The Influence of Certain Taxonomic and Environmental Parameters on Biomass Production and Triterpenoid Content in the Leaves of Centella asiatica (L.) Urb. from Madagascar

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by **Voninavoko Rahajanirina***[a])[b]), **Soaharin'ny Ony Rakotondralambo Raoseta**[a])[b]), **Edmond Roger**[a])[b]), **Harena Razafindrazaka**[a]), **Sarah Pirotais**[c]), **Marie Boucher**[c]), and **Pascal Danthu**[b])[d])

[a]) Département de Biologie et Ecologie végétales, Université d'Antananarivo, BP 906 Antananarivo, Madagascar

[b]) CIRAD DP Forêts et Biodiversité, BP 853, Antananarivo, Madagascar (e-mail: voni_rahajanirina@yahoo.fr)

[c]) ONG L'Homme et l'Environnement, Lot II M 98 Antsakaviro, Antananarivo, Madagascar

[d]) CIRAD, UPR 105, Campus de Baillarguet, FR-34398, Montpellier cedex 5

Centella asiatica (Apiaceae family; *Talapetraka* in Malagasy) is a tropical and subtropical plant with leaves containing glycotriterpenoids (asiaticosides) used in traditional and modern medicine. *C. asiatica* is collected exclusively in natural stands. It is Madagascar's second most important indigenous plant export. The objective in this study is to provide data which will make it possible to optimize the harvest and thus effectively develop this resource. Two foliar morphotypes were identified: morphotype A with small reniform leaves (leaf area *ca.* 4.5 cm^2), found in the east of Madagascar, and morphotype B with large round leaves (up to 7.5 cm^2) found in the west, with sympatric zones in the central part of the island. Morphotype A produces a higher biomass, and is twice as rich in asiaticosides as morphotype B. Significant variations in biomass yield and asiaticoside content are observed depending on the date of collection: higher during the rainy season (December to April) and lower during the dry season (June to August). Inter-annual variations are also observed. Populations located at around 800–1400 m altitude on the eastern side of Madagascar, in a sub-humid climate, appeared to be more productive. These results provide more precise information to the economic sector, which confirms the empirical choices made by collectors. They represent the first elements towards sustainable management of the resource, and maybe even domestication.

Introduction. – *Centella asiatica* (L.) Urb. (Apiaceae), or *Talapetraka* in Malagasy, is a medicinal plant found in tropical and subtropical regions, and it is recognized all over the world particularly for its wound-healing properties [1–4]. *C. asiatica* is a perennial stoloniferous herb, found mostly in moist zones. Although it reproduces by seed, the most competitive mode is by vegetative propagation through stolons [5][6].

In Madagascar, *C. asiatica* can be found throughout the island, except in the semi-arid region in the South and above 2000 m [7][8]. It exists in two forms, which we will call morphotypes, because the taxonomy of the *Centella* genus seems unclear in Madagascar [7][9]. *C. asiatica* with reniform leaves is found in the east and center of the island (morphotype A; *Fig. 1,a*), which may correspond to the variety *typica* var. *nov.* as defined by *Boiteau* [7]. In the west, *C. asiatica* has rounder, fleshier leaves (morphotype B), possibly close to *C. asiatica* (L.) Urb. var. *abyssinica* Gandoger pro

