



***In vitro* biological control of *Ceratobasidium ramicola* by using tannin extracts from *Acacia villosa*, *Myristica fragrans*, *Acacia mangium*, and *Calliandra calothyrsus* leaves**

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Abstract

Ceratobasidium ramicola is a fitopathogenic fungus that harmful and causes various levels of damage on several types of forestry and horticultural crops. The purpose of this study was to examine the effect of *Acacia villosa*, *Myristica fragrans*, *Acacia mangium* and *Calliandra calothyrsus* leaf extracts as tannin sources related to the *in vitro* inhibition of *Ceratobasidium ramicola* growth. The *in vitro* inhibition was performed by employing solid potato dextrose agar (PDA) medium to obtain the radial inhibition, while liquid potato dextrose broth (PDB) medium was used to obtain the biomass inhibition. Experimental design was based on in time nested-completely randomized design and statistical analysis was carried out with SAS software version 9.1. The result of radial growth inhibition of *Ceratobasidium ramicola* showed that tannin extracts of *A. mangium* and *M. Fragrans* were not significantly different to each other. Treatment of tannin extracts from *A. villosa*, *M. fragrans*, *A. mangium* and *C. Calothyrsus* with a concentration of 1% were significantly different with other concentrations and resulted the greatest inhibition values. Tannin extract of *A. Mangium* at 1% concentration produced the greatest radial inhibition by 33.2%. In most cases, the effective inhibition from tannin extract occurred at the 24h of incubation. The greatest biomass inhibition was produced on 1% tannin extract of *A. mangium* by 64.3%, while the lowest was produced from 1% tannin extract of *M. fragrans* by 27.0%.

Keywords: fungicide, polyphenol, fungal attack, deuteromycetes.

Controle biológico *in vitro* de *Ceratobasidium ramicola* usando extratos tânicos de folhas de *Acacia villosa*, *Myristica fragrans*, *Acacia mangium* e *Calliandra calothyrsus*

Resumo

Ceratobasidium ramicola é um fungo fitopatogênico prejudicial que causa vários danos em diversas culturas florestais e agrícolas. O objetivo deste estudo foi examinar o efeito dos extratos de folhas de *Acacia villosa*, *Myristica fragrans*, *Acacia mangium* e *Calliandra calothyrsus* como fontes de tanino relacionadas à inibição do crescimento *in vitro* de *Ceratobasidium ramicola*. A inibição *in vitro* foi realizada empregando o meio de ágar batata dextrose para obter a inibição radial, enquanto o meio de caldo de batata dextrose líquido foi usado para obter a inibição da biomassa. O projeto experimental foi fundamentado *in time*. O delineamento utilizado foi inteiramente casualizado, e a análise estatística foi realizada com o software SAS, versão 9.1. O resultado da inibição do crescimento radial de *Ceratobasidium ramicola* mostrou que os extratos de tanino de *A. mangium* e *M. fragrans* não diferiram significativamente entre si. Os tratamentos de extratos de tanino de *A. villosa*, *M. fragrans*, *A. mangium* e *C. Calothyrsus*, utilizando uma concentração de 1%, foram significativamente diferentes em comparação com outras concentrações e resultaram em maiores valores de inibição. O extrato de tanino de *A. mangium* a 1% de concentração produziu a maior inibição radial, com taxa de 33,2%. Na maioria dos casos, a inibição efetiva do extrato de tanino ocorreu em 24 horas de incubação. A maior inibição de biomassa foi produzida em 1% de extrato de tanino de *A. Mangium*, com taxa de 64,3%, enquanto a menor foi produzida a partir de 1% de extrato de tanino de *M. fragrans*, com taxa de 27%.

Palavras-chave: fungicida, polifenol, ataque fúngico, deuteromicetos.

1. Introduction

Rhizoctonia sp. fungus is a type of pathogen that causes varying levels of damage to various types of forestry and horticultural plants. *Ceratobasidium ramicola* causes leaf blight disease on *Toona sureni* Merr. (*Meliaceae*) (Achmad et al., 2015) and *Pinus palustris* (Barnard, 1996), it also causes dumping off on various conifer species (Sneh et al., 1996) and strawberry (Zeeler, 1932), spot, blotch, and leaf blight on tobacco, peanut and soybean, stem rot on celery, leaf rot on onion and root rot on maize (Kucharek, 2000). Various types of damage and vulnerable host to the *Ceratobasidium ramicola* require an effort to prevent crop production loss.

