Original Article

Effects of Fertilizer Application and Successive Harvesting on Clipping Yield, Phytochemical Contents and Antioxidant Activity of *Cynodon dactylon* (L.) Pers.

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Abstract

The present study aimed to investigate the effects of fertilizer application and successive harvesting on phytochemical contents and antioxidant activities of *Cynodon dactylon*, a medicinal Bermuda grass in Sabah (Malaysia). Three fertilizers of two nitrogen concentrations were used in the experiment. The grass was harvested successively three times at five-weeks interval. Grass treated with 25 kg N/ha/month from the first harvest was found to have the highest clipping yield. Successive harvesting decreased the dry matter production of the grass irrespective of N concentration applied. Total saponin and alkaloid contents of the grass were increased by a combination treatment of fertilizer type × rate × harvesting; total flavonoid content was increased by fertilizer type × harvesting treatments; however, total phenolic content was not affected by any of the treatment or combination of the treatments. Both of the antioxidant assays (DPPH and FRAP) indicated that antioxidant activity of the grass was increased by fertilizer rate × harvesting treatments. There was a significant correlation found between total phenolic and flavonoid contents and antioxidant activities, suggesting that these two secondary metabolites may contribute to the antioxidant property of the grass. Overall, the obtained data indicated that the described treatments could be used to manipulate the production and accumulation of bioactive compounds of *C. dactylon*.

Keywords: Bermuda grass; cutting frequency; nitrogen rate; organic/ inorganic fertilizer

Introduction

The application of organic and inorganic fertilizers improves soil fertility and thus plant growth and development. More specific, the chemical composition and concentration of the fertilizers will affect the biosynthesis and composition of secondary compounds in the plants (Hallmann and Rembiałkowska, 2012). The manipulation of fertilizer application has been reported to increase the phytochemical and bioactive contents of medicinal plants, such as *Cosmos caudatus, Hypericum perforatum* and *Ocimum basilicum* (Hassan *et al.*, 2012; Farshchi *et al.*, 2014; Onofrei *et al.*, 2017). In organic or inorganic fertilizers, nitrogen (N) has been one of the major components important for plant growth and development, meaning this element has an important role in affecting the phytochemical and bioactive contents of plants (Vashisth *et al.*, 2017; Barroso *et al.*, 2018). In addition, successive harvesting has also been one of the factors that affects chemical properties and dry matter yield of medicinal plants. Frequent harvesting has been reported to affect the

phytochemical contents and antioxidant activities of *Stevia rebaudiana* Bertoni (Tavarini *et al.*, 2015) and *Chicorium spinosum* L. (Petropoulos *et al.*, 2017). Successive harvesting at 35-day and 30-day interval has been found to decrease the dry matter yield of *Moringa oleifera* Lam (Amaglo *et al.*, 2007) and *Talinum triangulare* (Jacq.) Willd (Brasileiro *et al.*, 2015) respectively. Other than nitrogen application and harvesting frequency, planting time (Brasileiro *et al.*, 2015), geographical location and maturity stages (Gull *et al.*, 2012), cultivar and harvesting time (Zou *et al.*, 2012) could also affect the biosynthesis of secondary metabolites in plants.

Cynodon dactylon is one of the important medicinal plants documented in traditional medicine system around the world and has been claimed by tribe or traditional communities to have various medicinal values (Nagori and Solanki, 2011). It has also been reported to have contributed to pharmacological development in recent years, such as, in the production of antioxidant, antimicrobial, wound healing, antidiabetic, anti-cancer, anti-malarial and anti-chikungunya agents (Khlifi *et al.*, 2013; Chandel and Kumar, 2015; Murali *et al.*, 2015).

There are many ecotypes of *C. dactylon* found in Sabah (Malaysia) (Gobilik *et al.*, 2013). Several ecotypes have been reported to show antimicrobial activities against clinical pathogens (Abdullah *et al.*, 2012). To date, however, there is no study carried out to explore the effect of agricultural practices on the secondary metabolites production and antioxidant activity of this grass.