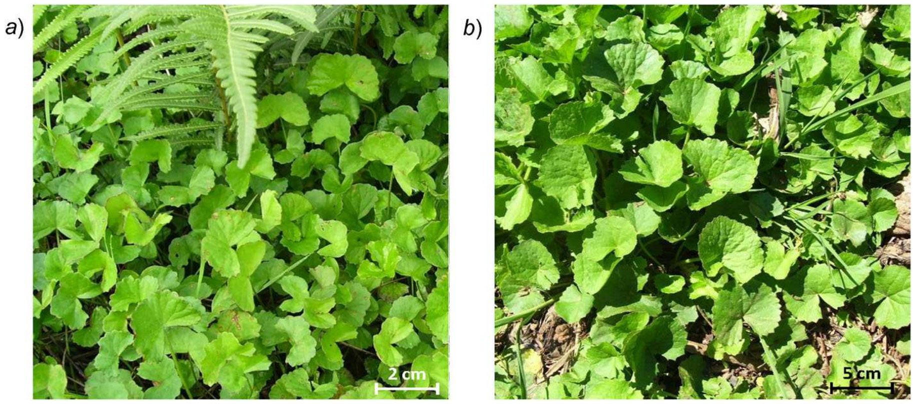

Fig. 1. *Foliar morphologies of Malagasy* C. asiatica*:* a) *morphotype A with reniform leaves;* b) *morphotype B with round leaves*

sp. [7] (*Fig. 1,b*). These two morphotypes are genetically distinguishable by chloroplastic markers [8].

C. asiatica has long been used in the traditional Malagasy, Asian, and Chinese pharmacopoeia for healing various types of wounds, including stomach ulcers, and the skin lesions present in leprosy [1][4][10–12]. In Madagascar, it is the active ingredient of *Madecassol®*, a proprietary medicine used internally and externally for its healing properties. It is also used by the cosmetic industry as a basis of anti-ageing, antioxidant, and restructuring creams, as well as for treating stretch marks [13]. It is used to treat vascular and nervous disorders [4].

Since the studies by *Bontemps* [14], the biological activity of *C. asiatica* has been accounted for by the presence of triterpene acids in the leaves (asiatic acid and madecassic acid) and their respective glycosides: asiaticoside and madecassoside [4][12]. Other chemical components such as vitamins B and C, carotenes, and minerals (calcium, magnesium, and zinc) are present in the leaves attributing it nutritional value [12][15]. *C. asiatica* contains essential oils rich in sesquiterpenes with antibacterial properties [4][16].

C. asiatica is the second most important medicinal plant exported from Madagascar, which is the world's number one producer [17]. The leaves of *C. asiatica* are collected for the most part by hand from the moist low-lands bordering rice paddies, uncultivated rice paddies, and *zetra* (undeveloped land destined for rice growing) in the region of Alaotra-Mangoro and around Antananarivo [18][19]. The specimens of morphotype A used in this study were collected exclusively from this zone. In the 1980s, the annual harvest represented between 20 and 97 tons of dry matter [20]. Currently, harvest predictions reach several hundred tons [21]. Since the 1980s, the price per kilogram of leaves paid to collectors has increased tenfold. The harvest of leaves presently constitutes a vital source of revenue for poor rural farmers, who collect *C. asiatica* during the lean season [22][23].

C. asiatica is currently not at risk [17], but with the growing demands of the international market for natural products, over-exploitation could threaten the resource and consequently the economic sector. All the more so, since the possibilities for increasing growing surface are limited by the rate of development of *zetra*. Consequently, short-to-medium-term shortfalls in the harvests are likely to occur, which will leave the demands of the international market unsatisfied [23].

The aim of this study is to provide information which will make it possible to manage the resource of Malagasy *C. asiatica* more efficiently. Two specific aims are envisaged: *i*) to study the possibility of extending the collection to include the round-leaved morphotype in the west by evaluating its productivity in terms of biomass and yield in active matter (by comparison with the harvests of the reniform morphotype in the east); and *ii*) to study a number of environmental factors which could have an impact on the productivity of *C. asiatica* in the regions from which it is presently collected.

Results and Discussions. – *Biogeographic Distribution of* C. asiatica *in Madagascar.* Our surveys confirm that *C. asiatica* inhabits the dry, sub-humid, and humid bioclimatic layers defined by *Cornet* [24] and is absent from the sub-arid layer in the south and south-west (*Fig. 2*).

