The application of biotic elicitor on *Artemisia annua* L. to increase artemisinin content

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Abstract. Artemisinin-based Combination Therapy (ACT) has been recommended by WHO as an alternative to treat malaria overcoming drug resistance. The secondary metabolic products in plants, including artemisinin, can be increased by utilizing biotic elicitor from fungi. The research was conducted in Gunung Putri Research Installation, Cipanas, West Java from 2010 to 2011. *Phytophthora sp*. from eggplant and *Colletotrichum* sp. from *Artemisia annua* were applied as biotic elicitor. The types of biotic elicitor applied to the plants were 1) the medium of potato dextrose broth were inoculated with fungi and harvested after 10 days (filtrate), 2) powdery mycelium of both fungi. There were 16 treatments: control negative, control positive (uninoculated medium) 1%, 2%, 3% (v/v)], *Phytophthora* sp. filtrate [1, 2% and 3% (v/v)], *Colletotrichum* sp. filtrate [1, 2% and 3% (v/v)], *Phytophthora* sp. mycelium [1%, 2% and 3% (w/v)], *Colletotrichum* sp mycelium [1%, 2% and 3% (w/v)]. The elicitor application increased plant production by 26.21% and artemisinin yield by 72% compared to control. Furthermore, the artemisinin production of the plants treated with medium inoculated with 2% filtrate of *Phytophthora sp* (FP2) (25.19 kg/ha) and 1% powdery mycelium of *Colletotrichum sp* (MC1) (26.42 kg/ha) were higher than control (K) (11.17 kg/ha).

Keywords: *biotic elicitor, Artemisia annua L., artemisinin content*

1. Introduction

Artemisinin is active compound of *Artemisia annua* L. which is sub-tropical plant. It is introduced from China and spread in Vietnam and Malaysia. Drugs contain artemisinin have been proved particularly effective to treat severe malaria, even for multi drug-resistant malaria [1]. Currently, WHO recommended malaria drug artemisinin combination based therapy (ACT) to treat malaria [2].

Malaria is one of the most important tropical diseases [3], causes great human suffering and loss of life in affected countries. Indonesia is one of the countries with high number of malaria infection, hence requires high demand of artemisinin drug for malaria. However, in Indonesia, artemisia has not been cultivated widely and is planted in limited area only as trial.

Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge isolated from artemisia plants was effective against both drug–resistant and cerebral malaria caused by *Plasmodium falciparum* [4] [5]. Artemisinin had efficacy as antiviral, antiparasitic, antiinflammmation and anticancer [6]. In addition to being used as a drug, artemisinin also has phytotoxic activity as natural herbicide. The artemisinin application was reported could decrease the radicle length and seedling vigor index of *Arabidopsis thaliana* [7].

Artemisinin is obtained by extracting the leaves and stems of artemisia plants. These compounds belong to sesquiterpen lactone groups with endoperoxide bridges that are rarely encountered in nature. Artemisinin is a compound difficult to synthesize. Thus, the easiest and cheapest way to obtain artemisinin were to extract directly from the plant [8]. In Indonesia, the planted accessions had very low artemisinin content, ranged from 0.25-0.41% [9]. Thus, it considered inefficient to develop the plants in industrial scale.

In recent years, several elicitors have been used for stimulating artemisinin biosynthesis to increase artemisinin content. The elicitors can be divided into two groups, biotic and abiotic elicitors. Abiotic elicitors consisted of metal ions and inorganic components. The biotic elicitors included fungi, bacteria, viruses, herbivores, the components of plant cell walls, chemical compounds released by plants parts attacked by pathogens or herbivores, miconazole, chlorocholine chloride, homobrassinolide and crude extracts of fungal mycelia of *Verticillium dahliae, Rhizopus stolonife* and *Colletotrichum dematium* [10]. Elicitors may lead to cellular processes and regulatory principles for the activation of secondary metabolite biosynthesis. This led to the presence of extracellular or intracellular signals captured by receptors on the surface of plasma membrane or endomembrane. The elicitor signal was perceived as the initiation of the transduction signal to form de-novo activation or biosynthesis, or was a transcription factor expressing the biosynthetic gene regulation involved in plant secondary metabolism. Further, it yielded an enzyme for secondary metabolite biosynthesis [11]. The use of elicitors was proved more effective to increase secondary metabolite content because it was more natural, hence easier to enter the plant metabolism system. The types of elicitors commonly used were mycelial extract, filtrate or toxin from the fungi. The use of mycelial extract from fungus *Colletotrichum* sp. increased artemisinin content from 0.8 mg to 1 mg per dry weight [12]. The objective of this research was to obtain the type of elicitor and its concentration to increase artemisinin content.