In the present study, the effects of two common agricultural practices, fertilizer application and harvesting, on the production of secondary metabolites and antioxidant activities of *Cynodon dactylon* in Sabah (Malaysia) are reported.

Materials and Methods

Grass planting, maintenance and dry matter yield assessment
The experiment was carried out in rain shelter as a factorial tray-experiment of 3 fertilizers (F) × 2 N application rate (R) and 5 replicates arranged in a Completely Randomized Design. The harvesting (H) treatment was included as a within subject factor to the fertilizer treatment. The stolons of the grass were collected from a wild population of *C. dactylon* that has already been reported to produce medicinally important metabolites (Abdullah *et al.*, 2012). Twenty-five stolons were planted per tray (46 cm × 34 cm × 15 cm) on a medium consisting of top soil and sand (1:9). Inorganic NPK 15-15-15 (F1), and two types of organic NPK 8-8-8 (F2), and NPK 5-5-5 (F3) were used as the fertilizers. The fertilizers were applied monthly at two rates: 12.5 kg N/ha (R1) and 25.0 kg N/ha (R2). Grass sprouted from the stolon was let to grow for 5 weeks before it was trimmed. New shoots were let to resprout in the next 5 weeks. From week 10 and onwards, grass in each tray was harvested three times (H1, H2 and H3) at 5-week interval; all stolons of 4-cm above the medium were harvested. At each harvest, grass collected from each tray was cleaned under running tap water and dried in an oven at 50 °C until attained a constant weight or at least 24 hours to obtain the dry matter weight of the clippings.

Preparation of extract for phytochemical and antioxidant activity analyses
The dried grass was grinded to powder at 3,000 rpm using a mechanical grinder, kept in air tight container and stored at 4 °C. For the quantification of total saponin and alkaloid, the crude extract was prepared following the procedures described by Mohadjerani *et al.* (2014). A total of 50 g of the grass powder was macerated at room temperature in 100 ml of 80% (v/v) ethanol for 24 h. The extract was filtered and concentrated at 55 °C under reduced pressure using rotary evaporator (Heidolph). The crude extract was kept at -20 °C for further use. For the quantification of total phenolic and flavonoids, the crude extract was prepared following a slightly modified method explained by Ibrahim *et al.* (2013). The powder (0.1 g) of the grass was soaked in 10 ml of 80% (v/v) ethanol and kept in water bath shaker at 50 °C and 120 rpm for 2 h. The extract was filtered using Whatman filter paper No.1 (Whatman Ltd., England). The extract was directly used for the total phenolic, total flavonoid and antioxidant analyses.

Total saponin and alkaloid contents
The total saponin content (TSC) of the crude extract was assessed using the method described by Sudha and Srinivasan (2013). It was expressed as diosgenin equivalent per gram of the crude extract (mg DE gm^{-1} crude extract) used in the test. The total alkaloid content (TAC) of the extract was determined using the technique explained by Shamsa *et al.* (2008). It was expressed as atropine equivalent per gram of the crude extract (mg AE gm^{-1} crude extract) used in the test.

Total phenolic and flavonoid contents
The total phenolic content (TPC) of the crude extract was measured following the method described by Singleton *et al.* (1999). It was expressed as mg gallic acid equivalent per gram of the grass dry sample (mg GAE g^{-1} dry sample) used to prepare the crude extract. The total flavonoid content (TFC) of the crude extract was quantified using the technique explained by Ibrahim *et al.* (2013). It was expressed as mg quercetin equivalent per gram of the grass dry sample (mg QE g^{-1} dry sample) used to prepare the crude extract.

2, 2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity (DPPH-RSA)
The DPPH-RSA of the crude extract was assessed to evaluate its antioxidant activity. The evaluation was carried out following the method explained by Fook and Kheng (2009) with a slight modification of the wavelength used, i.e., 518 nm. The final antioxidant activity (AA%) data were expressed as IC$_{50}$ values. The IC$_{50}$ values were interpreted as the concentration of the samples that produced 50% scavenging DPPH radical (mg/ml).