They also confirm the occurrence of two morphotypes of *C. asiatica* (*Figs. 1* and *2*) in Madagascar. Morphotype A (reniform leaves) is found in the east and on the central high plains of the island, between sea level and 1400-m altitude (Ambanitsena site), in humid and sub-humid climates. Morphotype B (round leaves), however, occurs only in the west and on the high plains below 1200 m (Analavory site), in dry and sub-humid climates (*Table 1*). In certain sites, such as Ambohitromby and Analavory, the morphotypes are sympatric, although no intermediary leaf forms were detected.

Biometric and Biochemical Variations Associated with Morphotypes. In *Table 2*, biomass yields, leaf area, and the composition of active ingredients of the two morphotypes of *C. asiatica* harvested from their natural environment are compared. Results represent the means calculated from 32 sampling sites for morphotype A and 13 sites for morphotype B.

Morphotype A from the east has smaller leaves than morphotype B from the west (4.5 *vs.* 7.5 cm^2). However, the biomass collected per unit of surface area is 2.5 times greater for morphotype A, and its content in triterpenoids, including asiaticoside and madecassoside, is significantly greater.

In *Table 3*, it is shown that morphotype A produces significantly more stolons than morphotype B (+75%), and more leaves (+30%) when cultivated in an identical and non-limiting environment.

Biometric Variations along a East/West Transect of Madagascar. In *Fig. 3*, the evolution of yield per unit of surface area and the size of blades found along a west/east transect is illustrated. Three explanatory parameters have been isolated: morphotypes (A or B), altitude, and bioclimate (as described in *Table 1*).

The biomass production per unit of surface area and the size of blades of morphotype B remain more or less stable across regions I and II (*ca.* 10.7 g dry matter/m^2 and 8.7 cm^2, resp.; *Fig. 3*).

However, in regions III, IV, and V, morphotype A shows opposite variations of the production of biomass per unit of surface area and the size of blades. Whilst in region IV, the size of blades is significantly lower than in regions III and V (3.9 ± 1.3 *vs.* $5.2 \pm$

