Screening for antifungal-producing bacteria from *Piper nigrum* plant against *Phytophthora capsici*


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**Abstract**

*Piper nigrum*, commonly known as black pepper, is one of the most important spice crops with high demand by the world market. However, diseases like foot rot and stem blight cause by *Phytophthora capsici* have become the important production constraints in black pepper industry. The frequent application of toxic fungicides to counter the diseases in pepper plantations has raised certain environmental issues. In order to mitigate the use of fungicides, biological approach to control *P. capsici* has been suggested. In this study, endophytic bacteria were isolated from six *P. nigrum* roots and screened for *in vitro* antagonistic activity against *P. capsici* through dual culture, mycelial growth, spore germination and double plate assay. The antagonism testing involved the secretion of volatile and diffusible bioactive compounds by the endophytic bacteria. Out of 19 isolates tested, two isolates DB(2)7 and SB(2)6 produced volatile bioactive compounds and these two isolates showed highest antagonism against *P. capsici* mycelia with the percentage of inhibition up to 47.63% and 43.33%, respectively. Diffusible compounds from isolates DB(2)7, DB(2)9 and SB(2)6 produced clear zones in spore germination test with radii measurements of 10.0-17.0 mm. Three isolates with promising antifungal activity were further characterised through 16S rDNA sequencing. The analysis of their sequences via National Center for Biotechnology Information (NCBI) suggests close identity towards *Enterobacter cancerogenus*, *Enterobacter cloacae* and *Enterobacter asburiae*. This research study demonstrated that these endophytic bacteria isolates are potentially to be used as biocontrol agent in pepper cultivation.

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**Introduction**

Black pepper (*Piper nigrum* L.), synonym with the title “king of spices”, is a type of flowering vine belong to the family Piperaceae which originate from Malabar’s coast of Southern India (Ravindran, 2000; Augstburger *et al*., 2001; Nazeem *et al*., 2008). It was spread by the Hindus trader and travellers to Malaysia and Indonesia. Nowadays, it is commercially cultivated in tropical areas including Malabar, Thailand, Malaysia, Indonesia, Brazil, Sri Lanka, Vietnam and People’s Republic of China to support the world populations’ demand for black pepper (Sivaraman *et al*., 1999; Augstburger *et al*., 2001; Farhana *et al*., 2013). In Malaysia, pepper cultivation is concentrated in Sarawak (90%) with the remaining grown in Peninsular Malaysia (Johor and Melaka) and Sabah (Anita *et al*., 2013). According to the report published by State Planning Unit, Chief Minister’s Department of Sarawak in 2013, a total of 26,000 tonnes of white and black pepper were produced from 14,900 hectares of planted area in Sarawak (Farith *et al*., 2015). However, foot rot disease is still the main constraint in the black pepper production and it has been one of the main disease which cause an estimated about five to six million ringgit losses in export or 5-10% of annual crop loss in Sarawak alone (Kueh, 1990).

*Phytophthora capsici* is an oomycete plant pathogen that commonly regarded as causal agent of foot rot and stem blight in black pepper (Anandaraj and Sarma, 1995a; Babadoost, 2005; Nguyen, 2015). The foliage, leaves and fruits are susceptible to the *Phytophthora* infection. It is known as a type of virulent, hemi-biotrophic and wet weather pathogen that inflicted significant loses reported from western hemisphere, Asia and Europe (Anandaraj and Sarma, 1995a; Gevens *et al*., 2008; Lamour *et al*., 2012a, 2012b; Fisher *et al*., 2012). Relative humidity which is more than 79% with intermittent showers and low temperature are conducive for the initiation and spreading of *P. capsici*, thus *Phytophthora* diseases are prevalent during wet period of the year which may varies from countries to countries (Babadoost, 2005; Sarma *et al*., 2013).

