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Centelloside content of cultivated pegagan (*Centella asiatica*) with application of phosphorus fertilizer

N S Vinolina

Department of Agrotechnology, University of Sisingamangaraja XII, North Sumatera, Indonesia

Email: noveritasitumorang@yahoo.com

Mobile number of corresponding author: +628126052933

Abstract. *Centella asiatica* was one of the wild plants that widely exploited from nature. The objective of this study was to obtain potential plant material that could be used as seeds with high centelloside content and obtain plant responses to biomass and centelloside production on application of phosphorus fertilizer. This research conducted at experimental field was used Non Factorial Randomized Block design, single factor, with six levels of P₂O₅ fertilizer (0, 10, 20, 30, 40, 50 kg P₂O₅/ha) with 5 replication to determine the treatment effect on the content of centelloside, with harvest time 84 DAP (day after planting). If the treatment showed a significant effect on the observed variables then continued with the DMRT test. The plant material was used Pegagan Deli Serdang accession. Previous research found that the centelloside pattern (asiaticoside, madecassoside and asiatic acid) influenced by the condition of media and phosphorus content of media. The results show that at lowland of North Sumatera, phosphorus fertilizer dose level did not affect significantly on vegetative growth of *Centella asiatica* but affect the pattern of centelloside. Phosphorus treatment gave the highest production of asiaticoside in leaves was on the phosphorus treatment of 50 kg/ha.

1. Introduction

This study discovered that the content of centelloside in pegagan plant was very useful to human life for various health problems. Because the benefits of *Centella asiatica*, it was necessary to have pegagan can be used as food as vegetable, and in some areas in Indonesia have been used as fresh food or eaten fresh. Some previous research that has been done that phosphorus, harvest time and methyl jasmonate will affect the content of pegagan's centelloside. This research will help researchers to find the role of phosphorus that had not been explored by many researchers in the biosynthesis of centelloside remains unclear. Thus a new theory of centelloside biosynthesis can arrive at a clearer stage. Thus, a new theory about the role of this phosphorus micronutrient, and possibly other combinations, may be more revealing about the response of the pegagan plant, especially to centelloside biosynthesis of the amount of each bioactive produced.

Basically, knowledge on medicinal herbs in Indonesia was originated from hereditary knowledge from generation to generation. Common peoples was not familiar with medicinal herbs and their use as medicine [25, 26]. Efforts has been conducted to conserve medicinal herbs In neighboring countries such as Japan, China, Taiwan, Hong Kong, Korea and other Eastern countries. Japan pays attention to the sustainability of medicinal and aromatic plants and seeks for sustainable harvesting. One of the wild plants that are widely exploited from nature was *Centella asiatica*. Japan imports medicinal and



aromatic plants from China and India. China was the largest exporter of medicinal and aromatic plants [1].

Pegagan was still categorized as wild plants that had not been domesticated. Some known chemical ingredients, among others contain several saponin compounds, including asiaticoside [17]. The asiaticoside bioactive compound could accelerate wound healing process and useful for leprosy and tuberculosis treatment [15, 16]. *Centella asiatica* was cooling, had the function of cleaning the blood, blood circulation, diuretics, antipyretic, stop bleeding (haemostatic), improve the nerve memory, antibacterial, tonic, antispasmodic, anti-inflammatory, hypotension, insecticide, allergy and stimulant [7]. Saponins could also inhibit the production of excessive scar tissue (inhibits the occurrence of keloids) [15].

Demand for simplicia derived from wild plants could make these plants scarce and even endangered. Presently, pegagan was still harvested from nature. Large scale development of pegagan could be conducted by cultivation efforts and high quality of pegagan could be obtained using high quality plant material [4, 8, 25, 26]. Plant material was important to produce a simplicia which had high content of centelloside [25, 26].