Control of fitopathogen fungal is generally performed by using chemical fungicide. Synthetic fungicide is widely used for uncontrollably pathogen such as soil-borne pathogen. Despite the use of synthetic fungicide is relatively convenient, however, its excessive use may lead to negative impacts on human health and environment. The second resurgence of pathogen, resistant bodies, as well as the death of the natural enemies that is indirectly disrupt the ecosystem stability may occurs due to continual and excessive use of synthetic fungicide. An effort in improving environmental quality to control plant diseases is through biological control.

Biological control is defined as efforts in controlling the causal of disease or decreasing the number and effect of pathogen which is related to the mechanism of organism, except human (Campbell, 1989). Biological control may also be defined as an effort to reduce the density of the inoculum or the activity of both pathogen and parasite during its active or dormant phase using one or more organism. This activity can be done naturally through manipulation of environment, host, or antagonist by adding one or more antagonists (Baker and Cook, 1982; Achmad and Arshintia, 2014).

One of biological control efforts that can be done and need to be examined is the use of biological material or natural compound such as tannin to inhibit the growth of pathogen (Achmad et al., 2015). Tannin is the result of secondary metabolites produced by plant as a form of natural protection mechanism against tissue invasion by microorganisms (fungi and bacteria) and damage by herbivores (insects, birds, and animals). Every plant can produce tannin but it may have a different structure and concentration based on the species (Norton, 2000). According to the Jayanegara et al. (2011), *Acacia villosa*, *Myristica fragrans*, *Acacia mangium*, and *Calliandra calothyrsus* leaves are classified as the types of forestry plant leaves that contain considerable amount of tannin. They are classified as angiosperms in the botanical family of plantae kingdom. Hence, this study was conducted to examine the effect of *A. villosa*, *M. fragrans*, *A. mangium* and *C. calothyrsus* leaves as tannin sources to inhibit *Ceratobasidium ramicola* growth as performed *in vitro*. It was hypothesized that these tannin extracts were able to inhibit the growth of the fungal pathogen by causing hyphae lysis.

2. Material and Methods

2.1. Sample preparation

Samples of *A. villosa* leaves were collected from the International Research Institute for Animal Production in Ciawi, while the samples of *M. fragrans*, *A. mangium* and *C. calothyrsus* were collected from Bogor Agricultural University, Dramaga, Bogor. The leaves were collected on mature stadia leaves from adult tree. They were immediately wilted at room temperature for 24 h and oven-dried at 50 °C for another 24 h. The leaves were then ground by using a hammer mill to pass a 20 mesh (0.85 mm) screen.

2.2. Tannin extraction and measurement

Extraction of tannin was conducted by following the procedure of Makkar (2003). Firstly, 50 g of *A. villosa*, *M. fragrans*, *A. mangium*, and *C. calothyrsus* leaves were added with 300 ml of methanol 50%. The samples were then extracted using a sonicator (Barnstead Lab Line Aqua Wave 9377) for 30 min at room temperature. The filtrate was performed using a filter paper, while the sludge was re-extracted using addition of 200 ml of methanol 50%. Both extracts were pooled and evaporated using a rotary evaporator (Buchi R-200) at the temperature of 50 °C. They were subsequently freeze dried for 48 h.

Tannin contents of *A. villosa*, *M. fragrans*, *A. mangium* and *C. calothyrsus* were measured using Folin-Ciocalteu method (Makkar, 2003). Polyvinyl polypyrrolidone (PVPP) was used to separate between non-tannin phenol and tannin phenol. Tannic acid was used as the standard for quantification of total phenol and total tannin. Total tannin was obtained from the difference between total phenol and non-tannin phenol. Briefly, 0.5 ml sample extract was added with 1.25 ml Na₂CO₃ (20% v/v) and 0.25 ml folin reagent. The mixture was vortexed and kept in dark for 40 minutes. The absorbance was measured using a UV-VIS spectrophotometer with $\lambda = 725$ nm.

