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Correlations between Leaf Nutrient Content and Production of Metabolites in Orange Jessamine (*Murraya paniculata* L. Jack) Fertilized with Chicken Manure

Siti Rahmah Karimuna*[A], Sandra Arifin Aziz[BC], Maya Melati[B]

[A]Postgraduate School, Agronomy and Horticulture, Bogor Agricultural University, Bogor, Indonesia 16680
[B]Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University, Bogor, Indonesia 16680
[C]Biopharmaca Research Center, Bogor Agricultural University, Bogor, Indonesia

*Corresponding author; email: sitirahmahkarimuna@rocketmail.com

Abstract

Plant secondary metabolites are unique sources for pharmaceuticals and food additives. Orange jessamine (*Murraya paniculata)* contains secondary metabolite that is beneficial to human health including lowering blood cholesterol levels, anti-obesity, and has the antioxidant capacity. Leaves of orange jessamine have several chemical constituents including L-cadinene, methyl-anthranilate, bisabolene, β-caryophyllene, geraniol, Carene, 5-guaiazulene, osthole, paniculatin, tannins, eugenol, citronelled, coumurrayin and coumarin derivatives. This study aimed to determine the correlation between leaf concentrations of N, P and K with leaf bioactive compounds following chicken manure application to the plants. The experiment was conducted at IPB Experimental Station at Cikarawang, Bogor (6°30' - 6°45' S, 106°30'-106°45' E) from March to November 2014 (250 m above sea level). The study used a randomized block design (RBD) with rates of chicken manure as a treatment, i.e. 0, 2.5, 5 and 7.5 kg per plant. Chicken manure was applied at 30 months after planting (MAP). Leaves were harvested by pruning the plants to a height of 75 cm above the soil surface. The results showed that the optimum rates of chicken manure to produce maximum fresh and dry leaf weight were 3.1 kg and 6.5 kg per plant, respectively. This rate was sufficient for leaf production at the first harvest (34 MAP) but was insufficient for the second harvest (38 MAP). K concentration of the leaves from different positions within the plant and leaf age positively correlated with leaf dry weight ($P < 0.01$), but negatively correlated with total flavonoid content ($P < 0.01$). Leaf P concentration was negatively correlated with dry weight of the 5th young leaves, or mature leaves from all positions. Leaf K concentration was categorized very high (3.59-4.10%), whereas leaf P concentration was high (0.28-0.29%) to very high (0.33-0.35%). The 5th mature leaves determined plant K requirements.

Keywords: antioxidant, bioactive compounds, leaf position, organic, NPK leaf concentrations

Introduction

Orange jessamine (*Murraya paniculata* L. Jack) belongs to Rutaceae family and usually found to grow wild in thickets, forest edges, or grown as a medicinal plant or hedge (Mattjik 2010). The plants are used as raw material for craft, cosmetics, and insecticide. *Murraya paniculata* is originated from Southeast Asia and Australia, and from mainland India, South Asia. Regional distribution of *M. paniculata* includes Java, especially in Central Java and East Java, Sumatra, Bali, West Nusa Tenggara, East Nusa Tenggara, Sulawesi, and Maluku based on herbarium specimens in Bogoriense Herbarium and the National Herbarium of Leiden (Heyne 1987).

Murraya paniculata contains secondary metabolite that has long been used in pharmacology to lower blood cholesterol levels (Pane 2010), as anti-obesity (Iswantini et al. 2011), and the metabolites are reported to have antioxidant capacity (Rohman and Riyanto 2005). Orange jessamine leaves contains several chemical constituents including i.e. L-cadinene, methyl-anthranilate, bisabolene, β-caryophyllene, geraniol, Carene, 5-guaiazulene, osthole, paniculatin, tannins, eugenol, citronelled, coumurrayin and coumarin derivatives (Kardono et al. 2003).