2. Materials and methods

Phytophthora sp was isolated from eggplant and endophytic fungi (*Colletotrichum* sp) from artemisia. Both fungi were propagated for the examination of their capability as elicitor on artemisia plant in the field. The endophytic fungi was propagated by culturing the fungi in 50 ml dextrose potato broth in the erlenmeyer flask and was incubated at room temperature for 30 days. The mycelium was then separated from the filtrate, washed several times with sterile distillation water, grinded using mortar and centrifuged for 5 minutes. The supernatant was suspended with sterile distillation water following the concentration treatments, then autoclaved for 20 minutes at 120°C. The mycelium and filtrate (the media used to culture *Phytophthora* sp and *Colletotrichum* sp) were used as elicitor.

Artemisia seeds were germinated in the nursery for one month and transplanted into polybag, then planted in the field after 2 months. The basic fertilizer was applied one month after planting (MAP), consisted of 0.5 kg manure, 3.5 g Urea, 1.5 g SP-36 and 1.5 g KCl/plant. Plant maintenance such as weeding was performed once a month and pest control was done if it was necessary.

The elicitor was applied by spraying 2 months-old plants (2 MAP). Water was used as control negative whereas un-inoculated dextrose potato broth as control positive. The elicitor application was performed every week until two weeks before harvesting. The plants were harvested at 6 MAP or when 10% of the population started flowering.

The trial was arranged in randomized block design consisted of 16 treatments: control negative (water); control positive (1%, 2%, 3% (v/v) of un-inoculated medium); *Phytophthora* sp. filtrate (1, 2%, 3% (v/v); *Colletotrichum* sp. filtrate (1, 2%, 3% (v/v)); *Phytophthora* sp. mycelium (1, 2%, 3% (w/v)); *Colletotrichum* sp mycelium (1, 2%, 3% (w/v)). Every plot consisted of 20 plants, repeated three times.

Parameters observed were plant height, leaf dry weight, stem dry weight, total biomass dry weight and artemisinin content. The data were analyzed with ANOVA and contrast orthogonal for further analysis if the treatments were significantly affected the parameters.

3. Result and discussion

3.1. Plant growth

Naturally, plants were able to express their natural defence and triggered the defence signal through intermediary receptor by recognizing microbe structure known as pathogen-associated molecular patterns (PAMPs) or elicitor [13]. Elicitor was considered as signal molecule activated transduction signal wave (Cascade) and caused the activation and expression of gene related to secondary metabolite biosynthesis [14].

The data was analysed using orthogonal contrast and indicated significant differences among the treatments at 5% level of significance (Table 1). The parameters affected by elicitor application showed significant differences compared to both control negative and control positive, except for plant height. However, there was no significant effect between the elicitor from the filtrate of *Phytophthora* sp. and *Colletotrichum* sp on parameters observed (Table 1). The biochemical compound from fungi secretion on growth medium might have low concentration or the fungi secreted no chemical compound into growth medium. The pathogen lived in host plant rich in carbohydrate would arrange the production of polysaccharide-degrading enzyme. A small alteration in composition, structure or accessibility of host-plant carbohydrate was able to change the composition of polysaccharide-degrading enzyme secreted by pathogen, both qualitatively and quantitatively [15]. The phytohormones involved in plant defence, such as gibberellin [16] and salicylic acid [17], also possessed important role in artemisinin accumulation. However in this experiment, chemical compound secreted by fungi on filtrate was unable to give signal to induce secondary metabolite increase in artemisia. Filtrate treatment might be unable to increase gibberellin which functioned to trigger plant growth. Thus, plant growth parameters represented by plant height and total biomass dry weight, were not significantly different (Table 1).

Elicitor from both fungi mycelium gave significant effect on stem and biomass dry weight. The *Phytophthora* mycelium 2% gave higher stem and biomass dry weight than other mycelium treatments (Table 1). Mycelium was not dissolved in water. However, proper handling such autoclave treatment or alkali application could dissolve active elicitor from mycelium cell. The dissolve mycelium-cell comprised of 1,3-glucan or glycoprotein contained mannan. The chemical composition of mycelium linked to plant cell-wall was detected by plant cell during phatogenesis process. The un-dissolved elicitor was released from fungi mycelium wall soon after contacted with plant tissue. This process indicated that the released-elicitor was responsible for initiating the accumulation of phytoalexin and pythohormone in fungi-infected plants [18]. The increase of phytohormone would enhance biomass weight of the plant.