2.3. Examination of Tannin Extract on PDA (*Potato Dextrose Agar*) medium

An amount of 1 mL tannin extract, 14 mL PDA (*Potato Dextrose Agar*) and DMSO (Dimethyl sulphoxide) of 10% were mixed in petri dish under the Laminar Air Flow, and kept for 30 minutes to become solid. The control treatment was an addition of 1 mL solution without containing any tannin extract. Isolate of *Ceratobasidium ramicola* (obtained from isolate collection of Pathology Laboratory, Department of Silviculture, Faculty of Forestry, Bogor Agricultural University), which had been rejuvenated earlier on PDA, was inoculated into the media using cork borer. The species of isolate had been previously identified by morphological and molecular technique, and published in a separated paper (Firmansyah et al., 2018).

The petri dish was then wrapped with plastic wrap to avoid contamination and incubated under room temperature. Observation was conducted every 12 h for 7 days by measuring the diameter in x-axis and y-axis. The growth of radial diameter of *Ceratobasidium ramicola* was determined by averaging the diameter of x-axis and y-axis (both in cm).

The percentage of inhibition of *Ceratobasidium ramicola* colony can be determined using the following formula: $RI = \frac{D1 - D2}{D1} \times 100\%$, where RI is relative inhibition (%), D1 is diameter of control colony (cm) and D2 is diameter of treated colony (cm). This examination was conducted in five replicates (one dish per replicate).

2.4. Testing of Tannin Extract on PDB (Potato Dextrose Broth) media

An amount of 1 mL tannin extract, 79 ml PDB (Potato Dextrose Broth) and DMSO 10% were mixed in jar bottle under the Laminar Air Flow. *Ceratobasidium ramicola* isolate was then inoculated to the medium and incubated at room temperature. Observation was performed at day 7 after incubation. Colony growth examination was conducted through the separation between mycelia and PDB medium using filter paper. The mycelia of colony found on the filter paper was oven-dried for 24 h at 60 °C. The biomass of mycelia was determined using the following formula: $BM = (B1 + B2) - B1$, where BM is biomass of mycelia (g), B1 is dry weight of paper filter (g), and B2 is dry weight of mycelia (g). The percentage of inhibition of *Ceratobasidium ramicola* colony was determined using the following formula: $RI = \frac{B1 - B2}{B1} \times 100\%$, where RI is relative inhibition (%), B1 is biomass of control mycelia (g) and B2 is biomass of treated mycelia (g). This test was conducted in five replicates (one bottle per replicate).

2.5. Data analysis

Data were analyzed using the analysis of variance (ANOVA) based on “in time” nested-completely randomized design (Montgomery, 2005). When the ANOVA result showed significant different at $P < 0.05$, a post-hoc test namely Duncan’s multiple range test was employed to distinguish among treatment means. The statistical analysis was performed by using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

Tannin contents of extracted leaves of *A. villosa*, *M. fragrans*, *A. mangium*, and *C. calothyrsus* were varied (Table 1). The greatest total phenol was observed in *C. calothyrsus*, while the greatest total tannin content was found in *A. villosa*.

Growth inhibition of *Ceratobasidium ramicola* was characterized by lower growth by addition of tannin extract in comparison to control. Tannin extracts of *A. villosa*, *M. fragrans*, *A. mangium* and *C. calothyrsus* produced the highest inhibition on *Ceratobasidium ramicola* at concentration of 1% in PDA medium. The greatest inhibition of tannin

extract at a concentration of 1% was found in *A. Mangium* by 56.5%. Tannin extract that resulted the longest period of inhibition of 156 h was *A. mangium* (Figure 1).