The infection associated with *P. capsici* was first reported in Lampung, Indonesia in 1885 and then identified by Muller in 1936 (Drenth and Guest, 1936).
2004; Sarma et al., 2013). Between November 2010 and January 2011, thirteen major pepper growing orchards located in four divisions of Sarawak state of Malaysia were surveyed for Phytophthora foot rot disease and the severity of infection incidence during an outbreak (Farhana et al., 2013). Ulu Sarieki in Sarieki had the highest disease incidence reported (75%) followed by Pasai Siong in Sibu (70%) and the lowest at Tatau in Bintulu (5%) (Farhana et al., 2013). Meanwhile, the highest disease severity was recorded at Ulu Sarieki (70%) followed by Pasai Siong (62%) and the lowest at Tatau (4%) (Farhana et al., 2013). The causal agent of foot rot disease of black pepper was confirmed as \( \text{P}. \text{capsici} \) Leonian.

Increased awareness of public on the deleterious effect of chemical fungicides to human health, environment and ecosystem had promoted the development of several safe management alternatives to control Phytophthora diseases (Anandaraj and Sarma, 1995b; Marin et al., 2014). In order to mitigate the application of fungicides, biological approach has been suggested. The objective of this study was to screen potential endophytic bacteria from \( \text{P}. \text{nigrum} \) plant that can be used to inhibit the growth of \( \text{P}. \text{capsici} \).

Materials and Methods

Root sample collection

Roots of \( \text{P}. \text{nigrum} \) L. were collected from different \( \text{P}. \text{nigrum} \) farms with \( \text{P}. \text{capsici} \) infection history within Kota Samarahan District, Sarawak, Malaysia in December 2013.

Isolation of endophytic bacteria

Approximately 100 g of root samples were weighted, washed with sterile distilled water, surface disinfected by soaking in 2% (v/v) sodium hypochlorite for 10 min, followed by washing with 70% (v/v) ethanol for one min and later rinsed five times with sterile distilled water. The samples were then ground aseptically in phosphate buffered saline (PBS) solution (gL\(^{-1}\): \text{NaCl} 8, KCl 0.2, \text{Na}_2\text{HPO}_4 \text{1.44} and \text{KH}_2\text{PO}_4 \text{0.24, } \text{pH} \text{ 7.4}) and centrifuged at 60 x \text{g} at 4°C for 1 min. The supernatants were serially diluted up to \( 10^3 \), spread plated on tryptic soy agar (TSA) and then incubated at 28 °C for 4 days. The population of bacteria in the grinded root samples was expressed as colony forming units (CFU) of tissue. Five individual bacterial colonies from each agar plate were randomly selected and purified on TSA plate. A set of the pure culture was kept in slant agar stored at 4°C and another set was stocked in glycerol kept at -20°C.

Dual culture assay

Dual culture assay was performed as described by Živković et al. (2010) and Jeyaseelan et al. (2012) with slight modification. A total of 129 isolates of endophytic bacteria were streaked at approximately 1 cm from the edges of potato dextrose agar (PDA) plates, meanwhile a \( \text{P}. \text{capsici} \) culture on agar plug with the size of 1 cm\(^2\) was placed at the opposite of the bacteria streaks. Plate containing the fungus without any bacteria inoculation was used as negative control. The cultures were incubated at 30°C for six days and the percentage inhibition of radial growth (PIRG) was calculated as follows:

\[
\text{PIRG} \ (%)= \left[ \frac{\text{R1}\text{-R2}}{\text{R1}} \right] \times 100\% \quad \ldots \ldots \ (1)
\]

Whereby, \text{R1}: The radial growth of \( \text{P}. \text{capsici} \) in control plate; \text{R2}: The radial growth of \( \text{P}. \text{capsici} \) towards the antagonist endophytic bacteria.

The percentages inhibition of radial growth were categorized from low to very high antifungal activity; \(<30\% = \text{low antifungal activity}, 30 \text{-} <50\% = \text{moderate antifungal activity}, 50 \text{-} <70\% = \text{high antifungal activity}, \geq70\% = \text{very high antifungal activity} \) Those bacteria isolates with more than 30% of inhibition from this dual culture assay were selected and kept for further studies.

