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Full Length Research Paper

Growth and photosynthesis responses of *Rosmarinus officinalis* L. to heavy metals at Bougrine mine

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Heavy metals pollution is considered as one of the most dangerous environmental problems in agricultural soil, particularly neighboring mining sites. Phytoremediation based on green plants use seems to be an interesting biological solution to detoxify these soils. This study focuses especially on heavy metals effects on *Rosmarinus officinalis* L. morphological and growth parameters. The site of our research is "Bougrine" mine which represents different zinc, lead and cadmium amounts by geological layers. In opposite to amounts obtained by previous studies made for mining exploration, we thought that zinc content was more important at transition zone than Cenomanian Turonian level. Nevertheless, this element is not the most determinant in plant growth and its morphological parameters. Moreover, *R. officinalis* L. volume and vigor decreased when soil's heavy metals content increases. However, this reduction is more related to soil lead content. Despite this decrease, we do not observe any visual phytotoxicity symptom. This aromatic and medicinal species, belonging to Lamiaceae family, could be used as alternative crops in polluted soils.

Key words: *Rosmarinus officinalis* L., zinc, lead, growth, photosynthesis, "Bougrine" mine.

INTRODUCTION

Heavy metals can be considered dangerous environment pollutants (Kassassi et al., 2008). They can affect crop productivity with toxic effect in high concentrations and then can attempt human health (Houston, 2007).

In Tunisia, agricultural soils near mining area are often excessively polluted with trace metallic elements that contaminate crops and would be transferred to animal and humans. Physico-chemical methods' decontamination, such as pollutant leaching or excavation and off-site treatment, are very expensive (Cunningham et al., 1995) and may damage considerably the soil constitution. Phytoremediation is a reliable biological cleansing process of polluted area. It is defined as the use of green plants to remove pollutants from environment or to render them harmless (Raskin et al., 1997). This method is divided into several approaches. The most important of them are phytoextraction and phytostabilization (Salt et al., 1998). First technology consists of installing hyperaccumlating plants to remove metals or organics from soil by concentrating them in the harvestable parts (Salt et al., 1995, 1998). It can be more profitable by Phytomining which is metals recycling technology (Brooks, 1998). In theory, phytoextraction seems to be the most interesting process for soils cleanup, but this theory is not really operational for much polluted soils. In fact, hyperaccumlating plants known until now have low aerial biomass then they need many cycles to decontaminate only weak contaminated soils (Salt et al., 1998; Robinson et al., 1998). Moreover,

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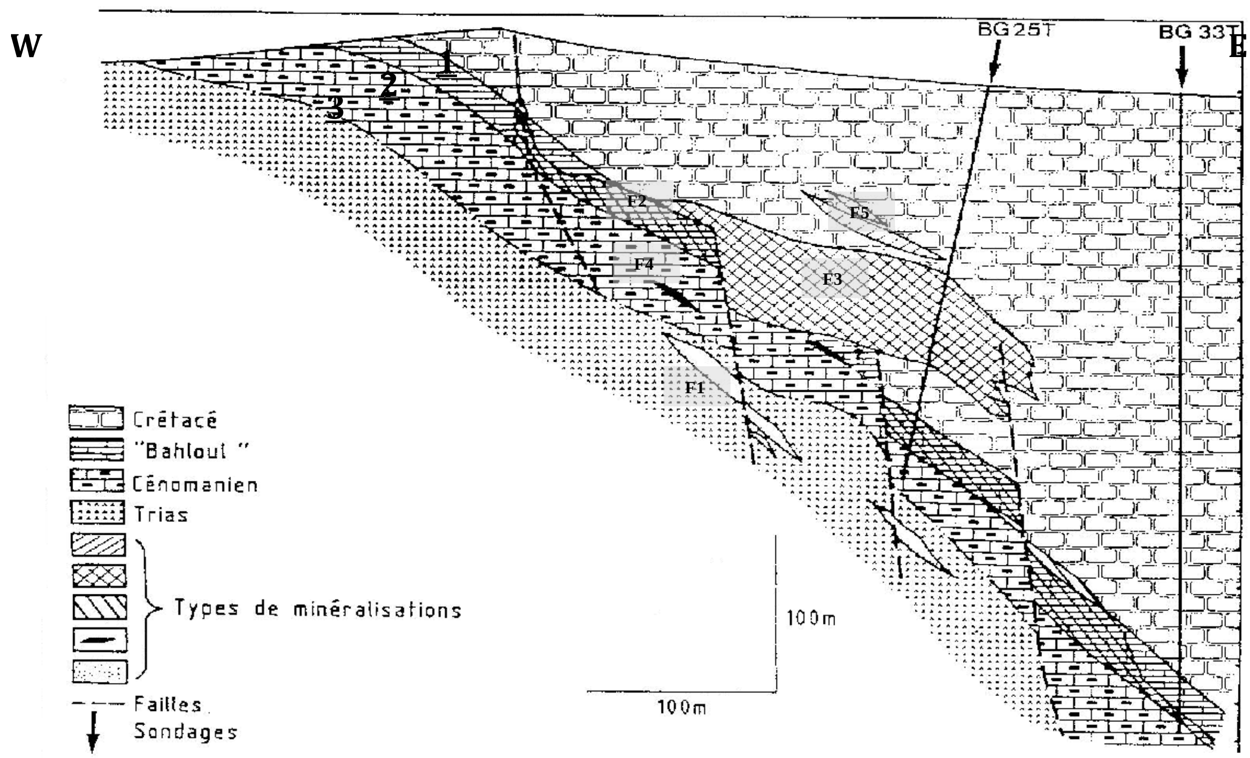

Figure 1. Schematic section W-E of Bougrine mining (Orgeval, 1986). Geological levels: 1, Cenomanian Turonian; 2, basic Cenomanian; 3, transition zone Triassic/Cretaceous.

phytostabilization is the most reliable and efficient biological method for phytoremediation. Its finality is to stabilize pollution to contaminated area rate and avoid polluting neighboring waters and soils by erosion, runoff or leaching. It uses heavy metals tolerant plants (Salt et al., 1998). Plants should have mechanic action (shoot and root parts) and chemical action (metals complexation attributed to root substances) (Berti and Cunningham, 2000). In this context, the use of tree is particularly attractive since root system is developed and deep, and evapotranspiration is very efficient so they limit leaching and runoff (Schnoor, 2000).

