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Effects of harvest frequency on leaf biomass and triterpenoid content of *Centella asiatica* (L.) Urb from Madagascar

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ABSTRACT

***Centella asiatica*, is a wild plant used extensively for its healing properties. In order to establish a rational management, the present study evaluates the effects of time and rate of collection on biomass yield and content of active ingredients. Six collection frequencies were considered (monthly, bi monthly, quarterly, four-monthly, semi-annual and annual). This study shows that the yield of leaf biomass and content of active ingredients vary seasonally according to rainfall, increasing during the rainy season (November to April).The reconstruction of the biomass is maximal if collections are spaced at least three to four months apart. The annual recurrence of repetitive collections causes a loss of productivity of leaf biomass, is significant in the second year of operation. These results offer the best compromise between least effort required for gathering by farmers and greatest yield of biomass and active material, advocating a rate of quarterly collection during the rainy season.**

KEY WORDS: *Centella asiatica*, inter-annual variability, leaf biomass, Madagascar, seasonal variability, triterpenoid yields.

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INTRODUCTION

Centella asiatica (L.) Urban, belonging to the Apiaceae family, is known primarily as a medicinal plant with a variety of therapeutic and cosmetic activities (Brinkhaus et al., 2000; Hashim et al., 2011; Bylka et al., 2014). Currently, it is highly sought after for its wound healing properties (Abdulla et al., 2010; Gohil et al., 2010), to regulate the vascular and nervous systems (Brinkhaus et al., 2000; Tiwari et al., 2008; Zhang et al., 2012) and as anti-oxydant (Zainol et al., 2003). Its therapeutic properties are essentially attributable to the presence of two triterpenoid components (asiaticoside and madecassoside) and their acid derivatives (asiatic acid and madecassic acid) (Brinkhaus et al., 2000). These components are found essentially in the leaves (Aziz et al., 2007; Puttarak and Panichayupakaranant, 2012). Consequently, there is a high demand for *C. asiatica* on the international market and Madagascar is one of the main exporters of this product (Péchard et al., 2005).

In Madagascar, the leaves of *C. asiatica* are harvested exclusively by collecting them from their natural habitat corresponding to the humid zones and low-lands, particularly in the region of Alaotra-Mangoro and the area around Antananarivo. Collection is carried out by local peasants (women and children in particular) and contributes considerably to the incomes of the most impoverished rural households (Chupin, 2010). *C. asiatica* is a stoloniferous creeping plant (Singh and Singh, 2002). In the natural environment, its regeneration and propagation occur through both seed and vegetative multiplication, the latter seeming to be the most efficient

(Singh and Singh, 2002). On account of the economic interest and physiological properties of *C. asiatica,* a number of studies have explored ways of improving the yield and quality of its products, which rely for the best part on the plants' active ingredient contents. The main areas of investigation have concentrated on searches for the best accessions or clones (Randriamampionona et al., 2007; Rasoeta Rakotondralambo et al., 2013) and defining the best ecological and growing conditions (Devkota and Jha, 2010; Puttarak and Panichayupakaranant, 2012; Rahajanirina et al., 2012).

Yet, despite a solid pool of information, the cultivation of *C. asiatica* seems not to be sufficiently managed to enable stable and optimal output of active molecules.

There still exist areas that have not yet been investigated. In particular, information concerning the long term effect (over successive years) of repeated collections on biomass yield and active ingredient content, is limited to a preliminary study by Antsonantenainarivony (2010), which showed that cutting the plant to the ground has a regressive effect on the regrowth size of leaves and length of petioles. Rosalizan et al. (2008) showed that the active ingredient content was greatest in the regrowth two months after collection.

Our study therefore aims to evaluate the effects of different frequencies of leaf collection on the renewal of biomass of *C. asiatica*. It also assesses the impact on the production of biomass and the triterpenoid content of *C. asiatica* over a long period (three years) of different harvest frequencies*.* The results enable us to suggest a means for rationalising the management of the resource taking into account the yield of biomass and active ingredients relative to the input of labour required to collect the crop.

