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Biological control of vanilla anthracnose using *Emericella nidulans*

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Emericella nidulans was proved to be antagonistic to *Colletotrichum gloeosporioides* (Penz) causing anthracnose of *Vanilla planifolia*. Eight isolates of *C. gloeosporioides* were proved for pathogenecity. With this, isolate VP8 gave the highest virulence for disease incidence. *E. nidulans* was isolated from fallen leaves of Vanilla and tested against mycelial growth and sporulation of *C. gloeosporioides* VP8 by bi-culture and crude extract methods. Bi-culture test showed that *E. nidulans* could inhibit mycelial growth and sporulation at 49.44% and 75.31%, respectively. Crude extracts were extracted from *E. nidulans* with hexane, ethyl acetate and methanol. Methanol crude extract inhibited the mycelial growth and sporulation at the concentration of 1,000 µg/ml and the effective dose (ED_{50}) were 2,910 µg/ml and 0.0001 µg/ml, respectively.

Key words: anthracnose, *Vanilla planifolia*, *Emericella nidulans*, *Colletotrichum gloeosporioides*

Introduction

Vanilla is a crop of great commercial importance as the source of natural vanillin, a major component of flavor industry. The genus *Vanilla*, is composed of about 110 species, distributed in tropical and subtropical regions and commercial vanilla, an important and popular flavoring material and spice. Natural vanilla flavor is derived from beans of the vanilla orchid, *Vanilla planifolia*. There are many compounds present in the extracts of vanilla, the compound is vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component of natural vanilla, which is one of the most widely used and important flovouring materials worldwide. (Podstolski *et al*., 2002; Walton *et al*., 2003; Besse *et al*., 2004; Divakaran *et al*., 2006; Waliszewski *et al*., 2006). It is also used in the fragrance industry, in perfumes, livestock fodder, and

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cleaning products. However, Vanillin has been used as flavor resources in confectionery, food, sensual desserts such as ice cream, sugar cookies, puff pastries, and butter creams and in pharmaceutical preparations (Bythrow, 2005; Waliszewski *et al*., 2006; Jadhav *et al*., 2009). Vanilla grows as a vine, climbing up an existing tree, pole, or other support (Ratanacherdchai and Soytong, 2004; Waliszewski *et al*., 2006). It usually grows under hot humid climate from sea level to an elevation of 1500 m. Vanilla cultivation is severely hampered by the incidence of various diseases. It is susceptible to many fungal diseases such as foot rot and wilting which caused by *Phytophthora meadii*, *Fusarium oxysporum*, *Calospora vanillae*, *Sclerotium* rot, leaf rot, blights and brown spots or anthracnose caused by *Colletotrichum gloeosporioides* (Divakaran *et al*., 2008). These diseases can be controlled by fungicides and biological control agents. The biological control is an additional method that can help in reducing the disease to economically levels, with a concomitant decrease in the use of chemicals. Presently, the researcher have been considerable efforts to find biological control agents for this disease and several potential candidates have been reported including *Actinomycetes* spp. (Crawford *et al*., 1993; Valois *et al*., 1996; Gesheva, 2002; Bressan, 2003; El-Tarabily and Sivasithamparam, 2006; Prabavathy *et al*., 2006), *Trichoderma harzianum* (Bae and Knudsen, 2005; Srinon *et al*., 2006), *Penicillium striatisporum* (Ma *et al*., 2008), *Verticillium lecanii* (Verhaar *et al*., 1999), some endophytic fungus (Lu *et al*., 2000; Cao *et al*., 2005; Inácio *et al*., 2006; Istifadah and Mcgee, 2006; Phongpaichit *et al*., 2006; Tan *et al*., 2006; Mejía *et al*., 2008; Pongcharoen *et al*., 2008; Rukachaisirikul *et al*., 2008; Qin *et al*., 2009), *Chaetomium globosum* (Soytong and Quimio, 1989; Soytong, 1991; Dhingra *et al*., 2003; Aggarwal *et al*., 2004; Park *et al*., 2005), *Chaetomium cochliodes* (Phonkerd *et al*., 2008), *Emericella variecolor* (Malmstrøm *et al*., 2002) and *Emericella nidulans* (Sibounnavong *et al*., 2008). This information would valuable to further study on biological control using antagonistic fungus. The aim of this research was to test antagonistic fungus, *Emericella nidulans* to inhibit *C. gloeosporioides* causing anthracnose disease of *Vanilla* species.

Materials and methods

Collection and isolation

Isolates of *C. gloeosporioides* were obtained from infected leaves of *V. planifolia*. Isolation of causing agent was done by using tissue transplanting technique into pure cultures.