Ferric Reducing Antioxidant Power (FRAP)
FRAP test was used to support the result of the DPPH-RSA explained above. FRAP was assessed following the procedures applied by Benzie and Strain (1996). The method used by those authors was based on the reduction of colourless ferric complex (Fe^{3+} tripyridyltriazine) to blue-colored ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. The reaction was monitored by measuring the change in light absorbance of the substance at 593 nm wavelength. The result was expressed as the concentration of antioxidant having a ferric reducing ability in 1 g of the grass dry sample (mM g^{-1}).

Statistical analysis
Mixed Analysis of Variance (Mixed ANOVA) was performed to infer the effects of fertilizer treatments and successive harvesting on the clipping yield, phytochemical contents and antioxidant activity of the grass. The comparison of between-subject-factor means (fertilizer treatments) was evaluated using Tukey test (at $p \leq 0.05$), and the comparison of within-subject-factor means (successive harvesting) was evaluated using Bonferroni test (at $p \leq 0.05$). All statistical analyses were carried out using SPSS® version 21.

Results

The summary of the effects of fertilizer application and successive harvesting on yield, total saponin, total alkaloid, total phenolic and total flavonoid contents and antioxidant activity of *Cynodon dactylon* is shown in Table 1.

Clipping yield

Combination of R × H had significantly increased the clipping yield (Table 1). The highest clipping yield was 4,386.02 kg/ha, which was found for grass under R2 at H1 (Fig.1). These findings, however, were authentic only at H1. The clipping yields for R1 and R2 decreased significantly after H2 and H3.

Total saponin content

The TSC of the grass was increased by F × R × H (Table 1). The highest TSC was 588.49 ± 17.08 mg DE/g of crude extract, which was found for grass grown under F1 at R2
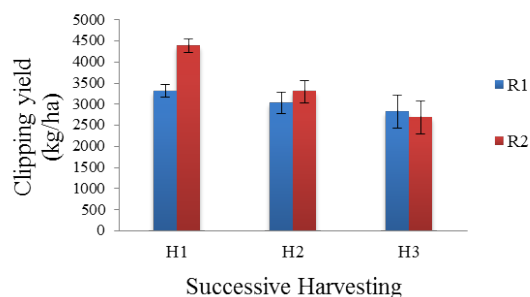

Fig. 1. Effect of fertilizer rate (R) and successive harvesting (H) on clipping yield. Note: R1: 12.5 kg N/ha; R2: 25 kg N/ha; H1: 1st harvest; H2: 2nd harvest; H3: 3rd harvest. Error bars represent standard error

and H3 (Fig. 2). This result also showed that TSC increased after H3 in F1, F2 and F3 treatments regardless of N application rate (R).

Total alkaloid content

The TAC of the grass was also increased by F × R × H (Table 1) with a little variation of responses. For F1, it increased with increment of R and H; for F2, there was no consistent response; for F3, TAC was the highest under R1 and H1 (Fig. 3). The highest TAC was 23.16 ± 3.18 mg AE/g crude extract, which was found for grass grown under F1 at R1 and H3.

Total phenolic and flavonoid contents

The production of TPC was not affected by any treatment or combination of the treatments. For TFC, it increased only under F × H (Table 1). TFC was the highest under F3 at every harvesting time (H1 – H3) (Fig. 4). The value of TFC under F3 ranged from 47.92 ± 1.58 to 49.17 ± 1.58 mg QE/g dry samples for H1, H2 and H3.

Antioxidant assay

The DPPH-RSA indicated that the IC_{50} of the grass was decreased (e.g. antioxidant capacity increased) by F × R and R × H (Table 1). For F × R, the IC_{50} decreased under F3 at both R1 and R2 and in fact, F3 led to the lowest IC_{50} in this study (Fig. 5A). Under F1 and F2, the IC_{50} decreased only under R1. Similarly, for R × H, the IC_{50} decreased significantly only under R1 (Fig. 5B). For R2, the IC_{50} was almost similar between H1, H2 and H3.

Meanwhile, the treatment of R × H also significantly increased the antioxidant activity by FRAP assay. The antioxidant activity of the grass under R1 was increased from H1 to H3 (Fig. 5C). The same pattern was observed for R2, however, the FRAP value was lower compared to R1.