Phytostabilization is applicable in all pollution levels inasmuch as the used species are sufficiently tolerant and present a particular interest. Aromatic and medical crops, including those belonging to Lamiaceae family, seem to present a real phytoremediation potential (Zheljazkov and Nielsen, 1996; Zheljazkov and Warman, 2003). Moreover, their essential oils are not contaminated, that confer those plants another economical interest. Angelova et al. (2007) demonstrated that *Lavandula vera* L. and *Ocimum basilicum*, belonging to Lamiaceae family, can be cultivated on heavy metals polluted soils. Furthermore, some researches concluded that aromatic plants such as *Mentha piperita* L., *O. basilicum* L., *Anethum graveolens* L., coriander, sage, dill, chamomile and lemon balm (Zheljazkov and Nielsen, 1996; Zheljazkov, 2006; Zheljazkov et al., 2008a) and medicinal crops such as *Marrubium vulgare* L., *Melissa officinalis* L. and *Origanum heracleoticum* L. (Zheljazkov et al., 2008b) can be an alternative solution to polluted soils.

In Tunisia, the “Bougrine” mine is a plomb-zinc geological deposit. It is the most important in North Africa lands. It presents significant layers of Rosemary (*Rosmarinus officinalis* L.) that may have a real potential of polluted soils stabilization throughout this mine.

The objective of our research was to study different amounts of heavy metals effects on Rosemary morphological aspect and growth.

MATERIALS AND METHODS

Site

Soil and plant samples were taken from the “Bougrine” mine. This is situated in septentrional part of Tunisian Atlas inside an important Zn-Pb deposit. Three geological levels were chosen because of their different zinc and lead content and their important density of Rosemary plants (Figure 1) (Orgeval et al., 1986):

(1) Cenomanian Turonian known as “Bahloul”: The passage to Bahloul facies constituted by dark brown to black limestones containing millemetric laminae is very distinctive because its flow in platelets and its richness in organic matter are brutal. This formation is typically attributed to the passage upper Cenomanian-lower Turonian. It covers most of the zinc mineralization at “Bougrine”

Table 1. Zinc, lead, cadmium and organic contents of three geological levels of Bougrine mining.

Metal	Cenomanian Turonian	Basic Cenomanian	Transition zone Triassic/Cretaceous
Zn ($mg.kg^{-1}$)	550.740 ± 172.390	1031.546 ± 229.328	10499.693 ± 850.281
Pb ($mg.kg^{-1}$)	69.030 ± 14.750	190.1 ± 33.129	185.2 ± 28.539
Cd ($mg.kg^{-1}$)	0.858 ± 0.166	1.258 ± 0.166	1.365 ± 0.476

mine, "beam 2 (F2)" and these mineralizations are located in the lodges of planktonic foraminifera (Orgeval, 1979).

(2) Cenomanian basal: Above, has developed a succession of marls with loaves and bioturbated argillaceous limestones that constitute the "beam 4 (F4)". The thicknesses are highly variable from one point to another of the massif. These variations in thickness are often very brutal and partly related to recent tectonic laminations (Orgeval, 1979). These two levels belong to upper Cretaceous.

(3) Transition zone between Triassic and Cretaceous: The contact between the triassic and the cretaceous series is most often abnormal. This contact is related to tectonic phenomena caused by the rise of the triassic to the surface. The transition zone has been defined on the site of "Bougrine" to characterize the particular features that make the connection between the triassic clay-gypsum and the upper cretaceous transgressive coverage basis (Orgeval, 1979).

Soil

Chemical characteristics soils' were determinate: samples taken in 30 cm deep were submitted to trace heavy metals analyses: zinc (Zn), lead (Pb) and cadmium (Cd). Heavy metals content were determined using inductive coupled plasma atomic emission spectrometer; ICP) (Model: Activa; Marque: Horiba Jobin Yvon) and expressed in $mg.kg^{-1}$ MS.

Plants

For each geological level, 18 Rosemary samples were taken at random, at 100 m interval. The following parameters were measured on each plant: tuft height (cm), number of branches per shoot, and tuft diameter. The bivolume was calculated: biovolume = $[(3/4)*(\pi)*(r^3)]/2$. Noddles number/leafed branch, leaves number/noddle, leaves number/Branch (= leaves number/Noodle * noddles number /leaved branch), inflorescences number/branch, flowers number/inflorescence, flowers number/branch (= flowers number/inflorescence * inflorescences number/branch), were measured on the 18 taken samples. The length and width of 13 leaves were measured on those samples. For photosynthetic pigments extraction (chlorophyll a and b, and Carotenoïds), 1 g of *R. officinalis* L. fresh leaves was dissolved into 30 ml acetone 80%; during 30 days in cold and darkness, until completely bleach. Optic density was determined using spectrophotometer (Biochrom Libra S6), pigments content was calculated with method of Arnon (1949).

Statistical analysis

Analysis of variance (ANOVA), discriminate canonic analysis, Mahanalobis distances matrix established on basis of morphological parameters and clusters deduced from those distances were carried out with Genstat statistical logical.

RESULTS

At "Bougrine" mine, zinc content differs according to geological layer; a difference between three levels was highly significant ($p < 0.001$). In fact, Cenomanian Turonian known as "Bahloul" present the lowest zinc content (550.740 $mg.kg^{-1}$) followed by Cenomanian basal (1031.546 mg. Kg^{-1}). Transition zone present the highest zinc content (10499.693 $mg.kg^{-1}$) (Table 1). Concerning lead content, the difference was highly significant between Cenomanian Turonian, which present the lowest content (69.030 $mg.kg^{-1}$), and the two other levels. Furthermore, there is not any significant difference ($p = 0.681$) between Cenomanian basal and transition zone that present respectively 201.859 $mg.kg^{-1}$ and 196.140 $mg.kg^{-1}$. Cadmium content did not differ according to geological level ($p = 0.085$). Its value was around 1 $mg.kg^{-1}$.

Analysis of morphological parameters will be carried according to "Bougrine" mine's geological levels, which differ essentially by their zinc and lead content. Moreover, cadmium content remains almost the same.

Morphological parameters of *R. officinalis* L.