Pathogenicity test

The anthracnose pathogen was isolated from diseased plant parts. Pure cultures were multiplied on PDA for inoculation. All isolates were tested for pathogenicity test on leaves of vanilla followed Koch's postulate. Inoculated leaves were kept in humid conditions at room temperature (28-32 ^{o}C). Data collected as lesion diameter (cm.) on leaves, and analyses of variance using completely randomized design (CRD) with four replications.

Bi-culture antagonistic test

In this experiment, bi-culture antagonistic test were conducted to evaluate the antagonistic fungus, *E. nidulans* against *C. gloeosporioides*. Pathogen isolates used in bi-culture antagonistic test were obtained from pathogenicity test which was the most aggressive isolate. Hyphyl plugs of *C. gloeosporioides* and *E. nidulans* were placed to the middle of a half of petri dishes (9 cm diameter) and incubated at room temperature. Data were colleted measuring colony diameter for 10, 20, and 30 days of *C. gloeosporioides*, sporulation and computed as percent growth inhibition. Percentage of growth inhibition (PGI) of pathogen was evaluated in the formula (cc-cd)/cc x 100; cc = colony diameter of plant pathogenic fungi in control petri dish and cd = colony diameter of plant pathogenic fungi on bi-culture in petri dish. Data were computed analyses of variance using completely randomized design (CRD) with four replications.

Test for antifungal metabolites

The microbial antagonist, *E. nidulans* were cultured in Potato dextrose broth (PDB) for 30 days, then filtered and dried mycelium mats were collected for extraction method using rotary vacuum evapotator. The crude extracts were tested in petri dishes which mixed to PDA at concentrations 0, 10, 50, 100, 500 and 1,000 µg/ml. Cultures of plant pathogenic fungi, *C. gloeosporioides* was grown for 7 days on PDA. Each pathogen was separately transferred the agar plug (0.3 cm diameter) to the center of petri dish containing crude extracts of microbial antagonist and incubated at room temperature. After 5 days of incubation, colony diameter and sporulation were recorded and computed growth inhibition (GI), Effecive dose (ED_{50}) and analyses of variance using completely randomized design (CRD) with four replications.

Results and discussion

Collection and isolation

Eight isolates of the anthracnose pathogen of *V. planifolia* were isolated and identified as *Colletotrichum gloeosporioides*. With this, Soytong and Rattanacherdchai (2008) also reported that this pathogen causing anthracnose of Vanilla (Fig. 1).

Pathogenicity test

All isolates of *C. gloeosporioides* were proved to be pathogenicity to the host species. The inoculated leaves showed the anthracnose symptoms within two weeks after inoculation. Symptoms were found as small brown spots initially appeared on leaves, and the spots gradually enlarged and coalesced (Nakamura *et al.*, 2008). The result showed that isolates of *C. gloeosporioides* VP8 gave the most aggressive causing anthracnose symptom (Fig. 2).

Bi-culture antagonistic test

Bi-culture showed that *E. nidulans* could inhibit *C. gloeosporioides* VP8 in term of mycelial growth (49.44%) and sporulation (75.31%). There are several reports on the potential use of microbial antagonists for biological control of anthracnose disease caused by *Colletotrichum* spp. such as *Chaetomium*, *Trichoderma* and *Penicillium* (Udomratsak and Soytong, 2004; Soytong *et al.*, 2005), *Nigrospora* sp. strain L-03 (Thongsri and Soytong, 2004). But this result was also expressed the potential of *E. nidulans* to control vanilla anthracnose caused by *C. gloeosporioides*. With this, Sibounnavong *et al*. (2008) also reported that *E. nidulans* could be reduced tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici.*

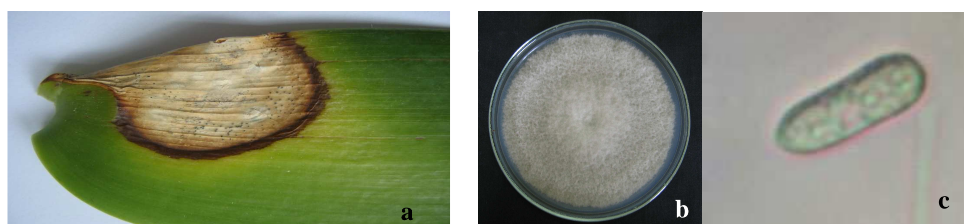

Fig. 1. Vanilla anthracnose caused by *Colletotrichum gloeosporioides* a) Symptom, b) Culture on PDA at 10 days, c) Conidium (40x).