MATERIALS AND METHODS

The study was carried out over a period of three consecutive years (year 1: June 2005 to May 2006; year 2: June 2006 to May 2007; year 3: June 2007 to May 2008 in Vohimana Reserve (Madagascar, region of Alaotra-Mangoro, 18° 55' 12" S, 48°31' 22" W, altitude: 705 m), in full light. The climate is tropical humid (Cornet, 1974), with annual rainfall of around 1500 mm, and a very rainy season between November and March, and a drier season from April to October. Annual rainfall rates recorded by the meteorological station at Andasibe, located around 10 km from Vohimana, during the period of the study are as follows: year 1: 1289 mm (with a monthly average of 224 mm for the rainy season and 24 mm for the dry season; year 2: 1848 mm (respectively 139 mm and 44 mm) and year 3: 1246 mm (respectively 199 mm and 32 mm)**.**

The experimental area is a 300 m² plot located in the low-land. It was established in 2003 by transplantation of *C .asiatica* collected in the immediate vicinity. At the beginning of the experiment, the entire plot was uniformly covered in *C. asiatica*. The plot was sub-divided into forty-eight (48) small plots each 2.25 m² (1.5 x 1.5 m) and eight collection frequencies were compared: monthly, bi-monthly, quarterly, four-monthly, semi-annually and annually (with three dates: every June, December or February). Each collection frequency was appointed to six randomly chosen small plots. At each date, the total number of leaf limbs present in each small plot was collected. This corresponds to the useful biomass harvested by the peasants (Rahajanirina et al., 2012). For the monthly, bi-monthly, quarterly and four-monthly frequencies, a prior cut was made before the beginning of the experiment, respectively in May, April, March and February 2005 in order that the measurement would not be falsified by the presence of biomass pre-existing the experiment. Two parameters were measured from each lot of leaves harvested: (i) yield, expressed by the leaf biomass produced per unit of surface area and (ii) active matter content, in particular asiaticoside and total terpenoid content.

The mass of dry matter produced per unit of surface area was assessed from aliquots taken from each harvest and put to dry in a heating chamber (105°C) for 24 h. The results are expressed as mass of dry matter (DM) harvested per square meter (g DMm^{-2}). The leaves collected were left to dry outdoors in the shade. Measurement of triterpenoids was carried out by High Performance Liquid Chromatography (HPLC), according to the method described by Rahajanirina et al*.* (2012). Chemical analysis itself was performed in the laboratory of Bionexx to Fianarantsoa. Ten grams of dry matter were finely ground with 200 ml of methanol R and then boiled under reflux for 16 h in a Soxhlet. After cooling, 200 ml of methanol R was added and the solution was filtered through a 0.45 µm mesh filter. The extract was diluted ten times, and then 20 µl samples were injected into a Zorbax SB-c18 column, 5 µm, 4.6 x 250 units, at a flow rate of 1.0 $ml.mn^{-1}$. The column was balanced by successive elutions of: 22% acetonitrile (solution A) and 78% O-phosphoric acid 0.3% (solution B) for 65 mn; 55% A - 45% B, 1 mn; 95% A - 5% B, 10 mn and 22% A -78% B, 9 mn.

The analyses were performed with a UV-vis detector (Merck Hitachi L-6200), at 200 nm, compared with a reference solution of asiaticoside. The active ingredient contents were expressed in grams of asiaticoside or triterpenoids per 100 g of dry matter (% DM). Means comparisons were performed by analysis of variance (ANOVA) with XLSTAT software. If the null hypothesis of means equality was rejected at the probability threshold of P = 0.05, the analysis was pursued using Tukey's test at a threshold of P = 0.05. In the Figures, the values with the same letter belong to the same homogeneous group.