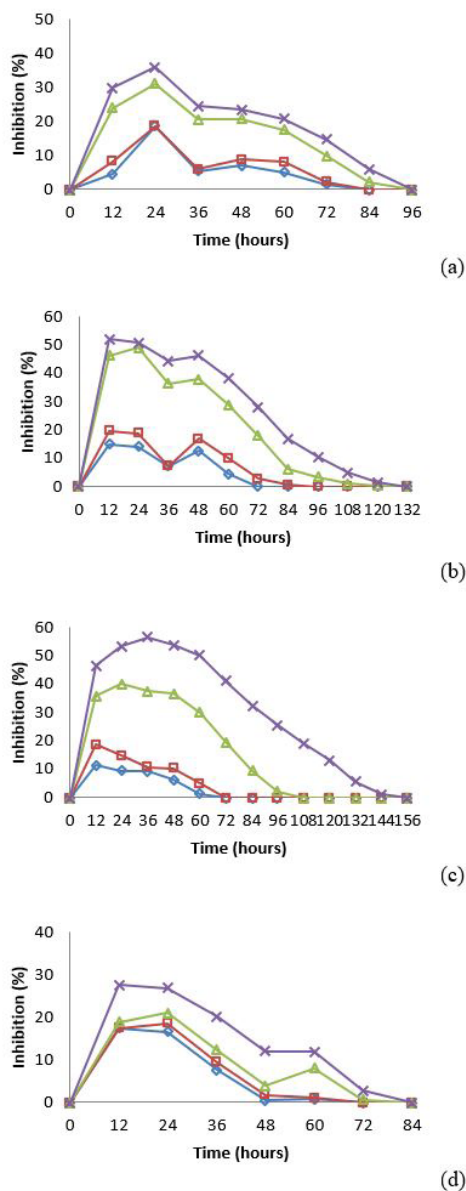


Figure 1. The average value of *Ceratobasidium ramicola* inhibition on tannin extract of *Acacia villosa* (a), *Myristica fragrans* (b), *Acacia mangium* (c) and *Calliandra calothyrsus* (d). Concentrations used are (◇) 0.05%, (□) 0.10%, (Δ) 0.50%, and (×) 1.00%.

Table 1. Total phenol and total tannin contents (dry matter basis) of leaf extracts from the studied plant species.

Species	Total phenol (%)	Non-tannin phenol (%)	Total tannin (%)
<i>Acacia villosa</i>	14.96	4.14	10.82
<i>Myristica fragrans</i>	4.77	4.02	0.75
<i>Acacia mangium</i>	3.29	2.27	1.02
<i>Calliandra calothyrsus</i>	15.98	7.46	8.52

The pattern of inhibition generally was an increase of inhibition in the early incubation period and gradually declined after reaching a certain time. The decrease of inhibition at tannin extract of *A. villosa*, *M. fragrans*, *A. mangium*, and *C. calothyrsus* started between 24 to 36 h, 12 to 24 h, 36 to 48 h, and 12 to 24 h, respectively.

Comparing among different tannin sources, effectively of *A. mangium* and *M. Fragrans* in inhibiting the growth of *Ceratobasidium ramicola* were similar but they were significantly different with *A. villosa* and *C. calothyrsus* ($P < 0.05$; Table 2). Tannin extract of *A. mangium* produced the highest percentage of inhibition by 14.7%. The effect of concentration of each tannin extract showed that concentrations of 0.10 and 0.05% were similar for *A. villosa*, *M. fragrans*, and *C. calothyrsus* (Table 3).

However, the concentrations of 1% and 0.5% were significantly different within each tannin ($P < 0.05$). Concentration of tannin extract that generated the greatest percentage of inhibition was 1%. The higher the concentration of tannin extract added to media, the greater the inhibition of *Ceratobasidium ramicola*. In PDB media, during one week incubation period, the biomass growth inhibition of *Ceratobasidium ramicola* occurred (Figure 2). The greatest inhibition was produced by tannin extract of *A. mangium* while the lowest inhibition was produced by tannin extract of *M. fragrans*.

4. Discussion

Plant produces various secondary metabolites such as tannin. Tannin is produced purposely by plants as a defense mechanism against pests and diseases. Tannin can be produced on various parts of a plant including the leaves. Difference on tannin content is influenced by the type of plant due to distinctive structure of each species

that is differentiate it from other species, particularly the composition of tannic cell (Escaray et al., 2008). In addition, tannin content variation is caused by different genotype and growth stage (Norton, 2000).

