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Study of Genetic Variability of Mastic Tree (*Pistacia lentiscus* L.) in Moroccan Areas – Macro-Biochemical Characterization

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ABSTRACT

This study focuses on the characterization of the macro-biochemical composition of leaves of *Pistacia lentiscus* L. from 11 natural populations in Morocco, in order to allow genetic differentiation of this species. This characterization of the studied population allows research and identification of the most informative markers and analysis of relatedness between populations and their origin sites. The results of ANOVA showed that there were significant differences ($p = 0.05$) in total sugar content, dry matter content and fiber content between the studied populations. Organic matter, dry matter and total nitrogen contents were positively and significantly correlated with energy values ($r = 0.92$ and 0.962). In contrast, negative significant correlations were found between dry matter, minerals, fat content and carbohydrates ($r = -0.217, -0.379$). The results of principal component analysis showed that the 11 studied populations were dispersed among four groups, regardless of their geographical proximity. This grouping is confirmed by hierarchical classification

Keywords: Pistacia lentiscus, wild populations, biochemical parameters, marco-biochemical characterization, Moroccan areas.

INTRODUCTION

Lentisk, or Mastic tree (*Pistacia lentiscus* L., Anacardiaceae) grows wild in Morocco, is named in Morocco as "Drou" or "Tidit (Ait Mohand et al., 2020); is widely endemic to the Mediterranean regions and distributed in the extreme ecosystems of the Mediterranean basin (Ait Mohand et al., 2020). It has a large geographical and bioclimatic distribution, extending from the humid to the arid areas (Hayani et al., 2023). It can be regarded as an oleaginous plant with a high oil yield estimated at around 40% (Trabelsi et al., 2012). The lentisk and its unsaponifiable fraction extracted from is mainly used in the healing of skin (Trabelsi et al., 2012). Since antiquity, lentisk has been used as a spice, as a cosmetic agent but most importantly as a potent phytotherapeutic remedy, mainly for the treatment of gastrointestinal disorders (Pachi et al., 2020). Lentisk oil may partially help in the protection against mercury intoxication, and it could also be considered a safe nutritional source, at least by maintaining total cholesterol and LDL-cholesterol in their normal ranges (Maarouf et al., 2008). Also, the essential oil of Lentisk is extensively used in the perfumery and in food and pharmaceutical industries as reported by Dhifi et al. (2013).

Morocco has very important plant genetic resources for which it has made great efforts to sign and ratify conventions and bilateral, regional and international agreements on the protection and conservation of biological diversity (Kabiri et al., 2019). However, until now, no studies

have been made on the composition of the lentisk leaves, especially the chemical composition. In this study, regions having a particular forest on the middle, High Atlas Mountains and littoral, with a favorable microclimate for several plants with high added values and also with a vulnerable population of which this plant can constitute a source of income in a spirit of protection of the biodiversity, and these regions are characterized by a semiarid climate in the North and subhumid climate in the South. Therefore, the main objective of the presented work is to highlight the chemical composition of Moroccan lentisk leaves under the influence of pedoclimatic conditions of the study area. This study aims to characterize the biochemical composition of leaves of *Pistacia lentiscus* L. from 11 natural populations in Morocco to have an identification of this endemic species.

MATERIALS AND METHODS

Sampled areas and plant material

Eleven sites from three areas in three different geographic regions of Morocco were selected for this study. Zone I: Middle Atlas (five sites: 1, 3, 5, 8 and 10), zone II: High Atlas (five sites: 2, 4, 6, 9 and 11) and zone III: littoral region (one site: 7) (Fig. 1). Detailed description of the sampled sites is previously described in Bouta et al. 2020 and Bouta et al. 2022. From each site, three mature trees were randomly chosen and fully sun exposed mature leaves were randomly and carefully harvested and stored in -20°C until further use.

Biochemical traits analysis

Dry biomass

Leaves dry biomass (DM) was determined by oven drying the leaves at 105°C until constant weight. The humidity (H) was estimated according to the method of Simsek (2010) and Ionica et al. (2013) using the formula (1) given in Table 1, and dry mass (DM) by the formula (2) given by Audigie and Dupont, 1982. Table 1 shows the formulas used to estimate the biochemical traits tested for the characterization of Mastic tree (*Pistacia lentiscus*) leaves.