Confidence intervals were calculated with a threshold of

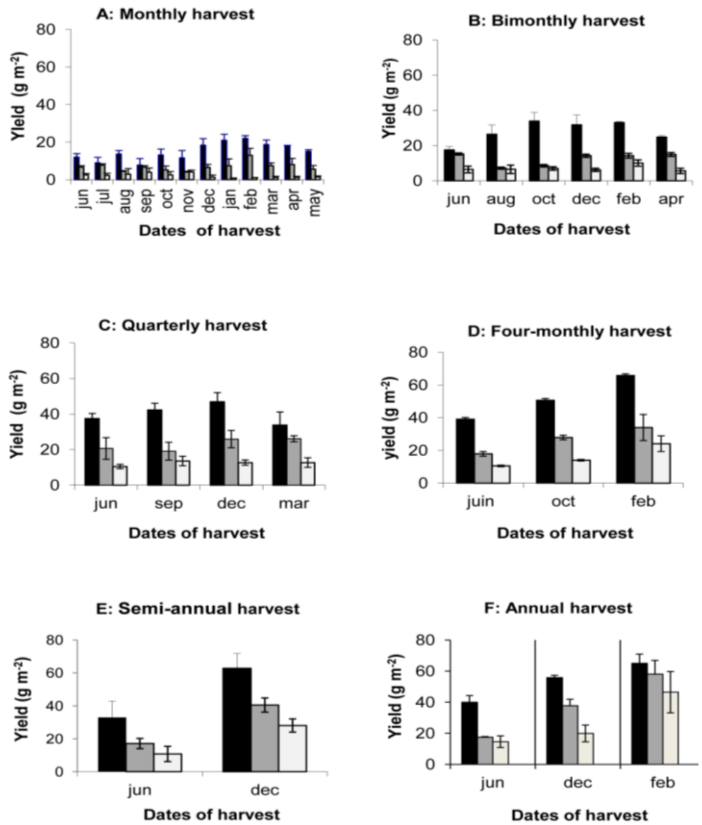

Figure 1. Biomass yield of *C. asiatica* harvested in the Vohimana reserve, by harvest frequency, **A**: monthly cut, **B**: bi-monthly cut, **C**: quarterly cut, **D**: four-monthly cut, **E**: semi-annual cut in June and December F: annual cut at three different dates (June, December and February). Measurements for three consecutive years: June 2005/May 2006 (black), June 2006/May 2007 (grey), June 2007/May 2008 (white). *F and P* value were obtained by one way analysis of variance (ANOVA). Confidence intervals at a threshold of $p < 0.05$.

P = 0.05. Pearson's correlation coefficient was calculated using R software with a threshold of P = 0.05.

RESULTS AND DISCUSSION

Figure 1 shows the variation in leaf biomass yield of *C. asiatica* according to frequencies of harvest and dates of harvest over a period of three consecutive years (June 2005 to May 2008). It shows that the maximum leaf biomass yield is acquired in December/February, in the middle of the rainy season, and the minimum in June/August, during the driest season (Figure 1A, 1B, 1C). This seasonal variation in yield is associated to rainfall: Pearson's correlation test between biomass yield and rainfall over the three years of harvest is positive with R = 0.71 and P = 0.008. When harvesting on a monthly basis, biomass yields are poor: 25 gm^{-2} (250 kg ha^{-1}) in the most productive month (December 2005) and 2 gm^{-2} in the least productive (April 2008) (Figure 1A). The yields increase correspondingly as the intervals between harvests increase. Peak yields are attained for collections made four-monthly or more (six-monthly or yearly) during the rainy season (Figure 1D, 1E, 1F) around 60 gm^{-2} of dry matter is harvested from the collections made between December and February in the first year.

Figure 2 compares (throughout the three-year study) the annual cumulative leaf biomasses harvested according to the different harvest frequencies. It shows that the cumulative biomasses are significantly higher for the greater harvest frequencies (monthly, bi-monthly, quarterly and four-monthly). Dry matter yields in this case were between 150 and 200 gm^{-2} the first year and 300 gm^{-2} for the three years cumulatively. If harvests are carried out at yearly intervals, at the most productive time of February, the yield is significantly lower: 60 gm^{-2} the first year and 120 gm^{-2} for the three years cumulatively. The same applies to the semi-annual harvest, in which the yield is 61 gm^{-2} the first year and 180 gm^{-2} for the three years cumulatively (Figure 2). The correlation test