There are several approaches to determine the optimum fertilizer rates for plants: soil analysis, plant analysis, and observation of nutrient deficiency

symptoms on greenhouse-grown or field-grown plants (Lozano 1990). Optimization of leaf nitrogen (N), phosphorus (P), and potassium (K) content for maximum biomass production and bioactive content can be performed by correlation test analysis on nutrient concentrations of the leaves. Experiment conducted by Hermanto et al. (2012) showed that the concentration of N, P, and K in *Centella asiatica* leaves decreased with increasing age, whereas increase in leaf N, P, and K positively correlated with leaf dry weight and leaf bioactive contents. The optimum harvest time for the highest fresh weight and asiaticoside content of highland *C. asiatica* leaves was five months (Hermanto et al., 2012). Very limited studies have been conducted on nutrient requirement and biomass production of *M. paniculata*. This research was conducted to determine 1) the optimum rates of chicken manure application for leaf production, leaf bioactive contents and N, P and K of the leaves from different positions within the plant; and 2) the correlation between concentrations of leaf N, P and K with the levels of leaf bioactive compounds following chicken manure application.

Materials and Methods

Plant Materials

The experiment was conducted from March to November 2014 at the IPB experimental station at Cikarawang, Bogor Agricultural University, Bogor, Indonesia (6°30' - 6°45' S 106°30'-106°45' East). The location is at 250 m above the sea level. The type of soil is latosol. Soil and leaf nutrient analysis were conducted at Soil Research Institute, Agricultural Research Campus Cimanggu, Bogor, Indonesia. Harvesting on fresh and dry weight of *M. paniculata* leaves was carried out in the Laboratory of Postharvest, Department of Agronomy and Horticulture, Bogor Agricultural University. Phytochemicals analysis was conducted at the Laboratory of Biopharmaca Research Center, Bogor Agricultural University Indonesia.

The experiment used 30-month-old-plants from seed of ± 100 cm height with plant spacing of 1 m x 1 m between plants. Other materials used were chicken manure and chemicals for analysis of leaf N, P, K and bioactive compounds (described below).

Treatments

The study used a randomized block design (RBD) with rates chicken manure as treatments, i.e. 0, 2.5, 5 and 7.5 kg per plant, applied at 30 MAP. Each experimental unit consisted of two plants with four replications. Chicken manure was applied into soil surrounding each plant. The plant parts for analysis were harvested at 75 cm above soil surface at 34 and 38 MAP.

Fresh leaves were harvested at 34 and 38 MAP and leaf dry weight was measured. Concentration of nutrients in the soil and fertilizer, concentration of leaf N, P, and K, total flavonoids, anthocyanin, total chlorophyll content and antioxidant activity were analysed. Analysis of leaf N, P, and K and bioactive compounds were performed on young and mature

