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Correlations between Leaf Nitrogen, Phosphorus and Potassium and Leaf Chlorophyll, Anthocyanins and Carotenoids Content at Vegetative and Generative Stage of Bitter Leaf (*Vernonia amygealina* Del.)

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Abstract

Vernonia amygdalina Del. (*Asteraceae*) is a nutritional and medicinal plant that is used widely throughout tropical Africa and other countries. The leaves are proved to have strong antioxidant property. The concentration of chlorophyll a, chlorophyll b, anthocyanins and carotenoids in five different leaf positions of vegetative and generative phases were studied. The aim of the study is to understand and to correlate the content of the three nutrients and the bioactive compounds along the five leaf positions at vegetative and generative stage of the crop growth. The study showed that the concentration of chlorophyll a and b was higher, and anthocyanin concentration was almost zero in the generative than in the vegetative stage. The leaf concentration of N, P, and K were higher in vegetative than in generative stage. At vegetative stage, N positively correlated with chlorophyll a at the 1st leaf; K had strong correlations with anthocyanins at the 2nd and 3rd leaf and with chlorophyll a at the 2nd and 5th, and with chlorophyll b and carotenoids at the 5th leaf. At the generative stage, P had a strong positive correlation with carotenoids at 4th and 5th leaves, and K positively correlated with chlorophyll a and b at 5th leaf. The findings suggest that bitter leaf is better to be harvested at vegetative stage, and that potassium level is important to increase anthocyanins and carotenoids content.

Keywords: bitter leaf, nutrient level¸ pigments, vegetables, vegetative and generative phases

Introduction

Bitter leaf, *Vernonia amygdalina*, belongings to the family *Asteraceae*, is perennial shrub or tree up to 10 m. In cultivation, it is often pruned to a shrub or hedge. Leaves blade is dark green, ovate-elliptical to lanceolate, cuneate or rounded at base, acuminate at apex, margin minutely toothed to coarsely serrate and finely pubescent. It can grow up to 2000 m altitude and on almost all types of soil (Grubben and Denton, 2004).

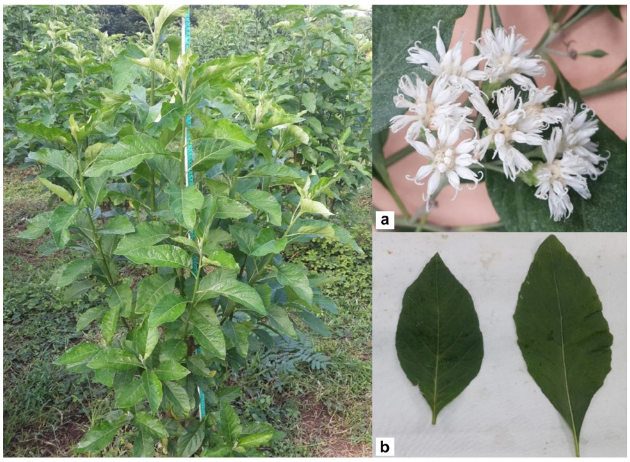

Figure 1. *Vernonia amygdalina* plant (left), flowers (a, top right) and leaves (b, bottom right)

Bitter leaf is commonly used in traditional medicinal plant in Africa and other countries around the world. The leaves is also well known and consumed as a vegetable. Bitter leaf is traditionally used to treat gastroenteritis, diarrhea, fever, parasite infections, dysentery, diabetes and hepatitis (Challand and Willcox, 2009; Vlietinck et al., 1995).

