International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 4, No. 2, p. 1-11, 2014

INNSPUB

RESEARCH PAPER OPEN ACCESS

Effect of some abiotic factors on the concentration of β-sitosterol of *Prunus Africana* (Hook.f.) Kalkman in the tropical forests of Cameroon

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Article published on February 25, 2014

Key words: *Prunus africana*, phenotypic character, soils, β-sitosterol, altitude.

Abstract

Prunus africana is a medicinal plant which develops in the mountains of several African countries. *β-sitosterol* can be used as a marker for the control of the product quality of the aforementioned plant in terms of phytotherapy. Farmers and public authorities do not have information on the influence of altitude and chemical characteristics of soils on the concentration of β-sitosterol of *P. africana*. To contribute to solve the problem, this research, carried out in Cameroon, aims to appreciate the effect of abiotic factors on the above phenotypic character. In nine composite samples of barks taken at different altitudes, the concentration of *β*-sitosterol is appreciated via qualitative analyses by Thin Layer Chromatography, High Performance Liquid Chromatography and quantitative analyses by Gas Chromatography coupled with the Mass Spectrometry. The chemical analyses of soils taken under the stems of the aforementioned trees were made. The statistics were carried out using the SAS software. The concentration of *β*-sitosterol in each population of *P. africana* varies from zero to 38.65 µg/ml. There is variability between the averages of the aforementioned concentration with respect to altitude and chemical elements of the soils but the differences are not significant. The Ascending Hierarchical Clustering distributes populations into three groups. These tools obtained are indispensable for the ground management, the products exploited from this tree species and the production of seeds for creating forest and agro-forest plantations.

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Introduction

Prunus africana (Hook. F.) Kalkman is a medicinal plant which develops in the forests of mountains at altitudes going from 700 m to more than 1000 m in several countries of Africa (Avery *et al.*, 2001). Its bark is exploited and marketed internationally because of its effectiveness in the treatment of benign prostates and hyperplasia (Watt and Bryer, 1962, Bankill, 1997, cit. by Hall *et al.*, 2000).

In pecuniary terms of value, one Kilogram of rough bark is bought at 300 F CFA from farmers and the drawn extract from the aforesaid Kilogram costs 500 000 F. CFA in pharmaceutical industries (Vockins, 2000, cit. Avana *et al.*, 2006).

P. africana is a component of biodiversity which represents 6 % of the species used for forest and agro-forest plantations in the agro-ecological zone of the high plateaus of the West of Cameroon (Tchouakionie *et al,* 2010).

To optimize the output of the plantations, it is necessary to take into account the biotic and abiotic factors relating to the *P. africana*. The small farmers and public authorities do not have formal information on the variability of the concentration of *β*-sitosterol, the principal active matter of *P. africana* with respect to altitude and the chemical characteristics of the soils of the aforementioned species plantations.

The general objective of this study is to appreciate the effect of some abiotic factors on the concentration of *β*-sitosterol of *P. africana* within mount Cameroon and the Bamenda high land areas. To achieve this goal, three specific objectives were formulated:

To evaluate the effect of altitude on the concentration of *β-sitosterol* of *P. africana;*
to appreciate the correlation between the chemical characteristics of the soils and the concentration of *β-sitosterol* of the aforementioned species;
to group the populations of *P. africana* according to their concentration in *β*-sitosterol.

In the biological context, *P. africana* belongs to the family of Rosaceae and are found practically only on mountains (Letouzey, 1982).The detailed description of the adult tree is materialized by characteristic phytogenetic parts (fig. 1).

The stem (Fig.1.A) is a seed-bearer in an agro-ecosystem in Akum, within Mezam Division, in the North-West of Cameroon. The base of trunk presents a simple footing of 8-10 cm (Fig.1. B). The young seedlings are visible under this tree. These seedlings come from two modes of pollination in particular: self-fertilization and cross-pollinated parents. Pollination here is primarily entomophilous. However the implication of certain birds was noted (Avana, 2006). Flowering is irregular in *P. africana* and occurs every 2 to 3 years. Fructification intervenes 2 to 3 months after the beginning of flowering. The number of fruits per inflorescence varies from 1 to 6 units (Fig. 1.C).

