Trypsin inhibitor activities as defense mechanism of sengon (Falcataria moluccana) against pest attacks

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Abstract. Sengon (Falcataria moluccana) plantation always suffers from a serious attack of stem borer, which is called Boktor (Xystrocera festiva Pascoe). This research aimed to investigate the presence of anti-pest substances, i.e. trypsin inhibitor on each part of a sengon tree, which might serve as a defense mechanism against the pest. Healthy and severely attacked sengon trees from Cianjur, Solo, and Kediri provenances were sampled for their leaves, barks, and woods. Trypsin inhibitor activity from each sample was assayed using synthetic enzyme and substrate in a spectrophotometer. Data were analyzed under Complete Randomized Design, using SPSS 11.5. Different provenances, tree condition, and part of tree gave significant effects on trypsin inhibitor activity. Healthy trees had a higher Trypsin Inhibitor Activity (TIA) value compared to the pest-infected tree. The sengon tree tissue that had the highest TIA is the bark from healthy trees of Solomon provenance, with a value of 730.0403 TIU/mg. Obviously the bark is the first defense against the stem borer pest.

1. Introduction
One of tree species that are widely utilized in the development of plantation forest and community forest in Indonesia today is sengon (Falcataria moluccana). As legume tree [1] sengon (F. moluccana) could improve soil fertility, has an economical value that is quite high and prospective, also easily cultivated and adapted to marginal land. However, when planted in monoculture sengon always suffers from a serious attack of stem borer, which is called Boktor (Xystrocera festiva Pascoe). The increase of sengon populations as main food of the stem borer resulted in overbreed of the pest (X. festiva) and expanding pest population thereby leading to considerable economic loss. So far there is not any effective and efficient control methods for this pest. Therefore, a resistant line of sengon tree is needed to manage the pest attacks. Observation in the field showed that although most sengon trees are suffering to the pest, several individuals showed some resistance against the pest. These seemingly resistant individuals showed very few damages in the main stem and less suffering from the pest attacks. These resistant individuals are important for tree breeding program of sengon trees.

Similar to other insects, inside Boktor digestive tract has trypsin enzyme among others, which has a linear pattern of enzyme activity [2]. Trypsin enzyme plays a role in the digestion of proteins in the insect diet into simple molecules which is ready to be absorbed by the cells [3]. Boktor pest usually lays eggs at the wounded main stem of sengon. When the eggs hatch the larvae would start chewing the outer
bark and move inside while eating the bark and eventually bore into the woody tissue. The larvae would eventually stay inside the wood until develop cocoon and hatch imago.

On the other hand, the seemingly resistant sengon trees are known to have compounds that can inhibit trypsin enzyme to work in boktor digestion process, known as trypsin inhibitor [4]. The inhibitor has different activities on each part of the sengon tree. Given the huge losses inflicted by Boktor’s attack, it is interesting to investigate which part of the sengon tree, as well as which provenance has the largest trypsin inhibitor activity. Such information can be used to obtain superior sengon seeds that contain the largest trypsin inhibitor activity that can inhibit the work of trypsin enzyme inside boktor digestive tract.

This research aims to determine the trypsin inhibitor activity in sengon leaves, bark, and wood from Cianjur, as well as the differences in trypsin inhibitor activity between healthy sengon trees and pest-infected trees from Solomon and Kediri provenances.

2. Materials and Methods

2.1. Preparation of raw materials
Observations on trypsin inhibitor are carried out using chemicals as substrate, i.e. synthetic benzoyl-DL-arginine-p-nitroanilid (BAPNA), buffer Tris-HCL, dimethyl sulfoxide (DMSO), NaOH, 30% acetic acid, trypsin enzyme from SIGMA 3.5 U/mg.

2.2. Determination of trypsin inhibitor activity
This method is based on the inhibitory hydrolysis of a synthetic substrate (benzoyl-DL-arginine-p-nitroanilid) by a synthetic trypsin enzyme, in the presence of an anti-trypsin or trypsin inhibitor. The compounds that should be prepared including reagents and extracts, as followed.

