strains were extracted using the method of Melo *et al.* (2006). PCR was performed using universal primers (ITS1/ITS4) following the method of Kamle *et al.* (2013) and primers of calmodulin (CAL) gene (CL1/CL2A) following the method of O'Donnell *et al.* (2000). The sequencing was analyzed by the National Center for Genetic Engineering and Biotechnology (NCBI) and compared with blastn program of NCBI database.

Statistical analyses

Data were analyzed using analysis of variance to evaluate the efficiency of avirulent strains against *C. capsici* Cc17. Duncan's multiple range tests was used to compare the means of the treatments in each experiment. All statistical analyses were conducted using SPSS software version 19.0.

Results

Isolation of Colletotrichum spp.

Thirty-four fungal isolates of *Colletotrichum* were isolated and divided into 2 groups. The first group showed cottony, dense grayish, white aerial mycelium covered with an orange conidial mass; the second group exhibited tufted, grayish green, sparse white aerial mycelia. Conidia of all isolates were found in 3 types; the first type was hyaline and cylindrical (11.37-12.26×3.38-3.54 μm); the second type was hyaline and falcate (11.25×3.52 μm); the last type was hyaline and fusiform (10.17×3.60 μm). Appressoria of all isolates were found in 2 types, clavate and brown to dark brown, and irregular and dark

brown. These characteristics were described for *Colletotrichum* spp. according to Sutton (1992).

Screening of avirulent strain of Colletotrichum spp.

Pathogenicity test on chilli fruits, seeds and seedlings was used to select avirulent strains. Among 34 fungal isolates, 11 isolates produced typically necrotic, and sunken anthracnose symptoms on detached green chilli fruits. Fourteen isolates produced anthracnose symptoms on detached red chilli fruits. Three isolates (R3121, G323 and G4211) did not produce any symptom on chilli fruits, showing less than 1 for DSR value (Figure 1) compared with the pathogen Cc17 (DSR=2.5 on green and DSR=4 on red fruits). Additionally, all 3 strains did not show any effect on percentages of seed germination which were 92.75, 99.25 and 95.25 for the treatments of R3121, G323 and G4211, respectively. Meanwhile, the Cc17 treatment showed only a 2% germination rate (Figure 2). To confirm the avirulent strain, seedling was sprayed with the conidial suspension of avirulent strains. The treated seedlings with G323 and G4211 exhibited healthy petiole without lesions (DSR=1) as the same in non-inoculated control (Figure 3). The R3121 showed petioles with lesions less than 3 mm (DSR=2.1), while the treated seedlings with Cc17 showed necrotic petioles and dead plant (DSR=5). The results indicated that G323 and G4211 proved to be avirulent strains that no harmful effects on chilli plants, while R3121 caused mild disease in chilli plants.

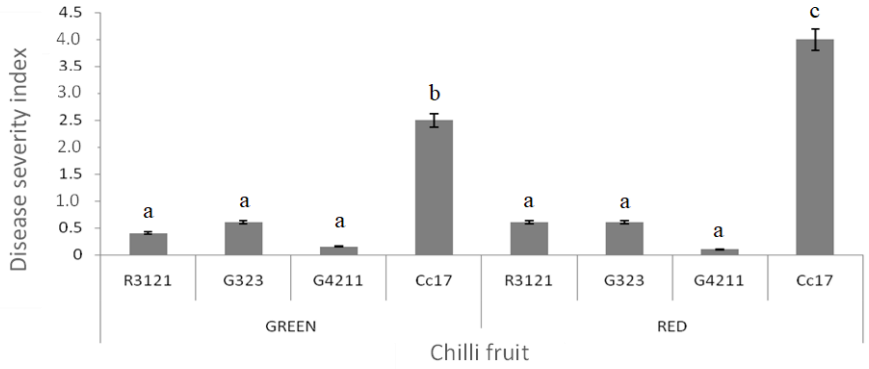

Figure 1. Disease severity index of green and red chilli fruits in response to inoculation with avirulent strains at 7 days after inoculation. Error bars represent the mean standard deviations of three replicates. Different letters on bars indicate significant differences ($p \leq 0.05$) according to Duncan's multiple range tests