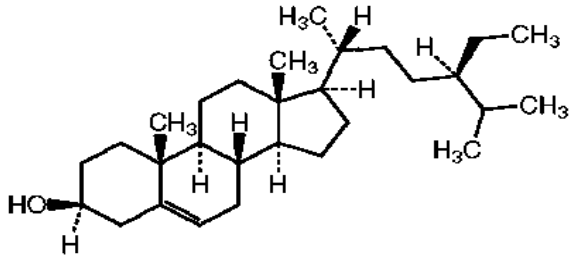
The natural ecological zone of *P. africana* in Cameroon is confined to the mountain and sub-mountain forests of altitudes ranging between 1500-3000 m (Vivien and Faure, 2011). The surface distribution of the forests of *P. africana* planted from 1976 to 2007, reached 625 ha with at least 1.526.430 trees (Kadu *et al.*, 2012). The peasants partly receive seeds from non-governmental organizations and official establishments.

Concerning the influence of biotic factors on the development of *P. africana,* analyses carried out by Dawson and Powell (1999) using molecular markers indicated that the variation is quite effective at the level of the genes (fig. 2).

There are quantitative and qualitative differences in chemical compounds from the barks of *P. africana* within geographically dispersed populations (Hall *et al.*, 2000). The concentration of *β*-sitosterol, the major component of the bark of *P. africana,* varies from 101 to 150 µg/ml between origins and 50 to 191µg/ml between individuals (Simons and Leakey, 2004, cit. Avana, 2006). One of the factors of the

environment which has an influence on the behavior of the plant is the physico-chemical composition of the soils (Sant' Anna, 1980).

Sudberg (2005) established that *β*-sitosterol is the most significant sterol chemical compound which exists in the extract of *P. africana*. It can be used as a marker for the control of the quality of barks of this species. Kadu *et al.,* (2012) established that most chemical components of the bark of *P. africana* are correlated between themselves. Environmental parameters such as temperature, precipitation and the altitude of the sites are not correlated with the concentration of the aforesaid components. The metabolic chart of *β-sitosterol* (Anonym, 2013) shows that its empirical formula is $C_{29}H_{50}O$ and developed:



In a natural environment, plants are nourished, from organic matter transformed beforehand into minerals by the organisms present in the soils (Larouche, 1983). These soils minerals influence the aspect of some phonotypic characters of the trees within its ecosystem.

Materiels and methods

Zone of study

The zones of study (fig. 3) were targeted in two administrative areas named the South-West and the North-West Regions of Cameroon, country of Central Africa.

The choice of these two areas is justified by the presence of natural regeneration and plantations of P. *africana*. In the South-West, the phytogenetic samples of materials and soils were taken in the Fako Division whose soils are ferralitic red. In the North-West, sample selection was made in the Mezam Division where soils are of the humid cambisol type (Valerie, 1968).

Experimental field works

The tools used for data collection on the field consisted of a GPS of GARMINT 5 mark used to record various altitudes, a numerical camera of mark HP Photosmart E 427, a digital meter of 7.5 m for appreciating the circumference of the stem, plastic bags for pocketing in a separate way the various samples and a cutlass to carve out the selected samples of bark and dig out soils.

The geographical co-ordinates of the sampled populations are given (Table 1).

The experimental scheme is made up of nine populations where nine composite samples of phytogenetic and ground materials were collected. The trees which were the subject of taking away of bark were beforehand identified with focus on those with ages varying between 19 and 22 years. Barking took place under a rainy weather. Each sample of bark (fig. 4) is removed on the stem, at the height of 1.30 m from the ground and on the face of the stem exposed to the rays of the sun after midday.

Extraction and isolation of β-sitosterol

The chemical analyses related to the extraction and isolation of *β*-sitosterol was carried out in laboratories. The protocol related to the matter is given (fig. 5).

The mechanical stages of the analysis related to the composite bark of each population concerned the splitting with the cutlass in bits of approximately 5 cm x 5 cm. The bits are dried on cemented ground or tarpaulin in the ambient air whose temperature varies between 25 with 27° C, for seven days. The dried bits are crushed for obtaining vegetable powder.

The stages of chemical handling related to steeping of the powder and to filtering it using a special filter paper. The various parameters of the rotary evaporator are regulated for obtaining the organic extracts of nine samples with ethyl-acetate (Fig.6).