Table 1. The effect of type and concentration of biotic elicitor on artemisia plant growth**.**

Treatments comparison	Plant Height (cm)	Leaf dry weight /ha (ton)	Stems dry weight /ha (ton)	Biomass dry weight /ha (ton)
Control vs Elicitor				
Control	176.33 a	0.72 a	0.73 a	1.45 a
Elicitor	178.17 a	**0.87 b**	**0.96 b**	**1.83 b**
Control negative vs Control positive				
Control negative	176.33 a	0.72 a	0.73 a	1.45 a
Control positive	182.16 a	0.66 a	0.70 a	1.35 a
Control negative vs Filtrate				
Control negative	176.33 a	0,72 a	0.73 a	1.45 a
Filtrate	182.28 a	**0.84 b**	**0.95 b**	**1.78 b**
Control negative vs Mycelium				
Control negative	176.33 a	0.72 a	0.73 a	1.45 a
Mycelium	172.96 a	**1.00 b**	**1.11 b**	**2.11 b**
Control positive vs Filtrate				
Control positive	182.16 a	0.66 a	0.70 a	1.35 a
Filtrate	182.28 a	**0.84 b**	**0.95 b**	**1.78 b**
Control positive vs Mycelium				
Control positive	182.28 a	0.84 a	0.95 a	1.78 a
Mycelium	172.96 a	**1.00 b**	**1.11 b**	**2.11 b**
Filtrate *Colletotricum* vs Filtrate *Phytophthora*				
Filtrate *Colletotricum*	179.89 a	0.88 a	0.98 a	1.87 a
Filtrate *Phytophthora*	184.67 a	0.87 a	0.95 a	1.82 a
Colletotricum mycelium vs *Phytophthora* mycelium				
Colletotricum mycelium	170.45 a	1.01 a	1.02 a	1.99 a
Phytophthora mycelium	177.00 a	1.03 a	**1.20 b**	**2.23 b**
1% *Colletotricum* filtrate (v/v)	191.33 a	0.86 a	1.12 a	1.99 a
2% *Colletotricum* filtrate (v/v)	172.67 a	0,86 a	0.97 a	1.83 a
3% *Colletotricum* filtrate (v/v)	175.67 a	0.89 a	0.86 a	1.75 a
1% *Phytophthora* filtrate (v/v)	189.67 a	0,85 a	0.93 a	1.78 a
2% *Phytophthora* filtrate (v/v)	181.33 a	0,86 a	1.02 a	1.88 a
3% *Phytophthora* filtrate (v/v)	183.00 a	0,89 a	0.89 a	1.79 a
1% *Colletotricum* mycelium (w/v)	165.67 a	1.09 a	1.13 a	2.22 a
2% *Colletotricum* mycelium (w/v)	181.00 a	1.03 a	1.08 a	2.11 a
3% *Colletotricum* mycelium (w/v)	164.67 a	0.90 a	0.84 b	1.74 b
1% *Phytophthora* mycelium (w/v)	186.33 a	0.99 a	1.13 a	2.12 a
2% *Phytophthora* mycelium (w/v)	174.67 a	1.04 a	**1.28 a**	**2.32 a**
3% *Phytophthora* mycelium (w/v)	170.00 a	1.07 a	1.19 a	2.26 a

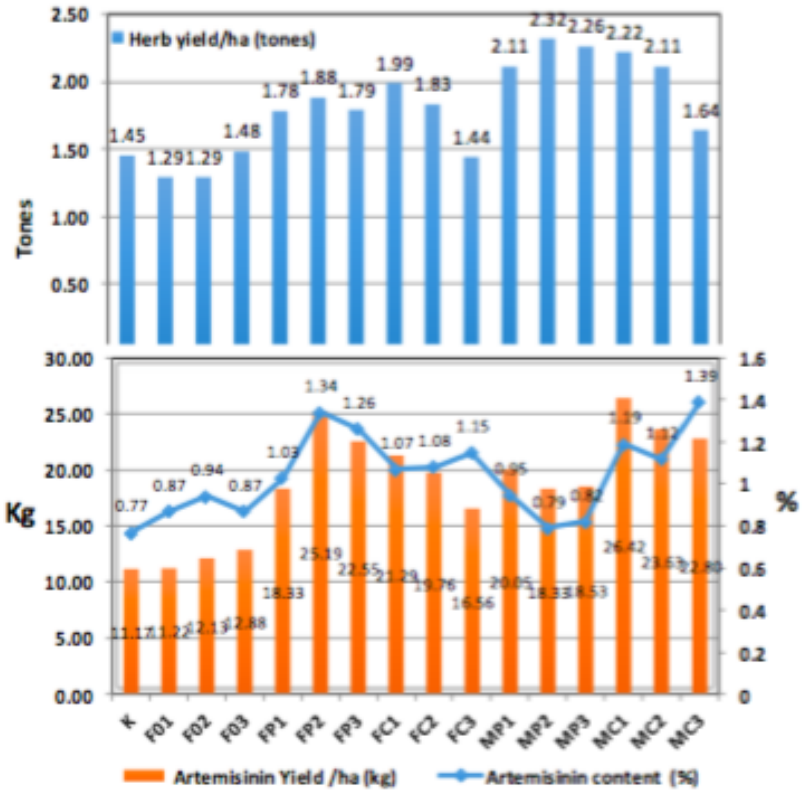
Note : Number followed by the same letters in the same column were not significantly different at 5% level of significance