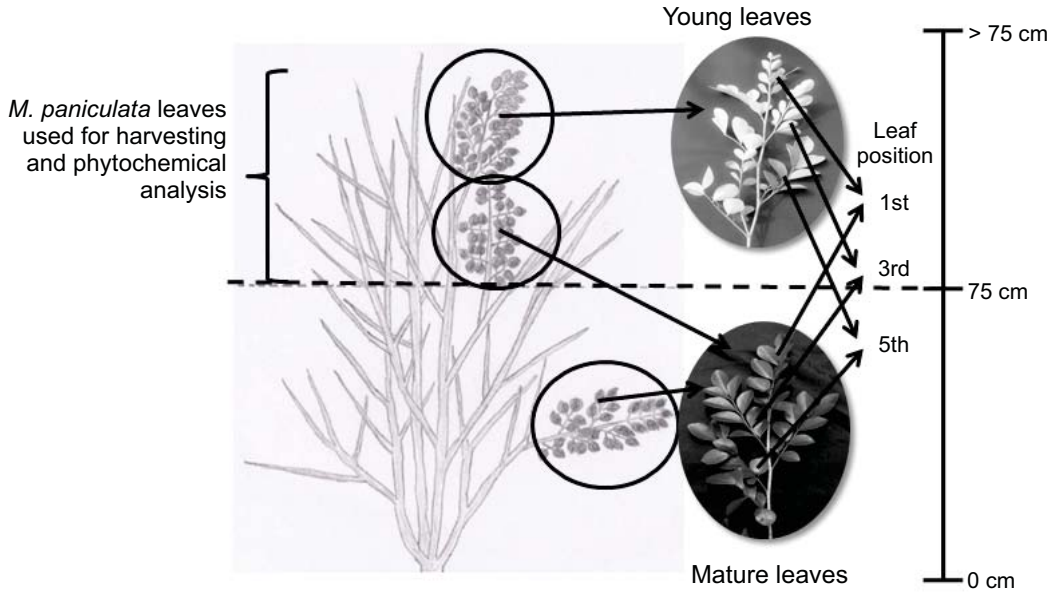

Figure 1. *M. paniculata* showing young and mature leaves of different positions within the plant.

fully developed leaves that grow at the 1st, 3rd and 5th position of the twigs branches (Figure 1) at 34 and 38 MAP. The first young fully expanded leaves are yellowish green; the third to fifth are light green, all mature leaves (first to fifth) are dark green (Figure 1).

Fresh and Dry Weight of Leaves

Data on leaf fresh and dry weight were obtained by harvesting the leaves 75 cm above the soil surface (Figure 1). The leaves were detached from the stems and the leaves were used for further analysis. The leaves were oven-dried (60°C) for 3 x 24 hours.

N, P, and K Leaf Analysis

Nitrogen, phosphorus, and potassium content of the 1st, 3rd and 5th young and mature leaves (Figure 1) were analysed. Total N was measured using semi-micro Kjeldahl method; P and K used dry ashing method (Anderson 1968). Phosphorus concentration was measured with Hitachi Spectrophotometer U-2010, and K was measured with Atomic Absorption spectrophotometer Agilent 240 FS AA.

Sample Preparation For Analysis of Total Flavonoid Content and Antioxidant Activity

The 1st, 3rd and 5th *M. paniculata* young and mature leaves (Figure 1) were used for phytochemical analysis. Freshly harvested leaves were washed with water, drained, and oven dried at 60°C for 3 x 24 h before crushed into powder using a blender. Plant powder of 0.1 g was extracted in 1 mL of methanol into a micro tube and heated for 60 min at 60°C, and was vigorously shaken every 20 minutes. The mixtures of liquid and solid phases were separated using a centrifuge at 12,000 rpm for 5 mins. The resulting supernatant was used for subsequent analysis.

Analysis of Leaf Total Flavonoid

Total flavonoids were analysed using a method by Chang et al. (2002) with slight modifications as follows: plant liquid extract of 0.1 mL was added with ethanol (1.9 ml), aluminum chloride (0.1 mL, 10%), potassium acetate (0.1 mL, 1 M), distilled water (2.8 mL) separately then mixed using a vortex. After incubation at room temperature (27°C) for 30 min the absorbance of the mixture was measured at a wavelength of 415 nm using Shimadzu UV-1201 UV-VIS spectrophotometer. Blank or control was ethanol (1.9 mL), aluminum chloride (0.1 mL, 10%), and potassium acetate (0.1 mL, 1 M) and distilled water (2.9 mL). Standard curve was constructed using 0-400 mL. L^{-1} quercetin in methanol (y = 0.038x + 0.091, R^2 = 0996). The result was expressed as milligrams of quercetin equivalents per gram of sample (quercetin per g of dry matter).

Analysis of Leaf Antioxidant Activity

1, 1-Diphenyl-2-picrylhydrazyl free radical scavenging assay (DPPH) was carried out following a modification of Brand-Williams et al. (1995) and Payet et al. (2005) as follows: 0.1 mL of methanolic extracts of leaves (using sample extract concentration of 50%, taking 50 mL of the supernatant was diluted with 50 mL of methanol) and standard (Ascorbic acid) at various concentrations (0, 25, 50, 75 and 100 $\mu g.mL^{-1}$) were added to 4.9 mL of DPPH in ethanol (with absorbance 1) and the reaction mixture was shaken vigorously. These solution mixtures were kept in dark for 20 min at 27°C and optical density was measured at 517 nm using Shimadzu UV-1201 UV-VIS spectrophotometer. Ethanol with DPPH was used as blank. The % scavenging activity was calculated using the formula:

Percentage of inhibition of DPPH activity = (A – B)/A x 100

Where A = optical density of the blank, and B = optical density of the sample.