Longitudinal and lateral growth

On Cenomanian Turonian (Bahloul), Rosemary tuft present the most important growth (49.056 cm height and 96.417 cm diameter) (Figure 3). This longitudinal and lateral growth decrease on Cenomanian basal (H = 40.056 cm and ∅ = 83.389 cm) and transition zone (H = 41.611cm and ∅ = 74.056 cm). According to fisher test, this decrease was highly significant between the three levels ($p < 0.001$). Nevertheless, Rosemary growth does not differ significantly between the last levels (p = 0. 508 longitudinal growth and $p = 0.255$ lateral growth), that present different zinc (Zn) contents but almost the same lead (Pb) content (Table 1).

Biovolume

Rosemary biovolume (Figure 4) was more important on Cenomanian Turonian (113.588 cm^3) than on the two other geological levels. In fact, Cenomanian basal and

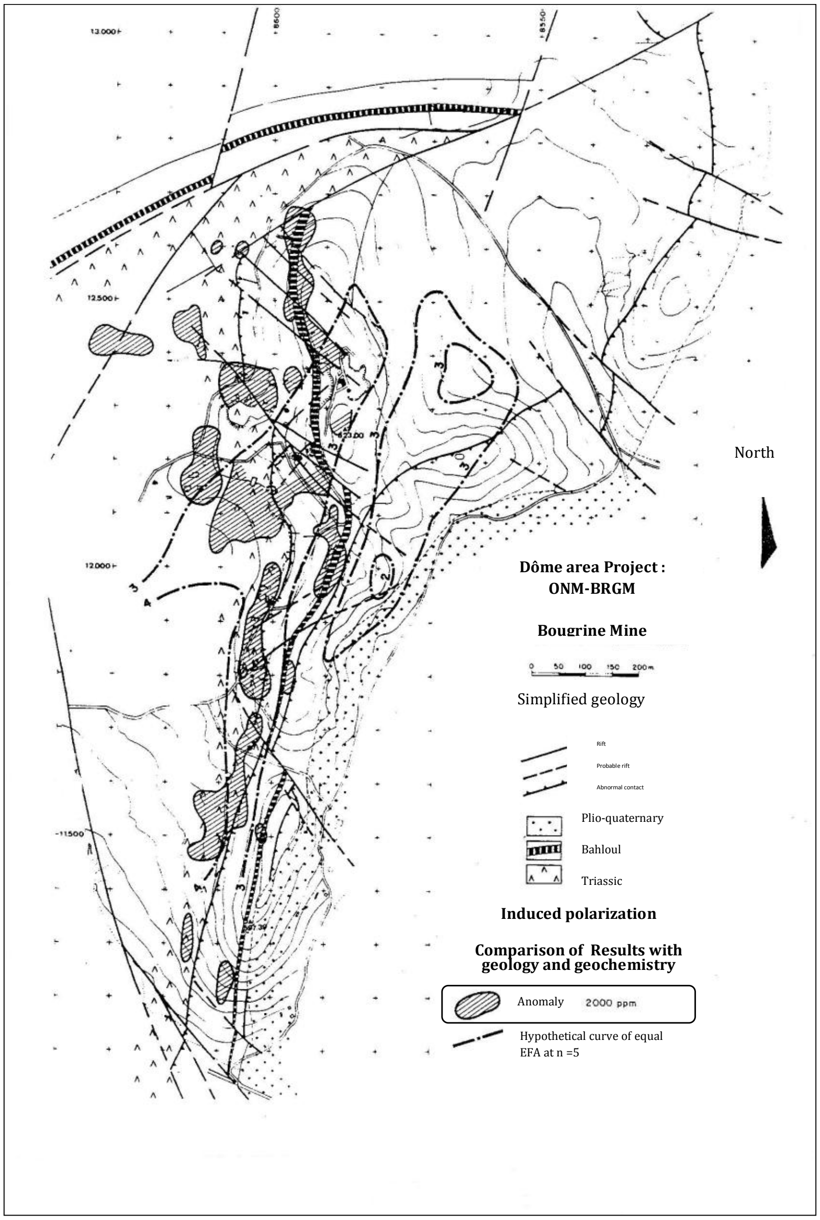

Figure 2. Bougrine Mining– Simplified Geology (Chettaoui, 1985).

transition zone present respectively 98.240 cm^3 and 87.245 cm^3 biovolumes. The difference between three levels is highly significant ($p < 0.001$) while it was not significant between the two last ones ($p = 0.105$).

Branches number per shoot

Branches number per shoot (Figure 5) was very important on Cenomanian Turonian (27.222), then it decreases by more the half on Cenomanian basal and transition zone. The difference was highly significant between the three geological levels ($p < 0.001$). This parameter differs ($p = 0.011$) between Cenomanian basal (12.889) and transition zone (10.000). Nevertheless, this difference can be neglected because the variation coefficient was relatively high (cv = 36.5).

Vegetation parameters

Numbers of noddles per leaved branch, leaves per noddle and leaves per branch

Noddles per branch number differs significantly from one level to another on "Bougrine" mine ($p = 0.002$ according