A. Preparation of reagent solution:
   1) Tris-HCL buffer 0.05 M, pH 8.2 containing 0.02 M CaCl₂: 6.05 g Tris (methyl aminometen hydroxy) and 2.94 g CaCl₂·H₂O dissolved in 900 mL distilled water. The pH was adjusted to 8.2 and the volumes of the solution were filled up to 1000 mL
   2) BAPNA solution (benzoyl-DL-arginine-p-nitroanilid): 40 mg BAPNA dissolved in 1 mL DMSO (dimethyl sulfoxide), then diluted to 100 mL by adding a Tris buffer that had a temperature of 37°C
   3) Trypsin Solution: SIGMA trypsin enzyme with the activity of 3.5 U/mg dissolved in 200 mL HCl 0.001 M
   4) 30% acetic acid solution (V/V)

B. Preparation of sample extracts:
   1) Leaf, bark, and wood samples were freeze-dried and then powdered
   2) As much as 0.2 g of each powdered sample was suspended in 50 mL of 0.1 N NaOH solution. After stirring for 3 hours at room temperature using magnetic stirrer then centrifuged at 2,000 x g or 5,000 rpm for 10 minutes at 5°C and the supernatant was separated
   3) The extract obtained was then diluted with distilled water as such that 1 mL of the extract will produce an inhibitory force about 40-60% of the trypsin enzyme activity used
   4) Procedures 1) and 2) were performed for leaves, bark, and wood powder from Cianjur, Solomon, and Kediri provenances

2.2.1. Procedure for analyzing trypsin inhibitor activity:
Series of extracts, i.e. 0; 0.2; 0.4; 0.6; 0.8; 1.0 mL were pipetted into the reaction tube. Distilled water was added to each reaction until the volume reached 2 mL. As much as 2.0 mL of trypsin solution was added to each reaction tube, then all tubes were incubated in a 37°C water bath and left for 5 minutes. As much as 5 mL of BAPNA solution 37°C was added into each tube, and the solution was mixed quickly using a vortex, and incubated in a 37°C water bath for 10 minutes. After 10 minutes, as much as 1 mL of 30% acetic acid is added to each tube and mixed quickly. When the solution was becoming clear, measurement on the spectrophotometer can be done immediately. Absorbance measurements of the extracts were done at a wavelength of 410 nm, using the above blank reagent containing 0 mL extract
as a reference. If necessary, in the case of colored extracts, the blank samples can be made in the following ways: adding 5 mL of BAPNA solution into 2 mL of extracts, the mixture is incubated at 27°C for 10 minutes, then add 1 mL of 30% acetic acid and 2 mL of trypsin solution.

One Trypsin Unit (TU) is defined as an increase of 0.01 units of absorbance at 410 nm per 10 mL reaction mixture under the conditions used. Trypsin inhibitors activity is expressed as one Trypsin Unit Inhibited (TUI). The trypsin inhibitor activity is calculated as follows:

\[
\text{TU} = \frac{\text{absorbance}}{0.01} \\
\text{TIU} = \text{TU} (S) - \{\text{TU} (C) - \text{TU} (BC)\} \\
\text{TIU/mL} = \frac{\text{TIU}}{2} \\
\text{TIU/mg Extract} = \frac{(\text{TIU/mL} \times \text{f.p})}{\text{sample weight}}
\]

where, C: example; S: Standard; BC: Example blank; TU: trypsin unit; TIU: trypsin inhibitor unit; f.p: the dilution factor.

2.3 Data analysis
Data on trypsin inhibitor enzyme activity were analyzed using factorial complete randomized design. The equation form:

\[
Y_{ijkl} = \mu + A_i + B_j + C_k + AB_{ij} + AC_{ik} + BC_{jk} + ABC_{ijk} + \epsilon_{ijkl}
\]

where:
i: 1 (Solomon), 2 (Kediri); k: 1 (leaves), 2 (bark), 3 (wood); j: 1 (healthy), 2 (attackted); l: 1, 2, 3, 4, 5
\(Y_{ijkl}\): response provenance influence to-i, tree condition to-j, part of tree to-k, and repeat to-l
\(\mu\): average general value
\(A_i\): provenances to-i
\(B_j\): tree condition to-j
\(C_k\): parts of tree to-k
\(AB_{ij}\): interaction of factor A to-i and B to-j
\(AC_{ik}\): interaction of factor A to-i and C to-k
\(BC_{jk}\): interaction of factor B to-j and C to-k
\(ABC_{ijk}\): interaction of factor A to-i, B to-j, and C to-k
\(\epsilon_{ijkl}\): trial error

3. Results and Discussion
3.1 Study on trypsin inhibitor activity in every part of a sengon tree
Trypsin inhibitor is compounds that have the ability to inhibit the proteolytic activity of trypsin enzymes. These compounds have been found in some crop plants, especially the types belonging to Leguminoseae family. The active compounds of this inhibitor are protein. Trypsin inhibitor has physiological functions as the plants’ abilities to defend against natural pest attacks [5].

