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Nitrogen Dosage and Application Time of Cytokinin on *Artemisia annua* L., a Traditional Antimalaria Herbal

Dosis Nitrogen dan Waktu Pemberian Sitokinin pada Tanaman Artemisia annua L., Herbal Anti Malaria

Usman Siswanto[1], Yuli Widiyastuti[2] dan Ruri Kustia[1]
[1]Department of Agroecotechnology University of Bengkulu
Jln. Raya Kandang Limun Bengkulu 38371A
[2]Medicinal Plant and Traditional Medicine Research and Development Office (B2P2TO2T)
Tawangmangu
usismanto@yahoo.com

ABSTRACT

Vegetative growth and artemisinin constituent may be stimulated by the application of nitrogen and cytokinin. This study aimed to determine the response of nitrogen fertilizer dosage and time of cytokinin application on growth and yield of *Artemisia annua* L. The research was undertaken at Kelobak Village, Kepahiang Regency, Bengkulu, from April to September 2009. The experiment used a Randomized Complete Block Design arranged factorially. The first factor was nitrogen fertilizer dosage, consisting of 0, 50, and 100 kg ha^{-1}; and the second one was time of cytokinin application, comprising 3, 6, 9 week after planting. Each treatment combination was replicated three times. Data were analyzed by analysis of variance and means were separated by orthogonal polynomial. Thin Layer Chromatography (TLC) was employed to trace artemisinin content of leaf. The results indicated that there was an interaction between nitrogen fertilizer dosage and time of cytokinin application on plant height and stem diameter. Nitrogen fertilizer dosage influenced both fresh weight and dry weight of upper plant part. Time of cytokinin application did not affect all observed variables. Nitrogen fertilization 60.27 and 62.49 kg ha^{-1} together with cytokinin application time 3 week after planting yielded both the highest plant height (102.23 cm) and the biggetst stem diameter (22.21 mm). Nitrogen fertilizer dosage 100 kg ha^{-1} resulted in heaviest fresh and dry weight of upper plant part, namely 421.23 g and 39.25 g, consecutively. Artemisinin was detected in leaf.

Key words: artemisinin, cytokinin, plant height, stem diameter, fresh weight

ABSTRAK

Peningkatan pertumbuhan vegetatif dan kandungan senyawa artemisinin dapat dipacu dengan pemberian nitrogen dan sitokinin. Penelitian ini bertujuan untuk mendapatkan dosis pupuk nitrogen dan waktu pemberian sitokinin terhadap pertumbuhan dan hasil *Artemisia annua* L. Penelitian dilaksanakan di Desa Kelobak, Kabupaten Kepahiang, Provinsi Bengkulu mulai bulan April sampai bulan September 2009. Percobaan menggunakan Rancangan Acak Kelompok Lengkap yang disusun secara faktorial. Faktor pertama adalah dosis pupuk nitrogen yang terdiri dari 0, 50, dan 100 kg ha^{-1}; dan faktor kedua adalah waktu pemberian sitokinin yang terdiri dari 3, 6, dan 9 minggu setelah tanam. Setiap kombinasi perlakuan diulang sebanyak tiga kali. Data dianalisis dengan analisis sidik ragam dan rata-rata dipisahkan dengan orthogonal polinomial. Untuk melacak keberadaan senyawa artemisinin digunakan Kromatografi Lapis Tipis (KLT). Hasil menunjukkan terdapat interaksi antara dosis pupuk nitrogen dengan waktu pemberian sitokinin terhadap tinggi tanaman dan diameter batang. Pupuk nitrogen berpengaruh terhadap bobot segar dan bobot kering bagian atas tanaman. Waktu pemberian sitokinin tidak memengaruhi semua parameter yang diamati. Pupuk nitrogen masing-masing 60,27 dan 62,49 kg ha^{-1} dengan pemberian sitokinin tiga minggu setelah tanam berturut-turut menghasilkan tanaman tertinggi (102,23 cm) dan diameter batang terbesar (22,21 mm). Dosis pupuk nitrogen 100 kg ha^{-1} menghasilkan bobot segar dan bobot kering bagian atas tanaman, masing-masing 421,23 g dan 39,25 g. Artemisinin terdeteksi dalam daun tanaman Artemisia.

Kata kunci: artemisinin, sitokinin, tinggi tanaman, diameter batang, bobot segar

INTRODUCTION

Malaria is a global health issue that will continue to spread with the increase in mosquito populations caused by the rise in global temperatures. Malaria is a major health problem in many parts of the world, especially Africa, South America and Southeast Asia. Between 300 and 500 million people are currently infected with malaria, with more than two billion at risk for infection. Experts project a continued increase in these numbers. Two million people die annually from malaria, including one million children. In Indonesia, 56.3 million people out of 70.0 million population living in endemic areas are at high risk of malaria. In 2003, malaria spread out in 30 Provinces covering 6,053 villages in which the highest mortality rate went to under five-year children (Depkes, 2007; Gunawan 2000). Latest report indicated that there are 424 regencies as malarial endemic regions. Malaria is an infectious disease causing a serious and complex predicament due to ineffective medication against resistant strain of *Plasmodium* parasite. Cerebral malaria, caused by *Plasmodium falciparum,* is the most deadly form of malaria, is particularly difficult to treat and results in high mortality. The available drug, however, is slow acting, and resistance to chloroquine and other currently used antimalarial drugs is widespread and increasing.