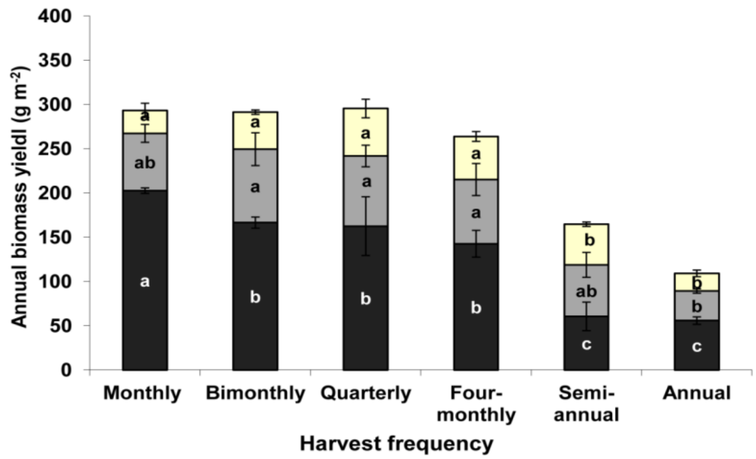

Figure 2. Annual cumulative dry leaf biomass yield of C. asiatica harvested in the Vohimana reserve, by harvest frequency for three consecutive years: June 2005/May 2006 (black), June 2006/May 2007 (grey), June 2007/May 2008 (white). F and P value were obtained by one way analysis of variance (ANOVA). Tukey's test at a threshold of 0.05 and confidence intervals at a threshold of $p < 0.05$.

Table 1. Annual variation rate (%) of dry leaf biomass of *Centella asiatica* in relation to the first year of harvest (June 2005/May 2006 = 100%), by harvest frequency. *F* and *P* value were obtained by one way analysis of variance (ANOVA). Tukey's test at a threshold of 0.05.

	Harvesting Frequency					
Periods	**Monthly**	**Bimonthly**	**Quarterly**	**Four-monthly**	**Semi annual**	**Annual**
June 2005 to May 2006	100 a	100 a	100 a	100 a	100 a	100 a
June 2006 to May 2007	35 b	48 b	49 b	67 b	68 b	43 b
June 2007 to May 2008	29 b	29 b	32 b	59 b	36 b	36 b
	F=37.88	F=54.05	F=27.30	F=52.68	F=44.52	F=113.05
Statistical values	P=0.000	P=0.000	P=0.001	P=0.000	P=0.000	P=0.000

between annual rainfall and cumulative annual biomass yield is not significant (R=-0.25, P=0.84). Biomass yields diminish year-on-year regardless of period and frequency of harvest.

This decrease is generally significant, especially between the first and second years, and across the entire set of measurements, the second year's yield representing between 35 and 68% of that of the first year, and the third years between 29 and 59% (Table 1). This year-on-year fall in yield does not correlate with annual recorded rainfall, as the second year was far wetter than the other two, the correlation test returning a value of R = 0.31 and P = 0.54. Chemical analysis of leaves shows that the highest active ingredient contents (total triterpenoids) are obtained during the months of November and April, and the lowest between May and October (Figure 3A). Triterpenoid contents vary between 5% (relative to the mass of dry matter) in September 2005 and 2006 (Figure 3C, 3F) and 12% in February and March 2006 (Figure 3A and 3F). Asiaticoside contents also vary according to the season (Figure 4). They are highest between November and May, between 3.5 and 6% DM (Figure 4A, 4B, 4D and 4F) and lowest during the dry season between June and October, between 2 and 3.5% DM (Figure 4A, 4B, 4C and 4D). Seasonal variation in the contents of triterpenoids and asiaticosides are related to rainfall: Pearson's correlation test between the contents of triterpenoids, asiaticoside and rainfall are, respectively, R=0.53, P=0.05 and R=0.77, P=0.003.

Comparison of Figures 3A to 3F does not reveal any evidence of a net effect of harvest frequency on total triterpenoid contents in the leaves of *C. asiatica*. For example, leaves collected in June, regardless of harvest frequency, always have triterpenoid contents of between 6 and 8%, whereas leaves collected in December generally have contents higher than 9%. The same observation can be made with regard to variations in asiaticoside contents: Figure 4 shows that regardless of the harvest frequency, the asiaticoside content is generally lower during the dry season (between 3 and 4%) than in the rainy season (between 4 and 5%) (Figure 4A to 4F). Table 2 confirms, in a synthetic way, that for the three harvest frequencies which give the best biomass yields (bi-monthly, quarterly and four-monthly),