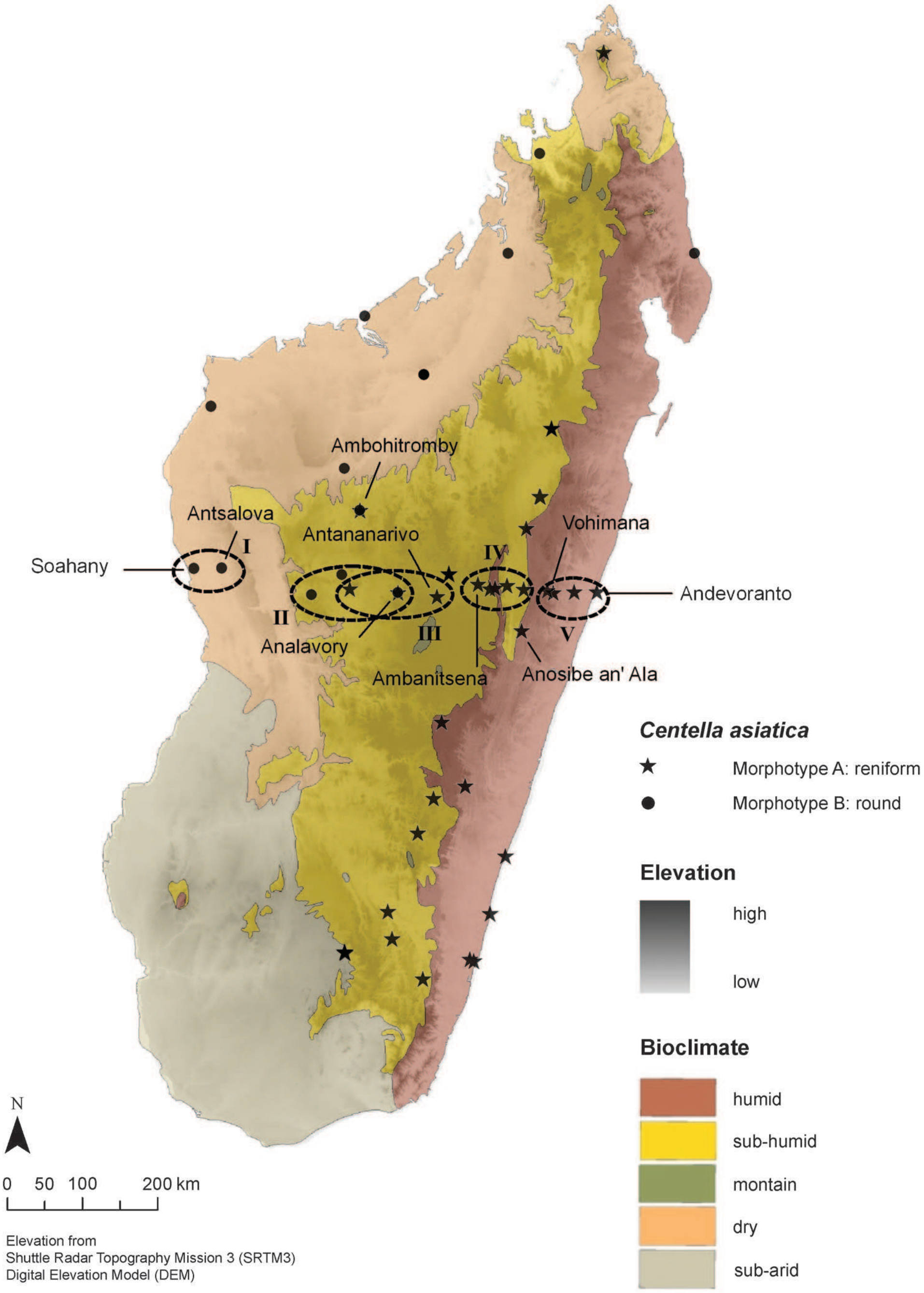

Fig. 2. *Distribution of collection regions and sites of two foliar morphotypes of* C. asiatica *found in Madagascar: by bioclimate* (simplified from *Cornet* [24]) *and altitude*. Morphotype A with reniform leaves; morphotype B with round leaves.

Table 1. *Characteristics* (altitude, ecological parameters (temp. and precipitation)) *of the Five Collection Regions of* C. asiatica *along a West/East Transect* (from Soahany to Andevoranto through Antananarivo (number of studied sites: 17))

Region of collection	Range of altitude [m]	Bioclimatic characteristics	*C. asiatica* morphotypes	Number of sites
I	0–800 (west)	Dry $6 < N^{a}) < 7$ $500\ mm < R^{b}) < 1600\ mm$ $T_m{}^{c}) > 20°$	round	2
II	800–1200 (west)	Sub-humid $5 < N < 7$ $1000\ mm < R < 1500\ mm$ $15 < T_m < 20°$	round	3
III	800–1400 (west)	Sub-humid $5 < N < 7$ $1000\ mm < R < 1500\ mm$ $15 < T_m < 20°$	reniform	3
IV	800–1400 (east)	Sub-humid (dry season reduced by winter fogs) $0 < N < 2$ $1500\ mm < R < 2000\ mm$ $10° < T_m < 15°$	reniform	5
V	0–800 (east)	Humid $N = 0$ $R > 2000$ mm $T_m > 20°$	reniform	4

[a]) *N:* Number of ecologically dry months per year. [b]) *R:* Annual rainfall. [c]) T_m: Minimal average temperature of the coldest month) [24][27].

Table 2. *Comparison of Biomass Production and Triterpenoid Contents in Two Morphotypes of Malagasy* C. asiatica (45 collections points, 32 for morphotype A and 13 for morphotype B, see *Fig. 2*). The triterpenoid content was determined from five samples collected between February 27th and March 4th 2008; ANOVA at threshold of $P < 0.05$ and *Tukey*'s test at the probability threshold of $P < 0.05$, if null hypothesis of equal means rejected, confidence interval threshold is $P < 0.05$. In each row, values followed by different letter (a or b) are significantly different