Before the isolation of the *β*-sitosterol, two processes

are used to be sure of the presence of this component in the bark of *P. africana*:

The Thin Layer Chromatography which is a physical technique of separation of chemical species. The samples resulting from the extracts with the ethyl-acetate are placed horizontally and on the same level on paper with a thin layer. Each sample containing one or more species is moved through a mobile current phase along the stationary phase constituted of polar paper with silica. The chemical species which migrate and stop at the same height as that of the standard sample of *β-sitosterol* informs about the presence of the aforementioned active matter in the samples under study;

The High Performance Liquid Chromatography (HPLC) with UV detection wave length at 272 nm was used for the isolation and qualification of *β*-sitosterol of the barks of *P. africana*.

The quantification of the compound was achieved by GC-MS through comparing the chromatographic and spectroscopic data with an authentic standard.

Samples of soils analyses

Concerning edaphic matters, nine composite samples of soils are obtained from 27 single samples collected under *P. africana* trees. Laboratory analyses were focused on seven characteristics of soils fertility.

Statistical data analyses

The statistical data treated with SAS software was focused on chi-square test and the factorial multivariable analyses.

Results and discusion

The effective isolation of *β*-sitosterol of the barks of *P. africana was* carried out in two phases.

The chromatogram obtained from the Thin layer Chromatography (CCM) is given (Fig. 7).

On the CCM plate, the compounds *β*-sitosterol of the various samples of the barks migrated and were stationed at the same height as that of *β*-standard sitosterol. It is thus established that each sample of bark taken in the area of South-West and the North-West contains at least some molecules of *β*-sitostérol.

Table 1. Geo-references of the populations sampled for the barks and soils. NW = North-West, SW = South-West, PEx = Population of bark x, where x represents the number of the aforementioned population.

N°	Populations	Longitude	Latitude	Altitude (m)	Date of plantation	Ecosystem or environment
1	Population PE1 SW/Bova quarter/ Buea	0521604	0451332	885	1992	Agro-forest plantation
2	Population PE2 SW/Bova quarter/ Buea	0521556	0451141	898	1992	Agro-forest plantation
3	Population PE3 SW/Kuma-Bokwago /Buea	0521257	0457584	963	1993	Agro-forest plantation
4	Population PE4 NW/ Majemba quarter/ Bambui	0642243	0661454	1249	1990	Agro-forest plantation
5	Population PE5 NW/Fely quarter/ Bambui	0642000	0661601	1412	1993	Agro-forest plantation
6	Population PE6 NW/Atunui quarter/ Bambui	0641448	0661653	1570	1992	Agro-forest plantation
7	Population PE7 NW/ Water catchment Bambui	0643760	0666762	1595	1993	Forest of water catchment protection
8	Population PE8 NW/ Water catchment Banbui	0642111	0666516	1596	1993	Forest of water catchment protection
9	Population PE9 NW/Akum	0628128	0652407	1639	1992	Agro-forest plantation
	Average			1312	1992	-

The HPLC chromatograms of nine samples of the extract from the bark of *P. africana* are produced in mobile phase and to 50% distilled methanol. The pH is 6.5 and the ultraviolet detector of rays (UV) was stabilized to 272 nm. The rays are optimized by spectrometer using the visible ultraviolet rays. The temperature is of 25°C and the volume of solvent injected into the HPLC system is 20 µl. The retention time for separating *β*-sitosterol of the barks of various populations is that which is closest to standard (fig. 8a.), which is 15.501 min. The chromatograms obtained (fig. 8b) show that, in general at least, the chemical compound of *β*-sitosterol exists in all the samples of barks of *P. afrucana* collected in the areas of the South-West and North-West. The superposition of nine chromatograms of the extract of the barks from the various sites shows an inking between those of the AE1, AE3, AE4, AE5, AE6, AE7 and AE8 whereas AE2 and AE9 resemble each other but present a light difference with the seven others. These qualitative analyses made it possible to be sure of the presence of *β*-sitosterol in the samples of the barks of P. *africana*. However, the precision on the concentration of the aforesaid chemical compound remains to be determined.

Table 2. Concentration of *β-sitosterol* in the barks of *P. africana* PEx = Population of bark x, where x represents the number of the aforementioned population.

Populations	PE1	PE2	PE3	PE4	PE5	PE6	PE7	PE8	PE9
Concentration of *β*-sitosterol (µg/ml)	0.00	0.00	14.95	38.65	12.23	15.35	0.00	6.61	18.15

Table 3. Concentration of Lupéol in the barks of *P. african* PEx = Population of bark x, where x represents the number of the aforementioned population.

