Potent Bioactive Compounds from *Nodulisporium* sp. NHL-L 6/6 for Control of Plant Pathogens

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Abstract: Bioactive compounds from *Nodulisporium* sp. NHL-L 6/6, a sterile endophytic fungus isolated from *Stemona burkii* leaf collected from Nahaew District, Loei Province, Thailand, exhibited high antimicrobial activity against many plant pathogens. The purpose of this study was to evaluate the *in vitro* antimicrobial activity of bioactive compounds extracted from *Nodulisporium* sp. NHL-L 6/6 against economically important plant pathogenic bacteria (*Erwinia caratovora*, *Pseudomonas solanacearum*, *Xanthomonas citrii*) and fungi (*Alternaria brassicola*, *A. porri*, *Penicillium sp.*, *Fusarium solani*, *F. oxysporum* and *Collectotrichum sp.*). The endophytic fungus *Nodulisporium* sp. NHL-L 6/6 was cultured in a liquid medium at 28 °C, 150 rpm for 6 days. The filtrate of the fermentation medium was extracted with ethyl acetate and concentrated to obtain a crude extract. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) of the crude ethyl acetate extract against plant pathogens were determined by using dilution method. The MIC values of the crude extract against all plant pathogens ranged from 31.25 to 125 µg/mL, while the MFC and MBC ranged from 125 to 500 µg/mL. The spectrum of antimicrobial activity observed in the present study revealed that secondary metabolite from *Nodulisporium* sp. NHL-L 6/6 could be a promising source of new natural bioactive agents for plant disease management.

Keywords: Endophytic Fungus, *Nodulisporium* sp. NHL-L 6/6, Bioactive Compounds, Antimicrobial Activity, Plant Pathogens

INTRODUCTION

Nowadays, agrochemical usages for protecting plants in agricultural area have negative effects on environment and human health [1]. The natural products that are highly effective, possess low toxicity, and have a minor environmental impact are required. The endophytic fungi, living asymptotically in healthy plant tissue, can protect their host from diseases and limit the damage caused by plant pathogens [2]. They have been reported to be a promising source of new natural bioactive agents, which strongly exhibit antifungal and antibacterial activity against plant pathogens [3].

Thailand is situated in a hot and humid climatic zone, which supports a variety of plants. *Stemona* (Non-Tai-Yak) the plant in family *Stemonaceae* have long been used traditionally for the treatment of respiratory diseases, enteric helminthes and as insecticides in Thailand. Ratnaranthorn *et al.* [4] isolated endophytic fungi from leaves and roots of *Stemona colinsae*, *S. burkii*, *S. tuberosa*, and *S. kerii*. Among endophytic fungi isolated, *Nodulisporium* sp. NHL-L
6/6 from *S. burkillii* leave showed highest activity against test plant pathogenic fungi. In this study, the crude extract of bioactive metabolite produced by *Nodulisporium* sp. NHL-L 6/6 was used to evaluate the *in vitro* antibacterial and antifungal activity against plant pathogens.

**MATERIALS AND METHODS**

**Microorganisms**

*Endophytic fungal strain*

An endophytic fungus, *Nodulisporium* sp. NHL-L 6/6, was isolated from *S. burkillii* leaves collected from Nahaew District, Loei Province, Thailand [4].

*Plant pathogenic strains*

The pathogenic strains used for antimicrobial activity test were bacterial pathogens (*E. caratovora*, *P. solanacearum* and *X. citrii*), and fungal pathogens (*A. brassicola*, *A. porri*, *Penicillium* sp., *F. solani*, *F. oxysporum*, and *Collectotrichum* sp.).

