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Growth and Secondary Metabolites Production of *Centella asiatica* (L.) Urb. Cultivated at Different Phosphate Application Rates in Acid Soil

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Abstract

Centella asiatica (L.) Urb. is a widely known medicinal plant for dermatological and non-dermatological diseases. The demand for *C. asiatica* extracts continues to increase but its availability is markedly depleted due to exploitation and limited cultivation. This study was conducted to evaluate the phosphate growth responses and variation of secondary metabolites production of *C. asiatica* cultivated at different phosphate application rates in acid soil in Indonesia. The soil pH used in this study was 2.76. Rates of phosphate application were 0 kg per plant as control plant, 20, 40, 60, 80 and 100 kg ha^{-1}, respectively. Results showed that the plants applied with 40 kg ha^{-1} phosphate resulted in the highest number of leaves, petiole length, total leaf area, and number of tendrils and stolons which lead to the higher biomass. *C. asiatica* produced various types of secondary metabolites, i.e asiaticoside, madecassoside, asiatic acid, saponin, tannin, phenolic, flavonoid, triterpenoid, steroid, and glycoside. The production of secondary metabolites was optimized by the application of 20 kg ha^{-1} phosphate. It can be concluded that the application low dosage of phosphate can enhance the growth and secondary metabolites production of *C. asiatica* cultivated in acid soil.

Keywords: Medicinal plant, Phytochemical, Asiaticoside, Madecassoside, Asiatic acid

Introduction

Centella asiatica (L.) Urb. is a perennial herbaceous plant found in swampy areas of tropical and subtropical countries. *C. asiatica* is a native plant in India, China, Nepal, Indonesia, Sri Lanka, Australia, and Southern and Central Africa [1]. *C. asiatica* is commonly known as *pegagan* in Indonesia, *mandukparni* or Indian pennywort or *jalbrahmi* in India, and *gotu kola* in China. *C. asiatica* has been used as a treatment for thousands of years and continues to draw wide attention for the benefits in the treatment of mild and chronic diseases [2].

Triterpenoid centellosides contents in *C. asiatica* i.e asiatic acid, asiaticoside, madecassic acid, and madecassoside have been reported to have many benefits. *C. asiatica* is especially used for dermatological and non-dermatological conditions. *C. asitica* healing small wounds, scratches, burns, hypertrophic wounds, and eczema [3,4], smoothing and improving skin condition [5]. *C. asitica* is also recommended for chronic venous insufficiency [6]; mental disability [7]; antinociceptive, antipyretic antibacterial, antiviral, anti-inflammatory [8]; and for improving cognition, relieving anxiety and anti-cancer agent [9].

The demand for *C. asiatica* extract is strongly grown due to its versatile medicinal properties. However, the wild stock of *C. asiatica* has been markedly depleted due to large scale and unrestricted exploitation with very limited cultivation [10]. Halimi [11] reported that *C. asiatica* can be grown in lowland, midland, and highland in Indonesia. However, *C. asiatica* required sufficient nutrient availability for obtaining high yield and maintaining the quality of its active principles [12].

Phosphorus (P) is one of the macronutrients required in large volumes by the plant. P involves many plant processes which affecting plant growth and physiology. P fulfill a structural and regulatory function in plant metabolism, energy transfer, the transformation of sugars and starches, and nutrient movement [13]. The supply of phosphate should be adequate with the demand of plant [14]. The deficiency of phosphate inhibits plant growth and reduces the quality and quantity of yield. Malhotra *et al.* [15]

estimated that the deficiency of the P element in soil reduces 30 - 40 % of crop yields in the world. While excessive application of phosphate will decrease the nutrient use efficiency and increase the risk of nutrient losses from soil to water resources and the atmosphere which harmful for the environment [16,17]. The previous study of reported that Deli Serdang accession of C. asiatica required 30.22 kg P_2O_5 ha^{-1} for obtaining optimum growth [18]. However, the requirement of external phosphorus application is depending on the P availability in soil solution. In acid soil, the P element is deficient because soluble inorganic P is fixed by Iron (Fe) and aluminum (Al) [19,20]. Therefore, the addition of P in form of phosphate fertilizer is necessary for acid soil. Thus, the present study was conducted concentrating the phosphate application rate on growth responses and secondary metabolites production of *C. asiatica* cultivated at acid soil in Indonesia.