Analysis of Leaf Anthocyanin and Total Chlorophyll

Leaf anthocyanin and total chlorophyll content were analysed using Sims and Gamon (2002) as a reference. Fresh leaf samples from different positions along the plants were crushed and each sample was added with 2 mL aceton tris (85:15 (%) and centrifuged at 14000 rpm for 10 minutes. Subsequently, 1 mL of supernatant was added with 3 mL of aceton tris and thoroughly mixed. Absorbance of the mixture was measured at wavelengths of 663, 647 and 537 nm.

Data Analysis

Data were analyzed with F-test, followed by Duncan Multiple Range Test at the 5% significance level. Statistical analysis was conducted using SAS Windows 9.1 System. Production of bioactive compounds weight was calculated using the following formulation:

Weight of bioactive compounds =
Dry weight of leaves (g per plant) x concentration of bioactive compounds (%)

Simple correlation tests were performed on (a) the levels of leaf N, P, or K at 34 and 38 MAP with weight of leaves and bioactive compounds production); (b) the levels of leaf N, P, or K of the 1st, 3rd, or 5th young and mature leaves with weight of leaves and bioactive compounds production.

The simple linear correlation model used is:
$\hat{Y} = a + bX$

where
$\hat{Y}$ = Production of anthocyanin on N concentration of 5th leaf
a = The value of Y when X = 0 (intercept)
b = Regression coefficient
X = N concentration of 5th leaf

Results

Soil and Fertilizer Chemical Properties

The results of soil chemical analysis (Table 1) showed that the soil was slightly acidic and low in organic matter content.

Effect of Chicken Manure Application on Crop Biomass Production

Chicken manure application increased leaf fresh weight and dry weight at 34 MAP ($P>0.05$), and at 38 MAP ($P<0.05$). Leaf fresh and dry weight at 34 MAP increased quadratically with the increase of chicken manure rates, whereas fresh and dry weight at 38 MAP increased linearly. *The* highest leaf dry weight at 34 MAP, and leaf fresh and dry weight at 38 MAP were obtained from plants applied with 7.5 kg chicken manure per plant, but the highest leaf fresh weight at 34 MAP were obtained from plants applied with 2.5 kg chicken manure per plant. The optimum rate of chicken manure application to produce a maximum leaf fresh weight and leaf dry weight harvested at 34 MAP is 3.1 and 6.5 kg per plant, respectively. Moisture content of harvested leaves of the plants treated with 7.5 kg chicken manure per plant was lower at 34 MAP than at 38 MAP, i.e.30.82 and 55.28%, respectively (Figure 2).

Effect of Chicken Manure Application on Leaf N, P, K Concentration and Metabolite Productions at 34 and 38 MAP

Leaf N and K decreased, whereas leaf P increased from 34 to 38 MAP (Table 2).

Table 1. Soil and fertilizer chemical properties[1)]

Chemical variables	Method	Soil Value	Criteria[2)]	Value fertilizer	Criteria[2)]
Extract 1:5					
pH H_2O	-	6.2	slightly acid	-	-
pH Kcl	-	5.7	slightly acid	-	-
Organic matter					
C-organic (%)	Walkey and Black	1.43	low	8.30	very high
N (%)	Kjeldahl	0.15	low	1.13	very high
C/N	-	10	low	7.34	low
HCl 25%					
P_2O_5 (mg 100 g^{-1})	Extractant Bray	99	very high	8.60	very low
K_2O (mg 100 g^{-1})	Extractant Morgan Wolf	9	very low	3.37	very low
P_2O_5 (ppm)	Olsen	50	high	-	-
Fe (ppm)	-	-		1408	very high

Note: [1)]Soil analysis was conducted by ISRI, Cimanggu, Bogor.
[2)]Based on criteria in Hardjowigeno (2010).