*Preservation of microorganisms*

For fungal culture preservation, fungi were grown on potato dextrose agar (200 g of fresh potato, 20 g of dextrose, 15 g of agar and 1000 mL of distilled water) and incubated at 26°C for 5 days, while bacteria were grown on potato sucrose agar (0.5 g of Ca(N03)2.4H2O, 2 g of Na2HPO4.12H2O, 5 g of peptone, 20 g of sucrose, 15 g of agar, 300 g of fresh potatoes and 1000 mL of distilled water) and incubated at 30°C for overnight. The stock cultures were maintained at 4°C until used and were subcultured in the same medium once a month. For long-term storage, a few small pieces of fungal mycelia or active bacterial cells were kept in 15% glycerol, and stored at -80°C.

**Endophytic Fungal Metabolite Production**

*Inoculum preparation for liquid culture*

For inoculum preparation, *Nodulisporium* sp. NHL-L6/6 was grown on potato dextrose agar (PDA) plates and incubated at 26°C for 5 days. Four plates of 5-day-old fungal culture and 100 mL of sterilized distilled water were blended at high speed in a sterile blender.

*Cultivation of endophytic fungus in liquid medium*

The endophytic fungus *Nodulisporium* sp. NHL-L 6/6 was cultured in a liquid medium containing 2.03 g/L yeast extract, 9.86 mL/L whey, 25.08 g/L sucrose and 12.99 g/L glucose. The medium was dispensed in 100 mL amounts in 250 mL Erlenmeyer flasks. The culture medium in each flask was inoculated with 5% (v/v) of inoculum and incubated at 28°C, 150 rpm for 6 days.

*Extraction of endophytic fungal metabolites*

The fermentation broth was harvested and filtered through a piece of nylon filter cloth to remove the fungal mycelia. The filtrate was extracted with one volume of ethyl acetate twice at room temperature. The pooled extract was evaporated in a rotary vacuum evaporator and weighed to constitute a crude extract. The crude extract was dissolved in 5% (v/v) solution of dimethyl sulfoxide (DMSO) to give a final concentration of 10 mg/mL as stock solution and kept at 4°C in a container wrapped with aluminum foil to protect the antimicrobial compound from light until use.
Determination of Minimum Inhibitory Concentrations (MIC)

The antimicrobial activities of the bioactive crude extracts were assayed against bacterial and fungal pathogens by using broth dilution and agar dilution method, respectively.

Bacterial susceptibility test

The two-fold serial dilutions of crude extract in 5% (v/v) DMSO were performed in the potato sucrose broth (PSB) medium to give the final concentrations of 15.625, 31.25, 62.5, 125, 250 and 500 μg/mL. The solutions of 5% (v/v) DMSO in PSB were used as control. Single colonies of bacterial pathogens was transferred into PSB and incubated overnight at 30 °C, 150 rpm. Bacterial liquid cultures were then added to PSB containing crude extract to obtain a final OD of 0.1 at 540 nm. A final volume of media used for each organism tested was 5.0 mL. All experiments were performed in duplicate. The samples were incubated at 30 °C for 24 hours. The MIC was defined as the lowest antimicrobial compound concentration at which bacterial growth was not observed.

Fungal susceptibility test

The stock crude extract in 5% (v/v) DMSO was added to molten potato dextrose agar (PDA) to make the final concentrations of 15.625, 31.25, 62.5, 125, 250 and 500 μg/mL. The agars and crude solutions were mixed thoroughly and poured into petri dishes. For the control plates, the PDA with a final concentration of 5% DMSO was performed. The agars were allowed to solidify at room temperature. The plates were placed in laminar flow hood for approximately 30 minutes with their lids open to quickly dry agar surface. Fungal pathogens were grown on PDA and incubated at 30°C for 7 days. The 7-day-old fungal pathogens were cut by using a 6 mm diameter cock borer, and then transferred on to PDA plates with or without antimicrobial solutions. All experiments were performed in duplicate. The culture plates were incubated at 30 °C for 5 days. The MIC was defined as the lowest antimicrobial compound concentration at which absence of growth compared to that produced by the control plate. Sensitivity of each fungal species to antimicrobial agent was calculated as percentage of mycelial growth inhibition, according to the formula described by Pandey et al. [5]: \( \frac{(dc-dt)}{dc} \times 100 \), where \( dc \) = average diameter of the fungal colony of the negative control and \( dt \) = average diameter of the fungal colony treated with the antimicrobial agent.