Table 1. Effects of fertilizer application and successive harvesting on yield, total saponin, total alkaloid, total phenolic and total flavonoid contents and antioxidant activity of *Cynodon dactylon*

Treatments	CY (kg dry weight ha⁻¹)	TSC (mg DE g⁻¹ crude extract)	TAC (mg AE g⁻¹ crude extract)	TPC (mg GAE g⁻¹ dry sample)	TFC (mg QE g⁻¹ dry sample)	DPPH-RSA (mg extract ml⁻¹)	FRAP (mM Fe (II)/g dry sample)
Fertilizer (F)							
F1	4,270[a]	301.89[a]	11.67[a]	29.10	39.52[b]	1.38[b]	559.55[b]
F2	2,960[b]	236.52[b]	12.75[a]	29.52	42.26[b]	1.25[b]	579.76[b]
F3	2,538[b]	214.01[c]	7.90[b]	31.20	48.68[a]	1.04[a]	650.85[a]
Rate (R)							
R1	3,056.3	242.65[b]	11.11	30.40	43.28	1.11[a]	630.77[a]
R2	3,455.1	258.96[a]	10.42	29.48	43.69	1.34[b]	562.67[b]
Harvesting (H)							
H1	3,850[a]	162.87[c]	11.06[b]	30.21	43.01	1.37[b]	546.58[c]
H2	3,164[b]	198.80[b]	6.68[c]	29.37	43.53	1.19[a]	597.25[b]
H3	2,753[b]	390.75[a]	14.57[a]	30.25	43.92	1.11[a]	646.33[a]
			Analysis of variance				
F	**	**	**	ns	**	**	**
R	ns	**	ns	ns	ns	**	**
H	ns	**	ns	ns	ns	**	**
F × R	ns	**	**	ns	ns	**	ns
F × H	ns	**	**	ns	**	ns	ns
R × H	**	**	**	ns	ns	**	**
F × R × C	ns	**	**	ns	ns	ns	ns

CY: Clipping yield; TSC: Total saponin content; TAC: Total alkaloid content; TFC: Total flavonoid content; TPC: Total phenolic content; F1: NPK 15-15-15; F2: NPK 8-8-8; F3: NPK 5-5-5; R1: 12.5 kg N/ha; R2: 25 kg N/ha; H1: 1st harvest; H2: 2nd harvest; H3: 3rd harvest; ns, **, *: non-significant, significant at $p \leq 0.01$, $p \leq 0.05$, respectively. Mean in a same column, not sharing a common alphabet were significantly different at $p \leq 0.05$
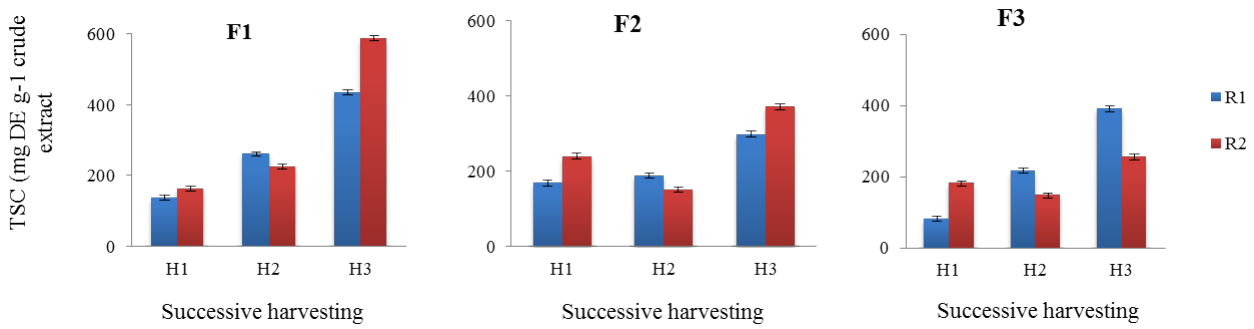
Fig. 2. Effect of fertilizer type (F), rate (R) and successive harvesting (H) on total saponin content (TSC). Note: F1: NPK 15-15-15; F2: NPK 8-8-8; F3: NPK 5-5-5; R1: 12.5 kg N/ha; R2: 25 kg N/ha; H1: 1st harvest; H2: 2nd harvest; H3: 3rd harvest. Error bars represent standard error
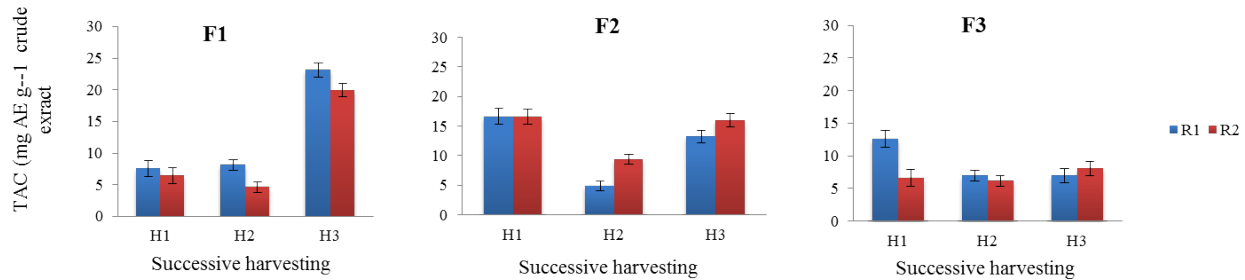