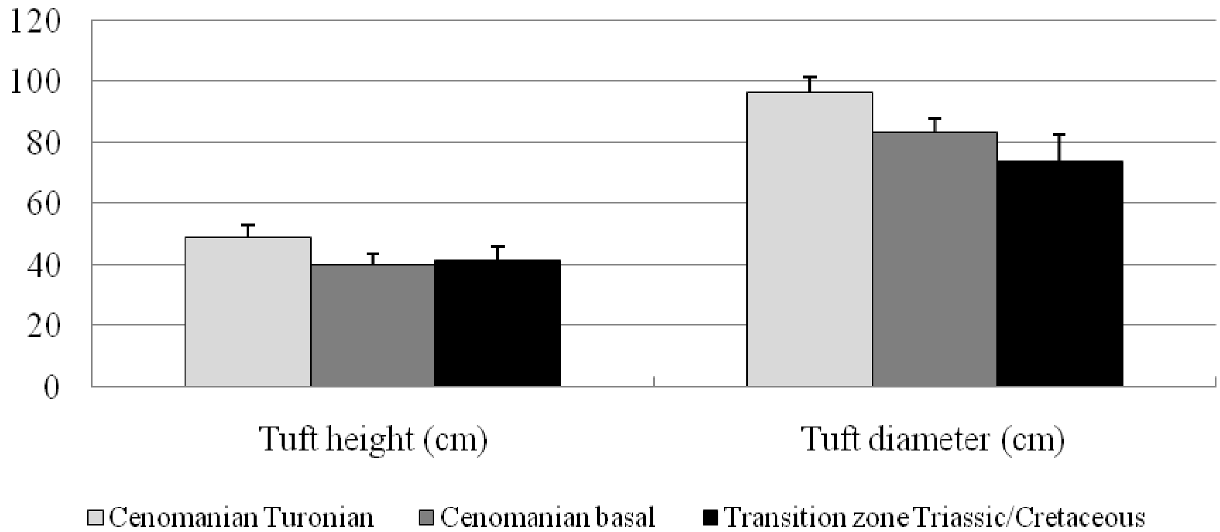

Figure 3. Height (cm) and diameter (cm) of *Rosmarinus officinalis* L. Tuft although geological levels in the Bougrine mining (■, Cenomanian Turonian; ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

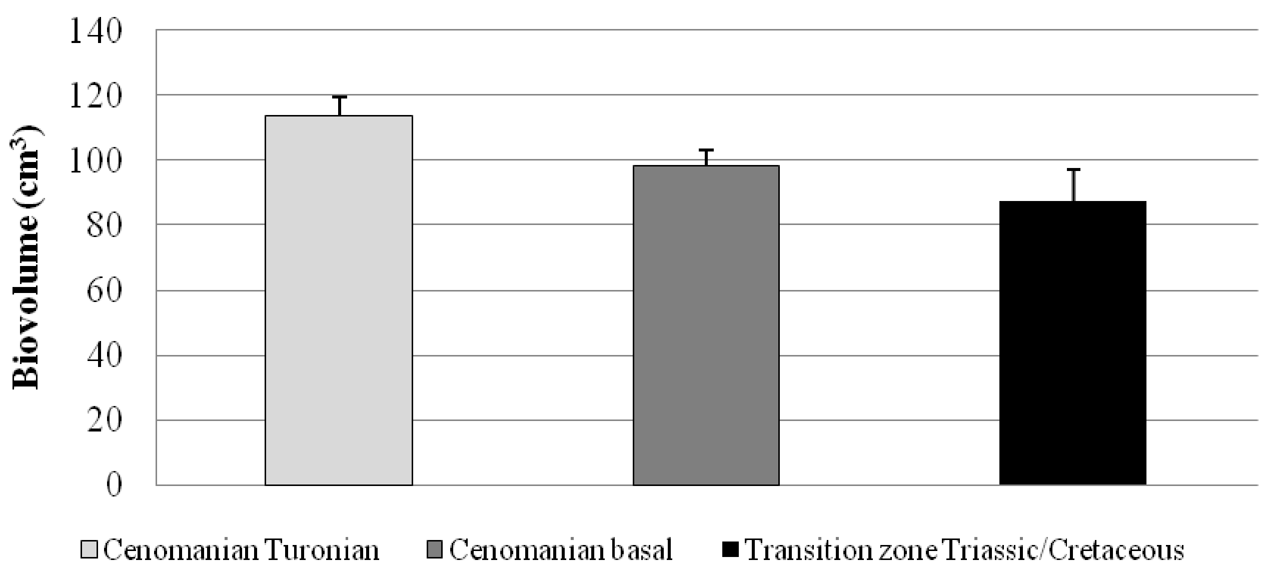

Figure 4. Biovolume of *Rosmarinus officinalis* L. Tuft (cm^3) although geological levels in the Bougrine mining (■, Cenomanian Turonian; ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

to the fisher test). Moreover, the number of leaves per noddle and of leaves per branch remains the same on three levels ($p = 0.713$ and 0.438, respectively) (Figure 6).

Leaf length and width

Leaves length differs between Cenomanian Turonian and the two other geological levels on the “Bougrine” mine. This difference was highly significant ($p < 0.001$) but it was not significant between Cenomanian basal and transition zone. Furthermore, leaves width remain constant on three levels ($p = 0.107$) (Figure 7).

Blossoming parameters

Rosemary flowers’ number per inflorescence changes significantly, between three geological levels on “Bougrine” mine. Concerning the number of inflorescences per 10 cm branch, the difference was

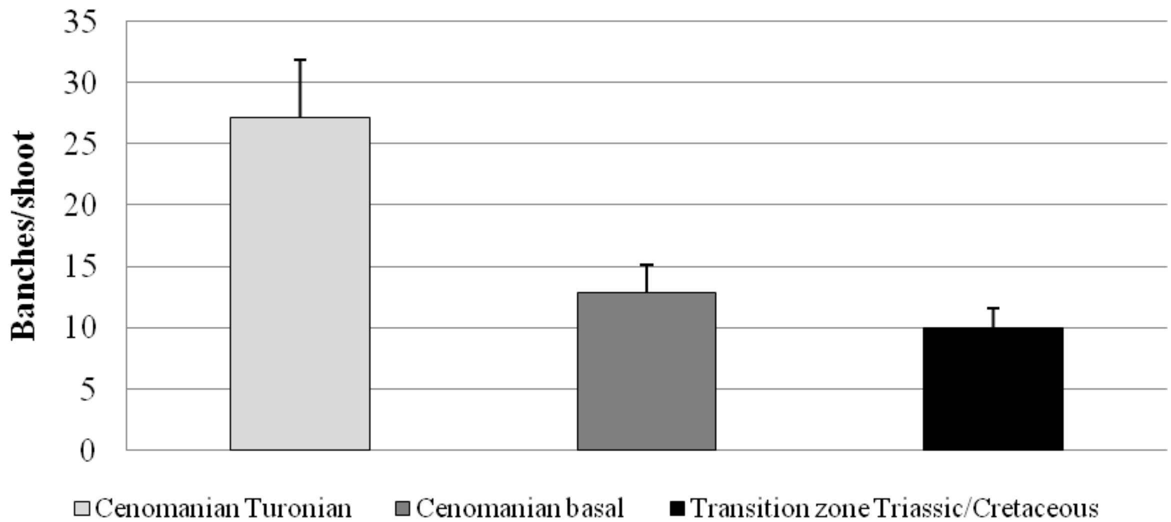

Figure 5. Branches number per shoot of *Rosmarinus officinalis* L. although geological levels in the Bougrine mining (■, Cenomanian Turonian; ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