	Morphotype A (reniform leaf)	Morphotype B (round leaf)	
Leaf area [cm^2]	4.5 ± 0.4 a	7.5 ± 1.2 b	$F = 34.91$; $P < 0.0001$
Biomass yield [g DM/m^2][a])	24.2 ± 1.9 b	10.0 ± 2.3 a	$F = 71.13$; $P < 0.0001$
Triterpenoid content [% DM]	7.8 ± 0.2 b	4.8 ± 0.4 a	$F = 63.86$; $P = 0.004$
Asiaticoside [% DM]	3.7 ± 1.1 b	2.0 ± 0.06 a	$F = 23.96$; $P = 0.016$
Madecassoside [% DM]	3.6 ± 0.1 b	2.0 ± 0.01 a	$F = 324.42$; $P = 0.0004$
Asiatic acid [% DM]	0.2 ± 0.008	0.4 ± 0.3	$F = 4.49$; $P = 0.124$
Madecassic acid [% DM]	0.2 ± 0.01	0.4 ± 0.3	$F = 3.79$; $P = 0.147$

[a]) DM = dry matter.

0.5 and 5.1 ± 1.0 cm^2), the biomass yield is higher (32.8 ± 3.0 *vs.* 23.6 ± 5.3 and 21.0 ± 3.6 g dry matter/m^2).

Table 3. *Comparison of the Development of two Morphotypes of Malagasy* C. asiatica *in a Nursery at Antananarivo* (ANOVA at threshold of $P < 0.05$ and *Tukey*'s test at the probability threshold of $P < 0.05$, if null hypothesis of equal means rejected). In each row, values followed by different letter (a or b) are significantly different.

	Leaf morphotype and origin		
	A – Anosibe an'Ala	B – Antsalova	
Number of nodes per m²	367 b	210 a	$F = 84.58$; $P < 0.0001$
Number of leaves per m²	923 b	709 a	$F = 34.17$; $P = 0.0004$

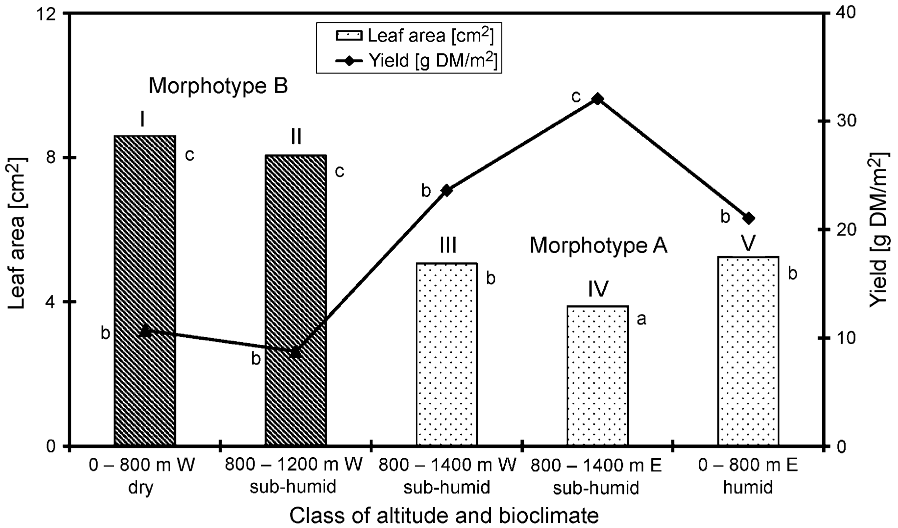

Fig. 3. *Variation in foliar biomass and leaf surface of Malagasy* C. asiatica *along a west/east transect* (according to altitude, environmental parameters (temperature and precipitation), and foliar morphotype – morphotype A: represented by dotted columns and diamonds; morphotype B: striped columns and triangles). Study of a total of 17 collection sites (gathered in five collection regions, see *Table 1* and *Fig. 2*) between January and April (2008, 2009, and 2010). For foliar biomass $F = 21.91$, $P < 0.0001$; for leaf surface $F = 34.29$, $P < 0.0001$). Values with the same letters belong to the same homogenous group defined by the *Tukey*'s test at the probability threshold of $P < 0.05$.