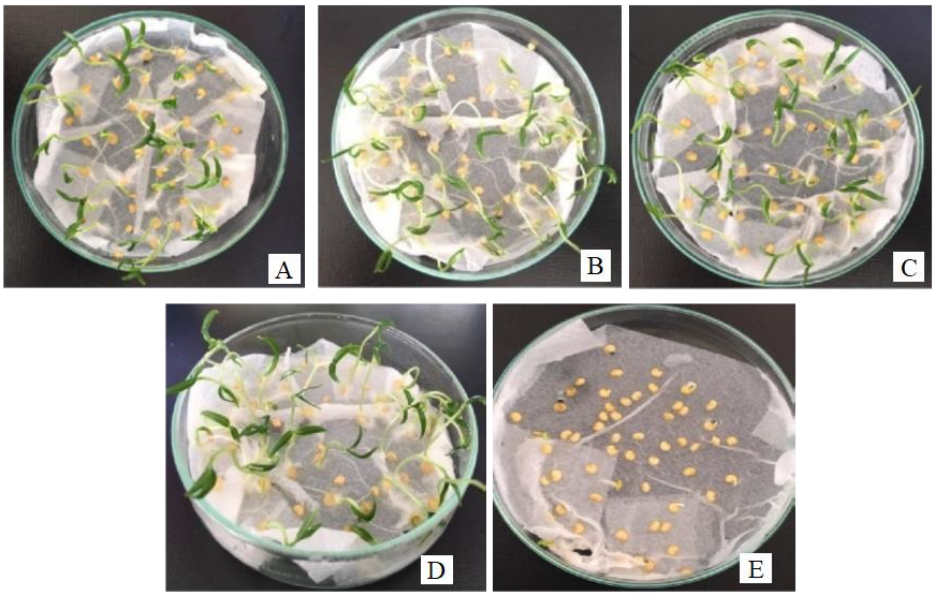

Figure 2. Seed germination for pathogenicity test under conditions treated with R3121 (A), G323 (B), G4211 (C), sterile water (D) and Cc17 (E) at 14 days after inoculation

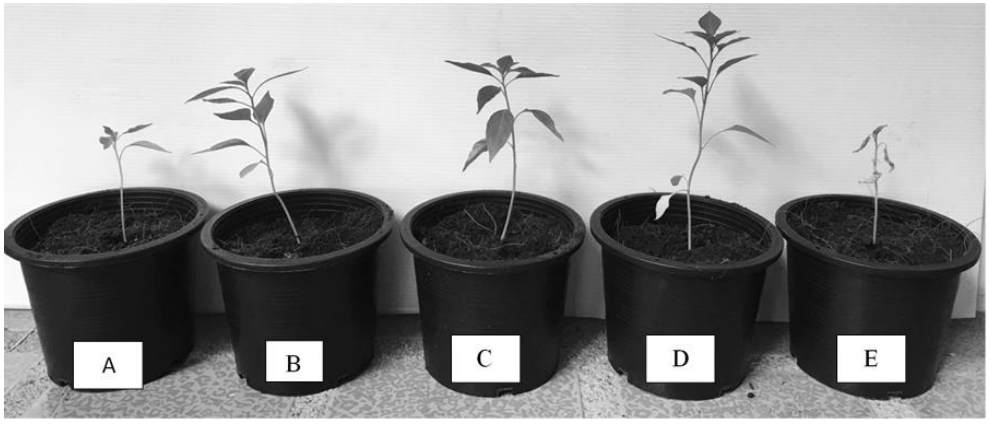

Figure 3. Phytopathogenicity test on seedling with isolate R3121 (A), G323 (B), G4211 (C), sterile water (D) and Cc17 (E) at 10 days after inoculation