The need for pegagan (*Centella asiatica*) reached 100 tons, PT. Sidomuncul need around 2-3 tons/month. The need for *Centella asiatica* at local factories reaches 25 tons per year, but can be supplied only 4 tons per year. In agribusiness perspectives, pegagan was promising commodity due to opening biopharmaceutical business opportunity where the demand was increasing every year for domestic needs and export [3, 8].

The objective of the study was to obtain potential plant material that could be used as seeds with high centellocide content.

2. Materials And Methods

This research was conducted at experimental field of Agricultural Faculty of Sisingamangaraja XII University Medan, Jamin Ginting Street, Medan at altitude 52 m above sea level, from January to August 2017. Soil type was Andosol located on N 3°31'22.20" and E 98°37'39.69". This research was used Non Factorial Randomized Block design, single factor, with six levels of P₂O₅ fertilizer (0, 10, 20, 30, 40, 50 kg P₂O₅/ha) with 5 replication, if the treatment showed a significant effect on the observed variables then continued with the Duncan's Multiple Range Test (DMRT).

2.1. Research Implementation

The plant material was used Pegagan accession of Deli Serdang. The planting space during preparation of plant material to obtain one stolon was 40 cm x 40 cm. The activities were started with soil sampling for soil chemical analysis in PPKS of North Sumatra. The activity continued with land clearing of weeds and soil cultivation. Furthermore, as much as 30 plots, each 1.0 m x 1.0 m in size, at an area of 100 m² were constructed. Distance among blocks was 50 cm, distance among main plot was 50 cm and distance among plot was 50 cm.

Fertilization was done when planting with P dose according to treatment, one third dose of Urea 300 kg/ha and KCl dose 220 kg/ha. Fertilizer was applied to the array around the planting hole. Urea fertilization was performed again at 20 and 40 days after planting (DAP), each one-third dose. Maintenance was performed by watering, replanting and weeding. Watering was conducted in the morning and afternoon, adjusted to the field conditions. Replanting was done 2 weeks after planting using seeds provided separately. Weeding was done every day manually that was by directly pulling by hand.

Harvest was done at once in accordance with the treatment that was at 12 DAP by dismantling all parts of the plant. Before harvesting, the soil was watered first to facilitate the dismantling of plants and no roots are left in the growing media. Observations begin at the first 1 to 12th by sampling 1 plant per plot, include wet weight, dry weight, leaf wet weight, leaf dry weight, wet weight of roots and tendrils, dry weight of roots and tendrils. Observations are made weekly.

Test of centelloside content of pegagan leaf which includes asiaticoside, made cassoside, asiatic acid was performed after the material collected to know the accumulation stage of asiaticoside, made cassoside, asiatic acid at the top (leaf) and bottom (root) [14]. Stages of analysis of asiaticoside, conducted in the Pharmacy Laboratory of USU (Figure 1).

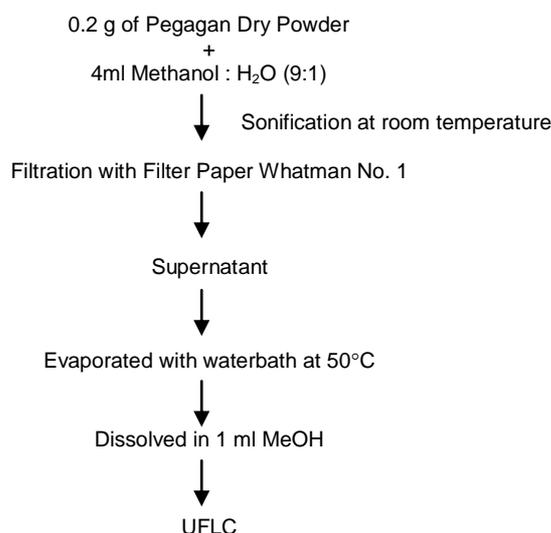


Figure 1. Working steps for asiaticoside, made cassoside and asiatic acid test with UFLC

Steps that must be done as follows: Start with 0.2 g dry powder, add 4 ml of 90% methanol (90 methanol : 10 water). Thoroughly mix and place on shaker for at least 5 hours, filter was used Whatman number 4 filter paper. Filtrate could be stored at -20°C , evaporate liquid under fume hood at 50°C . Weight 10 mg dry dark brown material and mix with 1 ml 90% methanol. It should completely dissolve. Filter the mixture using 0.45 μm filter adapter. Store the filtrate for HPLC analysis; would used 20 μl for HPLC injections.