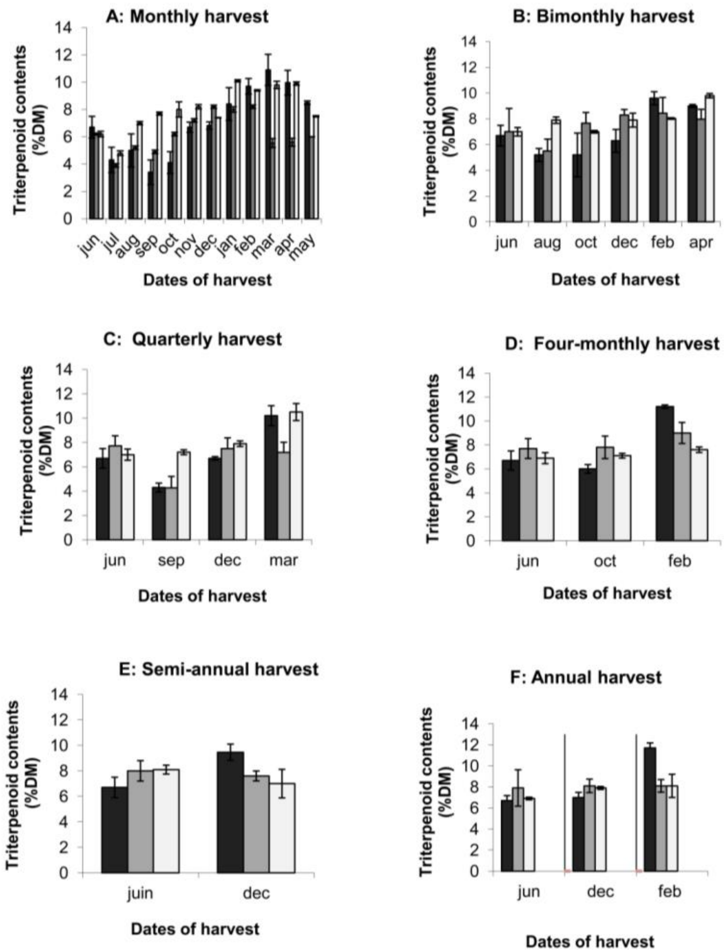

Figure 3: Variation in total terpenoid contents of leaves of *C. asiatica* harvested in the Vohimana reserve, by harvest frequency, **A:** monthly cut, **B:** bi-monthly cut, **C:** quarterly cut, **D:** four-monthly cut, **E:** semi-annual cut in June and December, **F:** annual at three different dates (June, December, February). Measurements for three consecutive years: June2005/May 2006 (black), June 2006/May 2007 (grey), June 2007/May2008 (white). F and P value were obtained by one way analysis of variance (ANOVA). Confidence intervals at a threshold of $p < 0.05$.

the annual and cumulative productions over three years in triterpenoids per unit of surface area remain unaffected by harvest frequency. However, it confirms the significant and progressive drop in year-on-year yields falling from 8 to 12 $kgha^{-1}yr^{-1}$ the first year (according to the harvest frequency), 4 to 8 the second and 2 to 4 the third.

Our results indicate that the cumulated annual harvest of *C. asiatica* leaves can reach around 1900 $kgha^{-1}$ dry matter the first year, and 3000 $kgha^{-1}$for the three years' cumulative harvest, an average of 1000 $kgha^{-1}$ of dry matter per year for the three years under study. These yields match those calculated by Raharison Ramiaramanana (1983), who estimated the annual harvest between 5 and 11 tonnes of fresh matter per year (1.0 to 2.2 tonnes of dry matter), according to the accessions selected in India and values given by Rosalizan et al. (2008) between 2.3 and 3.8 tonnes ha^{-1}. Our results also indicate that the biomass yields are subject to seasonal variation and correlate with rainfall variations. Maximum yields are obtained from November or December to April, corresponding to the rainy season, whilst yields fall in the drier season between May and October.

This confirms the prior observations of Raharison Ramiaramanana (1983) and Rahajanirina et al. (2012), who showed the same seasonal variations.