Comparing the two morphotypes reveals a significant difference not only in the size of leaves (greater for type B) but in the yield of matter, where morphotype A produces leaf mass per unit of area 2 to 2.5 times greater than morphotype B.

Seasonal Variations in the Foliar Biomass and the Active Molecule Content. In *Fig. 4*, variations in biomass yield and active molecule content of *C. asiatica*, morphotype A, harvested from the Vohimana plot between June 2005 and February 2008, are presented. The two parameters exhibit broadly parallel seasonal and inter-annual variations. The yield in dry matter of leaves increases during the rainy season (December to April) and peaks in February (February 2006: 79.9 ± 1.5 g dry matter/m²). It then falls during the drier, colder season (June to August), with a minimum

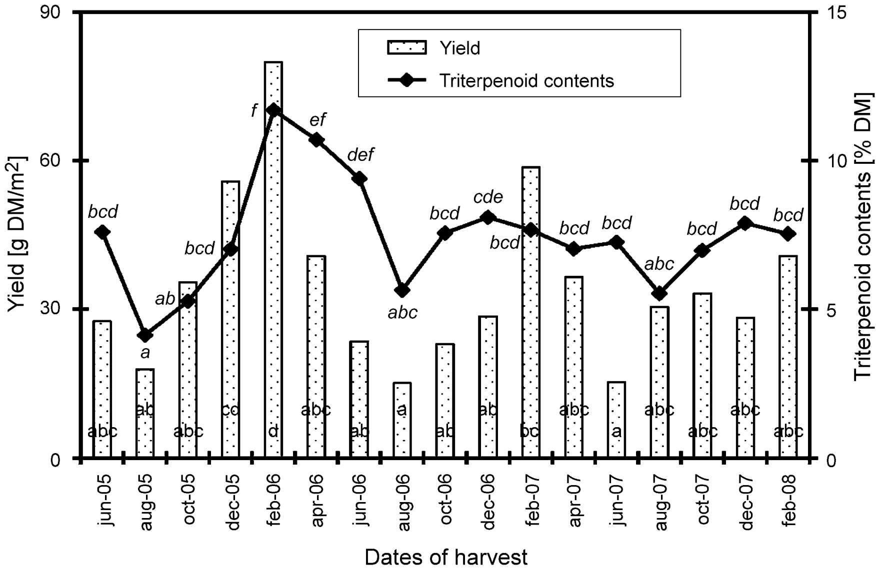

Fig. 4. *Every two months assessments of biomass yield and triterpenoid content of* C. asiatica *leaves* (morphotype A) *carried out in Vohimana from June 2005 to February 2008*. For foliar biomass, $F = 16.13$, $P < 0.0001$; for triterpenoid content, $F = 14.15$, $P < 0.0001$). Values with the same letters belong to the same homogenous groups defined by the *Tukey*'s test at the probability threshold of $P < 0.05$).

yield in August (August 2006: 15.1 ± 0.7 g dry matter/m^2). The content in active ingredients adheres to the same pattern as the leaf biomass, achieving a maximum value in February (February 2006: 11.7 ± 0.8%, 5.8% of which asiaticoside) and a minimum value in August (August 2007: 5.6 ± 1.1%, 2.7% of which asiaticoside).

Our results confirm studies by *Boiteau* [7] and *Rakotondralambo et al.* [8] as far as the distribution area of *C. asiatica* in Madagascar is concerned. The species has been found throughout the island except in the sub-arid zone in the south and south-west. Morphotype A, with reniform leaves, is present in the humid and sub-humid bioclimatic layers of the east and high plains, whereas morphotype B, with round leaves, inhabits the dry zone in the west and the sub-humid central Malagasy plateau.