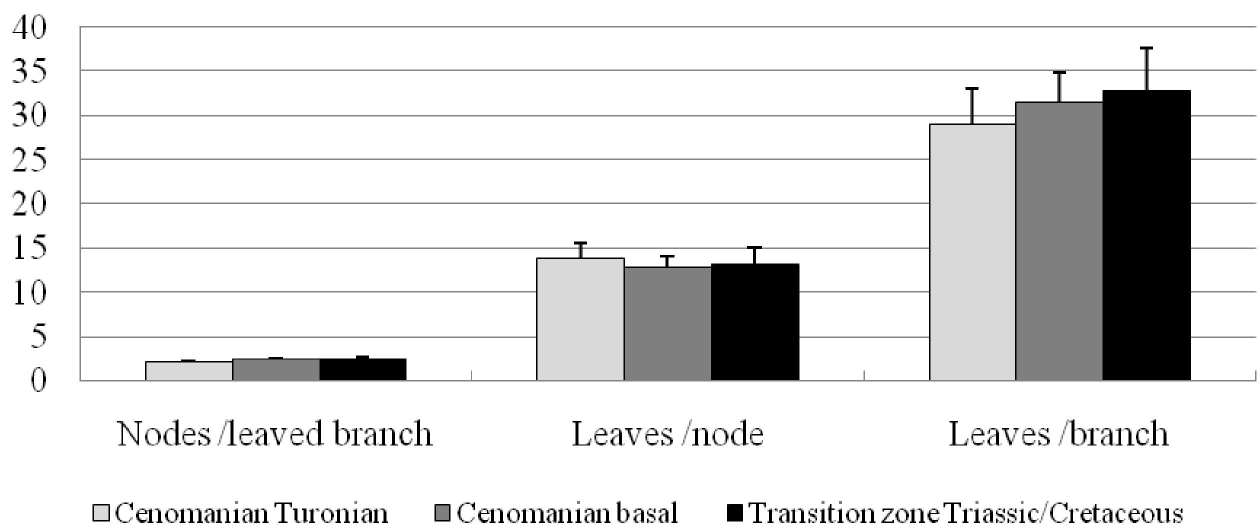

Figure 6. Nodes per leaved branch (1 cm), leaves per node and leaves per branch (1 cm) of *Rosmarinus officinalis* L. although geological levels in the Bougrine mining (■, Cenomanian Turonian, ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

significant between Cenomanian Turonian and the two other levels but it was not significant between the last ones. Moreover, the number of flowers per 10 cm branch does not vary between the three "Bougrine" mine levels (Figure 8).

Photosynthetic pigments content

The content of photosynthetic pigments (Figure 9) tend to increase quietly on Cenomanian basal, but this rise was not significant according to the fisher test ($p = 0.546$; 0.825; 0.775 and 0.113 for chlorophyll a, b and (a+b), and for carotenoïds, respectively).

DISCUSSION

Soil's zinc and lead contents were the lowest at Cenomanian Turonian "Bahloul". Zn content increases two times at Cenomanian basal and ten times at transition zone. But, Pb content increases almost three times at both the two other geological levels.

The lowest Zn content in the topsoil at "Bahloul" can be

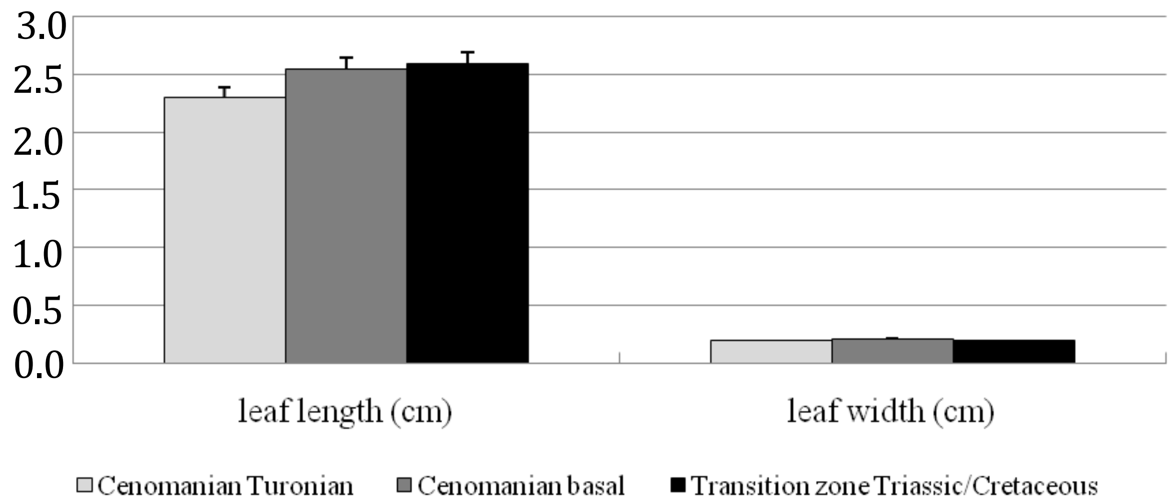

Figure 7. Leaf Length and width (cm) of *Rosmarinus officinalis* L. although geological levels in the Bougrine mining (■, Cenomanian Turonian; ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

