IN-VITRO ASSAY OF NEEM SEED FORMULATION AGAINST *Fusarium oxysporum*, CAUSAL AGENT OF BASAL PLATE ROT ON ONION

Ni Putu Ratna Ayu Krishanti* and Arief Heru Prianto

Research Centers for Biomaterials, Indonesian Institute of Sciences, Cibinong Science Center, Cibinong-Bogor 16911, Indonesia

*Corresponding author: ratna.a.krishanti@gmail.com

Abstract

Basal plate rot disease caused by *Fusarium oxysporum* is one of the most important diseases of onion in Indonesia. Application of fungicide such as soil drench will increase the cost of onion production and may be dangerous for environment. One of techniques to suppress the dispersal of pathogen can be implemented by natural resource which is effective, degradable, and environmentally friendly. Antifungal effect of neem seed formulation was examined by in vitro assay against *Fusarium oxysporum*. The results indicated that neem seed formulation inhibited the growth of fungi, although the rate of inhibition varied with different concentrations. It was indicated that neem seed formulation is potential to be developed as biofungicide

Keywords: antifungal activity, *Fusarium oxysporum*, basal plate rot, neem, onion

Introduction

Basal plate rot disease of onion caused by *Fusarium oxysporum* generates several losses of onion production in Indonesia. An alternative to suppress the dispersal of pathogen can be implemented by natural resource which is effective, degradable, and environmentally friendly. Several plant extract-based products are effective in controlling some plant diseases (Hanaa et al., 2011). Various plant products have been evaluated to manage the basal plate rot disease. Many plants are capable in producing secondary metabolites, which have an allelopathic effect. These phytochemicals are safer to the environment and human than conventional chemical. Some plants represent an abundant source of antimicrobial compounds such as flavonoid, phenol, unsaturated lactone, sulphur compound, and saponin (Bennett & Wallsgrove, 1994; Osbourne, 1996). Plant also become the source of natural pesticide which leads the development of new biopesticide (Dissanayake & Jayasinghe, 2013). One of the producing phytochemical plants as well as known for its pesticidal properties is neem (*Azadirachta indica*).

Indonesian farmers have been using neem to control pests and diseases for hundred years. The bioactivity of neem product has been attributed to various compounds which can be found in the seed and leaf, including nimbin, nimbidin and salannin, but the most important compounds appear as the tetranortriterpenoid molecules, which is azadirachtin (Salehzahde et al., 2003). These compounds possess insecticidal, ovicidal, antifeedant, and growth-inhibiting effects against many species of pests. Several research have been reported that azadirachtin also have huge antimicrobial activity (Lale & Abdulrahman, 1999). The antimicrobial activity of neem-derived products is uncontested (Pai et al., 2004), only few reports are available on action of neem against fungi especially plant fungal pathogen (Ramos et al., 2007; Sipahelut, 2015). Regarding the needs for an eco-friendly alternative to control plant fungal pathogen, it is believed that it is important to screen the antifungal effects of neem seed formulation. Since our concern is about controlling the pathogen of basal plate rot disease, so the objective of the present investigation is to evaluate the antifungi activity of neem seed formulation against *F. oxysporum* by in vitro assay.


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Materials and Methods

Neem extract production
Neem seed oil was obtained by pressing the seed with screw press. It is necessary to do a preliminary process that includes depulping (remove the seeds from the fruit), seeds drying, and decontating (peel the endocarp) before pressing the seeds. Seeds that have been cleaned and treated by the pre-treatment process will be pressed with the screw tool. The oil obtained was filtered in order to collect the pure neem oil.

Neem formulation
Biopesticide formulation which contains active neem seed oil was made by mixing neem oil extract with anionic and ionic surfactants. Combinations of the composition of each material were performed in order to obtain the best formulation which has a significant stability of emulsion. In this experiment, five (5) formulations of neem seed oil have been made. Formulas F1 to F5 were made in the similar procedure using cold process (without heating). Cold process have been selected because it is more efficient compared to the heat process. All formulations used two types of surfactants which were Geronol bc-5 (etoksilat alkil fenol) and Rhodakal 70 bc (calcium dodesilbenzen sulfonat), total surfactant content in variety of neem oil formulations was 10%.

Culture isolate and media preparation
Isolate of fungi pathogen, F. oxysporum was collected from Bogor Agricultural University culture collection laboratorium (IPBcc) and maintained in medium of potato dextrose agar. For in vitro assay, growth medium prepared for F. oxysporum was Potato Dextrose Agar (PDA). Then, 1 % of neem formulation from different concentrations (F1 – F5) added to each media to evaluate the antifungal activity. The medium was sterilized by autoclave at 121°C, 1 atm, for 15 minutes. PDA plates, without any neem formulation supplementation, acted as negative control.