Artemisinin, derived from *Artemisia annua* (family Asteraceae), which is used as an antimalarial in traditional Chinese medicine, has been found effective as an antimalarial drug (Gupta, 2005).

In the intact *A. annua* plant, artemisinin is synthesized from the mevalonate-terpenoid pathway in the glandular trichomes of the leaves, and artemisinin levels are highest in the shoots during the flowering stage One study investigating the distribution of artemisinin in five-week old *A. annua* plants found that the highest artemisinin content was found in the leaves of the upper parts of the plant (Shukla *et al.,* 1992).

Artemisinin (*qinghaosu*) is a sesquiterpene lactone of the cadinane series. In addition to a lactone group, artemisinin contains an endoperoxide bridge, which is rarely found in secondary metabolites, and is responsible for the antimalarial and anti-cancer activity. Complete chemical (*de novo*) synthesis of artemisinin was achieved by several research groups (Avery *et al.,* 1992). The procedures require several steps, and can start from different raw materials. A comprehensive review on the chemistry, synthesis and semisynthesis of artemisinin has been conducted. However, low yield, complexity and high cost indicate that the isolation of artemisinin from the plant is the most economically feasible method for its production at present (Zipper *et al.,* 1997).

Artemisinin is the raw material needed to manufacture antimalarial drugs such as dihydroartemisinin, arteether, artemether and artesunate. The flowering stimulus appears to be perceived at the apical meristem, and that flowering could be somewhat delayed by pinching the apical meristems and providing nitrogen fertilization. Very little published work exists on the vegetative growth responses of *A. annua* to the specific macronutrients nitrogen, phosphorus and potassium or of their effects on the concentration of artemisinin and related compounds (Ferreira and Janick, 1995).

Significant increase of total plant and leaf dry matter (1–3 ton ha^{-1}) was obtained in Mississippi, USA, where a complete fertilizer mixture containing 100 kg N, 100 kg P and 100 kg K ha^{-1} was broadcast and worked uniformly through the soil. Similarly in Tasmania, Australia, dry leaf yields of 6–12 ton ha^{-1} were obtained in experiments with a mixed fertilizer containing 60 kg N, 60 kg P and 50 kg K ha^{-1} pre-drilled in bands 150 mm apart and about 50mm below seed and 75mm below transplants (Laughlin *et al.,* 2002).

The concentration of artemisinin peaks around the time of flowering, although in some cases this may be just before flowering, and in other cases during full flowering(Ferreira dan Janick, 1995; Laughlin *et al.,* 2002). Field production of *A. annua* is presently the only commercially viable method to produce artemisinin because the synthesis of the complex molecule is uneconomic. Currently used selections reach the peak in artemisinin before flowering and at the end of vegetative growth, allowing maximal biomass accumulation of artemisinin before harvest (erreira and Janick, 2009).

Table 1. Summary of Fisher's-Test at Pd"0.05 for the effect of nitrogen fertilizer dosage and cytokinin application time on plant height (cm), stem diameter (mm), number of bud, chlorophyll content, leaf fresh weight (g), root fresh weight (g), leaf dry weight (g), root dry weight (g).

Components of growth and yield	Nitrogen (N)	Cytokinin (T)	N x T
Plant height (cm)	ns	ns	*
Stem diameter (mm)	ns	ns	*
Chlorophyll content	ns	ns	ns
Leaf fresh weight (g)	*	ns	ns
Leaf dry weight (g)	*	ns	ns

Note : *significant; nsnon significant.

The U.S. Agency for International Development is working in partnership with the WHO to increase artemisia cultivation, especially in Africa, and there is a pressing need to optimize artemisinin production per cultivated area, which will help meet the world demand of artemisinin-based combination therapies (ACTs) without intruding into areas originally cultivated with subsistence crops used to sustain a minimal nutritional level in African countries afflicted by malaria. However, there is little published information on individual nutrient requirements for growth and increased artemisinin production (Ferreira *et al.*, 2005).

Cytokinins are an important class of plant growth regulators, defined by their ability to promote cell division in tissue culture in the presence of auxins. Virtually all naturally occurring cytokinins identified to date are adenine species substituted at N6 with an isoprenoid or aromatic side chain. Cytokinins refer solely to the isoprenoid cytokinin bases and their sugar conjugates. Cytokinins affect many plant developmental processes including cell division, cell differentiation, chlorophyll senescence, and apical dominance (Astot *et al.*, 2000). Although exogenous application of cytokinins is mainly known for induction of spontaneous shoot formation in hairy root cultures, they can also impact growth and secondary metabolite accumulation. Indeed, low cytokinin to auxin levels have been shown to induce rapid disorganization in hairy root cultures of C. intybus as well as decrease root growth and the ability of root cultures of *C. intybus* to produce secondary products (Bais *et al.*, 2001; Weather *et al.*, 2005). Therefore, the objective of this study was to determine the response of nitrogen fertilizer and time of cytokinin application on growth and yield of *Artemisia annua* L.