Materials and methods

Plant material and study area

C. asiatica used in this experiment was Samosir accession. This accession commonly found in field and paddy field and characterized by kidney-shaped leaves and jagged edges. The study was conducted at an open field in Salaon Toba, Ronggur Nihuta, North Sumatra, Indonesia (2°19'47.784"N, 99°2'40.092"E). The altitude was 1025 m above sea level. The average temperature was 18 °C (night) and 25 °C (day) and 80 % of relative humidity. The soil in study area contained 0.44 % N, 0.21 % P, and 0.08 % K. Soil pH was 2.76 and cation exchange capacity (CEC) of 14.86 me 100 g^{-1} (**Table 1**). According to the rating suggested by Cottenie [21], the soil was extremely acidic with low cation exchange capacity and very low C/N and very low availability of P and K.

Table 1 Soil characteristics of the study area.

Parameters	Value
pH	2.76
N	0.44 %
Cation exchange capacity	14.86 me/100 g
P	0.21 %
K	0.08 %
C/N	6.21

Research procedures

The seedling of *C. asiatica* vegetatively propagated using rooted stolon with 3 leaves. The seedling of *C. asiatica* was planted on 100 cm length×100 cm width×25 cm height raised beds. Each raised bed consisted of 4 plants with the same treatment. The distance of each bead was 100 cm and planting distance within the bed was 40×40 cm^2. The layout of research design followed a randomized block design with 6 treatments and 3 replications. The different dosages of phosphate (P_2O_5) fertilizer were applied during planting. Rates of phosphate application were 0 kg per plant as control plant, 20, 40, 60, 80 and 100 kg ha^{-1}, respectively. Basal fertilizers equivalent to 60 kg N ha^{-1} and 35.2 kg K_2O ha^{-1} were applied along with phosphate. The addition of 60 kg ha^{-1} N was applied at 20 and 40 days after planting. All plants were well-watered during the study. The plants watered every afternoon to provide sufficient water except for rainy days.

Data collection

The effects of phosphate application on plant morphology were observed biweekly from the 2^{nd} until 12^{th} week after planting. The observed variable included the number of leaves, petiole length, leaf area, number of primary tendrils, number of secondary tendrils, length of tendrils and number of stolons. The observation of the leaf area was carried out using CI-202 Portable Laser Leaf Area Meter. The petiole length was measured from the base to the tip of petiole using a ruler. The primary tendrils counted for those appeared from the main plant, while secondary tendrils appeared from the primary tendrils. The length of tendrils measured from the base to the tip of tendrils using a tape measure. Plant biomass was measured during harvest at 12^{th} week after planting. Harvested plants were separated into the shoot and root then oven-dried at 50 °C for 72 h.

The accumulation of centellosides, i.e asiaticoside, madecassoside, and asiatic acid contents in above ground (leaves and petiole) and underground organs (roots and tendrils) were analyzed at harvest. The centelloside was determined using CAMAG® TLC Scanner version 3, Switzerland. The wavelength used for asiaticoside, madecassoside, and asiatic analysis were 276, 310 and 290 nm, respectively. The qualitative phytochemicals, i.e alkaloid, saponin, tannin, phenolic, flavonoid, triterpenoid, steroid, and glycoside were analyzed following Departemen Kesehatan Republik Indonesia [22].

Statistical analysis

Effects of phosphate application were tested by employing Microsoft Excel 2013. The data were analyzed using the Analysis of Variants (ANOVA) followed by the Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

Results and discussion

Plant growth

Soil nutrients availability are essential for improving the quality and quantity of plant. To evaluate the effects of phosphate application on shoot growth, leaf-related variables were observed biweekly during *C. asiatica* growing period. The number of leaves, petiole length, and total leaf area were gradually increased until 12 WAP. The rate of leaf growth was increased with the age of plant. The highest increment of leaf number and leaf area appeared from 10 to 12 WAP. Regarding phosphate application, plants treated with lower phosphate (20 and 40 kg ha^{-1}) resulted in a higher number of leaves and total leaf area (**Table 2**). Higher phosphate application rates decreased the number of leaves, petiole length, and total leaf area.