Jayanegara et al. (2011) observed that *A. villosa* is a species of forestry plant that contains the highest total tannin in its leaf by 22% (dry matter basis). The difference on tannin content is caused by several factors, such as different sources of the sample, the solvent for the extraction, sample size, climate, and season. Climatic and seasonal factors (such as temperature, rainfall, and defoliation) may affect the presence of herbivorous, such as insect. It leads to the adaptation of plant to produce tannin as a certain level of protection (Lehmann et al., 2015).

Tannin extracts of *A. villosa*, *M. fragrans*, *A. mangium* and *C. calothyrsus* have the *in vitro* inhibition ability on the growth of *Ceratobasidium ramicola* hyphae; it can be seen from all treatments on PDA and PDB that showed a slower growth than that of the control until a certain incubation period. The inhibition ability of tannin occurs due to the presence of phenol groups which have antimicrobial properties (Mailoa et al., 2014). Inhibition activity of tannin against a variety of microorganisms is related to its protein binding ability (Jayanegara and Palupi, 2010) including cell wall protein and enzymes released by fungi or other microbial species. The difference of various tannin extracts the inhibition of *Ceratobasidium ramicola* is potentially caused by the different concentration and may also be influenced by other functional compounds present in the extract.

Effect of incubation period on PDA media showed that the inhibition of tannin extract increased to a certain

Table 2. Relative inhibition tannin extracts on the growth of *Ceratobasidium ramicola*.

Tannin Extract	Relative inhibition (%)
<i>Acacia mangium</i>	14.7 ^a
<i>Myristica fragrans</i>	13.6 ^a
<i>Acacia villosa</i>	7.79 ^b
<i>Calliandra calothyrsus</i>	5.34 ^c

Different superscripts in the same column were significantly different at $P < 0.05$ by using Duncan's test.

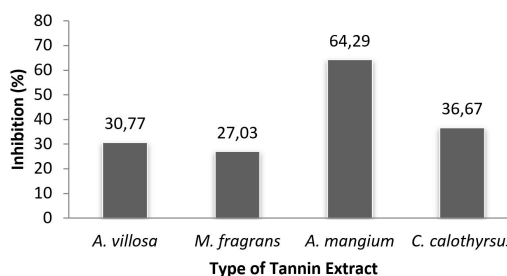


Figure 2. Comparison between the average biomass inhibition of *Ceratobasidium ramicola* and 1.00% tannin extract treatment on PDB (*Potato Dextrose Broth*) media.

Table 3. Relative inhibition of tannin extracts at various concentrations on the growth of *Ceratobasidium ramicola*.

Concentration (%)	Relative Inhibition (%)			
	<i>Acacia villosa</i>	<i>Myristica fragrans</i>	<i>Acacia mangium</i>	<i>Calliandra calothyrsus</i>
1.00	12.9 ^a	24.5 ^a	33.2 ^a	8.41 ^a
0.50	10.5 ^b	19.0 ^b	17.6 ^b	5.37 ^b
0.10	4.30 ^c	6.33 ^c	4.92 ^c	3.99 ^c
0.05	3.49 ^c	4.46 ^c	3.10 ^d	3.58 ^c

Different superscripts in the same column were significantly different at $P < 0.05$ by using Duncan's test.

time then continually decreased. Wright (1994) stated that tannin may cause lysis of the microbial cells, so that the tannin extracts of *A. villosa*, *M. fragrans*, *A. mangium* and *C. calothyrsus* were expected to cause the lysis on hyphae of *Rhizoctonia* sp., and in turn they inhibited the growth of the hyphae. *Ceratobasidium ramicola* might generate self-protection mechanism so that the lysis of cells can be immediately recovered. It is in accordance with Bouarab et al. (2002) which states that some species of fungi have the capability of protection against antimicrobial compounds to produce enzymes that can detoxify secondary metabolites compounds produced by the host.

5. Conclusion

Tannin extracts from *A. villosa*, *M. fragrans*, *A. mangium* and *C. calothyrsus* are able to inhibit the growth the pathogenic fungi *Ceratobasidium ramicola*, particularly at 1% concentrations for all extracts. Comparing among the different sources, apparently by far *A. mangium* tannin extract shows the greatest inhibition against the fungi.

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