3. Results and discussion

3.1. Production of Centelloside

Average data of centelloside production (mg) was showed at Table 1.

Table 1. Average production of pegagan centelloside at phosphorus treatments

Treatment	Centelloside (mg)					
	PML	PAAL	PAL	PMR	PAAR	PAR
P ₀	248.98	2482.50	63.47	400.59	70.90	120.53
P ₁	356.96	2634.69	84.99	665.53	74.74	153.04
P ₂	152.29	2092.81	47.1	164.93	33.32	59.98
P ₃	287.24	3523.06	76.21	388.90	73.89	143.89
P ₄	382.40	2669.15	137.66	1080.62	63.77	162.98
P ₅	370.85	3768.48	146.09	546.22	91.04	148.97

Note: Production of leaf asiaticoside (PAL), Production of leaf made cassoside (PML), Production of leaf asiatic acid (PAAL), Production of root asiaticoside (PAR), Production of root made cassoside (PMR), Production of root asiatic acid (PAAR) (mg)

3.2. Effect of Phosphorus Fertilization Treatment on Pegagan Production

Phosphorus treatment had no significant effect on observed parameters such as wet weight (g) and dry weight (g) either on leaf, petiole, roots and tendrils. However, the highest average of pegagan production found at phosphorus treatment 50 kg P₂O₅/ha (P₅). Soil analysis test before phosphorus application show that the available phosphorus content (P-av) in soil was 14.48 ppm, that was moderate according to PPKS assessment criteria. Available phosphorus in the soil was supported by high organic material C/N = 10 and neutral soil pH 5.8 as well as intensive care due to maintenance, causing the plant still capable to absorb phosphorus that was still available in the soil for growth and development of *Centella asiatica*. It was suspected that andosol soil fertility, both chemical and biological, in the study area was stable. Such condition provide very rapid growth and development of the plant at the beginning and end as well as increased root activity that led to high absorption of available nutrient solution in the soil and phosphorus fertilization and consequently the process photosynthesis increased to support the formation and number of stolons.

Phosphorus application had no significant effect on observed production parameters. It was suspected that the applied phosphorus was not yet optimally absorbed by the plant at the dosage level and the other possibility that the available phosphorus in the soil was sufficient for the growth of *Centella asiatica*, plus the availability of organic matter in the soil. Available phosphorus content in the soil was still able to be used by plant roots to support its growth. The initial soil analysis shows that soil pH (5.80) was neutral. The availability of inorganic phosphorus fertilizers was largely determined by soil pH and organic matter decomposition, as well as microorganism activity [19,23]. Thus the availability of phosphorus given through fertilization did not have visible effect.

Field research on the cultivation of *Centella asiatica* was very limited, so the reference articles that could be used very limited. However, the researchers tried to explain. Research was conducted by previous researchers also obtained the same thing, phosphorus fertilization had no significant effect on all variables of *Centella asiatica* plant growth [21]. Phosphorus fertilization had no significant effect on the number of primary vines, secondary vines, fresh weight and dried biomass, and tissue phosphorus content in *Centella asiatica* [20]. Although phosphorus application generally did not have a significant effect but there was a tendency for an upward trend to increase the yield of phosphorus doses given to the plants. The tendency that fertilized plants give higher yields than plants without fertilization. Parameters of wet weight and dry weight of leaves and petioles or roots and tendrils, the best results were obtained on phosphorus at 50 kg P₂O₅/ ha. The availability of sufficient nutrients will support plant growth [25]. Phosphorus was one of the macro nutrients needed by plants, which play an important role in various life processes, such as photosynthesis, carbohydrate metabolism, and energy flow processes in plants [19, 23].