Harvest frequencies of the leaves strongly influence the yield. Generally the greater the interval between harvest, the greater the yield of each crops is high. This result follows on logically from numerous studies of other plant models such as *Gliricidia sepium, Leucaena leucocephala* (Latt et al., 2000). This hypothesis is

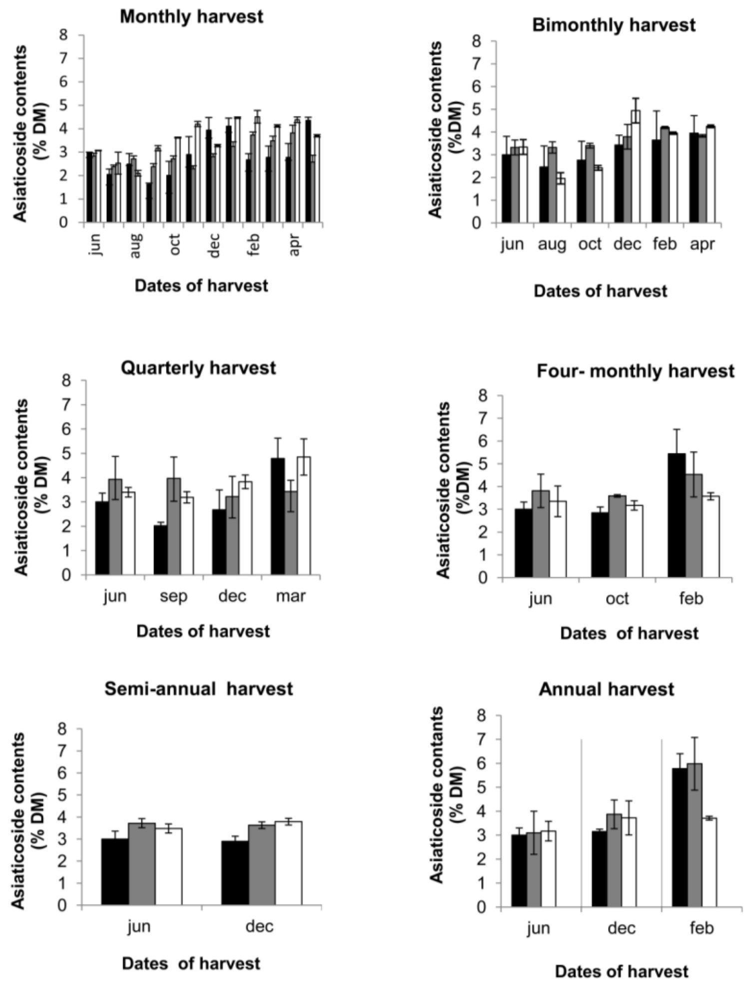

Figure 4. Variation in asiaticoside content of leaves of *C. asiatica* harvested in the Vohimana reserve by harvest frequency, **A**: monthly cut, **B**: bi-monthly cut, **C**: quarterly cut, **D**: four-monthly cut, **E**: semi-annual cut in June and December, F: annual at three different dates (June, December, February). Measurements for three consecutive years: June 2005/May 2006 (black), June 2006/May 2007 (grey), June 2007/May2008 (white). *F* and *P* value were obtained by one way analysis of variance (ANOVA). Confidence intervals at a threshold of p < 0.05.

Table 2. Annual yield of active matter (total triterpenoids) of *C. asiatica* per unit of surface area (gm^{-2}). *F* and *P* value were obtained by one way analysis of variance (ANOVA). *F* and *P* value were obtained by one way analysis of variance (ANOVA). Tukey's test at a threshold of 0.05.

	Harvesting Frequency			
Periods	**Bimonthly**	**Quarterly**	**Four-monthly**	**Statistical values**
June 2005 to May 2006	11.52 ± 2.0 a	8.12 ±1.2 a	10.30 ± 0.1a	*F*=3.2, *P*=0.1
June 2006 to May 2007	4.54 ± 2.0 b	5.54 ± 0.6 ab	7.80 ± 0.6 a	*F*=0.2, *P*=0.7
June 2007 to May 2008	2.47 ± 2.1 c	3.35 ± 2.6 b	3.56 ± 0.1a	*F*=4.5, *P*=0.6
Total	18.53 ± 2.4	17.01 ± 3.5	20.94 ± 4.6	*F*=0.4, *P*=0.7
Statistical values	*F*=29.11 *P*=0.008	*F*=9.90 *P*=0.012	*F*=4.56 *P*=0.062	