Populations	PE1	PE2	PE3	PE4	PE5	PE6	PE7	PE8	PE9
Concentration of Lupéol (µg/ml)	0.73	0.67	0.28	10.52	0.21	0.49	0.77	0.38	0.17

Gas Chromatography coupled with Mass Spectrometry (GC-MS) gave the result of the quantitative analyses of *β*-sitosterol of the samples of the barks of the populations of *P. africana*. The appreciation of the concentration of *β*-sitosterol in the barks of *P. africana* according to altitude is given (Fig.9).

Table 4. Correlation between the concentration of *β-sitosterol* of *P. africana* and the characteristics of the soils.

Chemical characteristics of the soils		Coefficient of correlation with the concentration of *β*-sitosterol
Organic matter	Total Organic matter (%)	0.01
	Organic carbon (%)	-0.45
	Total Nitrogen (%)	-0.42
	C/N (%)	-0.43
Acid phosphoric-Bray II	Assimilable phosphorus (mg/kg)	-0.08
Exchange acidity	Al^{3+} + H^{+} (cmole/kg)	-0.53
Exchangeable bases	Ca^{2+} (cmole/kg)	-0.40
	Mg^{2+} (cmole/kg)	-0.78
	K^{+} (cmole/kg)	-0.59
	Na^{+} (cmole/kg)	-0.14
	S (cmole/kg)	-0.77
	T(CEC) (cmole/kg)	-0.71
	V=S/T (100) (cmole/kg)	-0.55
Acid / Alkalinity	pH.eau-1/2,5	0.14
	pH.KCl- 1/2,5	-0.28

From Fig.9, it is established that:

In the population PE1, located at 885 m of altitude in the South-West area, the content of *β-sitosterol* is of zero µg/ml in the sampled barks. The concentration of *β-sitosterol* is also zero µg/ml in the barks of population PE2 located at 898 m of altitude in the South-West. This result shows that on the site of mount Cameroon, there are barks of *P. africana* which have excessively weak or even null concentrations of *β*-sitosterol.

Fig. 1. Various parts for the summary identification of the adult *P. africana*'s tree in situ. (A) Adult tree, (B) Base of trunk and section of bark, (C) Leaves and fruits.

In population PE3 located at 963 m of altitude on the mount Cameroon area, the concentration of *β*-sitosterol in barks of *P. africana*is is 14.95 µg/ml. It is released from this result that the concentration of *β*-sitosterol increases as altitude increases compared to the site of population PE1.

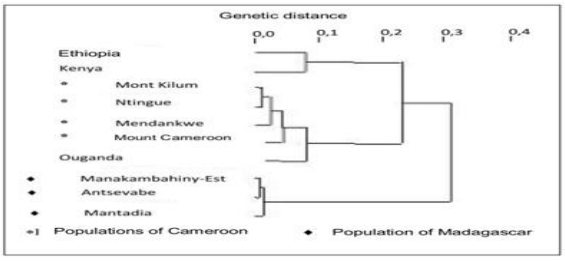


Fig. 2. Genetic bonds in *P. africana* (Dawson and Powell, 1999).

The barks of *P. africana* of the population PE4 at 1249 m of altitude have the biggest concentration of *β*-sitosterol, which is 38.65 µg/ml. This result establishes that the concentration of this active matter varies according to altitude. The optimum concentration of *β*-sitostérol is reached around 1249 m of altitude and beyond this point, the concentration starts to decrease;

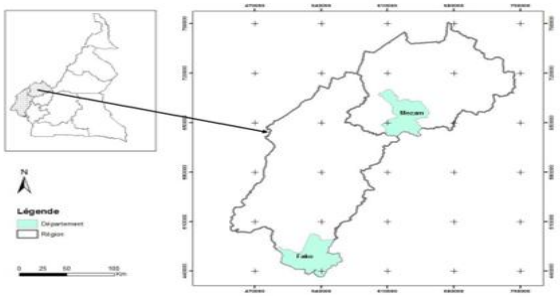


Fig. 3. Zone of study.

The barks of the population PE5 at 1412 m of altitude at Fely quarter in Bambui in the North-West Region, have a rate of 12.23 µg/ml. This concentration is in decrease compared to that of altitudes below 1412 m. This result shows that after the site of optimum concentration, *β*-sitosterol rates drop as one continues to go up in altitude.

Fig. 4. Cutting out of *P. africana*'s bark sample within population PE1.

