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Production of an antibacterial compound against the plant pathogen *Erwinia carotovora* subs. *carotovora* by the biocontrol strain *Gliocladium sp.* T.N.C73

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Hendra Saputra, Fifi Puspita and Titania Tjandrawati Nugroho (2013) Production of an antibacterial compound against the plant pathogen *Erwinia carotovora* subs. *carotovora* by the biocontrol strain *Gliocladium sp.* T.N.C73. Journal of Agricultural Technology 9(5):1157-1165.

Gliocladium sp. T.N.C73 was originally isolated as a biocontrol agent from suppressive soil against the phytopathogen *Phytophtora capsici.* Apart from producing chitinase, this biocontrol fungal strain also produces secondary metabolites having antibacterial properties against the Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aeureus.* In order to further explore the ability of *Gliocladium sp.* T.N.C73 in managing plant diseases caused by bacteria, ethyl acetate extracts of the fermentation medium of this strain was analyzed for its ability to inhibit the Gram negative *Erwinia carotovora* subsp. *carotovora* that causes soft rot disease in food crops. Disc diffusion bioassays showed that the extracts could inhibit *E. carotovora* subsp. *carotovora* growth. Depending on the initial spore concentration of the fermentation medium inoculum, several compounds were detected by thin layer chromatography (TLC) of the ethyl acetate extracts that fluorescence under UV light, and one compound that gave a bright red spot when sprayed with a *p*-anisaldehyde solution.

Keywords: *Gliocladium, Erwinia carotovora*, soft rot, biocontrol agent.

Introduction

Sustainable agriculture practices require the use of biological control agents (BCAs) to manage plant diseases, in place of synthetic chemicals. Several biocontrol mechanisms have been identified in BCAs, namely mycoparasitism involving cell wall degrading enzymes, nutrient competition, production of secondary metabolites acting as antibacterials or antifungals, and production of metabolites that can induce plant resistance (Punja and Utkhede, 2003; Harman, 2006; Shoresh *et al.*, 2010). Modeling and experimental studies

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have shown that effective BCAs should be able to perform more than one biocontrol mechanisms (Xu *et al.*, 2011).

Gliocladium sp. T.N.C73 was originally isolated as a natural biocontrol agent from suppressive soil against the phytopathogen *Phytophtora capsici*, and isolated based on its ability to produce chitinase. Production of chitinase rendered *Gliocladium sp.* T.N.C73 the ability to mycoparasitize the chitin containing plant fungal pathogen *Fusarium sp.* (Nugroho, 2006). Apart from that *Gliocladium sp.* T.N.C73 also produces secondary metabolites that can inhibit the growth of the Gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Nugroho *et al.*, 2006). Since the *B. subtilis* and *S. aureus* strains used in the study are not pathogenic to plants, we asked the question if the secondary metabolites produced by *Gliocladium sp.* T.N.C73 had the ability to inhibit Gram negative bacterial pathogens of plants. This could provide a second mechanism for the biocontrol activity of *Gliocladium sp.* T.N.C73 apart from its ability to produce chitinase. It would also provide evidence of *Gliocladium sp.* T.N.C73 as an effective BCA against fungal as well as bacterial plant pathogens.

Erwinia carotovora subsp. *carotovora*, also known as *Pectobacterium carotovorum* (Hauben *et al*., 1998), is a plant pathogen bacteria that causes soft rot disease in potatoes, and other economically important horticulture plants such as members of the *Brassica* family (Park *et al.*, 2005; Li *et al.*, 2011). Most BCAs reported to suppress *E. carotovora* are bacteria such as *Pseudomonas sp.* and *Paenibacillus polymyxa* that produce antibacterial compounds (Ghods-Alavi *et al.*, 2012; Niu *et al*., 2013). In this paper we demonstrate a biocontrol fungi from the genera *Gliocladium* also having the ability to produce antibacterial compounds that can inhibit *E. carotovora*. This provides an alternative BCA, that can be used for plant protection against Gram negative bacterial plant pathogens.