Mycelial growth test

Plugs of mycelium with approximately 1 cm\(^2\) were taken from the edge of six-days-old culture of \( \text{P}. \text{capsici} \) and dipped into broth cultures of the selected bacteria isolates for 30 min (Edward et al., 2013). The plugs were air dried in transparent chamber for 30 min. Each of the mycelia plugs was then transferred to the centre of PDA plates. \( \text{P}. \text{capsici} \) plug dipped in sterile distilled water served as negative control. The PDA plates were then incubated at 30°C for 4 days and the diameter of colony was measured after the incubation. Percentage inhibition of diameter growth (PIDG) was calculated as follows:

\[
\text{PIDG} \ (%)= \left[ \frac{\text{D1}\text{-D2}}{\text{D1}} \right] \times 100\% \quad \ldots \ldots \ (2)
\]

Whereby, \text{D1}: Diameter growth of \( \text{P}. \text{capsici} \) in control plate. \text{D2}: Diameter growth of \( \text{P}. \text{capsici} \) treated with endophytic bacteria.

Spore germination test

\( \text{P}. \text{capsici} \) spores from 2 days old potato dextrose broth (PDB) culture was spread over other potato dextrose agar (PDA) plates by swabbing and then allowed to dry at room temperature for 30 min. The
bacteria isolates were sub-cultured into nutrient broths and allowed to grow for 24 h. Double layers of sterile filter paper discs (0.6 cm in diameter) were inoculated with 65, 55 and 50 µl of bacterial cultures in a sterile petri dishes. The discs were then transferred onto the PDA plates after being air dried for 30 min. Discs loaded with 65, 55 and 50 µl of un-inoculated nutrient broth (NB) served as controls. The radii of clearing zones around the discs were measured after 24, 48 and 72 hours after incubation at 30°C.

**Double plate assay**

Bacteria and *P. capsici* cultures were grown on NA and PDA plates for 1 and 3 days, respectively. NA plate without inoculation served as the control. The PDA plate was inverted over corresponding NA plate without cover lids, and the two plates were sealed together with a parafilm strip. The diameter growth of fungus on PDA plates were first measured and then incubated at 30°C for another 4 days. The growth inhibition of *P. capsici* by endophytic bacteria was assessed based on PIDG values shown in equation (2) by calculating the difference in diameter of fungus culture before and after sealing together the two plates.

**Analysis**

The bacteria isolates that fulfilled most of the antagonistic characteristics towards *P. capsici* from in vitro antagonistic screening test (>40% of PIRG value from dual culture assay; >40% of PIDG value from double plate assay; >10% of PIDG value from mycelial growth test; >10 mm of radius forming clear inhibition zone) were chosen for further characterisation.

**DNA extraction and PCR amplification**

Bacterial DNA was extracted by boiled cell method as described by Freschi *et al.* (2005). The PCR was carried out using universal primer sets 27F and 519R in 50 µl of PCR reaction Master Mix containing 10 µl of 5× Green GoTaq® Flexi buffer, 6 µl 25 mM MgCl2, 3 µl 10 mM dNTPs, 8 µl sterile distilled water, 20 µl sample of 20-40 ng DNA, 1 µl GoTaq® Flexi DNA polymerase, 1 µl each forward primer and reverse primer. The thermocycling conditions consist of an initial denaturation at 95°C for 10 min, 26 amplification cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min 30 s and a final polymerization step of 72°C for 10 min. Each reaction was analysed by agarose gel electrophoresis in 1% (w/v) agarose gel and ethidium bromide staining. The agarose gel was electrophoresed at 90 V with 200 mA for 30 min and then visualised under UV transilluminator.

**16S rDNA gene sequencing and identification**

DNA fragments in gel were purified using Qiagen QIAquick® Gel Extraction Kit (Kathleen *et al.*, 2014). The purified DNA fragments were sent to 1st Base Laboratory Sdn. Bhd., Malaysia for sequencing. The sequences obtained were subjected to BLAST analysis and nucleotide sequence similarities were determined by comparing with National Center for Biotechnology Information (NCBI) databases.

**Results and Discussion**

Endophytic bacteria found in *Piper nigrum* are ubiquitous in most of the plant species (Hallmann *et al.*, 1997; Aravind *et al.*, 2009). It can latently reside or actively colonise the plant tissue locally as well as systematically without showing external symptom or bad effect on the plant host (Carroll, 1988; Wilson, 1995; Ryan *et al.*, 2007).