Effect of avirulent strains as biocontrol of chilli anthracnose

In this study, detached fruits technique was used to investigate whether the avirulent strains could induce resistance to anthracnose disease in chilli fruit. R3121, G323 and G4211 significantly suppressed necrotic lesion on fruit areas when applied for 24 h before facing with the virulent strain. G4211 showed the highest disease suppression on green and red fruits (Table 1), implying the antagonistic activity of this strain. Spraying technique was used to investigate the induction of plant resistance in seedlings. Inoculation with the avirulent strains for 48 h prior to the inoculation with Cc17, the plants reduced the lesion of anthracnose disease at 30 days after inoculation with the pathogen. G4211 showed the highest disease suppression (Table 1) which revealed small necrosis in seedling compared to the non-inoculated control with no symptoms of necrosis. The results confirmed that G4211 lacked virulent properties and possessed antagonistic activity for anthracnose disease.

Table 1. Disease severity rating (DSR) of avirulent strains for controlling anthracnose disease in chilli fruits and seedling caused by *C. capsici* Cc17

Treatments	DSR		
	Chilli fruit *		Seedling **
	Green	Red	
Inoculated with R3121 prior to the inoculation of Cc17	1.0[a]	1.2[a]	1.9[b]
Inoculated with G323 prior to the inoculation of Cc17	1.0[a]	1.0[a]	2.4[b]
Inoculated with G4211 prior to the inoculation of Cc17	0	0.2[a]	0.7[a]
Inoculated with Cc17 only	3.0[b]	4.2[b]	5[c]
Inoculated with sterile distilled water only	0	0	0

*Each value is the mean of ten replications.
**Each value is the mean of five replications.
Values within a column followed by the same letter do not differ at P=0.05.

Enzyme activity

The antagonistic activity of G4211 was determined by seedling inoculation for 48 h and followed by Cc17 inoculation. After this double inoculation, 1,3-β-glucanases and chitinase activities from plant leaves were significantly higher than the inoculated control treated with Cc17 (Table 2) at 72 and 120 h. The results implied that the chilli plants responded to the pathogen by mechanism of 1,3-β-glucanases and chitinase activities, producing more enzymes to control disease with triggering avirulent strain.

Table 2. Induction of avirulent G4211 for β-1,3- glucanase and chitinase enzyme in chilli seedling treated with *C. capsici* Cc17

Treatments	Enzyme activity (unit/mL)	
	β-1,3- Glucanase	Chitinase
72 h after double inoculation of G4211 and Cc17	5.47[a]	8.75[a]
120 h after double inoculation of G4211 and Cc17	5.84[a]	8.33[a]
72 h after inoculation with *C. capsici* Cc17 only	3.48[b]	5.27[b]
120 h after inoculation with *C. capsici* Cc17 only	3.77[b]	5.41[b]

Values within a column followed by the same letter do not differ at *P*=0.05.

Identification of an efficiency avirulent strain of Colletotrichum spp.

The effective G4211 showed white to pale gray mycelium with orange conidial mass on PDA (Figure 4A). Under microscopy, it presented septate hyphae, cylindrical conidia and clavate and brown to dark brown appressoria (Figure 4B and 4C). This strain was identified as *Colletotrichum siamense* with 98.77% identity to *Colletotrichum siamense* strain MFLU090230 (NR144784.1) from Genbank for ITS; moreover, the identification of CAL gene showed 99.85% identity to *Colletotrichum siamense* strain CSST1 (GQ849467.1). The G4211 was named *Colletotrichum siamense* strain G4211 with the accession number of MT330247.1.