Material and methods

Microbial isolates

Gliocladium sp. T.N.C73 used in this study is a fungal isolate from the rhizosphere of healthy cacao (*Theobroma cacao*) trees in an Indonesian plantation located in Riau Province, which is surrounded by trees infected by *Phytophtora sp.* This fungal strain is deposited at the University of Riau Biochemistry Laboratory Culture Collection, under the strain code LBKURCC3. It is also deposited at the Vienna University of Technology (TU Wien) Gene Technology and Applied Biochemistry Laboratory Culture Collection, under the strain code C.P.K. 3541. For bioassays the bacteria used

was a culture of *B. subtilis* obtained from the University of Riau Organic Laboratory Culture Collection. *E. carotovora* subsp. *carotovora* was isolated from infected Chinese Cabbage *Brassica rapa* var. *parachinensis* (*cai xin*).

Production and extraction of secondary metabolites

Fresh spores of *Gliocladium sp.* T.N.C73 were inoculated into flasks, containing 50 ml Potato Dextrose (PD) medium, giving a final spore concentration of 4 x 10^{10} spores/ml to 4 x 10^{11} spores/ml. This initial inoculum was incubated at 30^{o}C for 7 days on a rotary shaker (125 rpm). After 7 days, each 50 ml PD initial inoculum and biomass that had grown were transferred to new flasks containing each 100 ml of a different media with the following composition (pH 5.6-5.8): glucose 2%, yeast extract 0.5%, peptone 0.5%, $MgSO_4$ 0.05%, KH_2PO_4 0.1%. Incubation was continued with shaking on a rotary shaker (125 rpm) at 30^{o}C for 14 days. After 14 days fermentation, the mycelium was separated from the medium by filtration using a Whatman® GF/C filter. The filtrates were extracted twice with ethyl acetate, each time with half of its volume. The ethyl acetate extracts were then combined, dried with anhydrous Na_2SO_4, and the ethyl acetate evaporated at room temperature in a rotary vacuum evaporator. Residue obtained from every 1 l fermentation media was dissolved in 1 ml methanol (MeOH).

Thin Layer Chromatography and detection of compounds

Residue of vacuum evaporated ethyl acetate extracts of *Gliocladium sp.* T.N.C73 fermentation medium were redissolved in MeOH, and 2 µl of the MeOH solution were loaded on aluminum backed Silica gel 60 F254 Thin Layer Chromatography (TLC) plates (*Merck,* USA, Cat.no. 105554). Samples were air dried, and developed in CH_2CL_2/MeOH 80:20 (v/v). For detection of spots, dried plates were either visualized under UV light (366 nm), or sprayed with a 0.5% *p*-anisaldehyde solution in acidic alcohol (MeOH/H_2SO_4/AcOH 90:5:5 (v/v/v)), dried and heated at 110^{o}C for 20 minutes. The TLC plates were photograph under the respective visualization methods, and R_f values recorded.

Antibacterial bioassay

To determine the antibiotic activity of extracts against *B. subtilis* and *E. carotovora* subsp. *carotovora*, the disc diffusion method was used. Ten µl of redissolved ethyl acetate extracts of *Gliocladium sp.* T.N.C73 fermentation medium in MeOH were applied to sterile 6-mm antibiotic blank disc and left to dry at room temperature (30^{o}C), before transferring to a 9-cm diameter bacterial

inoculated agar plates (*B. subtilis* or *E. carotovora* subsp. *carotovora*). The cultures were incubated at 37°C for one day, and the inhibition zones, including the paper discs, were measured (mm). The assays were performed in duplicates. As positive controls, ten µl of a 3 µg/µl Amoxicillin (Amoxsan®, P.T. Sanbe Farma, Bandung, Indonesia) in MeOH were used. As negative controls 10 µl of the solvent MeOH were used.