Several research have shown that *V. amygdalina* leaves have cytotoxic (Kupcan et al., 1969), antibacterial and antitumor (Jisaka et al., 1992), antivirus (Vlietinck et al. 1995), antihelmintic (Alawa et al*.,* 2003), antimalaria/antiplasmodial (Abosi and Raseroka 2003; Sha'A et al., 2011), anticancer (Izevbigie et al., 2004), analgesic (Njan et al., 2008), antiinflammatory (Iroanaya et al., 2010), antioxidant (Erasto et al., 2007a), hypolipidemia (Adaramoye et al., 2008a), liver protective (Adaramoye et al., 2008b), antidiabetic (Michael et al., 2010; Asante et al., 2016) and antiparasitic properties (Leonidas et al., 2013). Leaves are mostly used as they contain sesquiterpene lactones (Erasto et al., 2006; Kupchan et al., 1969), steroid glycosides (Jisaka et al*.,* 1993) and flavonoids (Oriakhi et al., 2013; Igile et al., 1994). Flavonoids in leaf extract were proven to have antioxidant effects (Erasto et al., 2007a; Erasto et al., 2007b). An antioxidant is a scavenging agent that can prevent degenerative diseases (Gross et al., 2004), inhibit metastasis and tumorigenesis (Zhou, 2006) and constitute anti-inflammatory, antibacterial and antifungal properties (Christensen and Brandt, 2007). However, it is unclear which leaf position is best to produce certain secondary metabolites. Therefore the purpose of this research is to determine the leaf concentration of nitrogen, phosphorus, potassium, chlorophyll, anthocyanins and carotenoids of bitter leaf, and to correlate the concentration of N, P, K with chlorophyll, anthocyanins and carotenoids of different five leaf positions (top to base) at two different plant growth stages, vegetative and generative. This research also aimed to determine the stage of plant growth when the nutrition and bioactive compounds are the highest. Bitter leaf is usually pruned regularly to form short growing shrubs that stays vegetative so the plants do not flower. When the bitter leaf plants were not pruned they will continue to grow to small trees, set flowers, and can live to more than seven years.

Materials and Methods

Plant Materials

Plant materials were collected from Dramaga, Bogor, West Java, Indonesia. Leaves were obtained from flowering trees of > 10 year-old (generative) and from regularly pruned shrubs that have never flowered (vegetative). Leaf samples were collected from the 1st, 2nd, 3rd, 4th and 5th fully expanded leaves, counted from the shoot tip.

Methods

Leaf N concentration was analyzed analyzed using the Kjeldahl method, P concentration using Shimadzu UV-1800 Spectrophotometry, and K concentration using Atomic Absorption Spectrophotometry (AAS) Agilent 240 FS AA. The nutrient analysis was conducted at the Analytical Laboratory, Department of Agronomy and Horticulture, Bogor Agriculture University, Indonesia.

Chlorophyll, carotenoids, and anthocyanins were analyzed using the method of Sims and Gamon (2002) as follows: 20 mg fresh leaf was ground and added with 2 ml aceton tris (85:15)% then centrifuged at 14000 rpm for 30 seconds. One ml supernatant was added with 3 ml aceton tris and vortex thoroughly. Absorbance was measured at wavelengths of 470, 537, 647 and 663 nm. The analysis was conducted at the Department of Agronomy and Horticulture, Bogor Agriculture University, Indonesia.

Data Analysis

Data was analyzed with Pearson correlation and t-student test using STAR (Statistical Tool for Agricultural Research) Nebula, IRRI.

Results and Discussion

The concentration of chlorophyll a and b was higher in the generative plant than in the vegetative plants in all leaf positions (Figure 2). During the vegetative stage, the trend in both chlorophyll types was similar but the concentration of chlorophyll a was higher than chlorophyll b. The concentration of chlorophyll a and b slightly decreased from the 1st to 2nd leaf and was the lowest on the 3rd leaf stage. The same trend was recorded for chlorophyll a and b during the generative stage. The highest concentration of chlorophyll a and b was at the 2nd leaf. Meanwhile, anthocyanins were only found in the vegetative plants and it was were almost zero during generative stage, except on the 2nd leaf (Figure 3). The concentration of anthocyanins fluctuated from 1st to 5th leaf. Anthocyanins concentration of 1st and 3rd leaf was almost similar which was about 0.07 mg/100g, and it was the highest t the 5th leaf, i.e. 0.08 mg per 100g of leaf fresh weight.