Fig. 3. Effect of fertilizer type (F), rate (R) and successive harvesting (H) on total alkaloid content (TAC). Note: F1: NPK 15-15-15; F2: NPK 8-8-8; F3: NPK 5-5-5; R1: 12.5 kg N/ha; R2: 25 kg N/ha; H1: 1st harvest; H2: 2nd harvest; H3: 3rd harvest. Error bars represent standard error
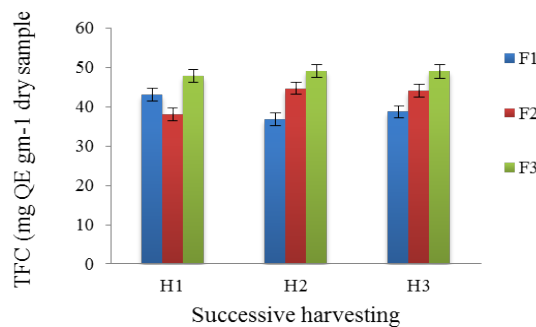

Fig. 4. Effect of fertilizer type (F) and successive harvesting (H) on total flavonoid content (TFC). Note: F1: NPK 15-15-15; F2: NPK 8-8-8; F3: NPK 5-5-5; H1: 1st harvest; H2: 2nd harvest; H3: 3rd harvest. Error bars represent standard error
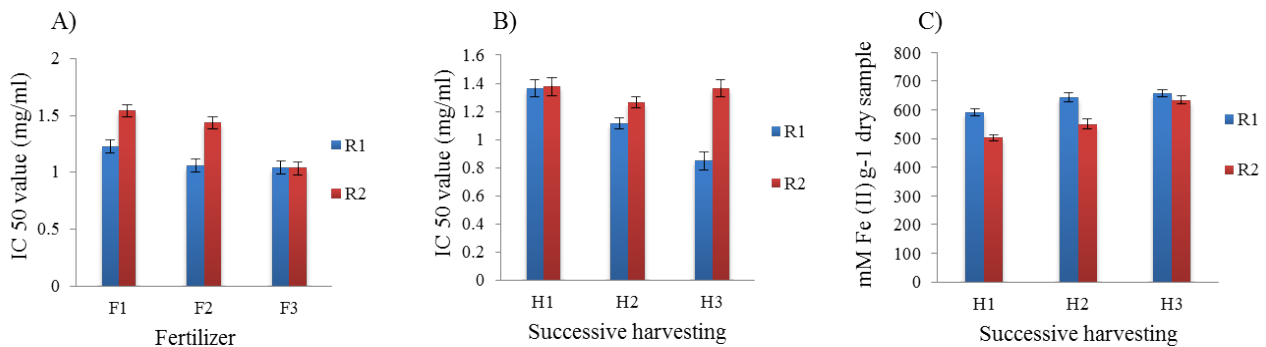