C. asiatica formed primary tendrils and secondary tendrils and stolons. Stolons were formed from 4 WAP and gradually increased and reach the peak at 12 WAP. A similar pattern is found in primary tendrils. Meanwhile, the secondary tendrils started to appear at 6 WAP and rapidly grew until 12 WAP. The number of secondary tendrils became higher than the primary tendrils from 10 WAP. The application of 40 kg ha^{-1} phosphate resulted in the highest number of primary and secondary tendrils and the number of stolons compared to other treatments (**Table 3**). The longest tendril was also formed by those plants treated with 40 kg ha^{-1} phosphate from 4 until 10 WAT that remain similar to under treatments at 12 WAP (**Figure 1**).

The growth improvement of *C. asiatica* by phosphate application was previously reported in Deli Serdang (Indonesia) accession planted in lowland area [18] and accession number 347492 (India) [23]. The growth of Deli Serdang accession and Indian accession were optimized by the application of 30.22 kg P_2O_5 ha^{-1} and 170 mg KH_2PO_4 l^{-1}. Similar results were also obtained in *C. asiatica* cultivated in hydroponic and verticulture systems [24]. The plant growth is highly sensitive to P availability. Inappropriate dosage of phosphorous fertilization unable to enhance the growth of *C. asiatica* in acid soil due to high concentration of Fe, Al, and Mn [25]. Sufficient availability of phosphorus elements enhances the photosynthetic rate and cell divisions which improves shoot and root growth and development [26]. Meanwhile, the excessive application of phosphate reduces the ability of plant to take up required micronutrients [27]. In addition, excessive P accumulation in leaf mesophyll cells causes necrotic symptoms due to reduction of Rubisco activation and inhibition of photosynthesis [28]. Thus, the application of 60 kg until 100 kg ha^{-1} phosphate decreased the plant growth.

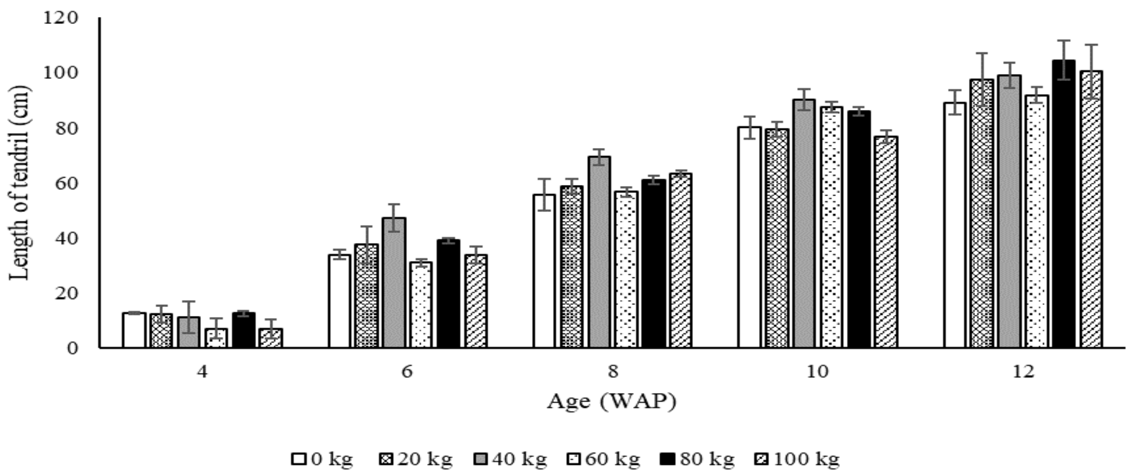

Figure 1 The length of tendril of *C. asiatica* from 4 to 14 weeks after planting (WAP) as affected by phosphate application.

Table 2 The number of leaves, petiole length, and total leaf area from 2 to 14 weeks after planting (WAP) as affected by phosphate application in *C. asiatica.*