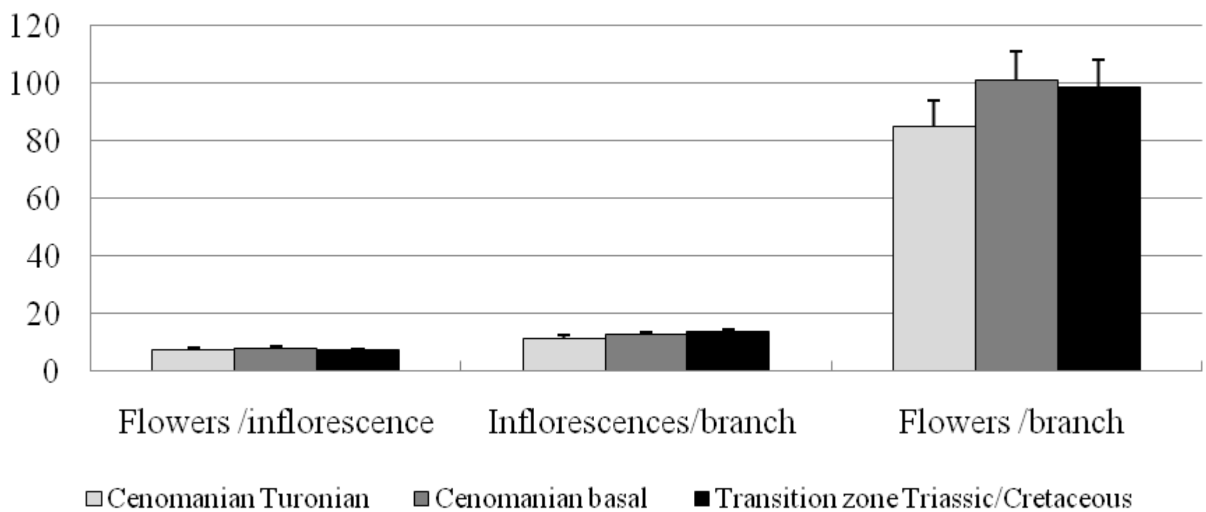

Figure 8. Flowers per inflorescence, Inflorescence and flowers per branch (10 cm) of *Rosmarinus officinalis* L. although geological levels in the Bougrine mining (■, Cenomanian Turonian; ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

explained by the fact that in stratiform disseminated mineralization of this type, nodes and areas of entanglement would be favorable to the accumulation of mineral type “clusters” or “body mass” (Chettaoui, 1985). This reasoning can be supplemented by researches of Sposito et al. (1989) who states that in the case of soil formed from bedrocks, particularly rich in heavy metals, content of metallic trace elements increases with depth. Also, the highest zinc content at transition zone may be attributed to disseminated mineralization related to abnormal contact between the Triassic and Cretaceous, that favors installation of spots with abnormally high levels in this area (Figure 2) (Chettaoui, 1985).

At 1000 ppm zinc in the soil, Rosemary yield seems to be affected. Baker and Walker (1990) suggested that plants actively take up the zinc, as they are required for metabolic (including photosynthetic) processes. When up-take exceeds metabolic requirements, however, a toxic impact may be expected. In spite of this, when it exceeds 1000 ppm and even reaches 10000 ppm in the soil, the zinc content has no apparent effect on these parameters. This finding can be explained by the fact that measurement of total Zn in soil does not show the real available fraction of this element to the plant. Therefore, more analysis concerning different fractions soil constitution, especially evaluation of the pool of soluble

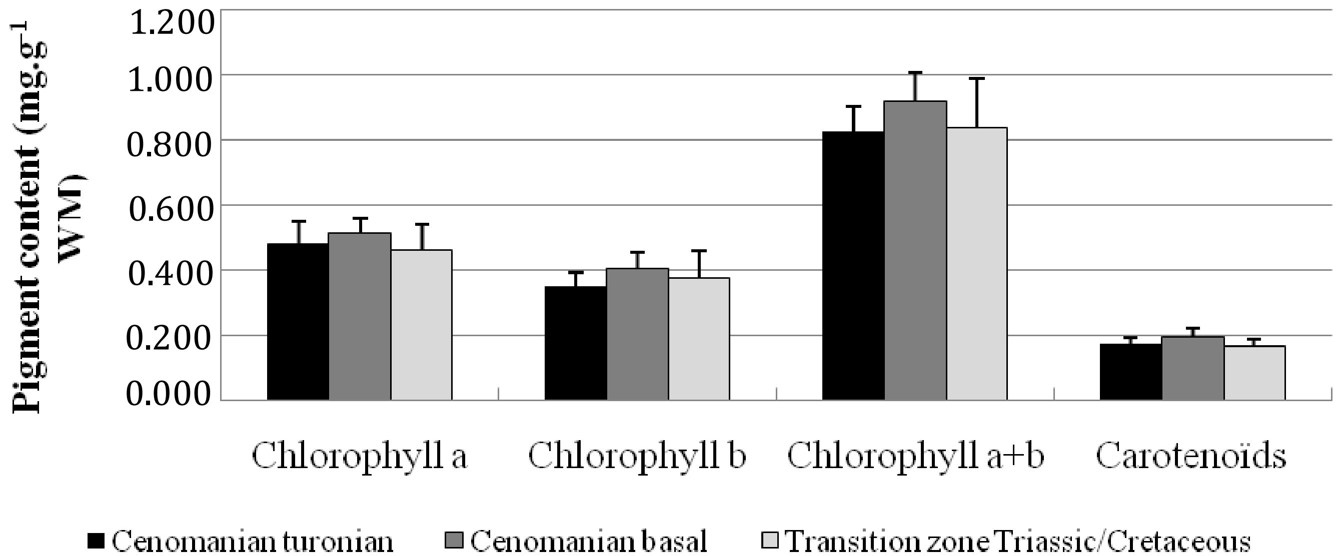

Figure 9. Pigments (chl a, b and a+b, and car) content of *Rosmarinus officinalis* L. although geological levels in the Bougrine mining (■, Cenomanian Turonian; ■, basic Cenomanian; ■, transition zone Triassic/Cretaceous).

(available) trace element in soil (Kabata-Pendias, 2004), would improve these results.

Most of measured parameters are much more dependent with lead content. In fact, they vary inversely with it. The Rosemary volume parameters represented by tuft's height, diameter and biovolume and its vigor represented by tuft branches' number per shoot and leaf length have most important values at "Bahloul". Then, these values decreased at Cenomanian basal and transition zone; however, they remained almost unchangeable between these two levels. We estimated Rosemary yield by the last analyzed parameters because fresh and dry matters analysis is a destructive method for natural Rosemary layers that we studied. The reduction of Rosemary volume and vigor at Cenomanian basal and transition zone has as obvious consequence a yield decrease. Our results are in agreement with previous researches such as those of Meers et al. (2007) and Zheljazkov et al. (2006), which thought that at high concentration, heavy metals can reduce yields of agricultural crops or other spices used for phytoextraction. Also, El Said-Deef (2007) concluded that biomass production of copper treated Rosemary plants decreased gradually above 200 ppm. Furthermore, Vartika Rai et al. (2004) observe a biomass reduction of *Ocimum tenuiflorum* under chromium stress. Zheljazkov et al. (2006) demonstrated that high concentrations of Cd, Pb and Cu would reduce the yields of crop plants such as copper phytotoxicity on dill (*Anethum graveolens* L.). Also, Zheljazkov et al. (2008b) had observe a yield decrease of five medicinal plants grown in soil at the immediate perimeter of trace metals (Cd, Pb, Cu and Zn) contaminated area; as herbage yields from *M. officinalis* L. grown on this soil decreased up to 38%. In another experiment, Zheljazkov et al. (2008a) demonstrated at the same site, that fresh yields of height aromatic crops and varieties were reduced with metallic stress. These different crops were, however, conducted in semi-controlled medium.