**Table 1.** Result of variance trypsin inhibitor activity using trypsin enzymes synthetic

| Factors                        | Various prints |
|--------------------------------|----------------|
| Part of tree from Cianjur provenance | ns             |
| Provenance                     | **             |
| Tree conditions                 | **             |
| Part of tree                    | **             |
| Provenance x Tree conditions    | ns             |
| Provenance x Part of tree       | ns             |
| Tree conditions x Part of tree  | ns             |
| Provenance x Tree Condition x Part of tree | ns |

Remarks: x: Interaction between factors; **: highly significant; ns: not significant
Boktor pest attacks sengon trees when the trees have formed woody tissues, preferably after 3 – 4 years old. Imago of the pest usually lays eggs on wounded tree bark, and when larvae is hatched, the larvae will start feeding on the bark [6]. As the larvae are getting older they start to bore the woody tissue in order to establish the place for pupation. As sengon is a legume tree, it was assumed that sengon would have trypsin inhibitor in each part of the tree as defense mechanism against the pest.

Statistical tests at 99% confidence level (Table 1) showed that the provenance, tree condition, and part of the tree, with exception of those coming from Cianjur provenance, had a highly significant effect on trypsin inhibitors activity. Figure 1 showed that although part of tree from Cianjur provenance did not differ of their trypsin inhibitor activities, the bark still had the highest activity, while the leaves were lowest.

![Figure 1. Average trypsin inhibitor activity of each sengon tree part from Cianjur provenance](image)

Remarks: *The same letter shows no significant difference at 95% confidence interval

Figure 1 shows that the average value of TIA found in the bark is 606.98 TIU/mg, followed by the wood at a value of 522.43 TIU/mg, and the lowest is in leaves of 510.57 TIU/mg. This result corresponds to Winarni [4] which also examined the activity of trypsin inhibitor in plus trees from local community forest in West Java, of which activity and its diversity in the wood was lower than the bark. This indicates that the bark is the first main protection of sengon tree from the boktor pest attack. Higher trypsin inhibitor activity is present in the bark because the bark contains many living cells compared to wood. Living cells have organelles especially ribosomes, which is active sites for protein manufacturing, most probably including anti-protein trypsin enzymes that work on boktor’s digestive system [7].

Figure 2 shows the results from experiments using different part of the trees, coming from two different provenances, with two different tree conditions. Contrary to the results of different tree parts from Cianjur provenance (Figure 1), the different parts of trees coming from Solomon and Kediri provenances showed highly significant difference (Table 1 and Figure 2). The reason for this apparently discrepancy results were different sample sizes, of which on the later experiments more samples were examined, therefore the results were highly significant. The samples of the first experiment only consisted of one provenance without considering the trees condition, whether the tree was healthy or suffer from pest attacks. Albeit the statistical analysis gave different results, the trend however, was still similar. Trypsin inhibitor activity was found highest in the barks compared to other part of the trees, regardless of tree conditions and provenances. The only slight differences in the results of the first and second experiment were the order of amount of trypsin inhibitor activity in the woods and leaves. In the first experiment the wood had higher trypsin inhibitor activity compared to leaves (Figure 1), whereas in the second experiment the leaves had higher trypsin inhibitor activity than the wood (Figure 2). Nevertheless, both wood and leaves were much lower than barks for their trypsin inhibitor activities.
Another important results as shown in Figure 2 are healthy trees, which were supposed to be resistant trees, always had higher value of trypsin inhibitor activities, regardless of the parts of tree and provenance origins, compared to attacked trees, which were assumed as susceptible trees. This experiment also showed that different provenance could have different resistance or susceptibility against pest attacks. As shown in Figure 2, Solomon provenance appeared to be more resistant to the pest attacks than Kediri provenance, as indicated by their higher trypsin inhibitor activities in every parts of the tree. The relative resistant of Solomon provenance compared to Kediri provenance still needs to be confirmed by comparison to some more other provenances.

The consistency of highest trypsin inhibitor activity in the barks of three provenances studied here, combined with higher activities in healthy, supposedly resistant trees compared to attacked, supposedly susceptible trees, has pin pointed to the role of trypsin inhibitor as defense mechanism in sengon tree against pest attacks. It would be interesting to find out later if this resistance is genetically based, by further identifying genes responsible for insect pest resistance, especially protease inhibitor genes.

4. Conclusion

Different provenances, tree conditions, and parts of the tree have highly significant effects on trypsin inhibitors activities. The highest Trypsin Inhibitor Activity (TIA) was consistently found in the barks from healthy, supposedly resistant trees. Trees from Solomon provenance apparently have higher TIA compared to trees Kediri provenance. Sengon tree that has the highest TIA is a healthy bark from tree of Solomon's provenance with the value of 730.0403 TIU/mg.

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