Phosphate Application (kg ha^{-1})	**Age (WAP)**					
	2	**4**	**6**	**8**	**10**	**12**
			Number of leaves			
0	4.67±0.33	10.67±1.76	25.00±5.68	46.67±1.66	65.33±2.18	110.67±21.49
20	4.33±0.33	14.33±3.17	25.67±6.35	53.67±6.98	82.33±15.62	183.00±76.33
40	5.00±0.00	10.67±0.66	30.00±2.64	54.00±1.00	80.33±13.09	181.00±46.45
60	4.00±0.00	11.67±1.66	23.33±4.37	59.67±6.17	81.00±7.02	119.67±16.37
80	4.67±0.33	10.67±2.18	31.33±5.81	57.33±12.87	80.00±14.5	154.33±35.43
100	4.67±0.33	7.33±0.66	22.33±2.60	51.33±2.33	69.67±3.52	144.33±22.37
			Petiole length (cm)			
0		3.50±0.28	5.83±0.16	7.00±0.28	7.65±0.15	8.40±0.30
20		4.00±0.76	5.33±0.83	6.37±0.81	7.20±0.76	7.87±0.85
40		3.67±0.16	6.67±0.44	7.60±0.37	8.77±0.18	9.77±0.64
60		3.33±0.33	5.17±0.72	6.83±0.44	7.37±0.27	7.80±0.20
80		3.33±0.16	5.17±0.33	6.43±0.47	6.90±0.55	8.27±1.21
100		3.67±0.33	5.67±0.60	7.07±0.23	7.90±0.26	8.67±0.33

Phosphate Application (kg ha^{-1})	Age (WAP)					
	2	4	6	8	10	12
	Total leaf area (cm^2)					
0	4.86±0.56	36.87±9.49	185.38±46.59	479.40±55.29	708.89±72.72	1409.52±150.17
20	4.74±1.20	50.76±17.38	168.07±32.77	496.37±22.14	804.97±108.73	2356.78±1044.92
40	5.25±0.65	33.18±3.28	204.72±40.02	514.80±30.18	808.34±80.84	2339.03±428.01
60	4.21±0.52	34.23±6.96	138.33±26.02	553.41±54.27	814.40±69.88	1516.15±148.60
80	3.69±0.26	31.61±8.35	176.38±13.59	468.57±118.48	742.40±151.31	1944.57±460.68
100	4.86±0.56	23.20±4.20	153.78±35.74	505.17±69.00	750.29±87.67	1683.57±27.57

Data are means of three replications ± standard error (SE)

Table 3 The effect of phosphate application on the number of tendrils and stolons of *C. asiatica*.

Phosphate Application (kg ha^{-1})	Age (WAP)				
	4	6	8	10	12
	Number of primary tendrils				
0	2.33±0.88	5.33±0.88	8.33±0.33	11.67±0.88	13.00±1.00
20	1.00±0.00	4.00±0.57	8.00±1.00	12.00±0.57	13.00±0.57
40	1.00±0.57	5.67±0.66	10.33±0.88	13.00±0.57	13.67±0.66
60	1.33±0.88	5.33±1.45	9.00±1.00	11.00±1.00	13.00±.057
80	1.67±0.33	5.33±0.33	8.67±0.66	11.00±0.57	11.33±0.33
100	1.67±1.20	5.33±0.88	9.33±0.33	12.00±0.57	13.67±0.33
	Number of secondary tendrils				
0	0.00±0.00	1.33±0.33	7.00±1.00	17.33±0.33	42.33±3.17
20	0.00±0.00	1.67±0.33	6.33±1.20	14.00±1.15	32.33±5.54
40	0.33±0.33	4.00±2.08	10.00±1.15	18.33±2.02	41.00±4.50
60	0.00±0.00	2.33±1.33	9.33±1.76	18.33±2.90	37.33±3.48
80	1.33±0.88	4.33±1.76	11.33±3.52	18.67±4.17	40.67±5.23
100	0.00±0.00	2.67±1.20	10.33±2.02	18.67±3.17	44.33±4.17

Phosphate Application (kg ha^{-1})	Age (WAP)				
	4	**6**	**8**	**10**	**12**
			Number of stolons		
0	0.67±0.33	3.67±0.88	11.33±1.85	26.67±3.66	44.00±3.60
20	0.67±.066	4.00±1.52	13.00±2.08	26.33±6.38	45.00±12.09
40	1.33±0.33	7.00±1.15	16.33±1.66	34.00±6.24	48.33±5.60
60	1.00±0.57	5.33±1.20	14.33±0.33	26.00±5.00	40.00±6.11
80	2.00±0.57	8.33±0.88	17.33±3.52	30.67±4.66	45.33±6.11
100	1.67±0.88	7.00±1.52	14.67±3.52	29.00±3.00	42.33±4.91

Data are means of three replications ± standard error (SE)