Despite the *R. officinalis* L. yield decrease, more dependent to Pb contamination, we do not observe any phytotoxicity symptom on plants. Similarly, no visual phytotoxicity was observed on many other aromatic (Zheljazkov, 2008a) and medicinal (Zheljazkov, 2008b) crops.

On the three geological levels of "Bougrine" mine, the presence of heavy metals (zinc, lead and cadmium) does not have any effect on the content of photosynthetic pigments in *R. officinalis* L. leaves. These observations are contrary to other researches such as those of Krupa et al. (1993), which stated that heavy metals cause a decrease on pigments, in particular, on chlorophyll content in *Phaseolus vulgaris* under nickel stress. Also, Zheljazkov et al. (2008b) explained that cellular alterations observed in morphology may be an indication of the toxic effect of heavy metals on transport solutes across plasma membrane and perhaps on photosynthesis, which may explain a yield reduction. They observed chloroplast alteration in plants was due to plants exposed to heavy metals. Vartika Rai et al. (2004) thought that chromium reduced the foliar contents of total chlorophyll, chl a and b contents in *O. tenuiflorum*. This might be attributed to the toxicity of this element to chlorophyll biosynthesis.

Moreover, Cu and Zn caused a significant decrease in pigments contents of *Avicennia marina*, but, no

Table 2. Discriminate canonic analysis *Rosmarinus officinalis* L. morphological parameters on Bougrine mining three geological levels: Axis and percentage of variability absorption.

Canonic axis	Axis definition		Adjustment term	Percentage absorption of variability absorption (%)	Cumulative percentage (%)
CAN 1	Leaf width	+0.1237	-1.502	81.68	81.68
	Number of branches / shoot	- 1.7625			
	Number de leaves / branch	- 0.1200			
CAN 2	Tuft height	+ 0.1970	+1.81	18.32	100
	Biovolume	+12.5081			
	Number of flowers/inflorescence	+0.0421			
	Number of flowers/branch	+0.2480			
	Number of nodes/ branch	-0.6664			
	Number of leaves / node	-0.4820			
	Leaf length	-2.5449			

Axis 1, *Rosmarinus officinalis* L. vigour. Axis 2, *Rosmarinus officinalis* L. volume and blossoming.

significant inhibitory effects on any photosynthetic pigments were evidenced on exposure to Pb (Macfarlane and Burchet, 2001) which has often been found to be less damaging to the photosynthetic apparatus unless applied in very high concentrations (Ahmed and Tajmir-Riahi, 1993).

Furthermore, accumulated Pb was possibly sequestered in vacuoles to minimize the toxic impact on photosynthetic pigments and to minimize membrane damage (Wozny and Krzeslowska, 1993). Also, Papazoglou et al. (2007) thought that chlorophyll content in giant reed plants remains unaffected and no significant differences were observed between treated and control plants. They stipulated that the examined metals were probably sequestered in a very effective manner within the plant.

In our study, we hypothesize that, lead as a cytoplasm toxic element, can be sequestrated in cell organelles, thus providing a powerful protection of the photosynthetic machine and not affecting the pigments synthesis. The yield reduction could then be explained by other mechanisms like cellular elongation decrease.

Canonic discriminate analysis

Axis and percentage absorption of variability are represented in Table 2. Axis one which represent the first canonic variable, absorb 81.68 % of totally variation. This component is correlated with leaf width, branches number per shoot and leaves number per branch. That can correspond to plant vigor. Axis two absorb 18.32 of total variation and it represent the second canonic component, which correlate with tuft height, biovolume, numbers of flowers per inflorescence, noddles per branch, leaves per noddle and with leaf length. This axis can correspond to *R. officinalis* L. volume.

The canonic analysis (Figure 10) revealed a one group divergence for Cenomanian Turonian. The two other geological levels (Cenomanian basal and transition zone) draw two other groups straddling.

Mahanalobis distances

The Mahanalobis distances permitted to measure the heavy metals content divergence between different geological levels (Table 3).

Clusters (Figure 11) deduced from those distances permitted to distinguish two groups, one group alone, which represent Cenomanian Turonian and a second group, which renferm transition zone and Cenomanian basal. The cluster confirm geological levels distinction, revealed by discriminate canonic analysis.

Conclusion

Despite the yield reduction under heavy metals stress (Zn, Pb and Cd), *R. officinalis* L., a Lamiaceae species, can be cultivated in contaminated soils without exhibiting any toxicity symptom. It might consequently offer feasible agronomic, environmental, and economic alternative to other commonly grown edible crops in the polluted area. Analysis of nutritional status, essential oil yield and composition will be conducted in future experiment to complete these results.