At 1570 m of altitude where the population PE6 is found in Majamba quarter, within the peripheral zone of the Bambui town, *β*-sitosterol concentration is 15.35 µg/ml. This rate remains weak compared to the one of this active matter in the barks collected on the site with maximum concentration. The tendency of the decrease of *β*-sitosterol rate as one moves up in altitude after 1240 m is confirmed.

In the population PE7 located at 1595 m of altitude, in a forest plantation created for the protection of a drinking water source locally called " Bambui Water catchment", the rate of *β*-sitosterol falls down to zero µg/ml.

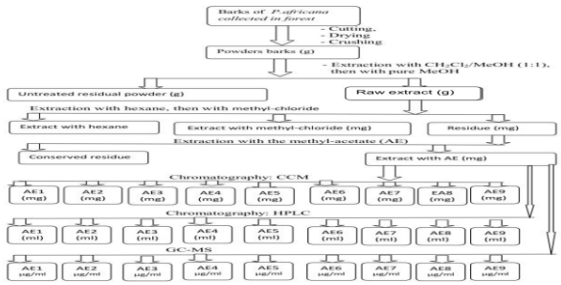


Fig. 5. General protocol of qualitative and quantitative analyses of *β*-sitosterol of *P. africana*. AEx = Extract with ethyl-acetate from bark x, where x represents the number of population, CCM = Thin layer Chromatography, HPLC=High Performance Liquid Chromatography and GC-MS = Gas Chromatography coupled with Mass Spectrometry.

At 1596 m of altitude where the population PE8 is located, still in another portion of the Water catchment's forest, the rate of *β*-sitosterol is 6.61 µg/ml. In spite of a light rise, the aforementioned content remains very weak compared to that of the optimum point:

The concentration of *β-sitosterol* of 18.15 µg/ml of the population PE9 is found at 1639 m of altitude in the Bamenda Highland forest. This concentration is high compared to that of the preceding site.

In general, the concentration of *β-sitosterol* of the nine composite samples of *P. africana*'s barks is given (Table 2).

To consolidate this result which shows that the content of *β*-sitosterol in the bark of *P. africana* varies according to the altitude of the forest plantation site, the quantitative analyses of Lupeol, generally correlated with the aforementioned active matter were carried out on the same samples. The results of the analyses made from the GC-MS is given (Tableau 3).

The optimum of the rate of Lupéol in the barks of *P. africana* is also at 1249 m of altitude in population PE4 like that of *β*-sitosterol. This second result confirms that the rate of *β-sitostırol* in the barks of *P. afrcana* varies according to altitude.

Fig. 6. Organic extracts of the nine samples with ethyl-acetate (AE).

The quantitative analysis carried out with GC-MS establish the absence of *β-sitostırol* in the samples of the barks of populations PE1, PE2 and PE7.

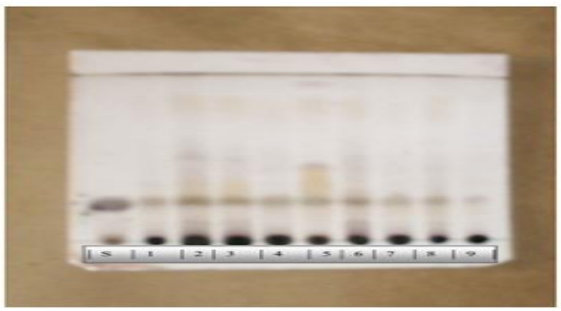


Fig. 7. CCM profile of nine samples of population's bark of *P. africana*. S = *β*-standard sitosterol, 1 = AE1, 2 = AE2, 3 = AE3, 4 = AE5, 5 = AE6, 7 = AE7, 8 = AE8, 9 = AE9 and AEx = Extracted with ethyl-acetate of bark x, where x represents it's number of the population.

The result of the analyses of the composites of soil samples taken under the stems of P. *africana* and bearing on 16 X 9 equivalents to 144 physic or chemical characters is obtained.

Statistical analyses carried out using SAS software were obtained. ANOVA analyses allowed for establishing that there is variability with respect to altitude between the averages of the concentrations of *β*-sitosterol of bark of *P. africana* throughout the populations; but the differences are not significant. The chi-square test confirms that locally, there is a very weak and non-significant correlation (r = 0.17) between the concentration of *β*-sitosterol of the bark

of *P. africana and* the environmental parameter which is altitude.