Dry weight biomass

The different rates of phosphate fertilizer application affected plant biomass as indicated by the dry weight of underground and above ground organs (**Figure 2**). Plants applied with 40 kg ha^{-1} phosphate obtained the highest dry weight of both under and above ground organs as compared to other treatments. The result showed that the accumulation of plant biomass was higher in underground organs than above ground organs. Phosphorus elements affecting from the cellular to whole plant level of plants' growth. P supply increases photosynthetic rates, thus enhancing carbon acquisition and further growth. P element enlarges leaf area and slow down the degradation of chlorophyll, phloem loading, and translocation as well as the synthesis of large molecular weight substances within plant organs [29]. In addition, phosphorus plays a vital role in the metabolism of sugars, energy storage and transfer, cell division, and enlargement and transfer of genetic information. In the present study, the application of 40 kg ha^{-1} phosphate increased the biomass of *C. asiatica*. The enhancement of plant biomass due to P application had been reported in many other crops. The enhancement of leaf biomass was reported in *Salvia officinalis* L. [30], *Lantana camara* [31], and *Gossypium hirsutum* [29]. Regarding root growth, the application P significantly increased the allocation of biomass to roots in *L. albus* and *L. micranthus* by 37 and 33 %, respectively [32].

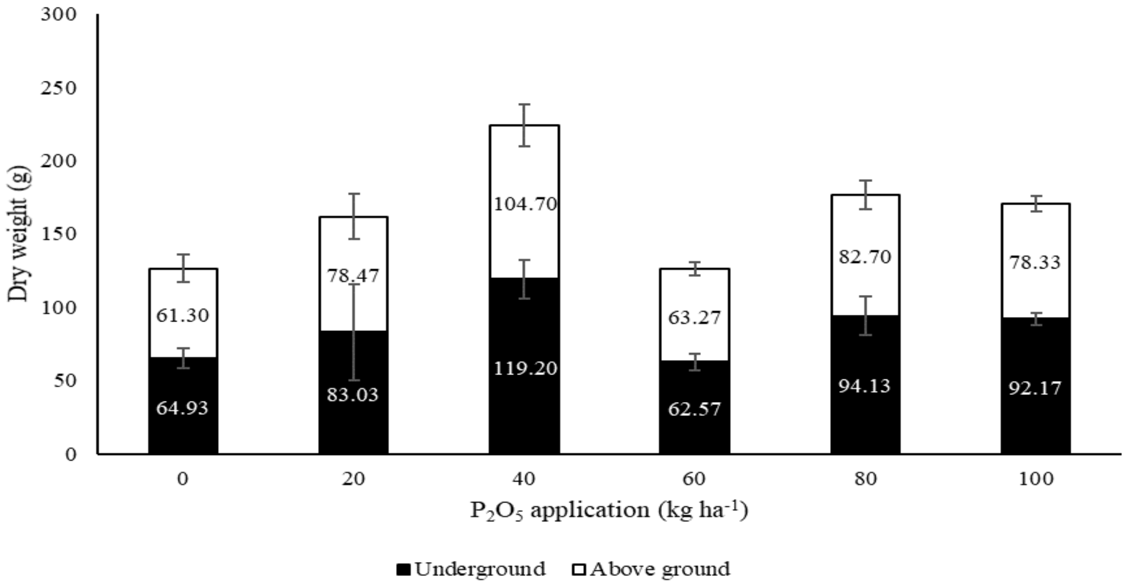

Figure 2 Above ground and underground organs' dry weight of *C. asiatica* affected by phosphate application.

The accumulation of secondary metabolites in above ground and underground organs

Phosphate application significantly affected the production of centelloside secondary metabolite of *C. asiatica* i.e asiaticoside, madecassoside, and asiatic acid (**Figure 3**). Madecassoside was the most produced metabolite, followed by asiatic acid and asiaticoside. The relationship between phosphate application rates with the concentration of centelloside in leaves and roots as indicated by R^2 value of polynomial regression were varied from $R^2 = 0.2152$ until $R^2 = 0.9986$. The production of centelloside was higher in above ground organs than underground organs except for asiatic acid with the application of 80 kg and 100 kg ha^{-1} phosphate. Results showed that the highest asiaticoside, madecassoside, and asiatic acid contents in aboveground organs were produced under the application of 20 kg ha^{-1} phosphate of 1.08, 2.24 and 1.25 %, respectively. The concentration of asiaticoside content in roots was decreased with the addition of phosphate from 0.98 % in control until 0.63 % under 100 kg ha^{-1} phosphate application. However, the pattern of madecassoside and asiatic acid under different phosphate applications remained unclear. The application of 60 kg ha^{-1} phosphate reduced madecassoside and asiatic acid but the higher application rate increased the production of these metabolites. The distribution of centalloside predominated in above ground organs was and less in underground organs. The present results agree with those of Aziz *et al.* [33] showing that asiaticoside and madecassoside content in leaves of *C. asiatica* higher than in roots. The constituent of centelloside affected by the availability of phosphorus elements. Kochan *et al.* [34] stated that phosphorus and phosphates are involved in the regulation of many biochemical and physiological processes in plants, including the constituent of nucleic acid and membranes, energy sources, transcriptional factors, or signaling molecules. Mumtazah *et al.* [35] stated that P content in plants greatly influences the formation of centelloside which is synthesized by the mevalonic acid pathway. The role of P in producing centellosides in *C. asiatica* including the formation of enzymes as precursor compounds and the formation of isoprene units [35,36].