Determination of Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

To evaluate the MBC, 100 μL aliquots from the antimicrobial compound dilution tubes with no visible growth were spread onto PSA. These plates were incubated at 30 °C for 24 hours. The lowest antimicrobial compound concentration that yielded three or fewer bacterial colonies was recorded as the MBC. To evaluate the MFC, the agar plugs of fungal pathogens with no visible growth were placed onto the center of new PDA plates and incubated at 30 °C for 5 days. The complete absence of growth on the agar surface at the lowest concentration of sample was defined as MFC. All experiments were performed in duplicate.

RESULTS AND DISCUSSION

In the present investigation, crude extract of *Nodulisporium* sp. NHL-L 6/6 was evaluated for antimicrobial activity against certain bacteria and fungi, which were regarded as plant pathogenic microorganisms. Several important plant pathogens were chosen, including bacteria (*P. solanacearum, X. citri*, and *E. carotovora*) and fungi (*Colletotrichum* sp., *F. solani*, *F. oxysporum, A. brassicola, A. porri*, and *Penicillium* sp.) in order to examine more closely the
antimicrobial activity of the fungal metabolite. These plant pathogens cause severe diseases in agricultural crops and ornamental plants, resulting in significant production loss.

The present work shows that crude extract exhibited strong inhibitory activity against all test plant pathogens. According to the results given in the Table 1, the minimum inhibitory concentrations (MIC) of the extract that inhibited both bacterial and fungal pathogens were found in the range of 31.25 to 125 µg/mL. The minimum fungicidal (MFC) and minimum bactericidal (MBC) concentrations were found in the range of 125-500 µg/mL.

<table>
<thead>
<tr>
<th>Plant Pathogens</th>
<th>MIC (µg/mL)</th>
<th>MFC/MBC (µg/mL)</th>
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<tbody>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
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<tr>
<td><em>Colletotrichum</em></td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td>sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td><em>A. brassicola</em></td>
<td>62.5</td>
<td>125</td>
</tr>
<tr>
<td><em>A. porri</em></td>
<td>62.5</td>
<td>250</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>62.5</td>
<td>250</td>
</tr>
<tr>
<td><em>Penicillium</em></td>
<td>125</td>
<td>500</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. solanacearum</em></td>
<td>31.25</td>
<td>125</td>
</tr>
<tr>
<td><em>X. citrii</em></td>
<td>31.25</td>
<td>125</td>
</tr>
<tr>
<td><em>E. caratovora</em></td>
<td>125</td>
<td>250</td>
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</tbody>
</table>

Bacterial pathogens were found to be susceptible to antimicrobial agent more than fungal pathogens. In comparison between bacteria, *P. solanacearum* and *X. citrii* were found to be the most susceptible bacterial pathogens to the antimicrobial agent with the MIC value of 31.25 µg/mL, while *E. caratovora* was the least susceptible bacterial pathogens with the MIC value of 125 µg/mL. The minimum bactericidal concentrations (MBC) of antimicrobial compound that completely killed *P. solanacearum* and *X. citrii* were found at 125 µg/mL, while *E. caratovora* was completely killed when exposed to 250 µg/mL.

In consideration of fungi, *Penicillium* sp. was less susceptible to antimicrobials with the MIC value of 125 µg/mL while other pathogenic fungi were more susceptible with the MIC value of 62.5 µg/mL. The minimum fungicidal concentrations (MFC) of antimicrobial compound that inhibited fungi completely depended on the strains. *Colletotrichum* sp., *F. solani*, and *A. brassicola* were completely killed when exposed to 125 µg/mL, *A. porri* and *F. oxysporum* were completely killed when exposed to 250 µg/mL, while growth of *Penicillium* sp. were completely prevented when exposed to 500 µg/mL of antimicrobial agent.