Effect of neem seed formulation on mycelial growth
Mycelial discs (Φ : 9 mm) from inoculant F. oxysporum were deposited into the center of PDA treated medium containing F1 – F5 formulations. The dishes were incubated at 37°C and photoperiod was performed for 12 hour day/ 12 hour night. The growth of mycelia was measured at the interval of 3 days and it was compared to the control treatment during nine days. Each formulation was triplicated. The percentage of inhibition is calculated by the formula as follows (Bragulat et al., 1991):

\[
\%\text{Inhibition} = \left(\frac{C - T}{C}\right) \times 100
\]

Where
C = Diameter of test fungus (Control)
T = Diameter of test fungus

Results and Discussion
Neem formulation
In this study, five active ingredient of formulations were made and they were contained neem seed oil as active ingredient. Formulations F1 to F5 made by the same procedure, but with different level of neem seed oil concentration and surfactant. Formula base comprised of 94% active ingredients which were neem seed oil and 6% surfactants (ionic and anionic). Formulations F1 to F5 used both types of surfactants in the total level of 6%. Further detail about formulation composition is presented in Table 1.
Table 1. Composition of five types of concentration of neem formulation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>F₁</th>
<th>F₂</th>
<th>F₃</th>
<th>F₄</th>
<th>F₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neem Seed Extract</td>
<td>94%</td>
<td>65.8%</td>
<td>32.9%</td>
<td>9.87%</td>
<td>2.96%</td>
</tr>
<tr>
<td>Anionic Surfactant (Genorol BC-5)</td>
<td>2%</td>
<td>1.4%</td>
<td>0.7%</td>
<td>0.26%</td>
<td>0.06%</td>
</tr>
<tr>
<td>Ionic Surfactant (Rhodakal BC-70)</td>
<td>4%</td>
<td>2.8%</td>
<td>1.4%</td>
<td>0.42%</td>
<td>0.13%</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>-</td>
<td>30%</td>
<td>65%</td>
<td>89%</td>
<td>96.85%</td>
</tr>
</tbody>
</table>

The results of water solubility testing showed that formulations F₁ to F₅ generated foam. The formation of foam occurred when the mixture became homogeneous due to dissolved surfactant in the water. The surfactant has a hydrophobic group (unlike water) and hydrophilic group (like water) that united in a molecule, so that the surfactant tended to be in between with the different phases of polarity (Fatima, 2005). Formulation of F₂ to F₅ formed two phases of solution, where the top phase solution consisted of foam with neem oil, while the low phase solution which was in white-colored. The formulation of F₁ only produced single phase solution which was in white ivory-colored with foam.

**Antifungal activity test**

The activity of neem seed formulation against mycelial growth of *F. oxysporum* is presented in Figure 1. It was observed that all neem formulations inhibited the mycelial growth of *F. oxysporum*. F₁ formulation showed the greatest inhibitory effect against mycelial growth than other formulations. Compared to the negative control, mycelial in treated medium grew slowly and it was secreted dark purple pigment. It was indicated that the mycelial was in the stage of stress condition.

![Figure 1](image_url)

**Figure 1.** Antifungal activity of neem formulation against *Fusarium oxysporum* after nine days of incubation. Picture description: (A) Control without treatment, (B) Treatment using F₁ formulation, (C) Treatment using F₅ formulation.

The mechanism of neem formulation action against pathogenic fungi of *F. oxysporum* was expected to be related to the cell wall, it has been observed that the neem formulation inhibits chitin synthesis in mycelial fungi (Maoz et al., 2000). Kavitha et al. (2014), found that neem seed methanolic extract was inhibit the ergosterol biosynthesis of *Aspergillus parasiticus*, this effect might be attributed to inhibition of enzyme which was involved in this process. Some of studies stated that beside of inhibit the mycelial growth, neem extract also effectively inhibits the germination of *F. oxysporum* spore as well as Mancozeb pesticide at concentration of 400 – 2000 µg/cm² (Govindachari et al., 1999). According to Da-Costa et al. (2010), based on the efficacy of neem seed extract against fungal pathogen, neem seed extract treatment had a higher inhibition percentage than neem leaf extract.

The result of antifungal activity of five formulations of neem seed oil against *F. oxysporum* showed that these formulations had the highest inhibitory in F₁ formulation generated the lowest...
mycelial growth of 31.4 mm on 9 days of incubation. While the lowest inhibition was found in the F₄ formulation with highest mycelial growth of 77.8 mm on 9 days incubation day (Figure 2). Formulations of F₂ to F₅ were not showing any significant different inhibition of mycelial growth. It was indicated that the composition of formula F₂ to F₅ were not as effective as formula F₁. The higher concentration of neem seed oil extract contained, the higher inhibition of cell growth of fungi obtained so that the strength of fungi cell will be decreased as well.