The triterpenoid centellosides, i.e asiaticoside, madecassoside, and asiatic acid are responsible for most of the pharmacological activities [37]. Asiatic acid ($C_{30}H_{48}O_5$) isolated from *C. asiatica* enhance intelligence and improve cognitive function [38,39]. Madecassoside ($C_{48}H_{78}O_{20}$) induces gene expression changes [40]. Asiaticoside ($C_{48}H_{78}O_{19}$) widely used for anti-inflammatory [41]; wound-healing agent by promoting fibroblast proliferation and collagen synthesis [42], neuroprotective activity [43] and induces cell proliferation and collagen synthesis collagen biosynthesis improvement [44]. The enhancement of centelloside content through phosphate application will contribute positively influence pharmaceutical needs supply.

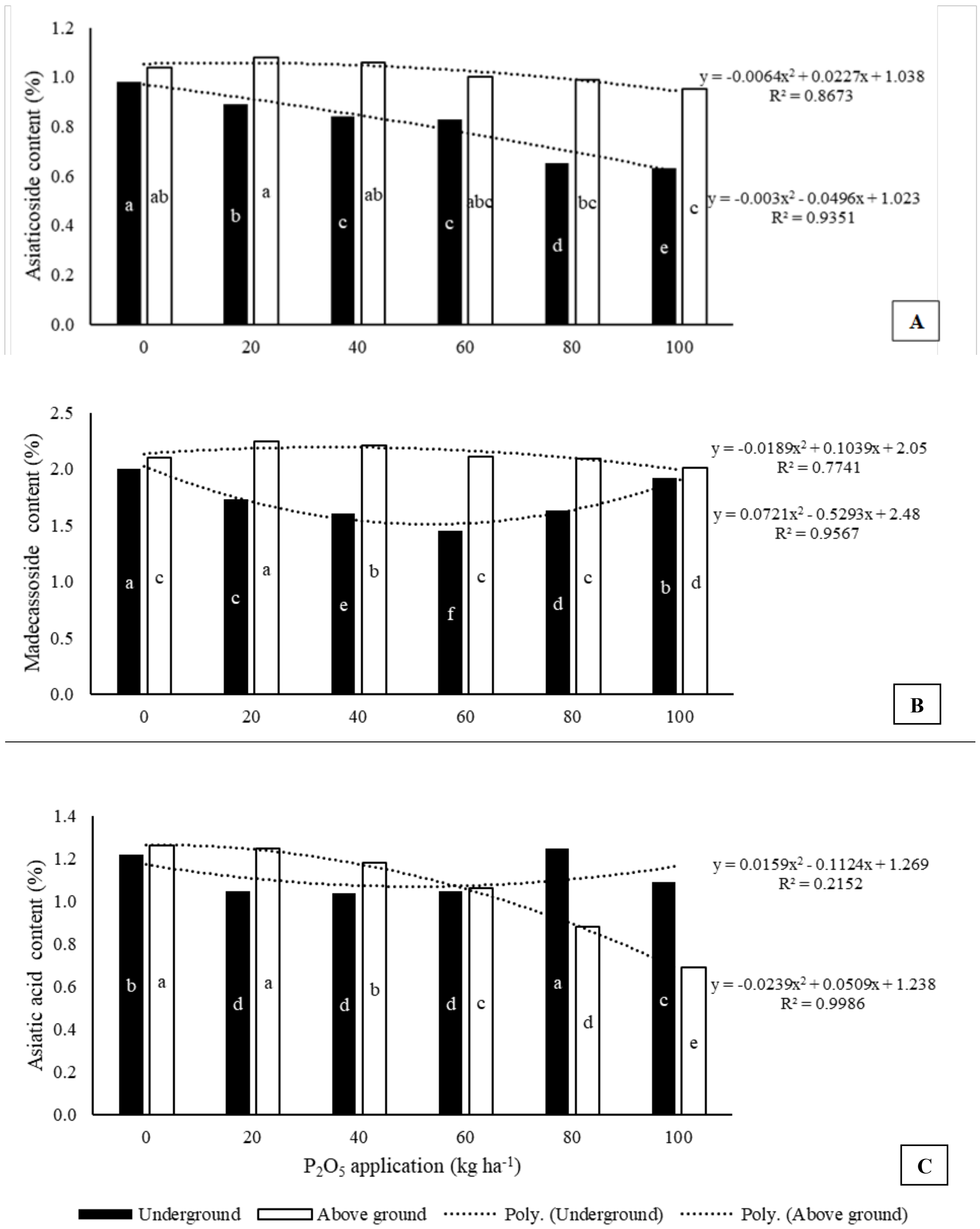

Figure 3 The effect of phosphate application on centelloside accumulation, i.e A) Asiaticoside, B) Madecassoside and C) Asiatic acid in *C. asiatica*. The different letters within bars indicated the significant different of each treatment based on the Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

A systematic review on the chemical constituents of *C. asiatica* reported the diverse and complex chemical constituents, including terpenes, phenolic compounds, polyacetylenes group, alkaloids, carbohydrates, vitamin, mineral, and amino acid [37]. This study revealed that the above ground and underground organs of *C. asiatica* Samosir accession contained saponin, tannin, phenolic, flavonoid, triterpenoid, steroid, and glycoside but did not contain alkaloid (**Table 4**). The results showed that the type of phytochemical produced by *C. asiatica* were not affected by the application rates of phosphate. The type of phytochemicals under control condition was identical with those plant treated with up to 100 kg ha^{-1} P_2O_5. The variation of chemical constituents of *C. asiatica* depending on the geographical distribution [40]. The quantity of each phytochemical identified in this study might be different as affected by phosphate application. However, it was not analyzed in the present study. A previous study by Yin *et al.