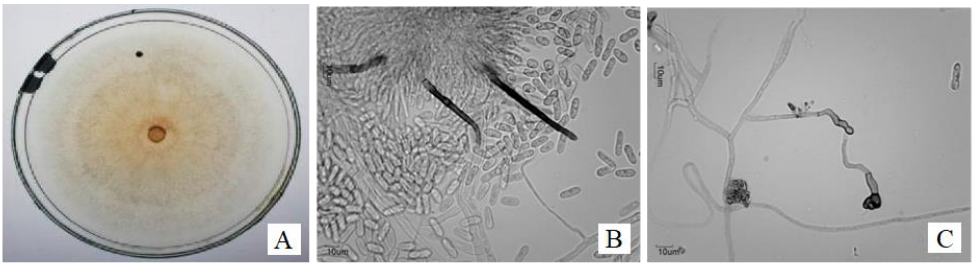

Figure 4. Morphology and cultural characters of *C. siamese* isolate G4211: Upper of cultures on PDA for 7 days after inoculation (A); (B) Conidia and setae; (C) Appressoria (Bars = 10 µm)

Discussion

As result, avirulent and pathogenic strains belong to the same genus which similar characteristics, enabling them to colonize and occupy plants; but the avirulent strain is able to compete successfully against their respective pathogenic strains in plants after double inoculation. The effective

Colletotrichum G4211, in the present study, had no harmful effect on seeds, seedlings and fruits of chilli compared with the virulent *Colletotrichum* Cc17 strain, which caused severe disease symptoms in pathological experiments. This G4211 strain proved to be an avirulent strain that has the ability to control the disease from the virulent *Colletotrichum* Cc17. It reported that an avirulent strain of *Colletotrichum fragariae* acquired strong resistance against virulent *C. acutatum* strain in strawberries (Salazar *et al.*, 2013). The possible mechanism against fungal pathogens of the G4211 might be the competition of habitats and nutrients as it had compatible interaction with plants the same as fungal pathogens. The pathogens penetrated to seed coat and peripheral layers of the endosperm in moderately colonized seeds and then produced abundant inter- and intracellular mycelia and acervuli in the seed coat, endosperm and embryo (Chitkara *et al.*, 1990) including *Colletotrichum* species forming acervuli and microsclerotia in seeds (Pernezny *et al.*, 2003). Moreover, *Colletotrichum truncatum* (formerly *C. capsici*) showed cuticle penetration and necrotrophic phases of colonization on leaves and fruits (Ranathunge *et al.*, 2012). The colonization of avirulent strains into plant cells may be reasonable for the induction of plant hormones against pathogens.

The management of chilli anthracnose is reported and reviewed by Saxena *et al.* (2016). *Trichoderma* species is well know as possessing the most potential as biocontrol. It offers good competition for nutrients, space and ability to induce systemic resistance. The related results of avirulent strains were a nonpathogenic strain of *Fusarium oxysporum* f. sp. *cucumerinum* that protected cucumber leaves from the fungal pathogen, *C. lagenarium* (Ishiba *et al.*, 1981). Therefore, the effective *Colletotrichum* G4211 showed high efficiency in controlling the virulent *Colletotrichum* Cc17 on chilli fruits and seedlings is a new strain for controlling chilli anthracnose which may have many advantages for application. Further studies are necessary to elucidate interval times between the treatments with the elicitor challenge before pathogens and study on the defense mechanisms in chilli plant after inoculation with an avirulent strain.

Genetic analysis for *Colletotrichum* species required multigene to identify this genus, such as Actin (ACT), β-tubulin (TUB2), Calmodulin (CAL), Glutamine synthetase (GS) and Glyceraldehyde 3-phosphate dehydrogenase (GPDH) as they are closely relationships among *Colletotrichum* species (Damm *et al.*, 2009). The ITS region is the most widely region for fungal identification; however, ITS sequence data revealed a high error rate and frequency of misidentification (86%) within *Colletotrichum graminicola* species complex (Crouch *et al.*, 2009). In this study, the high identity showed in ITS and calmodulin sequences and the closest gene exhibited in calmodulin.

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