Table 2. Leaf N, P, K (%) of the plants treated with different rates of chicken manure at 34 and 38 MAP

Nutrient content (%)		Rates of chicken manure application (kg per plant)							
		0		2.5		5		7.5	
		value	criteria[1]	value	criteria[1]	value	criteria[1]	value	criteria[1]
N	34 MAP	2.22	low	2.09	very low	2.40	optimum	2.51	optimum
	38 MAP	2.07	very low	2.08	very low	2.11	very low	2.21	very low
P	34 MAP	0.35	very high	0.29	high	0.27	high	0.27	high
	38 MAP	0.33	very high	0.35	very high	0.28	high	0.29	high
K	34 MAP	3.71	very high	4.10	very high	3.59	very high	3.83	very high
	38 MAP	1.33	high	1.62	high	1.66	high	1.84	high

Note: [1]Based on Embleton et al. (1973) criteria. MAP: month after planting

Mature leaves had more anthocyanin, total chlorophyll, and total flavonoid than young leaves, but the young leaves had more antioxidant activity than mature leaves.

Young leaves had higher P and K at 34 and 38 MAP, and N at 38 MAP than mature leaves (Table 3). Leaf anthocyanin, total chlorophyll and total flavonoid of the 5th leaf were higher than those of other leaf positions, both in young and mature leaves. The highest antioxidant activity was found in the 1st young leaves (at 34 and 38 MAP).

The leaf total chlorophyll, total flavonoid and antioxidant activity were higher at 34 MAP than at 38 MAP, except for anthocyanin (Table 3).

Plant biomass production and total flavonoid at 34 MAP were higher than at 38 MAP. These findings suggested that the secondary metabolites could be better synthesized if it was preceded by optimal synthesis of primary metabolites.