Fig. 5. (A) Effect of fertilizer type (F) and rate (R) on IC_{50} value; (B) Effect of fertilizer rate (R) and successive harvesting (H) on IC_{50} value; (C) Effect of fertilizer rate (R) and successive harvesting (H) on FRAP value. Note: F1: NPK 15-15-15; F2: NPK 8-8-8; F3: NPK 5-5-5; R1: 12.5 kg N/ha; R2: 25 kg N/ha; H1: 1st harvest; H2: 2nd harvest; H3: 3rd harvest. Error bars represent standard error

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Correlation of TPC, TFC and DPPH-RSA, FRAP

There was a significant negative-correlation between TPC and IC_{50} DPPH (r = -0.608, p < 0.05) and between TFC and IC_{50} DPPH (r = -0.575, p < 0.01) (Table 2).

There was also a significant, but this time positive-correlation between TPC and FRAP (r = 0.484, p < 0.01) and between TFC and FRAP (r = 0.469, p < 0.01).

Table 2. Correlation between antioxidant activities (DPPH and FRAP assays) with total phenolic, total flavonoid, total saponin and total alkaloid contents

Parameters	1	2	3	4	5	6
1. TPC	1.000					
2. TFC	0.572**	1.000				
3. TSC	- 0.211	0.031	1.000			
4. TAC	- 0.101	- 0.447	0.275	1.000		
5. DPPH (IC_{50} value)	- 0.608**	- 0.575*	-0.060	0.242	1.000	
6. FRAP assay	0.484*	0.469*	0.445	- 0.071	-0.638**	1.000

TPC: Total phenolic content; TFC: Total flavonoid content; TSC: Total saponin content; TAC: Total alkaloid content; * and ** Correlation is significant at $p \leq 0.01$ and 0.05, respectively (2-tailed)

Discussion

Effects of fertilizer application and harvesting frequency on dry matter yield

In the present study, it has been expected that plants have a higher dry matter yield at a higher N application, irrespective of the types of the fertiliser used. The yield has also been reported to decrease with successive harvesting. High cutting frequency has been reported to reduce the dry matter production of grass, for example, Napier grass and hybrid *Pennisetums* (Manyawu *et al.*, 2003), as well as perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) (Vinther, 2006). The removal of the above ground growth will limit the capacity of the grass to photosynthesizes, leading to a lacking of carbohydrate for root and rhizome development, as the grass will use up most of the carbohydrate produced to resprout (Aldous and Wilson, 1999; Turgeon, 2008).

Effects of fertilizer application and harvesting frequency on plant secondary metabolites

The results regarding the positive effect of F1 (NPK 15-15-15) especially at a higher N application rate (25 kg N/ha) on TSC of the grass could be associated with the fast release of N from inorganic fertilizer. It has been reported that the concentration of saponin in the rhizome of *Anchomanes difformis* (Blume) Engl. increases after an application of only 15 kg N/ha of NPK 15-15-15 (Rivai *et al.*, 2017). There are, however, some different results reported by other studies. Xia *et al.* (2016) found that the accumulation of saponin in the roots of *Panax notoginseng* [(Burk.) F.H. Chen] has been due to an addition of magnesium mineral fertilizer. Ibrahim *et al.* (2013) reported that an addition of organic fertilizer containing a high concentration of micro nutrients promotes the production of saponin in *Labisia pumila* (Benthis). Mary and Nithiya (2015) reported a similar trend of response to that of Ibrahim *et al.* (2013) for *Solanum nigrum* L.

Similar to TSC, the positive effect of inorganic fertilizer (NPK 15-15-15) on TAC of the grass could be associated with the fast release of N from inorganic fertilizer. Nitrogen application has been reported to promote alkaloid formation, as nitrogen is an essential element for alkaloid biosynthesis (Gholamhosseinpour *et al.*, 2011). Even so, the requirement has been found to be dissimilar between plants.