Leaf carotenoids content at different growth stages showed a different trend (Figure 4). The concentration of carotenoids of the 1st and 2nd leaves were similar and dropped to the 3rd leaf , but increased gradually in the 5th leaf. The highest content of carotenoids was found in the 4th leaf which was 0.57 mg per g of leaf fresh weight. In the generative plant, the carotenoids trend decreased from the 1st to the 5th leaf. The highest was in the 2nd leaf (0.7 mg/g), followed by the 4th leaf (0.68mg per g). The percentage of nitrogen was slightly higher in the vegetative than generative stage at all leaf positions, with the highest level found in the 1st leaf (5.17%) (Figure 5). The N percentage slowly declined after the 1st leaf (Figure 5). The pattern was similar to the generative stage even though the 2nd leaf contained slightly higher nitrogen level than the 1st leaf.

Similarly, the phosphorus level in the vegetative stage was generally higher than in the generative stage (Figure 6). The highest phosphorus level was at the 1st leaf which then started to decline gradually. The highest phosphorus level was from vegetative plants, i.e. 0.41%.

The potassium levels in vegetative and generative plants tended tend to be constant (Figure 7) and there were no significant differences amongst leaves of different positions. However, potassium level in the generative stage between was lower (4.07 to 4.39%) than the vegetative stage (5.33 to 5.87%).

There were no significance differences in the levels of chlorophyll a and b, anthocyanins, carotenoids and NPK contents among the different leaf positions in the vegetative and generative plants. In addition, no significant differences were found in the levels of chlorophyll a/b, anthocyanins, carotenoids and NPK contents between vegetative and generative plants.

The result of the correlation test showed that 1st leaf nitrogen correlated positively with chlorophyll a in the vegetative plants (Table 1). Potassium positively correlated with chlorophyll a and anthocyanins at the 2nd leaf. Moreover, it also had a positive correlation with anthocyanins at 2nd and 3rd leaf. At the 5th leaf potassium positively correlated with chlorophyll a, chlorophyll b and carotenoids. In the generative plant, phosphorous positively correlated with carotenoids at the 4th leaf while potassium correlated positively with chlorophyll a at the 5th leaf (Table 2).

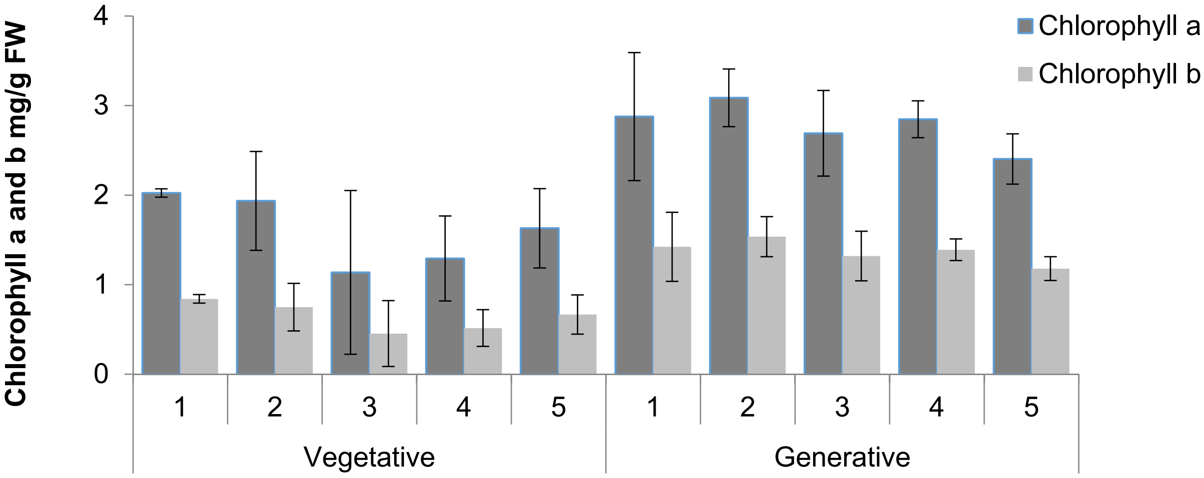

Figure 2. Chlorophyll a and b concentration of *V. amygdalina* leaves from five different positions along the stem of vegetative and generative plants