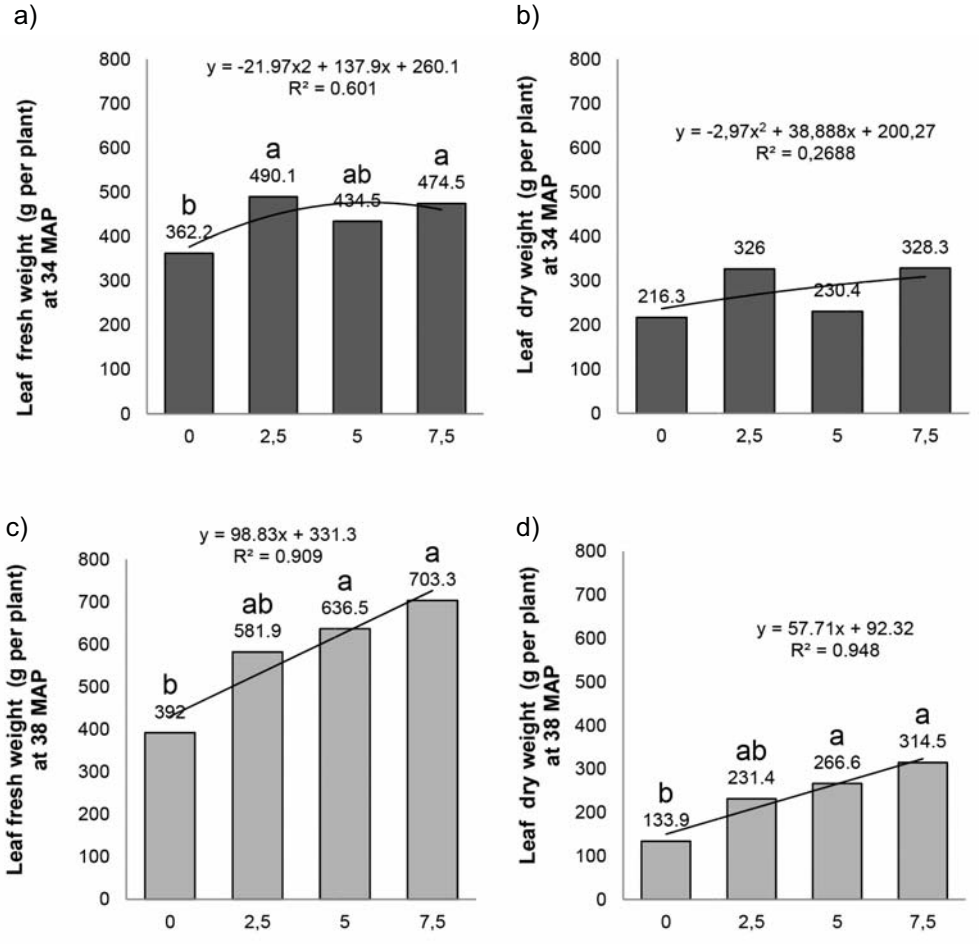

Figure 2. Effect of chicken manure application (kg per plant) on a) leaf fresh weight at 34 MAP, b) leaf dry weight at 34 MAP, c) leaf fresh weight at 38 MAP, and d) leaf dry weight at 38 MAP

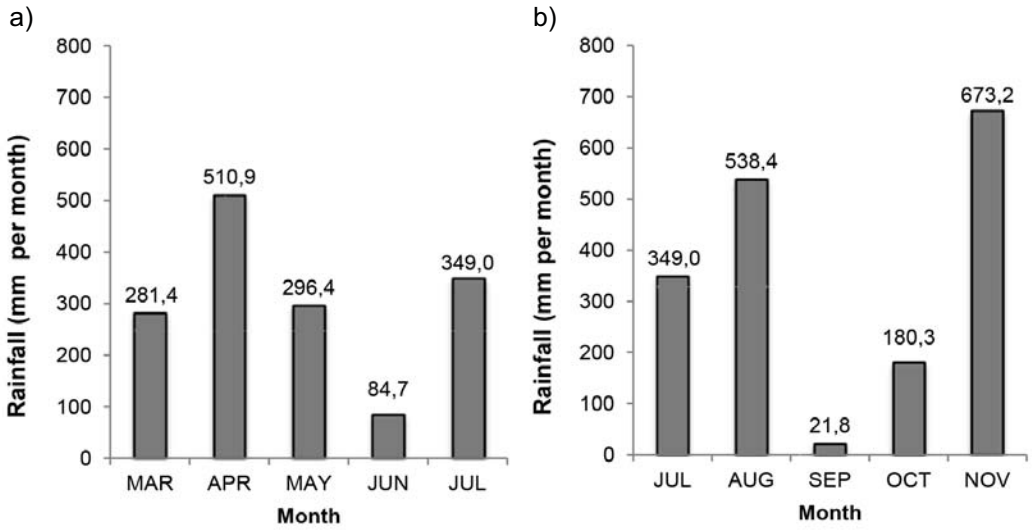

Figure 3. Rainfall (mm per month) at Cikarawang, Bogor during a) 30-34 MAP and b) 34-38 MAP. The arrows indicate the times of leaf harvesting.

Table 3. The leaf N, P, K and metabolite production at 34 and 38 MAP

Leaf position	N (%)	P (%)	K (%)	Anthocyanin (μmol. g^{-1})	Total chlorophyll ($mg.g^{-1}$)	Total flavonoid ($mg.g^{-1}$)	Antioxidant activity (%)
34 MAP							
Young leaves							
1st	2.19	0.31	4.00	0.0087	0.9123	1.43	77.60
3rd	2.07	0.32	3.92	0.0047	1.1255	1.48	72.14
5th	2.07	0.31	3.68	0.0128	1.6518	1.67	69.92
Mature leaves							
1st	2.48	0.29	3.83	0.0188	2.1825	1.76	54.98
3rd	2.52	0.28	3.86	0.0251	2.4265	7.80	52.70
5th	2.48	0.26	3.55	0.0224	2.4265	7.76	55.69
38 MAP							
Young leaves							
1st	2.33	0.33	1.81	0.1015	0.8363	0.78	71.22
3rd	2.02	0.30	1.41	0.0968	0.9580	0.72	62.69
5th	2.00	0.30	1.56	0.1195	1.5093	0.94	67.14
Mature leaves							
1st	2.19	0.31	1.42	0.1512	1.7779	0.87	62.82
3rd	2.15	0.30	1.74	0.1660	1.9230	0.81	58.38
5th	2.03	0.32	1.74	0.1723	2.0357	0.95	59.48

Note: DM: dry matter; MAP: month after planting

Correlation of N, P and K Leaf With Leaf Dry Weight and Metabolites Production at 34 and 38 MAP

At 34 MAP concentration of leaf K (all positions) and leaf age positively correlated with leaf dry weight ($P<0.01$), but negatively correlated with total flavonoid content ($P<0.01$). This indicated that the increase of K concentration in the leaves increased biomass production with lower total flavonoids (Table 4).