3.2. Artemisinin production of artemisia

Gibberelin (GA) was diterpenoid compound and involved in plant defense response and plant growth. GA was synthesized following terpenoid pathway, the same pathway as artemisinin biosynthesis. Artemisinin was synthesized through farnesyl diphosphate (FPP), which was the first intermediary com-

pound. On the other hand, GA was produced from geranyl diphosphate (GGPP), which was condensed product from isopentenyl diphosphate (IPP) and geranyl diphosphate (GPP) [16]. Therefore, GA increase would enhance plant growth, represented by increase in biomass weight. The same pathway in biosynthesis of GA and artemisinin caused the competition in GA and artemisinin production within the plant. The increase in GA biosynthesis, indicated by high biomass production, would be followed by artemisinin content decline. As shown in Figure 1, filtrate treatments generated lower biomass weight (1.44 -1.99 ton/ha) than mycelium treatments (1.64-2.32 ton/ha). On the contrary, the low biomass weight (on filtrate treatments) was followed by higher artemisinin content (1.03%-1.34%) than mycelium treatments (0.95%-1.39%). The exogenous GA_3 application on artemisia increased artemisinin contents [16]. This was linked to the enhancement of key enzyme expression in biosynthesis pathway of artemisinin. Furthermore, exogenous GA continuously increased artemisinin content from vegetative phase until flower initiation and produced higher leaf weight than control plants. Consequently, artemisinin content of plants applied with exogenous GA was much higher than control plants.

Artemisinin yield related to biomass dry weight because it was the multiplication of biomass dry weight and artemisinin content. Generally, artemisinin content tended to be low on plants with high biomass dry weight. However, plants with high biomass dry weight was also able to produce high artemisinin content as indicated by *Colletotrichum* sp mycelium 1% treatment (MC1), resulted in high artemisinin yield (26.42 kg/ha) (Figure 1).



Note :

K		Control negative (water)
F01	:	Filtrate (un-inoculated medium) 1%
F02	:	Filtrate (un-inoculated medium) 2%
F03	:	Filtrate (un-inoculated medium) 3%
FP1	:	*Phytophthora* sp filtrate 1%
FP2	:	*Phytophthora* sp filtrate 2%
FP3	:	*Phytophthora* sp filtrate 3%
FC1	:	*Colletotrichum* sp filtrate 1%
FC2	:	*Colletotrichum* sp filtrate 2%
FC3	:	*Colletotrichum* sp filtrate 3%
MP1	:	*Phytophthora* sp mycelium 1%
MP2	:	*Phytophthora* sp mycelium 2%
MP3	:	*Phytophthora* sp mycelium 3%
MC1	:	*Colletotrichum* sp mycelium 1%
MC2	:	*Colletotrichum* sp mycelium 2%
MC3	:	*Colletotrichum* sp mycelium 3%

Figure 1. The application of *Colletotrichum sp.* and *Phytophthora sp* elicitor on biomass dry weight, artemisinin content and artemisinin yield of artemisia.

4. Conclusion

Artemisinin content of artemisia could be enhanced by biotic elicitor application derived from fungi. The type of elicitor was important because not all of elicitor type were well-responsed by plants. Elicitor derived from mycelium of *Phytophthora* sp at 2% concentration increased biomass dry weight by 60% compared to control negative. The highest artemisinin yield was produced by *Colletotrichum sp* powdery mycelium 1% treatments (MC1) (26.42 kg/ha), was 136.5% higher than control (K) (11.17 kg/ha).