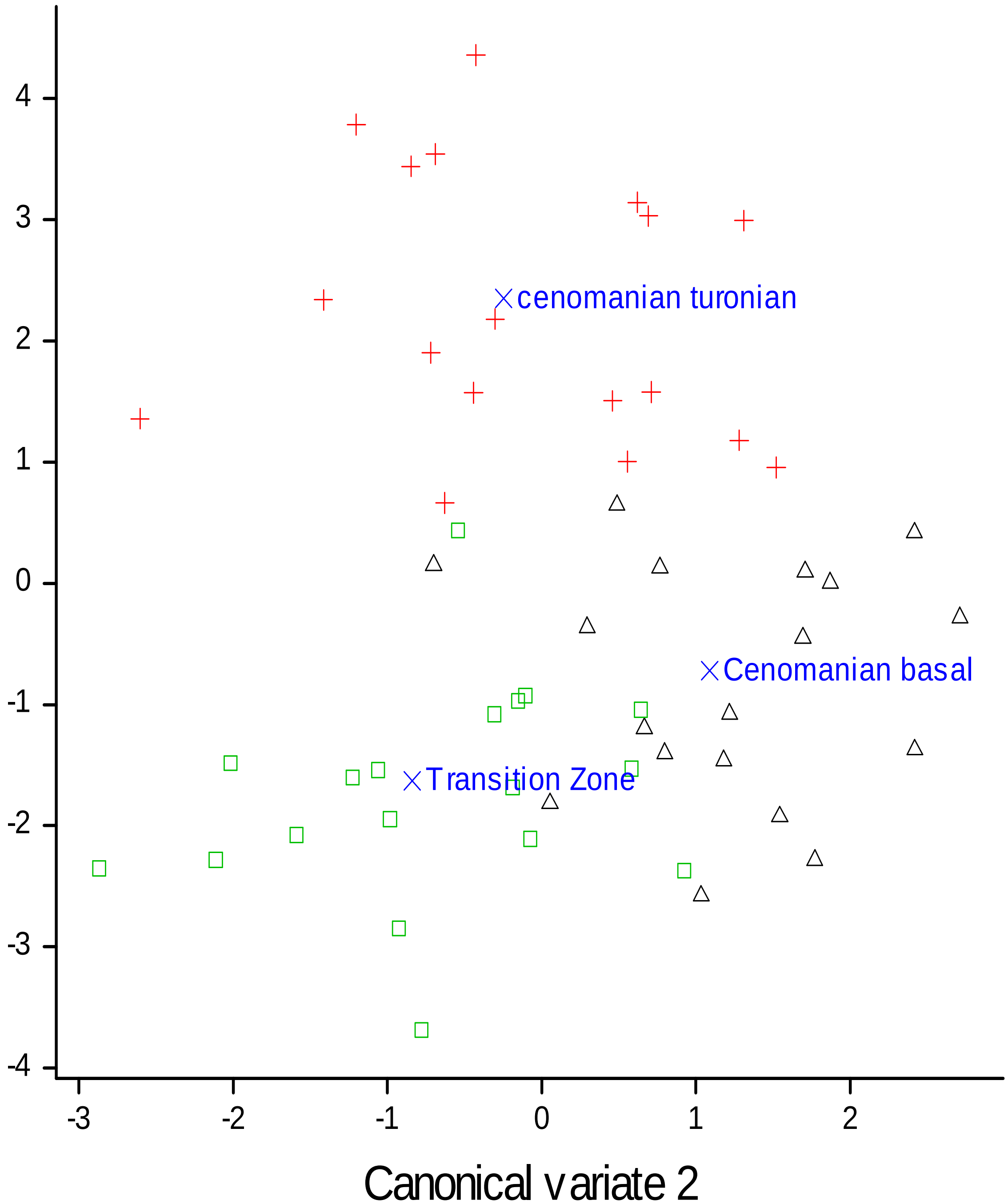

Figure 10. Discriminate canonic analysis of *Rosmarinus officinalis* L. morphological parameters on the Bougrine mining three geological levels: CAN DISC.

Table 3. Mahalanobis distances.

Parameter	Cenomanian Turonian	Transition zone, Triassic/ Cretaceous	Cenomanian basal
Cenomanian Turonian	0.000		
Zone de transition Triassic/ Cretaceous	3.351	0.000	
Cenomanian basal	4.024	2.131	0.000

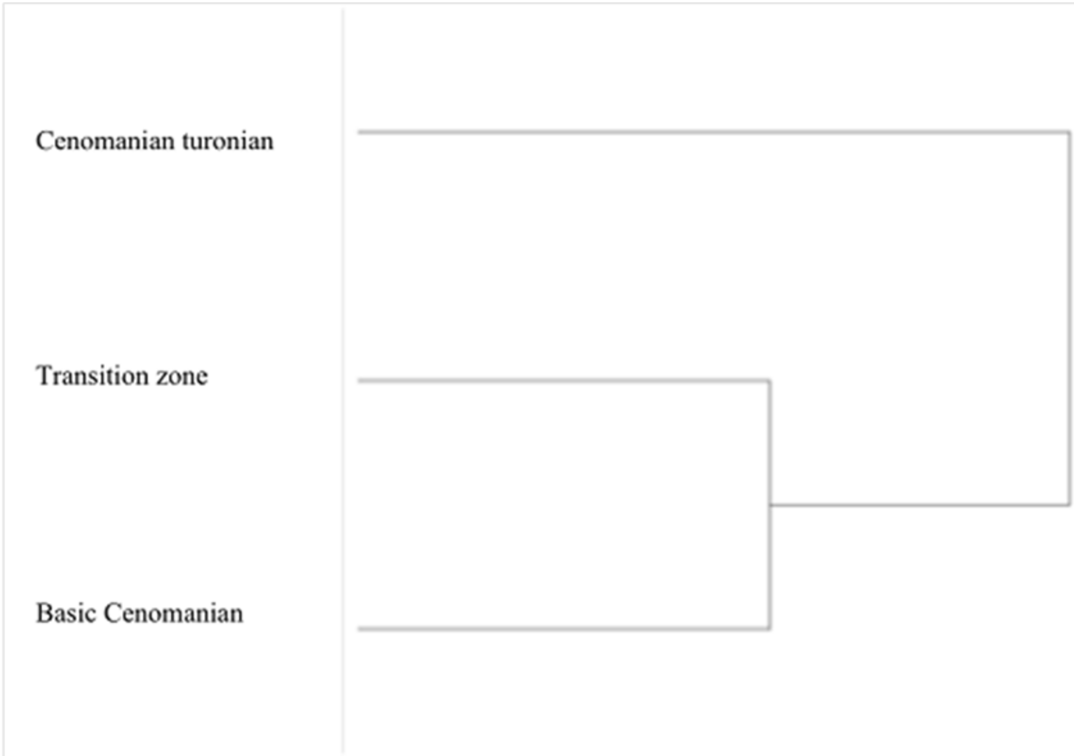

Figure 11. Cluster deduced from Mahalanobis distances.

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