The production of *Centella asiatica* centelloside, the phosphorus treatment gave the highest production of asiatic acid and asiaticoside to the highest in leaves was on the phosphorus treatment of 50 kg/ha (Table 5). While madecassoside production both in leaves and roots, the highest was in application of phosphorus 40 kg/ha. The highest production of asiatic acid in root was found in phosphorus treatment of 50 kg/ha while the highest asiaticoside in root was found in phosphorus 40 kg/ha. Application of phosphorus affect the content of centelloside. This was supported in a previous study that the soil P content affected centelloside levels of *Centella asiatica* [25]. Phosphorus functions in stimulating the formation of better roots so that the absorption of nutrients and water increases, the increase in the amount of leaf chlorophyll (the amount of leaf chlorophyll increased with the increase of phosphorus), the plant could photosynthesize well to produce photosynthate, and was suspected to increase the content of asiaticoside compounds. Phosphorus was never reduced in plants, still as phosphate either in free form or bound to organic compounds as esters. Ester phosphates were formed with sugars, alcohols, acids, or other phosphates (polyphosphates).

Centelloside production, either leaf asiaticoside (PAL), leaf made cassoside (PML), leaf asiatic acid (PAAL), root asiaticoside (PAR), root made cassoside (PMR), root asiatic acid (PAAR) was showed in Table 5. The dynamics of centelloside production between asiaticoside, made cassoside and

asiatic acid was different on leaves and roots. Acidic acid of tendril root was lower than leaf petiole but made cassoside and asiaticoside content was higher at tendril root.

Centelloside biosynthetic pathway could be seen in Figure below where the last step of the centelloside biosynthetic pathway was not known clearly so it is necessary to do various research to know the response of *Centella asiatica* in the content and also production of centelloside was very useful. Centelloside (asiaticoside, madecassoside, asiatic acid and madecasic acid) is a type of ursane saponin [2, 5, 15, 16].

Previous researchers, they had found several related genes in the triterpenes saponin biosynthesis pathway in *Centella asiatica* such as β -amryn synthase (CabAS), cycloartenol synthase (CaCYS), squalene synthase (CaSQS) and farnesyldiphosphate synthase. Farnesyldiphosphate synthase (FPS) plays an important role in the development of organs in plants. Farnesyldiphosphate synthase was identified as a key regulatory enzyme in triterpenine biosynthesis [11]. This biosynthesis was thought to take place in leaves where the asiaticoside content increases with time [16].

Phosphorus was also an essential part of various sugar phosphates that play a role in the reactions in the dark phases of photosynthesis, respiration, and other metabolic processes. Phosphorus was also a part of nucleotides (RNA and DNA) and membrane composite phospholipids. In addition, phosphorus acts as a constituent of metabolites and complex compounds, activators, cofactors or enzyme constituents, and plays a role in the physiological process. Phosphorous nutrients play an important role in the storage and transfer of energy. Phosphorus elements can increase the production of plants or dry matter and improve yield quality [18]. Level of mRNA CabAS (*C. asiatica*, β -amyrinsynthase) in the leaf reached its peak at 2-3 weeks and decreased after 4 weeks, but the leaf's asiaticoside content increased over time [11].

Pegagan (*Centella asiatica*) was the time to be cultivated because of many herbal concoctions that contain pegagan herbs [3]. The need for *Centella asiatica* reaches 100 tons, PT. Sidomuncul reaches 2 - 3 tons / month. The obstacles faced by the Indonesian herbal medicine industry (agromedicine) are the cultivation of plants, the problem of material quality uniformity which impacts on different product quality, production process, research and product development and marketing [8, 21].