A rate of 450 kg N/ha is required to increase the alkaloid content of guinea grass, *Panicum maximum* (Jacq.) (Onyeonagu and Ukwueze, 2012). On the other hand, the production of alkaloid in *Datura innoxia* (Mill.) has been reported to decrease with increment of N application (Al-Humaid, 2005). The study appears to support the interaction between N application and production of alkaloid in the present study; it was higher at the lower N application.

The application of organic fertilizer has been reported to improve phenolic and flavonoid production in broccoli (*Brassica olaracea,* var. Italica) (Naguib *et al.*, 2012), *Labisia pumila* Benth (Ibrahim *et al.*, 2013) and *Lycopersicon esculentum* Mill. (Ilupeju *et al.*, 2015). In the present study, the production of TPC in the grass, however, was not affected by either fertilizer type, rate or harvesting frequency. There is no clear explanation for this result based on the information available to the authors. For TFC, the positive effect of the organic fertilizers (NPK 8-8-8 and NPK 5-5-5) is in line with the previous findings. In other words, organic fertilizers could be used to increase flavonoid production in *C. dactylon*. Perhaps, the availability of major and minor elements in the organic fertilizers has contributed to higher production of flavonoid in the grass; this is contrary to the inorganic fertilizer, which consists only N, P and K.

With regard to the effects of successive harvesting on TSC, TAC, TPC and TFC, stress from the cutting may have induced the grass to produce more secondary metabolites. It has been reported that plants produce a higher amount of secondary metabolites as a response to stress, such as, injury stress (Dixon and Paiva, 1995; Jacobo-Velázquez *et al.*, 2015). Mechanical wounding activates phenylpropanoid metabolism and enhances the production of phenolic compounds (Jacobo-Velázquez *et al.*, 2015). In a plant–environment interaction, flavonoids play a major role for the survival of plants including in defending against biotic and abiotic stresses, such as UV radiations, pathogens and insects (War *et al.*, 2012). Other study has reported that a fortnightly instead of weekly cutting increases the level of alkaloid in horse grass (Zhang *et al.*, 2011). In fact, alkaloid production in grass was reported to be affected even by mowing height (Salminen *et al.*, 2003) and maturity stages (Onyeonagu and Ukwueze, 2012). There is also incidence, however, that successive cutting decreases the antioxidant

capacity of plants, such as the case in *Stevia rebaudiana* (Bertoni) and *Chicorium spinosum* (L.) reported by Tavarini *et al.* (2015) and Petropoulus *et al.* (2017), respectively. In the last two studies, the highest antioxidant activity was found for plant extract from the first rather than from the second or third harvest.

Effects of fertilizer application and cutting frequency on antioxidant activities

In DPPH-RSA assay, a lower IC_{50} value indicates a higher ability to scavenge free radicals (Kedare and Singh, 2011) which means a higher antioxidant activity. In FRAP assay, a higher value of ferric reducing ability (mM Fe (II)/g dry sample) indicates a strong antioxidant activity (Wong *et al.*, 2006). Thus, both the results of the DPPH-RSA and FRAP assays in the present study indicated that lower N application and successive harvesting increased the antioxidant activity of the grass. This phenomenon is probably due to the presence of various phenolic and flavonoid compounds in the grass. Fernandez-Panchon *et al.* (2008) reported that those compounds contribute to a better antioxidant activity of a plant. In fact, in this study, there was a significant negative correlation between TPC and IC_{50} DPPH and between TFC and IC_{50} DPPH, but a significant positive correlation between TPC and FRAP and between TFC and FRAP (Table 2), indicating that the increment of TPC and TFC in the grass due to the treatments has led to the higher antioxidant activity of the grass.

Conclusions

The fertilizer type, fertilizer application rate (as N rate) and successive harvesting could increase dry matter yield, phytochemical contents and antioxidant activities of *C. dactylon*, in specific treatments. Specifically, there is a valuable gain to use those treatments to manipulate the production and accumulation of bioactive compounds of *C. dactylon* for pharmaceutical purposes.

Acknowledgements

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