Results

Two different spore concentrations of *Gliocladium sp.* T.N.C73 were used for the initial inoculum for production of the secondary metabolites, resulting in two different final extracts obtained in MeOH, and giving two slightly different TLC profiles (Fig. 1). The final extracts we will further designate as extract A, for the extracts obtained from an initial inoculum spore concentration of 4 x 10^{10} spores/ml, and extract B, for the extracts obtained from an initial inoculum spore concentration of 4 x 10^{11} spores/ml.

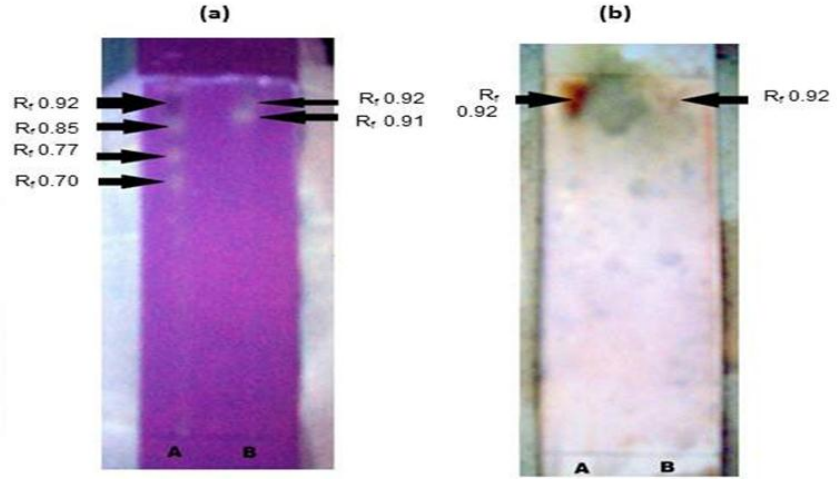

Fig. 1. TLC of ethyl acetate extracts of *Gliocladium sp.* T.N.C73 fermentation medium. Spotted in lanes A are extracts from media with an initial inoculum concentration of 4 x 10^{10} spores/ml (extract A). Spotted in lanes B are extracts from media with an initial inoculum concentration of 4 x 10^{11} spores/ml (extract B). Panel (a) Spots detected under 366 nm UV light. Panel (b) Spots detected after spraying with 0.5% *p*-anisaldehyde. Shown by arrows are Rf values for each spot.

As shown in Fig. 1, under 366 nm UV light, TLC of extract A gave three fluorescent spots with R_f values ranging from 0.7 to 0.85. These three fluorescent spots were absent in the TLC results of extract B. However, two other fluorescent spots could be detected in extract B with R_f values of 0.91 and

0.92. The spot corresponding to R_f 0.92 did not give any detectable fluorescence in lanes containing extract A. When the TLC developed plates were sprayed with an acidic 0.5% solution of *p*-anisaldehyde, a corresponding pink reddish spot in both extracts A and B could be detected with an R_f of 0.92.

Extract A and extract B were tested for their ability to inhibit growth of *E. carotovora* subsp. *carotovora*, as a plant pathogen and Gram negative bacteria, and compared to its ability to inhibit *B. subtilis* representing Gram positive bacteria. As shown in Table 1 and Fig. 2, extract A and B could inhibit growth of *E. carotovora* subsp. *carotovora.* Extract A gave significantly higher inhibition zones ($p < 0.05$) than extract B and Amoxicillin, to both bioassays against *E. carotovora* subsp. *carotovora* and *B subtilis.* Extract B gave significantly higher inhibition zones toward *B. subtilis* ($p < 0.05$), than toward *E. carotovora* subsp. *carotovora*, but had the same inhibition zone as the positive control Amoxicillin toward *B. subtilis.* No inhibition zones were detected in the negative controls.