Mineral and organic content

The dosage of mineral content (ash matter) is carried out according to the instructions of the French standard (AFNOR, 1977) and calculated according to the formula (3, Table1). Moreover,

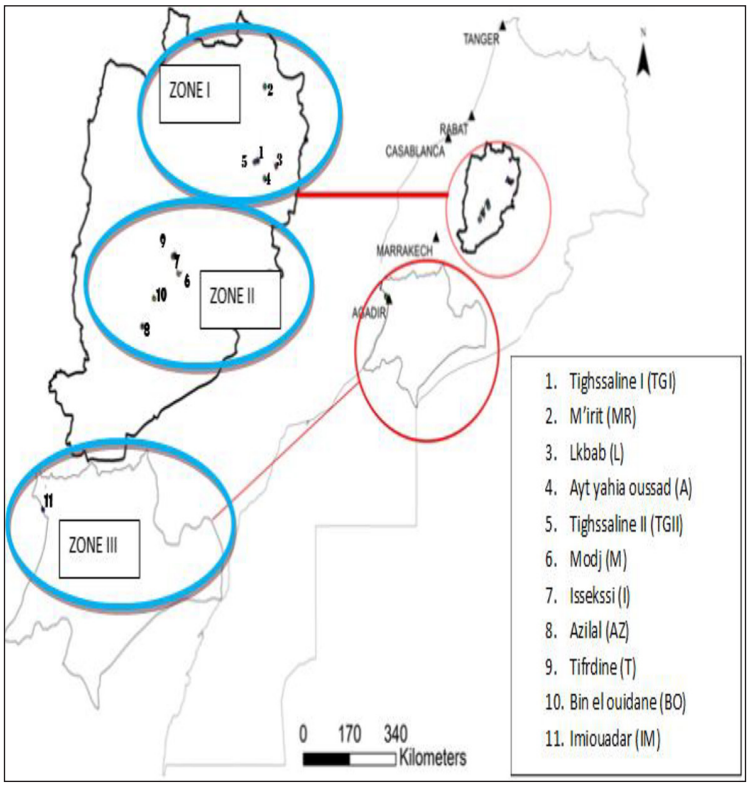

Fig. 1. Map of Morocco showing locations of the *P. lentiscus* populations analyzed

from the dry matter and ash content, the organic matter (OM) content is determined using the formula (4, Table1).

Quantitative determination of primary metabolites

Determination of oil content

It was carried out according to the ISO 659, 1998 method using Soxhlet type apparatus for 8 hours and the hexane as organic solvent extraction. The quantity of the oils is determined by the difference in the mass of the sample before and after the extraction compared to the initial mass of the sample using the formula 5 (Table1).

Estimation of total sugar content

The total sugar content (TSC) was estimated according to Babu (2002) and Dubois (1956). For this, 0.5 g of fresh leaves was ground with 2 ml of 80% ethanol subsequently introduced into suitable tubes for centrifugation. After centrifugation (2000 rpm/40 min), 50 µl of extract were added to 0.5 ml of 5% phenol and 1.5 ml of sulfuric acid solution (H_2SO_4) and heated in a water bath for 5 min at 100° C. After cooling in melting ice, the optical density (DO) was read at 485 nm. A range of glucose from a stock solution of 1 mg mol^{-1} was used as standard. The formula (6) was used to calculate the sugar content and values are expressed in mg g^{-1} of dry matter.

Estimation of total crude proteins

The crude protein (Prot) was estimated by estimating the total N content (TNC) according to the Kjeldahl method (Kjeldahl, 1883) and a nitrogen-protein conversion factor of 6.25.

Crude fiber content of fresh leaves

Crude fiber content was estimated according to Aryapak et al. (2014). Thus, five grams of crushed fresh leaves were digested in 100 ml of

Table 1. Formulas used to calculate some biochemical parameters tested of *P. lentiscus*