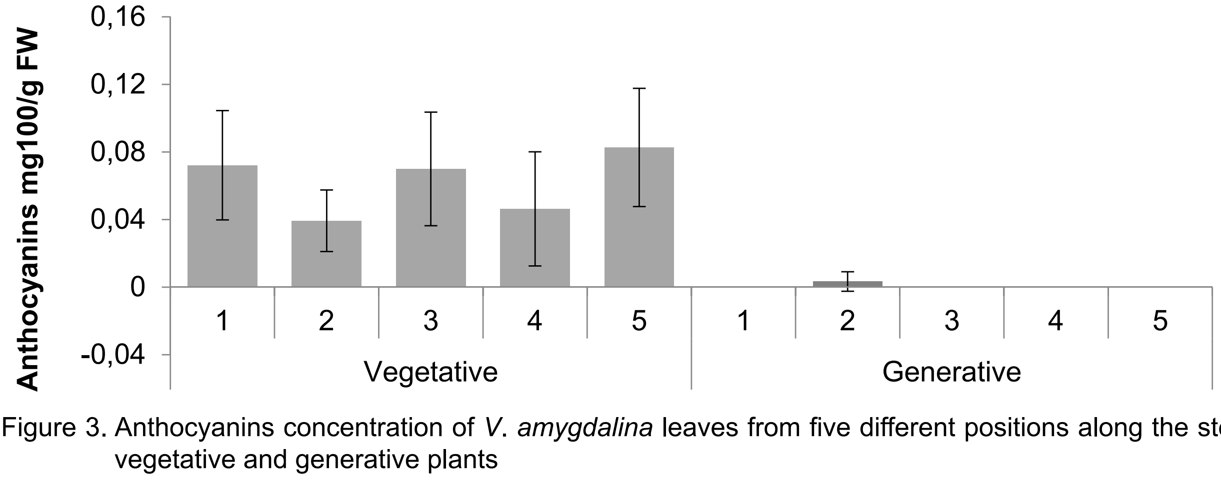

Figure 3. Anthocyanins concentration of *V. amygdalina* leaves from five different positions along the stem of vegetative and generative plants

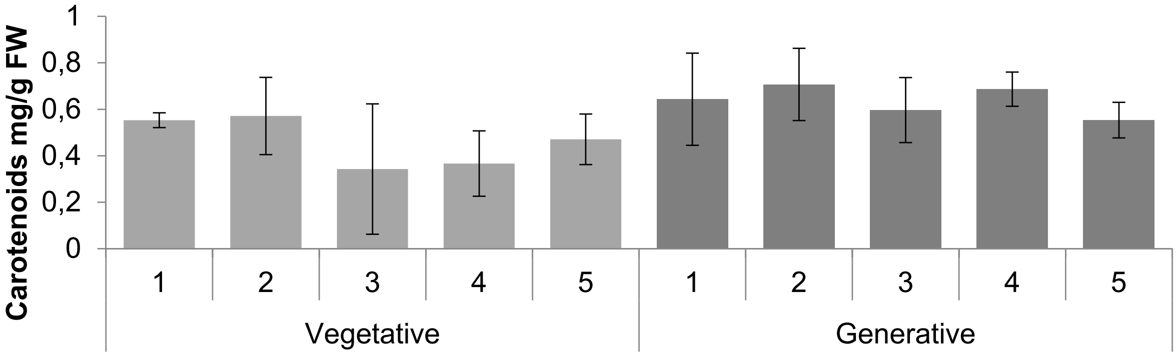

Figure 4 Carotenoids concentration of *V. amygdalina* leaves from five different positions along the stem of vegetative and generative plants