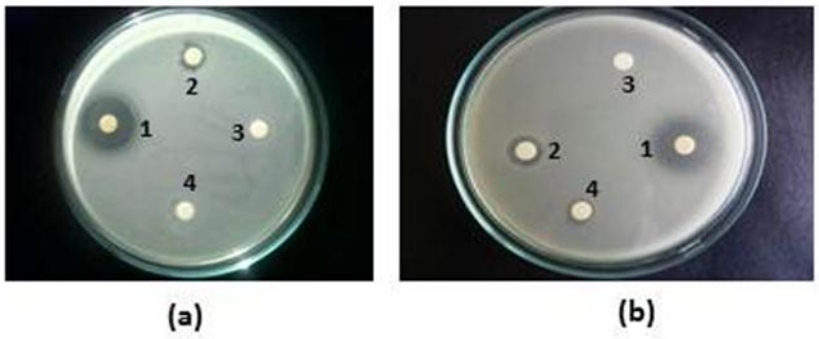

Fig. 2. Antibacterial bioassay of *Gliocladium sp.* T.N.C73 fermentation medium extracts against (a) *B. subtilis;* and (b) *E. carotovora* subsp. *carotovora*, by the disc diffusion method after incubation at 37°C for 24 hours. Disc 1: Extract A. Disc 2: Extract B. Disc 3: Negative controls of MeOH. Disc 4: Amoxicillin

Table 1. Antibacterial bioassay results of *Gliocladium sp.* T.N.C73 fermentation medium extracts by the disc diffusion method

Bacteria tested	**Average inhibition zone diameters (mm)**			
	Extract A	Extract B	Amoxicillin (positive control)	Solvent (negative control)
B. subtilis	$(19.1 \pm 0.2)^a$	$(10.6 \pm 0.6)^b$	$(10.2 \pm 0.6)^b$	n.d
E. carotovora subsp. *carotovora*	$(19.5 \pm 0.1)^a$	$(7.9 \pm 0.2)^c$	$(10.0 \pm 0.1)^b$	n.d

n.d. = no inhibition zone detected. Values of inhibition zones in brackets are the mean average of three bioassays followed by the standard deviation. Mean values followed by the same superscript letter outside brackets are not significantly different ($P = 0.05$).

Discussion

Biological control of plant pathogens is important for sustainable agriculture. The difficulty in applying BCAs to plant management in the field often lies in finding the correct BCA to use in different environments, and soil types, for a wide variety of plant pathogens. Several researchers have tried combining several BCAs for effective plant protection, however modeling and experimental studies have shown that the use of BCAs with more than one biocontrol mechanisms gives better plant protection than combining two or more BCAs, each with single biocontrol mechanisms (Xu *et al.*, 2011). Therefore the identification of biocontrol mechanisms by isolated BCAs are important to asses the potential use of a BCA in plant protection, before aplication in the field. In the present study, we have shown that *Gliocladium sp.* T.N.C73 that was originally isolated for its ability to suppress *Phytophtora sp.* , and for its ability to produce chitinase, also produces several compounds that can inhibit the plant pathogen *E. carotovora* subsp. *carotovora*. This makes *Gliocladium sp.* T.N.C73 a good candidate for an effective BCA against several kinds of plant pathogens, bacterial as well as fungal.

The present study also shows that the number, and type of compounds produce by *Gliocladium sp.* T.N.C73, as well as their effectiveness in controlling *E. carotovora* subsp. *carotovora* is dependent on the initial spore concentration inoculum in the fermentation media. It is possible that the compounds inhibiting the Gram positive bacteria *B subtilis*, may be different from those inhibiting the Gram negative bacteria *E. carotovora* subsp. *carotovora.* One indication that this may be true, is that the decrease of inhibition of *E. carotovora* subsp. *carotovora* compared to the decrease in inhibition of *B. subtilis* by extract B is significantly larger ($p < 0.05$) than by extract A. Further compound isolation, purification, chemical structure studies, and antibacterial assays of individual isolated compounds would address this question.