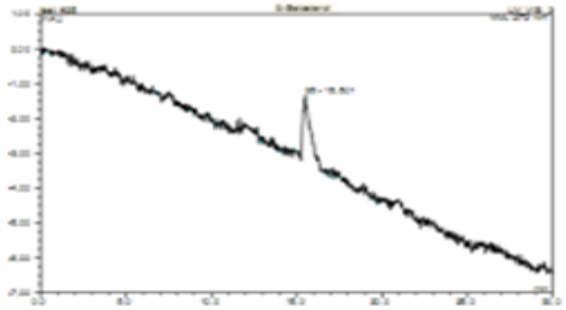


Fig. 8a. HPLC Chromatogram of the standard *β*-sitosterol.

The regrouping of the populations of *P. africana* sampled according to their concentration in *β*-sitosterol is carried out. The Analysis in Principal Components (PCA) targeted on the Ascending Hierarchical Clustering (CAH) was used for the data processing. The dendrogram resulting from the CAH is given (Fig. 10).

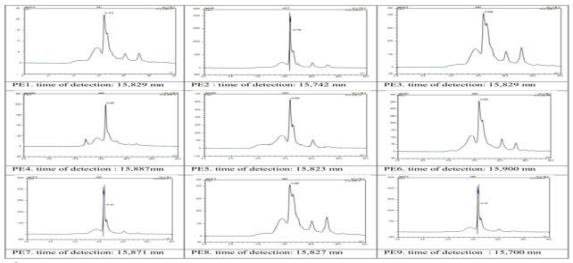


Fig. 8b. HPLC Chromatograms of nine samples of the *P. africana*'s bark extract. PEx = Population of bark x, where x represents the number of the aforementioned population.

The aforementioned dendrogram shows overall three groups of *P. africana*'s populations according to the rate of *β*-sitosterol of their barks.

The first group is made up of the stems of populations PE1, PE2, PE7 and PE8. The first two sites of this group are along the mount Cameroon in the South-West Region. The populations PE7 and PE8 are in the North-West Region. The barks sampled in these populations have the characteristic that their phenotypic characters materialized by the rate of β-sitosterol lies between zero and 6.61 µg/ml. The second group relates to populations PE3, PE5, PE6 and PE9. The barks of the tree of this group have a rate of β-sitosterol which varies from 12.23 to 14.95 µg/ml. The population PE3 is on the side of mount Cameroon whereas the other populations of this group, in particular PE5, PE6 and PE9 are in the area of the North-West. The third group consists of the population PE4. The barks of the forest present the strongest concentration of *β*-sitosterol which is 38.65 µg/ml. This site is in Bambui in the area of the North-West.

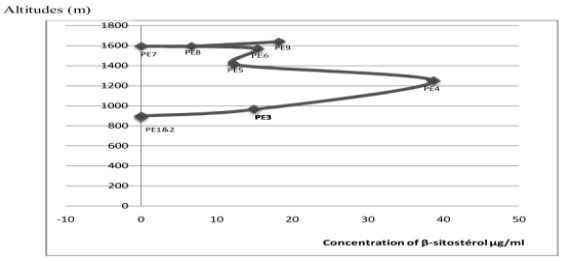


Fig. 9. Variation of the concentration of *β*-sitosterol of *P. africana* with respect to altitude. PEx = Population of bark x, where x represents the number of the aforementioned population.

In accordance with the regrouping resulting from the CAH, it is established that the best seed-bearing ones with respect to β-sitosterol concentration are in the populations of group II and III.

Correlation between the rate of *β*-sitosterol of *P. africana* and the chemical characteristics of the soils is given (Table 4.).

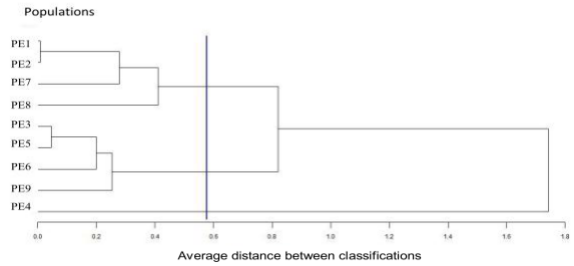


Fig. 10. Dendrogram resulting from the CAH of the concentration of *β*-sitosterol in the bark of *P. africana*.