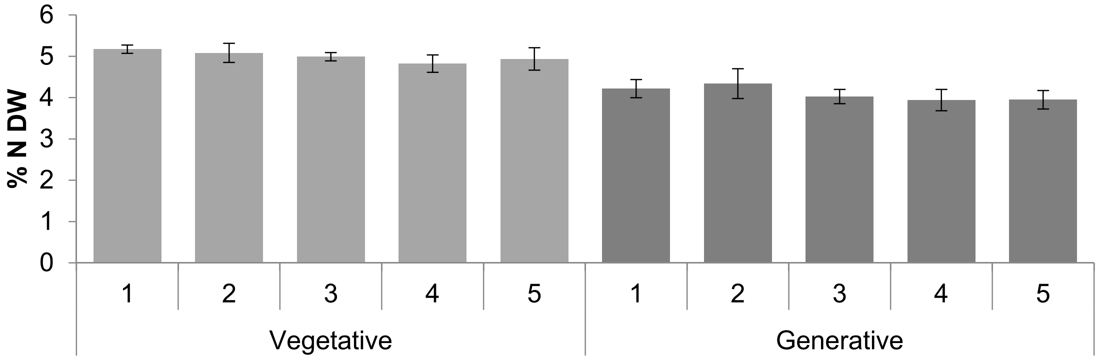

Figure 5. Nitrogen level of *V. amygdalina* leaves from five different positions along the stem of vegetative and generative plants

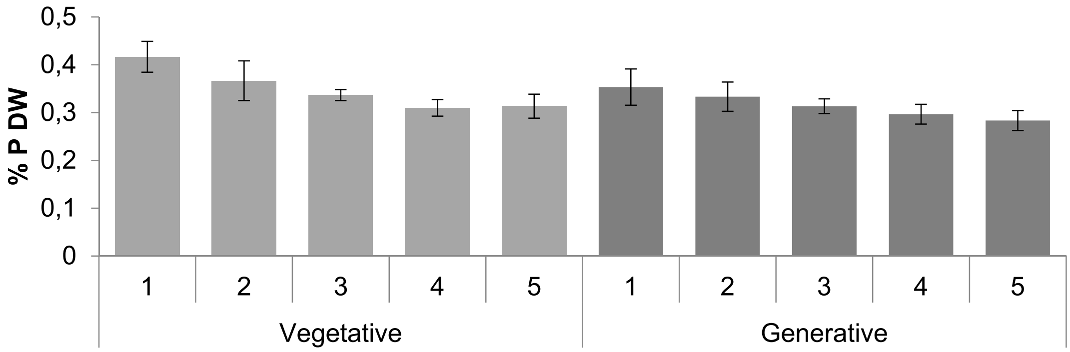

Figure 6. Phosphorus level of *V. amygdalina* leaves from five different positions along the stem of vegetative and generative plants

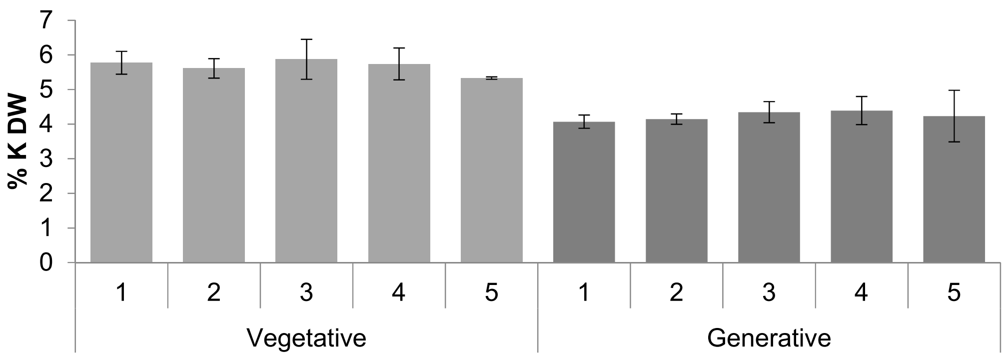

Figure 7. Potassium level of *V. amygdalina* leaves from five different positions along the stem of vegetative and generative plants