confirmed by Antsonantenainarivony (2010), who demonstrated that cutting *C. asiatica* to the ground results in a decrease in leaf surface area as well as dry leaf biomass on regrowth. Where *C. asiatica* is concerned, our results show however that if the interval between harvests exceeds three to four months the biomass collected is almost constant. Where the production of active matter is concerned, our results show that triterpenoid content of *C. asiatica* varies independently of collection frequencies. Total triterpenoid contents varied between 5 and 12% of the mass of dry matter, 1.6 to 6.0% of which is asiaticoside.

These values equal those measured by Randriamampionona et al. (2007) on samples of *C. asiatica* harvested in the central mid-altitude region of Madagascar (total triterpenoid content between 5.8 and 12.7% of dry matter and asiaticoside between 2.7 and 6.5%). Total triterpenoid and asiaticoside contents varied according to the collection season. These are greater in the leaves of *C. asiatica* during the rainy season (November to April) and lower during the dry, cool season (May to October). These results confirm those previously reported by Rahajanirina et al. (2012). They also concur with those of Puttarak and Panichayupakaranant (2012) and Alqahtani et al. (2015), which demonstrate maximum triterpenoid content in Thai and Australian accessions of C. asiatica between the months of December and March.

This could indicate that the content of active matter in the leaves of *C. asiatica* correlates positively with the metabolic activity which is associated in the plant to rainfall. Unlike the biomass, a significant difference in the concentration of active matter (total triterpenoids and asiaticosides) has not been recorded from one year to the next. In our study, yields in active matter (triterpenoids) are around 6 to 7 kg $ha^{-1}yr^{-1}$ for the whole three year period (10 to12 kg the first year and 2 to 4 the third). This result concurs with that of Mathur et al. (2000), who estimated the yield between 1.0 and 9.8 kg ha^{-1}. The difference in yield of active matter per hectare is primarily due to the loss of biomass. A significant and progressive fall in biomass production was observed over the three-year experiment, irrespective of the collection frequencies.

This decrease in production is not related to a progressive drop in rainfall over the period studied. It is most likely due, as suggested by Raharison Ramiaramanana (1983), to plant exhaustion. An application of compost and/or chemical fertiliser might remedy exhaustion and improve the biomass production of *C. asiatica,* as shown by Raharison Ramiaramanana (1983), Siddiqui et al. (2011) and Devkota and Jha (2013). However, it may have negative effects on the production of active molecules, as indicated by Devkota and Jha (2010), who showed that growing *C. asiatica* in poor substrate is more beneficial to the production of asiaticoside and madecassoside than growing it in compost or nitrogen-enriched substrates.

In the Malagasy context, where *C. asiatica* is a plant that is harvested only by collection in the wild (Péchard et al., 2005; Chupin, 2010), the only way to optimise the harvest is by adapting the rhythm of the harvests. Rosalizan et al. (2008) demonstrated that the optimum interval between harvests is seventy days, whilst the maximum active matter (asiaticoside, madecassoside) content is obtained at around two months. With our results, the best compromise for achieving a harvest which has optimal biomass and active molecules would be three to four monthly collections. And yet, the peasants usually go out collecting every two weeks during the rainy season (Chupin, 2010), which seems too frequent for useful plant regrowth.

Conclusion

Our results show that (i) quantity of leaf biomass of *C. asiatica* depends on the season (maximum in rainy season) and collection frequency; (ii) that the active matter content depends solely on the harvest period (maximum in rainy season and minimum in dry season); (iii) that replenishment of the biomass is optimal if the collections are spaced out at a minimum of three monthly intervals; (iv) that repeated harvests lead to the loss of biomass productivity noticeable from the second year of the plant's exploitation; (v) that optimal cumulative yield is achieved if the leaves are harvested at least three times a year. Our study would tend to show that quarterly harvests during the rainy season (three times a year, at intervals of three months between November and April) would be the best compromise between the effort required to harvest the crop and the yield of biomass and active matter.

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