The study on endophytic bacteria has shown that endophytes were able to prevent the disease development through endophyte-mediated de novo synthesis of certain compounds or metabolites with antimicrobial activity (Ryan *et al.*, 2007; Samuel *et al.*, 2014). Besides, some of the endophytic bacteria are considered mutualistic by stimulating the growth and development of hosts through synthesis of plant-growth-promoting hormone (Ahmad *et al.*, 2008). Hence, further study on endophytic bacteria is needed to search for new bioactive compounds that can be used to fight against *Phytophthora* foot rot diseases in *P. nigrum* and at the same time can promote the growth and development of plants.

In this research study, a total number of 129 endophytic bacteria were isolated from the root of *P. nigrum* and all of the isolates were screened for their *in vitro* antagonistic activity against *P. capsici* through dual culture, mycelial growth, spore germination and double plate assay.

**Dual culture assay**

Dual culture assay was carried out as preliminary screening test to select the endophytic bacteria with high antagonistic effects toward *P. capsici* (Oldenburg *et al.*, 1996). The information regarding the bioactive compound produced, such as specificity, potency or stability were obtained through this preliminary test (Oldenburg *et al.*, 1996). The radial growth of *P. capsici* toward the endophytic bacteria is shown in Figure 1. From dual culture assay, only 19 bacteria isolates showing more than 30% of inhibition were chosen for further analysis. Bacterial isolates KDKS5-49, KRBR-15 and BR(1)6 were observed to have significantly higher antagonistic effect towards
The calculated percentage of inhibition of radial growth of isolated bacteria

<table>
<thead>
<tr>
<th>Culture label</th>
<th>Radial Growth (cm)</th>
<th>Inhibition length (cm)</th>
<th>PIRG value (%)</th>
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<tr>
<td>Control</td>
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<td>-</td>
<td>-</td>
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<tr>
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</tr>
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</table>

\(P.\ capsici\) growth inhibition test

The mycelial growth test was carried out to study non-volatile bioactive compound produced by the bacteria isolates in broth media (Grover and Moore, 1962; Edward et al., 2013). Mycelial plugs were cut from the edge of the fungus by sterile forceps and directly dipped into the broth solution. This allowed non-volatile bioactive compound produced by the bacteria isolates to react antagonist directly on the mycelium plugs of \(P.\ capsici\) (Edward et al., 2013). Figure 2 shows the histogram of percentage of the inhibition by the bacterial isolates in the mycelial growth test after 4 days of incubation at 30°C. From the histogram, the highest PIDG value was produced by KDKBB-83 which is 30.19%, while the lowest was produced by HB(2)6 which is only 2.05%. This result indicates the presence of non-volatile bioactive compound in some of the inoculated broth solution which were able to inhibit mycelial growth of \(P.\ capsici\). For isolate KDKS5-49, although this isolate showed the highest PIRG value under the dual culture assay, it produces less non-volatile bioactive compound which specifically target by the mycelial growth test. Hence, the results obtained were paradoxically from the dual culture assay. In a study to investigate potential biocontrol for \(Verticillium\) wilt fungal disease, Lin et al. (2013) found the maximal zone of fungistasis of 29 mm and 26 mm by two bacterial strains KDRE25 and KDRE01, respectively.

**Spore germination test**

The results of the spore germination test was analysed by observing the inhibition zones formed by endophytic bacteria towards \(P.\ capsici\). The radii of the inhibition zones were measured from the centre of discs to the edge of the inhibition zone. The result was considered positive if inhibition zone is formed while the results was negative if the bacteria neither overgrown or could not produce inhibition zones. Figure 3 shows the radii of inhibition zone obtained in the spore germination test. The largest radii of inhibition zone obtained was 17 mm as shown by DB(2)7 and HB(2)6, while the shortest radii was recorded as 4 mm exhibited by isolate DB(2)8. The results were compared among the different bacterial isolates.
cultures age inoculated, incubation period and volume of bacteria culture inoculated.