References

[1] Pan WS, Zheng LP, Tian H, Li WY and Wang JW 2014 Transcriptome responses involved in artemisinin production in *Artemisia annua* L. under UV-B radiation. *J Photochem Photobiol B Biol* **40** 292–300. doi:10.1016/j.jphotobiol.2014.08.013.

[2] Ferreira JFS, Luthria DL, Sasaki T and Heyerick A 2010 Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* **15**:3135–70. doi:10.3390/molecules15053135.

[3] Hasugian AR, Tjitra E 2014 Artesunat-amodiakuin and klorokuin for vivaks malaria treatment in Puskesmas Kopeta, Maumere, Nusa Tenggara Timur, 2007 (in Bahasa) *Media Litbangkes* **24** 161–8.

[4] Devi S and Premkumar R 2012 Physicochemical analysis of groundwater samples near industrial area, Cuddalore District, Tamilnadu, India. *Int J ChemTech Res* **4** 29–34. doi:10.7904/2068.

[5] Zare Mehrjerdi M, Bihamta MR, Omidi M, Naghavi MR, Soltanloo H and Ranjbar M 2013 Effects of exogenous methyl jasmonate and 2-isopentenyladenine on artemisinin production and gene expression in *Artemisia annua*. *Turk J Botany* **37** 499–505. doi:10.3906/bot-1206-12.

[6] Ho WE, Peh HY, Chan TK and Wong WSF 2014 Artemisinins: Pharmacological actions beyond anti-malarial. *Pharmacol Ther* **142** 126–39. doi:10.1016/j.pharmthera.2013.12.001.

[7] Hussain MI, Reigosa MJ 2014 Evaluation of herbicide potential of sesquiterpene lactone and flavonoid: Impact on germination, seedling growth indices and root length in *Arabidopsis thaliana*. *Pakistan J Bot* **46** 995–1000.
[8] Aftab T, Ferreira JFS, Khan MMA and Naeem M 2014 *Artemisia annua* - Pharmacology and biotechnology doi:10.1007/978-3-642-41027-7.
[9] Gusmaini and Nurhayati H 2007 Potential of *Artemisia annua* L cultivation development in Indonesia (in Bahasa). *Perspective* **6** 57–67.
[10] Zhang B, Zou T, Lu YH and Wang JW 2010 Stimulation of artemisinin biosynthesis in *Artemisia annua* hairy roots by oligogalacturonides. *African J Biotechnol* **9** 3437–42.
[11] Vasconsuelo A and Boland R 2007 Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Sci* **172**:861–75. doi:10.1016/j.plantsci.2007.01.006.
[12] Wang JW, Zhang Z and Tan RX 2001 Stimulation of artemisinin production in *Artemisia annua* hairy roots by the elicitor from the endophytic *Colletotrichum* sp. *Biotechnol Lett* **23** 857–60. doi:10.1023/A:1010535001943.
[13] Mao J, Liu Q, Yang X, Long C, Zhao M, Zeng H, *et al* 2010 Purification and expression of a protein elicitor from *Alternaria tenuissima* and elicitor-mediated defence responses in tobacco. *Ann Appl Biol* **156** 411–20. doi:10.1111/j.1744-7348.2010.00398.x.
[14] Gadzovska-Simic S, Tusevski O, Antevski S, Atanasova-Pancevska N, Petreska J, Stefova M, *et al* 2012 Secondary metabolite production in *Hypericum perforatum* L. cell suspensions upon elicitation with fungal mycelia from *Aspergillus flavus*. *Arch Biol Sci* **64** 113–21. doi:10.2298/ABS1201113G.
[15] Albersheim P, Jones TM and English PD 1969 Biochemistry of the cell wall in relation to infective processes. *Annu Rev Phytopathol* **7**:171–94. doi:10.1146/annurev.py.07.090169.001131.
[16] Banyai W, Mii M and Supaibulwatana K 2011 Enhancement of artemisinin content and biomass in *Artemisia annua* by exogenous GA_3 treatment. *Plant Growth Regul* **63** 45–54. doi:10.1007/s10725-010-9510-9.
[17] Pu G Bin, Ma DM, Chen JL, Ma LQ, Wang H, Li GF, *et al* 2009 Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L. *Plant Cell Rep* **28** 1127–35. doi:10.1007/s00299-009-0713-3.
[18] Yoshikawa M, Matama M and Masago H 1981 Release of a soluble phytoalexin elicitor from mycelial walls of *Phytophthora megasperma* var. *sojae* by soybean tissues. *Plant Physiol* **67** 1032–5.

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