MATERIALS AND METHODS

The research was undertaken at Kelobak village, Kepahiang regency, Bengkulu province, from April to September 2009. The experiment used Randomized Complete Block Design arranged factorially. The first factor was nitrogen fertilizer dosage consisting of 0, 50, 100 kg ha^{-1}; and the second one was time of cytokinin application comprising 3, 6, 9 week after planting. Each treatment combination was replicated three times. Data were analyzed by analysis of variance and means were separated by orthogonal polynomial. Thin Layer Chromatography (TLC) was employed to trace artemisinin content of leaf.

RESULTS AND DISCUSSION

Enhancing the production of artemisinin in *A. annua* has been a goal for many research groups. The low yield of artemisinin within the *A. annua* plant is a serious constraint on the mass production of artemisinin-based drugs. Artemisinin can also be chemically synthesized; however, its production is complicated and unfavorable due to very poor yields and extremely high costs. Therefore, increasing yield in intact plants is a more cost-effective production method compared to commercial synthesis. The following table is summarizing the result of Fisher's test indicating the effect of nitrogen and cytokinin application on *A. annua* growth. In the intact *A. annua* plant, artemisinin is synthesized from the mevalonate-terpenoid pathway in the glandular trichomes of the leaves, and artemisinin levels are highest in the shoots during the flowering stage. One study investigating the distribution of artemisinin in five-week old *A. annua* plants found that the highest

artemisinin content was found in the leaves of the upper parts of the plant (Shukla *et al.,* 1992; Jaziri *et al.,* 1993).

Table 2. Mean of chlorophyll content

Treatment (kg ha^{-1}, wap*)	Chlorophyll content
0.3	3.78
0.6	3.57
0.9	3.98
50.3	3.27
50.6	3.46
50.9	3.66
100.3	3.39
100.6	3.79
100.9	3.58

Note : *wap: week after plant

Results indicated there was an interaction between nitrogen fertilizer dosage and time of cytokinin application on plant height and stem diameter. Nitrogen fertilizer dosage influenced both fresh weight and dry weight of upper plant part. Time of cytokinin application did not affect all observed variables.

There is some work on the response of *A. annua* to nitrogen under field conditions, and phosphorus and potassium in tissue culture. This technique, if applied early enough, will cause plants to branch out and potentially increase leaf biomass if the season is 4–5 months long (Ferreira dan Janick, 1992). Increasing N application did not significantly affect artemisinin content or yield. In Madagascar, a field crop with three plants m^{-2} that received 97 kg ha^{-1} of N increased dry leaf production from 2420 (control) to 4690 kg ha^{-1}, while the concentration of artemisinin dropped from 1.11 to 0.87%. Also, a mean 4.7 ton ha^{-1} increase (19%) in total fresh plant biomass, cultivated in densities varying from 27.8 to 111.1 thousand plants/ha, was reported with the addition of 67 kg ha^{-1} N, but the artemisinin concentration was not reported, and the total fresh plant biomass was reduced from 750 (at the lower density) to 275 g $plant^{-1}$ (at the higher density) (Ayanoglu *et al.,* 2002; Simon *et al.,* 1990).

One of the responses of plants to cytokinins is stimulation of shoot growth. Considering that this enzyme may be a rate-limiting step in cytokinin biosynthesis in tumorous plant tissues, overexpression of the isopentenyl transferase gene (ipt) gene should elevate cytokinin levels in plants thereby yielding more shooty tissue and possibly artemisinin. Constitutive expression of the ipt gene in *A. annua* was tested to gain a better understanding of the relationship between chlorophyll and cytokinins, and cytokinin function in growth, development, and production of artemisinin. This work suggested a direct correlation between shoot growth (chlorophyll content), cytokinins, and artemisinin (Sa *et al.*, 2001; Pamela *et al.*, 2006). The increase in plant growth with the addition of cytokinin canbe explained by the hormone's ability to trigger growth factors and activate the defense pathway within the plant. Because of this, one of the immediate effects of cytokinin treatment on *A. annua* is an increase in shoot growth and height.

CONCLUSION

Both the highest plant height (102.23 cm) and the biggest stem diameter (22.21 mm) were acquired by nitrogen fertilization 60.27 and 62.49 kg ha^{-1} combined with cytokinin application 3 week after planting. Nitrogen fertilizer at 100 kg ha^{-1} resulted in heaviest fresh and dry weight of upper plant part, namely 421.23 g and 39.25 g, consecutively. Qualitatively, artemisinin was detected in leaf.

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