Discussion

The vegetative and generative plants showed similar pattern of chlorophyll and carotenoids concentrations which were higher at the 1st and 2nd leaves (near shoot tips) than the rest of the leaf positions. This finding might indicate that the mature leaves near the tip function as the source of photosynthate for the growing tips (Taiz and Zeiger, 2002). In soybean, higher pigment concentrations are related to higher photosynthetic rate (Butterly and Buzzell, 1977; Fleischer, 1935). It could be implied that at the 1st and 2nd leaves had higher photosynthesis rate so they produced higher photosynthate relatively to the other

Table 1. Correlation between N, P and K level of the 1st, 2nd, 3rd, 4th and 5th leaf with chlorophyll a, chlorophyll b, anthocyanins and carotenoids in the vegetative plants

Leaf position	Nutrient	Chlorophyll a	Chlorophyll b	Anthoyanins	Carotenoids
1st	N	0.99*	0.99	0.89	0.99
	P	0.25	0.16	0.66	0.34
	K	0.42	0.34	0.79	0.50
2nd	N	0.86	0.93	0.79	0.77
	P	-0.03	0.11	-0.16	-0.19
	K	0.99*	0.98	0.99*	0.99
3rd	N	-0.96	-0.95	-0.99	-0.98
	P	0.26	0.29	0.11	0.20
	K	0.98	0.98	0.99*	0.99
4th	N	-0.03	0.04	0.74	-0.06
	P	0.76	0.70	-0.01	0.77
	K	0.98	0.99	0.77	0.98
5th	N	-0.91	-0.9	-0.01	-0.87
	P	-0.84	-0.82	0.15	-0.80
	K	0.99*	0.99*	0.47	0.99*

Note: *indicates significant correlation; +/- indicates positive or negative correlation

Table 2. Correlation between N, P and K level of the 1st, 2nd, 3rd, 4th and 5th leaf with chlorophyll a, chlorophyll b, anthocyanins and carotenoids in the generative plant

Leaf position	Nutrient	Chlorophyll a	Chlorophyll b	Anthocyanins	Carotenoids
1st	N	0.93	0.94	-	0.98
	P	0.33	0.35	-	0.48
	K	0.73	0.75	-	0.83
2nd	N	0.78	0.76	-0.77	0.81
	P	0.48	0.50	0.75	0.43
	K	0.34	0.37	0.84	0.29
3rd	N	0.04	0.06	-	0.08
	P	0.87	0.88	-	0.89
	K	0.97	0.97	-	0.97
4th	N	0.25	0.21	-	0.62
	P	0.92	0.91	-	0.99*
	K	0.66	0.69	-	0.30
5th	N	0.09	-0.00	-	0.49
	P	0.94	0.90	-	0.99
	K	0.99*	0.98	-	0.93

Note: *indicates significant correlation; +/- indicates positve or negative correlation to support the strong sink (shoot tips). The indication that the 1st and the 2nd leaves as the source for the tips was also supported by similar trend of nitrogen, phosphorus, and potassium concentrations in both plant phases. The higher chlorophyll concentration at the 1st and the 2nd position was also followed by higher nitrogen concentration and this may be due to the fact that nitrogen is one of the structural component of chlorophyll (Marschner, 2012). The high concentration of NO_3^-, PO_4^-, and K^+ could increase the rate of photosynthesis by increasing the absrobtion of CO_2 in cotton (Longstreth et al., 1980).