Extract B was produced with a ten fold higher *Gliocladium sp.* T.N.C73 spore concentration in the initial inoculum, but gave lower bacterial inhibition zones compared to extract A. This suggest that the secondary metabolites produced in the fermentation medium that has antibiotic activity is effected by the population density, and growth or developmental stage of the fungus. This in turn may also be an indication that the antibiotic compounds are produced not only for antagonisms or defensive purposes of *Gliocladium sp.* T.N.C73 againsts other organism, but instead may have other biological function for the fungus. It is also an indication that developmental regulation of the compounds exist. A different spore concentration in the initial innoculum, would cause differences in the developmental stage of the fungus after 14 days growth in the

fermentation medium, thus causing a difference in the fermentation product profile.

The fungus *Trichoderma,* a close relative of *Gliocladium,* produces peptide antibiotic compounds called peptaibols, which have been suggested to be of use to the fungus to induce sporulation (Kubicek *et al.*, 2007). A study by Chutrakul *et al.* (2008), showed that peptaibol production was dependent on the culture age, reflecting the growth stage of the fungal producer. Chutrakul *et al.* (2008) found that production of peptaibols increased in 1 to 4 day old cultures, and subsequently decreased until day 13, before increasing again. The differences in the TLC profile in extract A and B suggest that this same developmental regulation may take place in the antibiotic production by *Gliocladium sp.* T.N.C73.

Comparison of the TLC profiles of extract A and B shows that UV fluorescent compounds with R_f 0.7 to 0.85 in extract A, is not present in extract B. On the other hand two other UV fluorescent compounds appears in extract B not observed in extract A, having a R_f value of 0.91 and 0.92. Since extract B would be a more dense culture than extract A, due to its tenfold higher initial spore concentration, either compounds with R_f 0.7-0.85 are degraded or reabsorb by the fungus, and further metabolized to produce other compounds, such as the compounds with the Rf values of 0.91-0.92.

Does *Gliocladium sp.* T.N.C73 produce peptaibol like antibiotics? The peptaibol antiamoebin I has been reported to be produced by some *Gliocladium* species (Shenkarev *et al*., 2013). Peptaibols, are linier peptide antibiotics, having a high number of non-proteinogenic amino acids or lipoamino acids, possess a N-terminus that is acylated, and a C-terminus with an alcohol group (Kubicek *et al.*, 2007). Peptaibols can be extracted in MeOH or ethyl acetate. In TLC assays peptaibols give red spots seen under normal visible light when sprayed with 0.5% *p*-anisaldehyde (Berg *et al*., 2003; Chutrakul *et al*., 2008). In this study, both extracts A and B contained a compound that gave a red spot when sprayed with 0.5% *p*-anisaldehyde, having an R_f of 0.92 in the TLC assays. This compound could be a candidate peptaibol. However peptaibols inhibit only Gram positive bacteria (Xiao-Yan *et al.*, 2006), so that if the compound with R_f 0.92 is a peptaibol, it would not be the inhibiting antibiotic of *E. carotovora.* Plant protection by peptaibols against Gram negative bacteria stems more from its ability to induce plant defense responses (Viterbo *et al.*, 2007).

Non-peptaibol peptide antibiotics exist that can inhibit Gram negative bacteria. Recently a peptide antibiotic, polymyxin, which has a cyclic peptide chain and hydrophobic tail was reported as an inhibitor of *E. amylovora* and *E. carotovora.* (Niu *et al*., 2013) Some *Gliocladium* species also produce cyclic

peptides with antibacterial properties, such as diketopiperazine (Koolen, *et al.*, 2011). Other compounds having antibiotic activities produce from several other *Gliocladium* species include *p*-terphenyl (Guo *et al.*, 2007) and polyketides (Kohno *et al.*, 2000). The antibiotic activity inhibiting *E. carotovora* subsp. *carotovora* produced by *Gliocladium sp.* T.N.C73 is more likely to be a diketopiperazine or other form of cyclic peptide, terphenyl or polyketide, rather than the linier peptaibol.

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