* [45] showed that lower phosphate concentrations were favorable for the content of flavonoid and glycyrrhizic acid of *G. uralensis*. Furthermore, Chea *et al.* [46] stated that the low application rate of P increases plants secondary metabolites such as anthocyanin, proline, flavonoids, and amino acids for preventing the cellular structures from oxidative damage. The production of phytochemicals in *C. asiatica* not only beneficial for plant defense but also for the pharmaceutical, agriculture, cosmetic, and other sectors for their health-promoting properties and treatment of diseases.

Table 4 The effect of phosphate application on the availability of phytochemicals in *C. asiatica*.

Phytochemical	**Availability**					
	0 kg ha^{-1} P_2O_5	**20 kg ha^{-1} P_2O_5**	**40 kg ha^{-1} P_2O_5**	**60 kg ha^{-1} P_2O_5**	**80 kg ha^{-1} P_2O_5**	**100 kg ha^{-1} P_2O_5**
Alkaloid	−	−	−	−	−	−
Saponin	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Phenolic	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+
Triterpenoid	+	+	+	+	+	+
Steroid	+	+	+	+	+	+
Glycoside	+	+	+	+	+	+

\+ indicated available; − indicated not available

Conclusions

The application phosphate enhanced the growth and secondary metabolites production especially asiaticoside, madecassoside and asiatic acid in *C. asiatica*. The application of 40 kg ha^{-1} phosphate optimized the growth of above ground and underground organs indicated by leaf-related variables, tendrils, and stolons as well as plant biomass. *C. asiatica* produced centelloside secondary metabolites namely madecassoside, asiatic acid and asiaticoside, and others phytochemicals including saponin, tannin, phenolic, flavonoid, triterpenoid, steroid, and glycoside. The addition of external phosphate did not change the variation of secondary metabolites but the application of 20 kg ha^{-1} increased the production secondary metabolites of quantitatively. Thus, the application of phosphate at low dosage (not more than 40 kg ha^{-1}) is suggested of *C. asiatica* cultivation in acid soil.

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