Table 4. The correlation coefficients between concentration of leaf N, P and K and leaf age with leaf dry weight, anthocyanin, total chlorophyll, total flavonoid and antioxidant activity at 34 MAP

Leaf position	Nutrient content	Leaf dry weight	Anthocyanin	Total chlorophyll	Total flavonoid	Antioxidant activity
Young leaves						
1st	N	-0.28	-0.29	-0.34	0.14	0.39
	P	-0.29	0.04	0.10	0.17	0.49
	K	-0.74**	0.28	-0.31	-0.71**	-0.28
3rd	N	0.04	0.05	0.07	-0.00	0.26
	P	-0.30	0.29	0.12	0.02	0.50
	K	0.71**	0.41	-0.27	-0.74**	-0.52*
5th	N	-0.17	-0.31	0.02	0.22	0.26
	P	-0.42	-0.02	-0.00	0.25	0.50
	K	0.84**	0.08	-0.42	-0.68**	-0.23
Mature leaves						
1st	N	-0.14	-0.15	0.19	0.12	-0.08
	P	-0.43	0.17	0.30	0.16	0.33
	K	0.70**	0.28	-0.35	-0.67**	-0.31
3rd	N	0.21	-0.33	0.07	0.18	0.09
	P	-0.33	0.18	0.20	0.08	0.27
	K	0.76**	0.22	-0.36	-0.70**	-0.28
5th	N	0.08	-0.52*	-0.21	0.31	0.28
	P	-0.43	0.17	0.14	0.20	0.32
	K	0.79**	0.27	-0.49	-0.63**	-0.30

Note:**P <0.01, *P <0.05, +: positive correlation; -: negative correlation; MAP: months after planting

Different trend of correlation was recorded at 38 MAP; the P concentration was negatively correlated with dry weight of the 5th young leaf and of the mature leaves at all positions (Table 5). This showed that high concentration of P in the leaves correlated with low leaf production.

Discussion

Leaf fresh weight at 34 MAP was lower than at 38 MAP, but the dry weight at 34 MAP was higher than that at 38 MAP. This was possibly related to the differences in rainfall distribution during the observation periods. During 30-34 MAP the total rainfall was 1522.4 mm whereas during 34-38 MAP the total rainfall was 1762.7 mm (Figure 3a and 3b). Although the total rainfall during 34-38 MAP was higher than during 30-34 MAP, there were two months with monthly rainfall of less than 200 mm during 34-38 MAP. It is possible that these two relatively drier months had inhibited leaf growth so that the new leaves after pruning (harvested at 34 MAP) were smaller and were slower to develop to mature leaves, thus the plants at 38 MAP had more young leaves than at 34 MAP. This data is supported by the lower chlorophyll content in the leaves at 38 MAP. The young leaves harvested at 38 MAP had higher moisture content and consequently lower dry weight compared with those harvested at 34 MAP.

The nutrients for plants can be derived from the soil and from the applied chicken manure. The chemical analysis of soil and fertilizer showed that the soil in this study has low N, very high P and very low K concentration, and the chicken manure had a very high N, very low P and very low K concentration (Table 1). The nutrient supplies from chicken manure and from the soil were possibly sufficient for leaf production harvested at 34 MAP so that biomass production increased quadratically with increasing chicken manure rates. However, at 38 MAP increasing rates of chicken manure led to the formation of a linear curve, which indicated that source of nutrients for the plant might had been fully utilized and was limiting for plant growth until 38 MAP. Therefore, it can be suggested that more fertilizer should be applied after every harvest or pruning.