According to the results, it was found that the bioactive metabolite showed the property of bacteriostatic or fungistatic agent at low concentration (≤ 125 µg/mL). Upon transfer of fungal culture plug and bacterial suspension to antimicrobial-free medium, the fungi and bacteria
usually start to grow again. There is not always a precise distinction between biostatics and biocides; high concentrations of some biostatic agents are also biocidal, whereas low concentrations of some biocidal agents are biostatic [6]. In this case, the antimicrobial agent showed the biocidal property that killed the fungal and bacterial cells at high concentrations ranging from 125-500 µg/mL.

**Table 2** Effect of crude extract of *Nodulisporium* sp. NHL-L 6/6 culture broth against fungal plant pathogens

<table>
<thead>
<tr>
<th>Plant Pathogens</th>
<th>% Mycelial Growth Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration of crude extract (µg/mL)</td>
</tr>
<tr>
<td></td>
<td>15.625</td>
</tr>
<tr>
<td><em>A. porri</em></td>
<td>12.5±2.1</td>
</tr>
<tr>
<td><em>A. brassicola</em></td>
<td>12.6±2.2</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>11.1±2.8</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>22.1±2.4</td>
</tr>
<tr>
<td><em>Penicillium</em> sp.</td>
<td>4.8±0.0</td>
</tr>
<tr>
<td><em>Colletotrichum</em> sp.</td>
<td>34.6±3.8</td>
</tr>
</tbody>
</table>

* Mean percentages of mycelial growth inhibition ± S.D.

It was found that high concentrations of antimicrobial agent reduced the colonial growth of fungi significantly. As shown in Table 2, the extract of *Nodulisporium* sp. NHL-L 6/6 at the concentration of 15.625 µg/mL showed weak inhibitory effects (less than 40% inhibition) on the growth of *A. porri*, *A. brassicola*, *F. solani*, *F. oxysporum*, *Penicillium* sp. and *Colletotrichum* sp. The crude extract at the concentration of 31.25 µg/mL showed moderate inhibitory effects (41-55%) on the growth of *A. porri*, *A. brassicola*, *F. solani*, *F. oxysporum*, *Penicillium* sp. and *Colletotrichum* sp. The crude extract at the final concentration of 62.5 µg/mL showed moderate inhibitory effects on the growth of *Penicillium* sp. (54.0±3.2%). The mycelial growth of all plant pathogenic fungi was found to be inhibited with 100% efficacy at the concentrations of 62.5 to 125 µg/mL.

This study is a preliminary evaluation of bioactive compounds produced by *Nodulisporium* sp. NHL-L 6/6. The inhibitory effects of the culture extract of *Nodulisporium* sp. NHL-L 6/6 on both bacterial and fungal phytopathogens may be indicative of the presence of more than one broad-spectral antimicrobial compound or normally secondary metabolite. When compared the growth inhibitory effect on each group of phytopathogens (i.e. bacteria and fungi), MIC varied with different microbial species. This could be due to the different ecological niches of these plant pathogens since they were isolated from different infected plant species and tissues and moreover, their different protective mechanisms.

**CONCLUSION**

The development of natural antimicrobial metabolite would help to decrease the negative effect of synthetic agents, such as residues, resistance and environmental pollution. In this respect, natural antimicrobials may be effective, biodegradable, and less toxic to the environment.
as well as agriculture industries. As the crude extract of bioactive metabolites from *Nodulisporium* sp. NHL-L 6/6 showed broad spectrum of antimicrobial activity against plant pathogens, thus, it can be concluded that the antimicrobial compounds from *Nodulisporium* sp. NHL-L 6/6 are promising novel types of natural pesticides to control several plant pathogens causing severe diseases in crops and vegetables.

REFERENCES