Generally, morphotype A leaves have smaller blades than morphotype B, but twice the biomass yield and twice the concentration of active ingredients. These significant differences can be explained by morphotype A's greater capacity for vegetative propagation by stolons, which improves its ability to occupy the soil. This behavior could be attributed to genetic variability.

Yields seem, in particular, to be maximized in the eastern site of Madagascar at medium altitude (800–1400 m), with a sub-humid climate. This parameter could be explained by the ecological context predominating in sub-humid zones in the east (high rainfall and elevated temperature), which is favorable to the growth and development of *C. asiatica*.

These observations confirm the conclusions of *Randrimampionona et al.* [18] and corroborate the empirical choices of collectors, who concentrate their collections on morphotype A, in the middle altitude regions to the east of Madagascar, along the eastern cliff (in the region of Alaotra-Mangoro around Moramanga) and on the high plains surrounding Antananarivo [19] [23].

This study has revealed a major cause for the variation in *C. asiatica* productivity, which is associated with the season of collection. For morphotype A (reniform leaves), biomass yield and active ingredient content peak during the rainy season (between December and April) and diminish significantly in the dry season. Biomass production adheres to the same pattern. This variation confirms the influence of the season of collection already known empirically to collectors [23].

Our study has not, however, removed doubts concerning the taxonomy of our two morphotypes, as the *Centella* genus seems, for the time being, to be unstable. Morphotype B (round leaves) seems to be widely distributed across Southeast Asia and Africa and could be the type species [25]. Morphotype A (reniform leaves), contrary to that stated by *Boiteau* [7], seems more specific to Madagascar.

The observation of greater active ingredient content in morphotype A compared to morphotype B may support the findings by *James et al.* [25], who estimated the triterpenoid content of leaves of morphotype B harvested in South Africa at between 1.8 and 5%. These findings corroborate those of *Rouillard-Guellec et al.* [3], who estimate that leaves of Malagasy origin (morphotype A) could be three to seven times richer in triterpenoids than those of Indian origin. *Randriamampionona et al.* [18] have found Malagasy samples to contain between 5.8 and 12.7% triterpenoids (of which 6.4% are asiaticosides). This result is very close to our estimation, which depending on the season of collection varies between 5.6 and 11.7% triterpenoids (of which 5.8% are asiaticosides). As a comparison, *Brinkhaus et al.* [12] assessed the triterpenoid content of leaves of Asiatic origin at between 1 and 8%, whereas *Devkota et al.* [26] estimated *C. asiatica* collected in Central Nepal (Gorkha region) to contain asiaticosides ranging from 2.7 and 8.1%, at least equal to that of Malagasy leaves. These disparities could be due to differences in ecological context or collection dates (often not specified in the articles) which, as we have demonstrated, significantly influence this parameter.

Conlusions. – This study thus gives grounds, in conjunction with previously published findings [3] [18], to surmise that the small reniform leaves of morphotype A of *C. asiatica* are more productive (in terms of biomass yield and active ingredient content) than the large-leaved variety found in the west of the island. This is particularly the case if collection is carried out at medium altitude (800–1400 m) and during the height of the rainy season (December to April). However, the origin of the variation remains to be verified. Where leaves of Malagasy origin are concerned, genetic studies will undoubtedly be required to eliminate the taxonomic ambiguities.

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Experimental Part

Study Site. Evaluation of the natural diversity of *C. asiatica* was carried out throughout the whole of its area of presence in Madagascar. Sampling and biometric measurements were performed at 45 collection points (32 for morphotype A, 13 for morphotype B; see *Fig. 2*). These collections were carried out during the rainy season from January to April of 2008, 2009, and 2010.

Particular attention was paid to a series of 17 sites selected on the basis of high presence of *C. asiatica* and distributed along a transect running west-east between Soahany (18°41′05″S, 44°21′57″E) on the west coast and Andevoranto (18°46′01″S, 49°13′19″E) on the east coast, *via* Antananarivo (18°52′44″S, 47°33′24″E). These sites were grouped in five ecologically and climatically homogeneous regions [24][27] (*Fig. 2* and *Table 1*).