The experiment showed that potassium (K^+) had consistent positive correlations with leaf dry weight and negative correlation with total flavonoid of leaves from all positions and leaf ages (Table 4). The importance of K for plant growth has

Table 5. The correlation coefficients between leaf N, P and K and ages of leaves (MAP) with leaf dry weight, leaf anthocyanin, total chlorophyll, total flavonoid and antioxidant activity at 38 MAP

Leaf position	Nutrient content	Leaf dry weight	Anthocyanin	Total chlorophyll	Total flavonoid	Antioxidant activity
Young leaves						
1st	N	0.24	-0.30	0.06	0.09	0.31
	P	-0.47	-0.17	-0.10	0.40	0.44
	K	0.34	-0.02	0.20	0.24	0.15
3rd	N	0.40	-0.18	0.48	0.05	0.03
	P	-0.49	-0.20	-0.31	0.30	0.55*
	K	0.22	-0.16	0.05	0.10	0.40
5th	N	0.40	-0.23	0.51*	0.18	-0.18
	P	-0.60*	-0.10	-0.27	0.44	0.47
	K	0.23	0.05	0.20	0.19	0.13
Mature leaves						
1st	N	0.62*	-0.43	-0.07	-0.42	0.03
	P	-0.52*	-0.04	-0.10	0.46	0.43
	K	0.25	-0.20	-0.06	-0.24	0.28
3rd	N	0.22	-0.18	-0.02	-0.26	0.42
	P	-0.57*	-0.15	-0.07	0.44	0.46
	K	0.01	-0.13	0.01	0.05	0.19
5th	N	0.08	-0.04	-0.27	-0.34	0.09
	P	-0.68**	-0.25	-0.25	0.21	0.62*
	K	0.33	-0.15	0.09	-0.12	0.18

Note:**P <0.01, *P <0.05, +: positive correlation; -: negative correlation; MAP: months after planting

been widely reported, for example by Shabala and Pottosin (2014), that potassium is one of the most important macronutrient for all organisms, represents 2–10% of the dry biomass and plays important roles in crucial processes including plasma membrane polarization, stomatal aperture, and adaptation to environmental changes. The current experiment showed that leaf K had a negative correlation with leaf total flavonoid (Table 4) which indicated that K was utilized by plants mainly for biomass production during 30-34 MAP so that the production of secondary metabolites (flavonoid) was reduced.

Effects of P on *M. paniculata* growth in this study were unclear. The current study showed leaf-P concentration negatively correlated with leaf weight, which indicated that increasing P level in the leaves lowered leaf production at 38 MAP (Table 5). Leaf P concentrations were high to very high (Table 2). These findings were contradictory to Havlin et al. (2005) who reported that phosphorus has essential roles in regulating carbohydrate metabolism as a function of the distribution of photosynthesis between the source and reproductive organs, formation of the cell nucleus, cell division and multiplication, and formation of fat and albumin. However, a report by Marschner (1995) supported this study in that high P concentrations can inhibit Fe and Zn absorption. Furthermore, Hardjowigeno (2010) reported that Fe is important for plant growth, including formation of chlorophyll, oxidation reduction in respiration, and preparation of enzymes and proteins, and Zn is important for formation of growth hormone, catalyst formation and maturation of seed proteins. Further studies are required to determine the effects of high P in *M. paniculata* leaves on absorption of other micronutrients.

Conclusion

The optimum rates of chicken manure to produce maximum fresh and dry leaf weight at 34 MAP (four months after manure application) were 3.1 kg and 6.5 kg per plant, respectively. These rates, however, were not sufficient for the second harvest at 38 MAP. K concentration of the leaves of all positions and leaf age correlated positively with leaf dry weight (P <0.01), but correlated negatively with total flavonoid content (P <0.01). Leaf P concentration was negatively correlated with the dry weight of the 5th young leaves and of the mature leaves from all positions. Leaf K

concentration is categorized as very high (3.59-4.10%), and leaf P concentration as high (0.28-0.29%) to very high (0.33-0.35%). The 5th mature leaves could be used to determine plant's K requirements.

Acknowledgements

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