![Figure 2](image.png)

**Figure 2.** The growth of mycellia (mm) *F. oxysporum* on PDA medium containing neem formulation during nine days of incubation. Strong inhibition showed by F₁ formulation compared to control treatment. F₂-F₄ formulation showed growth inhibition but not significant among treatment.

**Inhibition of neem formulation on mycelial growth**

Percentage of inhibition analysis of pathogenic fungi *F. oxysporum* against neem seed oil formulation F₁-F₅ showed that the greatest percentage of inhibition was found in formula F₁ with 65.11% followed by F₂, F₃, F₄, and F₅ formulations (Table 2). Reduction of neem seed oil concentration and addition of ionic as well as anionic surfactant concentration makes the inhibition of mycelial growth becomes not optimal. Based on the data, it is necessary to re-optimize the composition of the formula which gave the greatest inhibition of pathogenic fungi growth. Further investigation on purified components of neem seed oil needs the proper insight of active ingredients responsibility for controlling the dispersal of diseases.

The high value of percent inhibition that produced by neem seed formulation against fungal mycelial of *F. oxysporum* can not be separated from the role of bioactive compounds contained. Based on the analysis performed by preparative HPLC, Suresh et al. (1999), found that there are at least 10 peaks that indicate active compounds in neem seed oil. Analytical HPLC revealed that peaks 1 and 2 contained mainly azadirachtins A, B, D, H and I. Peak 1 and 2 did not show any appreciable inhibitory activity. Peaks 3 and 4 yielded small amounts of material, with little or no activity against *F. oxysporum*. It was summarized that azadirachtins do not possess any antifungal activity. Peak 5 was identified as 6-deacetylnimbin and showed appreciable inhibition against *F. oxysporum* (49.2%) at 1000 ppm. Peak 7 (nimbin as the major constituent), peak 8 (salannin as the major constituent) showed excellent inhibitory antifungal activities at 1000 ppm. Peak 9 showed moderately active against *F. oxysporum* at 1000 ppm. Peak 10 (epoxyazadiradione as the major constituent) was most effective against *F. oxysporum*. 
Table 2. Anti-fungal activities of neem formulation against *F. oxysporum* (Mean ± SD).

<table>
<thead>
<tr>
<th>Neem Formulation</th>
<th>Diameter of Mycelial Growth (mm)</th>
<th>Percentage Inhibition of Mycelial Growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.00 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>F₁</td>
<td>31.40 ± 1.14</td>
<td>65.11 ± 1.27</td>
</tr>
<tr>
<td>F₂</td>
<td>70.00 ± 0.71</td>
<td>22.22 ± 0.79</td>
</tr>
<tr>
<td>F₃</td>
<td>72.40 ± 1.82</td>
<td>19.56 ± 2.02</td>
</tr>
<tr>
<td>F₄</td>
<td>76.00 ± 2.00</td>
<td>15.56 ± 2.22</td>
</tr>
<tr>
<td>F₅</td>
<td>77.80 ± 2.28</td>
<td>13.56 ± 2.53</td>
</tr>
</tbody>
</table>

While, Epoxyazadiradione in pure form exhibited no inhibitory activity against fungi. It is possible that in pure form the major triterpenoids from oil, have very low or no antifungal activity, while in combination they show excellent activity against the fungi, suggesting additive / synergistic effects. There is a need further investigations on purified components of neem seed oil to have depth knowledge of the active ingredients responsible for controlling the spread of the diseases.

**Conclusion**

Five types of neem seed oil formulation had antifungal activity and they could inhibit the growth of mycelial pathogenic fungi, *Fusarium oxysporum*, causal agent of basal plate rot disease on onion effectively. Based of the antifungal activity test analysis, F₁ formulation gave the highest percentage of inhibition of 65.11%. Formulations that gave the most stability described by the ratio of ionic surfactan : anionic formulation : neem seed extract were 4% : 2% : 96%. The results indicated that neem seed formulations were potential to be developed as biofungicide and it needs a further analysis about actives compound that plays important role in fungal suppression.

**Acknowledgment**

This work is financially supported by Research Center for Biomaterials LIPI through DIPA 2016. Therefore, we are grateful for this funding and support for this research.

**References**


The 6th International Symposium for Sustainable Humanosphere
Humanosphere Science School 2016
Bogor, 15 – 16 November 2016