In previous exploratory research, the asiaticoside content of pegagan accession were tested as follows: accession of Deli Serdang (2.38%), Kabanjahe (1.43%), Medan (1.38%), Berastagi(1.38%), Samosir with shade (0.28%) and accession Samosir without shade (0.24%) [25]. Based on the results of the survey, the highest content of asiaticoside found in lowland, Deli Serdang accession. In this research, there was a correlation of phosphorus (P) element to asiaticoside. Result of soil chemical analysis, P content in Deli Serdang soil was 31,30 ppm (very high), Medan was 15,60 ppm (medium), Kabanjahe was 14,25 ppm (medium), Samosir 9,97 was ppm (medium), and Berastagi was 3.03 ppm (low) [25]. This was associated with energy-rich phosphate compounds mediating the energy transfer phosphorylation in the process of plant organ growth and in producing secondary metabolites [12].

Increased availability of P could be cultivated with the application of P_2O_5 fertilizer. The other researchers that in the highlands, P fertilizer application could decrease the length of the flower stem, increase the leaf color, the weight of the petiole, the leaf tendrils, the harvest weight, and the asiaticoside content [8]. The highest harvest weight was obtained at the treatment of 72 kg P_2O_5 / ha, while the highest asiaticoside content was obtained at 36 kg P_2O_5 / ha. In lowland areas with Latosol soil type, P fertilization could decrease the number of leaves, length of tendrils and the length of the flower stem, but increase the length of the stem in pegagan age 2 months and increase leaf tendrils but not affect leaf color. The highest harvest weight was obtained at the treatment of 108 kg P_2O_5 / ha, while the highest asiaticoside content was obtained at 36 kg P_2O_5 / ha [21].

3.3. Phosphorus Mechanism in Increasing Bioactive Content

Phosphorus functions as stimulating the formation of better roots for nutrient and water absorption, an increase in the amount of chlorophyll, could photosynthesize well to produce photosynthate, so that the energy-rich compounds absorbed by the roots transported through the xylem to the canopy allegedly increase the asiaticoside content. Phosphorus was never reduced in plants and remains as

phosphate (both free and bound) to organic compounds as esters [19]. Esther phosphates were formed with sugars, alcohols, acids, or other phosphates (polyphosphates). The energy-rich compound may be thought to be a pentosa phosphate trace intermediate of the primary metabolite and derived from the precursor to the secondary metabolite. Pegagan plants contain the most compounds of the thyroid genome. Triterpenoids were derived from primary metabolite precursors biosynthesized by the path of mevanolate, resulting in a geranyl-geranyl pyrophosphate was the primary metabolite that forms monoterpenoids and their derivatives, whereas farnesyl pyrophosphate increases the formation of sesquiterpenoids and the conversion of squalene to triterpenoids and steroids. While the pyrophosphate geranyl becomes the precursor of the dihydride and carotenoids [24].

The asiatic acid ($C_{30}H_{48}O_5$) had the characteristics of triterpenoidsaponin which was also present in *Centella asiatica*. Given the benefits, some countries had cultivated, such as Hawaii. Even in Oregon, USA, the plant was cultivated in greenhouses by Pacific Botanicals, an organic herb farm. However, most of the market supply comes from India whose quality was less good and usually brownish. The ingredients of active ingredients were still quite good if processed in fresh or fresh condition. The benefits of asiatic acid had been studied, among others, in malignant glioma was one of the most damaging and incurable tumors. According to research that asiatic acid suggested its usefulness against malignant glioma [10] and there was an effects of asiatic acid on spatial working memory and cell proliferation in the adult rat hippocampus [22].

4. Conclusion

Phosphorus treatment gave the highest production of asiatic acid and asiticoside in leaves was on the phosphorus treatment of 50 kg / ha while madecassoside production both in leaves and roots, the highest was in application of phosphorus 40 kg / ha.

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