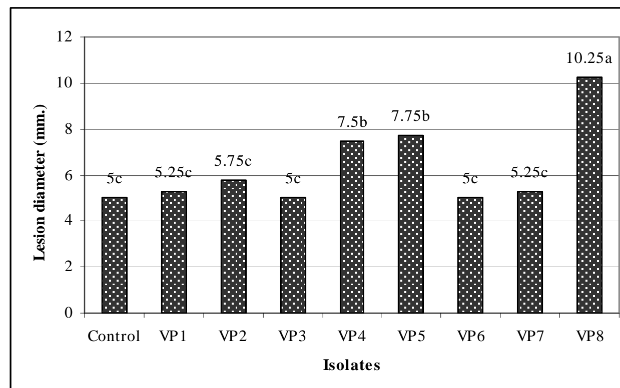

Fig. 2. Lesion diameter of vanilla anthracnose causing by *Colletotrichum gloeosporioides* on *Vanilla planifolia*.

Test for antifungal metabolites

The inhibitory effect of antifungal metabolites from *E. nidulans* on mycelial growth of *C. gloeosporioides* VP8 in the medium amended with methanol crude extract showed that the concentration of 1,000 µg/ml strongly inhibited the mycelial growth and sporulation as 47.50 and 95.85%. The ED_{50} values were 2910 and 0.0001 µg/ml, respectively. The antifungal metabolite extracted from ethyl acetate and hexane could inhibit the mycelial growth of 35.5 and 31% which the ED_{50} values were 4,922 and 5,249 µg/ml, respectively. The hexane and ethyl acetate extracts inhibited the sporulation of *C. gloeosporioides* VP8 as 97.34 and 68.67% which the ED_{50} were 0.002 and 434 µg/ml, respectively. Abnormal conidium of a pathogen was broken and then the protoplast was released from fungal cell when compared normal conidium. (Table 1 and Figs. 3 and 4). Similar results reported by Sibounnavong *et al.* (2008) that bioactive compound from *E. nidulans* strain EN could inhibit sporulation of *Fusarium oxysporum* f.sp. *lycopersici* causing tomato wilt at the concentration of 1,000 µg/ml with ED_{50} 211 µg/ml. With this, Moosophon *et al.* (2009) reported that *E. nidulans* strain EN could produce antibiotic substances e.g. emericellin, sterigmatocystin, demethylsterigmatocystin that inhibited human diseases such as gastric cancer, breast cancer, lung cancer and oral human epidermal carcinoma. It is interested that those antibiotic substances produced by E. nidulans may possible act as antibiosis as control mechanism. It is proved that *E. nidulans* become the promising antagonistic fungus as a biological agent against plant pathogenic fungi.

Table 1. Effect of crude extracts of *Emericella nidulans* for inhibition of mycelial growth and sporulation of *Colletotrichum gloeosporioides* VP8.

Concentration (µg/ml)	Hexane		Ethyl acetate		Methanol	
	% MGI[1/]	% SL[2/]	% MGI	% SL	% MGI	% SL
0	0.00	0.00	0.00	0.00	0.00	0.00
10	3.00c[3/]	87.64b	27.00c	76.33a	19.50c	57.61b
50	14.00b	93.52ab	33.00b	79.95a	20.50c	70.45ab
100	18.00b	90.25ab	33.50b	85.61a	29.00b	79.47a
500	27.00a	95.37ab	38.50a	88.96a	29.50b	80.85a
1,000	31.00a	97.97a	40.50a	92.93a	43.00a	88.47a
CV. (%)	14.95	4.46	3.74	8.82	7.35	10.63
ED_{50}	5249	0.002	4922	434	2910	0.0001

[1/]MGI = mycelial growth inhibition
[2/]SL = sporulation
[3/]Average of four replications. Means followed by the same letter in a column were not significantly different by DMRT at P = 0.01.

Fig. 3. Testing for methanol crude extract from *Emericella nidulans* to inhibit *Colletotrichum gloeosporioides* causing vanilla anthracnose.

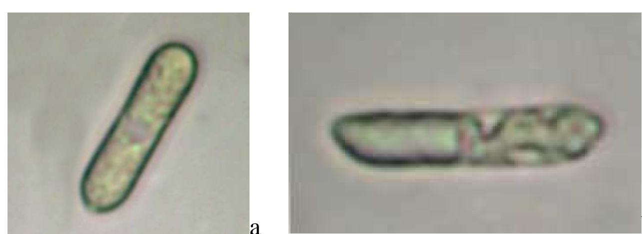

Fig. 4. Showing control mechanism of *Emericella nidulans* against *Colletotrichum gloeosporioides*. Normal conidium (a), abnormal conidium (b).

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