The table shows 15 coefficients of correlation between the chemical characteristics of the soils and the concentration of *β-sitosterol* of *P. africana*. With the exception of two coefficients, particularly that between the total organic matter (r = 0.01) as well as pH KCL (r = 0.14) and the content of *β*-sitosterol

which are positive but non-significant, the thirteen others which vary from -0.78 to -0.14 are negative and non-significant. It is thus established that in the natural environment, there is no relevant correlation between the chemical elements of the soils and the concentration of *β–sitosterol.* The phenotypic character materialized by the concentration of *β*-sitosterol of *P. Africana* is thus influenced much more by genetic parameters than environmental factors. In other words, the abiotic factors have a weak influence on the content of *β*-sitosterol of this forest species.

Discussion

Contrary to the results of Sudberg, (2005) who found a concentration in *β*-sitosterol from 29.10 to 32.43 µg/ml in three samples of the bark of *P. africana*, the present study highlights that rate of *β*-sitosterol in the extract of the bark of *P. africana* varies from zero (0) to 38,65 µg/ml. This result is relevant as the barks collected on the stems of populations PE1, PE2 and PE7 do not contain *β*-sitosterol and cannot consequently give the satisfaction discounted within the framework of phytotherapy. The genetic bond can justify the bringing together of populations PE1 and PE2 having a rate of zero in a site in the South-West and population PE7 in the North-West area. The seeds which were used with the installation of the above-mentioned populations can result from the same source. This study confirms the result of Kadu *et al.,* (2012) which established that the environmental parameters are not generally correlated with the concentration of the chemical components of bark of *P. africana*. It even adds that the chemical elements of the soils have a very weak influence on the concentration of *β*-sitosterol of *P. africana*. Pilate (2002) establishes that for forest trees, characters targeted for selection such as the volume of the barrel or the density of wood are strongly or fairly influenced by the environment. This means that they are less heritable; but the concentration of *β-sitosterol* could rather be a phenotypic character with heritable prevalence.

Conclusion

The qualitative analyses of *β*-sitosterol of barks of *P. africana* shows the presence at least in trace of this bioactive constituent in all samples of the plants collected in the areas of the South-West and the North-West of Cameroon. The quantitative analyses however establish that the populations of *P. africana*, PE1, PE2 and PE7 have barks which do not contain the *β*-sitosterol. This result is relevant because the barks collected within the above-mentioned populations cannot give the satisfaction discounted within the framework of phytotherapy. Locally, there is no significant correlation between the concentration of *β-sitosterol* of barks of *P. africana* and the environmental parameter which is altitude.

The CAH set out the populations in three groups. The first group is made up of the stems of populations PE1, PE2, PE7 and PE8. The phenotypic character materialized by the concentration of β-sitosterol of this group varies from zero to 6.61 µg/ml. The second group relates to populations PE3, PE5, PE6 and PE9. The rate of *β*-sitosterol of the tree barks of this group varies from 12.23 to 14.95 µg/ml. The third group is made up of the population PE4 whose concentration of *β*-sitosterol reaches 38.65 µg/ml.

There is no relevant correlation between the chemical elements of the soils and the concentration of *β*-sitosterol of *P. africana*. Consequently, the aforementioned phenotypic character is influenced much more by genetic parameters than environmental factors.

The tools thus obtained are essential for the management of the ground and the exploited products of this species, as well as the production of the seeds for the installation of forest and agro-forest plantations.

In prospect, the creation of the plantations of *P. africana* starting from seeds coming from the stems of populations PE1, PE2 or PE7 could lead to the establishment of forest trees whose barks do not have the principal bioactive matter, characteristic of this medicinal plant. In the short term, it will be advisable to carry out the sensitizing of the peasants and public authority for the collection of the seeds preferably

within the populations of the second and third group. On the long run, it is necessary to carry out a research on the production of seeds by in vitro culture whose explants will come from better stems of populations PE3, PE4, PE5, PE6 and PE9 and thus, improve the current practice of sylviculture in Cameroon.

Acknowledgements

We present our gratitude to the Center of Environmental and Social Management (CEGES), with the members of the scientific teams of the Laboratory of Plant Biotechnology and Environment of the University of Yaounde I and those of the Laboratory of the Natural Substances of the Higher Teachers' Training College (ENS) of Yaounde all in Cameroon, as well as the Institute of Environmental Research (INFU), Department of Chemistry and Biology, Chair of Environmental Chemistry and Analytical Chemistry in Germany, for their multiform contributions.

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