The spore germination test revealed strong inhibition ability of \( P. \) capsici by isolates DB(2)7, HB(2)6 and DB(2)9 with the radii of inhibition measured 17 mm, 17 mm and 14 mm, respectively. The result of the inhibition of the fungus in the spore germination test in this study is higher than study reported by Edward et al. (2013), where they reported inhibition zone ranged from 12.5 to 15 mm. Despite achieving higher PIRG value in dual culture assay, the bacteria isolates KDKS5-49, KRBR-15, BR(1)6, HB(2)7 and KRS6-84 showed limited or no suppression on the spore germination of the \( P. \) capsici. This contradictory results obtained might be attributed to the nature or chemical properties of bioactive compound produced by the bacteria isolates which might have low potency, stability and diffusible ability. Besides, the spore germination test was tested on the different volume and days of incubation of the bacterial inoculated onto the disk. It shows that the filter paper disk inoculated with higher volume of bacteria broth culture caused bacteria to overgrow and less positive results were obtained from the test. By reducing the volume inoculated into the filter paper disk, there were more positive results obtained from the test. On the other hand, the inhibition zone obtained by majority of the bacteria isolates were larger in size in the first day broth (lower bacteria concentration) compare to second day broth (higher bacteria concentration) as shown in Figure 3.

**Double plate assay**

The result of the double plate assay is displayed in Figure 4. By calculating the percentage of inhibition of diameter growth (PIDG) after 4 days of incubation period at 30°C, DB(2)7 showed the highest PIDG value (47.63%) followed by SB(2)6 (43.33%) and KDKS5-49 (38.29%). This result indicates the ability of the isolates to synthesise and release volatile gas which aids in the inhibition of the growth of \( P. \) capsici. On the other hand, the negative result obtained by DB(2)8 (-20.58%), BR(1)6 (-8.58%) and HR(2)6 (-7.54%) from the double plate assay indicated that the bacteria isolates might produce the volatile gases that stimulate the growth of \( P. \) capsici (Figure 4).

The double plate assay was designed mainly to target and detect the volatile gas produced by the bacteria isolates. The antimicrobial compound in the form of volatile gas can move faster to far distance due to their physical properties compared to diffusible compound (Edward et al., 2013). Besides, it has also been reported that the volatile compound were able to exhibit stronger antagonistic effects toward rhizospheric strains of \( B. \) subtilis and \( Pseudomonas \) corrugate compared with the diffusible compound (Sood et al., 2007).

**16S rDNA gene sequencing and identification**

Three endophytic bacteria isolates DB(2)7, SB(2)6 and DB(2)9 were chosen for the DNA sequencing. These bacteria isolates have moderately high PIRG value (more than 40%) from dual culture assay test, large radii of inhibition zone in spore germination test and large PIDG value from both the mycelial growth...
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Test and double plate assay. From NCBI database, the endophytic bacteria that have high similarity with the bacteria isolates from root samples were identified as Enterobacter cancerogenus, Enterobacter cloacae and Enterobacter asburiae. Nelson and Maloney (1992) reported that Enterobacter cloacae can inhibit the growth of seed rotting fungus Pythium ultimum. Pal and Gardener (2006) discovered volatile bioactive compound like ammonia produced by Enterobacter cloacae can suppressed Pythium ultimum that induced the damping-off of cotton. Besides, Rani et al. (2011) tested the activity of Enterobacter cancerogenus against pathogenic fungi Macrophomina phaseolina and Fusarium udum and they found positive antagonistic effects.

Conclusion

This research reported antifungal producing bacteria found living within the root samples of P. nigrum plant. A total of 19 endophytic bacteria isolated from the root samples of P. nigrum was tested positive in primary screening which can inhibit the growth of Phytophthora capsici. Mycelial growth test, spore germination test and double plate assay which specifically test the bacteria isolates on the direct antagonism of bioactive compound on the fungus, production of diffusible antifungal compound and production of volatile antifungal compound. Three bacteria were sequenced and identified as Enterobacter cancerogenus, Enterobacter cloacae and Enterobacter asburiae. These bacteria were able to produce various types of metabolites in liquid or volatile gaseous, therefore these bacteria isolates are potentially used as biocontrol agent in pepper cultivation.

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References


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