Assessments of seasonal variations in the production of *C. asiatica* (morphotype A) were carried out every two months between June 2005 and February 2008, in the Vohimana Reserve (18°55′12″S, 48°31′22″E, altitude 705 m with rainfall ranging from 1500 and 2500 mm and the possibility of an ecologically dry period in September/October; *Fig. 2*). The measurements were performed in a 300 m^2 plot planted by transplantation in 2003. During this trial, samples were taken from the same place on the same date each year.

Growth comparison of morphotypes A and B was carried out in a controlled environment in the nursery at Ambatobe (Antananarivo), during the rainy season of 2005.

Measurements of Biometric Data. At each site and at each date, measurements were carried out as follows:

i) The determination of leaf biomass (corresponding to the useful biomass exploited by the collectors who gather only the leaf blades) per unit of surface area was measured using a hoop (made from a PVC tube; 177 cm in circumference staking out an area of 0.25 m^2). This was thrown randomly into the plot under evaluation and the blades within the zone collected. After drying the leaves (at 105° for 24 h), the mass of dry leaf matter was reported by unit of surface area (g MS/m^2). Sampling was repeated three times.

ii) Comparison in nursery growth was carried out on a population of *C. asiatica* of morphotype A native to Anosibe an'Ala and a population of morphotype B native to Antsalova. Measurements were taken of the number of nodes and the number of leaves per unit of area assessed after three months of growth (initiated by transplantation of 16 plants bearing two leaves per m^2). Experiments were carried out in the nursery at Ambatobe (Antananarivo) and were repeated five times.

iii) Approximate leaf area was determined in the following manner: five leaves were sampled from within the surface area marked out by the hoop in order to establish the average leaf area. In selecting the leaves, the five largest were avoided and the next five largest collected. The area of the blade of each leaf was measured with a planimeter (*Li-3000 A* portable area meter) and expressed in cm^2.

iv) Leaves were divided into lots from which measurements of active ingredients were taken (see below).

Measurement of the Triterpenoid Content of Leaves. Three lots of *ca.* 100 g of fresh leaves were sampled from each site and/or on each collection date. The leaves collected were dried at air temp., in the shade, then stored in a dry place until analyzed.

Measurements were carried out using HPLC (High Performance Liquid Chromatography) in the laboratory of *INNOVEXX* (previously INDENA Madagascar), at Fianarantsoa (Madagascar).

Ten grams of dry matter were crushed with MeOH (200 ml) and then heated under reflux for 16 h in a *Soxhlet* extractor. Once cooled, MeOH (200 ml) was added and the soln. was filtered using a filter with a mesh measuring 0.45 μm.

The extract obtained was diluted ten times and 20 μl were injected into a *Zorbax SB-c18* column (5 μm, 4.6 mm × 250 mm), with an output of 1.0 ml min^{-1}. Equilibration of the column was carried out by successive elutions using:

i) 22% MeCN (soln. *A*) and 78% phosphoric acid 0.3% (soln. *B*) for 65 min,

ii) 55% *A*/45% *B*, 1 min,

iii) 95% *A*/5% *B*, 10 min,

iv) 22% *A*/78% *B*, 9 min.

Analyses were carried out using a *Merck Hitachi L-6200*, UV/VIS detector at (200 nm), in comparison with a reference soln. of asiaticoside. The composition in active ingredients was expressed in grams of asiaticoside (or other active ingredients) per 100 g of dry matter (DM).

Statistical Analysis of Data. The analysis of variance (ANOVA) using XLSTAT software, was applied to compare the effects of morphotype, origin, and harvest date on leaf biomass and size, plant density, and active molecule contents. If the null hypothesis of means equality was rejected with a probability threshold of $P < 0.05$, the analysis was pursued using *Tukey*'s test of groups of homogenous means at a probability threshold of $P < 0.05$. In the tables and figures, the values with the same letter belong to the same homogenous group. Confidence intervals were calculated to the threshold of $P = 0.05$.

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