There was a pattern showing the fluctuation of anthocyanins at the vegetative plants. However, according to Hughes et al. (2007) the concentration of anthocyanins are higher at young leaves than at the mature leaves. Anthocyanins would be present until chlorophyll reaches about 50% and carotenoids about 49% . In the early development of leaves, anthocyanins functioned as photo protection because chlorophylls had not fully developed to absorb excessive sunlight (Hughes et al., 2007). The absence of anthocyanins in the generative phase plant might be replaced by carotenoid as it had higher concentration than in the vegetative plants. Carotenoids are accessory pigments that function as photoprotection in the process of photosynthesis; it helps to return the activated chlorophyll, Chl*, to its ground state when it absorbed excessive light (Demmig-Adams dan Adams, 2000). A study by Andrzejewska et al. (2015) in chokeberry demonstrated that as the trees get older, especially above ten years, the anthocyanins content will decrease about 16 to 18% compred to the younger trees. The generative plants in this study is about 15-year-old, which might explain the lower anthocyanins concentrations than the vegetative stage.

The levels of three major elements during both growing stage was considered being normal compared to those from the same family of leafy vegetables, romaine, and head lettuce (Uchida, 2000). The recommended N, P and K levels for head lettuce are 3.8–5%, 0.45–0.8% and 6.6–9%, while the recommended N, P and K levels for romaine lettuce are 3.5–4.5%, 0.45–0.6% and 5.5–6.2% (Uchida, 2000). From these recommendations, the N, P and K level in the vegetative plants was sufficient, while in the generative plant the N and P was sufficient, but the amount of K was below the recommended status. However, the nutrient concentration of generative plant was slightly lower than the nutrient concentration in the vegetative plants. According to Marschner (2012) remobilization of nutrients to reproductive organ occurs as the development progress from vegetative to reproductive stage. As a result, the root activity and nutrient uptake decline because the carbohydrate supply to the root decreases, and the vegetative organs also experience the decline of nutrient concentrations. Nitrogen plays an essential role as a component of the structure of protein, enzymes, nucleic acids, phytohormones and other secondary metabolites. It is one of the main structural components of chlorophyll (Marschner, 2012). The increase of leaf nitrogen, phosphorus and potassium levels could increase the concentration of chlorophyll (Chenard et al., 2005; Onanuga et al., 2012; Oosterhuis and Bednarz, 2001; Zhao et at., 2001). In addition, carotenoids concentrations could be elevated by the increase in leaf nitrogen (Kopsell et al., 2007). In contrast, the decrease in the leaf nitrogen level (Ibrahim et al., 2012) and phosphorus deficiency (Ulrychov and Sosnova, 1970) could increase leaf anthocyanin concentration. Shaikh et al. (2008), however, reported that anthocyanins concentration was not affected by leaf nitrogen levels. Under nitrogen and phosphorus deficiencies, anthocyanins were found to increase as the plants' response to stress. This is because under stress conditions anthocyanins function as an antioxidant to scavenge free radicals (Chalker 1999). Therefore, it is important to supply the crops with nitrogen and potassium fertilizers to increase leaf anthocyanin concentration (Susanti et al., 2011). As excessive nitrogen has negative effects on the formation of anthocyanins, potassium can compensate that effect by benefiting phenolic compounds indirectly (Delgado et al., 2006). Potassium plays essential roles in protein synthesis, osmoregulation, enzyme activation, photosynthesis, stomatal movement, energy transfer, phloem transport, cation-anion balance and stress resistance (Marschner, 2002).

The results of this study demonstrated that bitter leaf would be best harvested at the vegetative stage, or the crops should be kept vegetative to have higher nutrition levels and more complete bioactive compounds in the leaves. Potassium correlated positively with leaf carotenoids and anthocyanins levels during the vegetative stage, therefore it can be inferred that bitter leaf production could benefit from potassium fertilizer application.

Conclusion

Bitter leaf for vegetable consumption should be harvested in the vegetative phase. Potassium of the 2nd and 3th leaf from the shoot tip had positive correlations with leaf anthocyanins levels, whereas correlation with leaf carotenoid level was recorded at the 5th leaf.

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