Biochemical parameters	Equation	Formula used	Formula components	References
Humidity	(1)	$H = (P\text{-}P1)/ M \times 100$	P – fresh weight P1 – dry weight M – weight of plant material	Simsek (2010) et par Ionica et al. (2013)
Dry biomass	(2)	$DM = 100 - H$		Audigie and Dupont, 1982
Mineral content	(3)	$MM = \frac{m2-m0}{m1-m0} \times 100$	m0 – mass of the crucible (g) m1 – mass of the crucible-sample (g) m2 – mass of the calcined crucible-sample (g)	AFNOR, 1977
Organic content	(4)	$OM = DM - MM$	DM – the dry matter content in % MM – ash content in %	
Oil content	(5)	$Oil\ (\%) = \frac{P2 - P1}{S} * 100$	P1 – weight of the empty balloon. P2 – weight of the flask after evaporation. S – mass of the test sample	ISO 659, 1998
Sugar content	(6)	$\text{Sugar content} = (DO \times V_{ethanol})/ (a \times V_{extract} \times FW)$	FW – fresh weight	Dubois, 1956
Fibers	(7)	$Fibers(\%) = \frac{M2 - M3}{M1} * 100$	M1 – Initial weight (g) M2 – Weight of residue before incineration (g) M3 – Weight of residue after incineration (g).	Ranganna S, 1977
Carbohydrates	(8)	$\text{Carbohydrates (\%)} = 100 - (\text{Protein (\%)} + \text{Lipids (\%)} + \text{Ash (\%)})$		Grosso et al., 2000)
Energy	(9)	$\text{Energy (kcal/100g)} = (4\times \text{protein (g)}) + (3.75\ \text{carbohydrates (g)}) + (9\times \text{lipids})$		Grosso et al., 2000)

1.25% H_2SO_4. The solutions were boiled for 45 minutes and then were filtered and washed with hot distilled water. The filtrates were digested in 100 ml of 1.25% NaOH solutions. These solutions were heated for 60 minutes, filtered and washed with hot deionized water and over dried and measured. The final oven-dried residues were ignited in a furnace at 550 °C. The weights of the left after ignition were measured as the fiber contents and were expressed in term of the weights of the samples before ignition and calculated according to Equation 7 (Table 1) (Ranganna, 1977).

Estimation of carbohydrate content

The carbohydrate content (Carb) is estimated by the difference with respect to the other constituents (Equation 8, Table 1): proteins, lipids, ash and moisture (Grosso et al., 2000). The following formula is used to calculate the carbohydrate content.

Estimation of energy value

The energy value is calculated by multiplying and summing the values obtained for proteins, carbohydrates and total lipids multiplied by 4.00, 3.75 and 9.00, respectively (Durucasu and Tokusoglu (2007) (Equation 9, Table 1). The contents are expressed in kcal g^{-1} of dry matter.

Statistical analysis

All the assays were carried out in triplicate and the results are expressed as mean values and standard error of the mean. The data obtained are subjected to several statistical analyzes including an analysis of variance (ANOVA) and a Pearson correlation (p=0.05) using the SPSS software (IBM SPSS Statistics 25). Then, the ordination and the classification of the populations are carried out by the principal component analysis (PCA) using the XLSTAT software (2017) and the ascending hierarchical analysis according to the UPGMA aggregation method by the SPSS (IBM SPSS Statistics 25).

RESULTS AND DISCUSSION

Dry matter and humidity

The analysis of variance showed that there is a significant effect of the population for the two parameters (DM and H) ($p<0.05$) (Table 2). The dry matter (DM) content varied from 29.30% for the population of Ait Yahia Oussaad (AO) to 47.533% for the population of Immi Oudar (IO), with an overall average of 39.26%. These results are in agreement with those obtained by Emile (2018) for woody forest species with a percentage of 36.9%. However, they are lower than that obtained by Mebirouk-Boudechiche (2014) for Algerian populations 49.33%. Concerning the humidity rate (H) ranged from 52.467% to 70.70%. The highest value is recorded in the population Ait Yahia Oussaad, while the population of Immi Oudar presents the lowest value, with an overall average of 60.74%. It is noted that the humidity rate obtained in the Moroccan populations is higher than the value obtained in the Algerian populations 49.33%, (Kadi and Zirmi-Zembri, 2016).

Ash content

The results of the ash content (MM%) of the 11 provenances are between 3.00%, recorded at the population Bine El Ouidane (BO), and 9.767% for Lkbab (LK), with an overall average of 5.17 % (Table 2). These results are similar to those reported by Kadi and Zirmi-Zembri (2016) for lentisk from El Taref in Algeria. But the results reported by Mebirouk-Boudechiche in 2014 and 2015 showed a somewhat higher content of the Algerian populations with 8.17% (Mebirouk-Boudechiche, 2014). But they are lower than those obtained in *Panicum maximum* and *Pennisetum purpureum* 11.90% and 15.20% (Ettian, 2018).

Organic matter content

This content is between 44.4%, recorded at the origin of Immi Oudar (IO) and 23.52% for Ait Yahia Oussad (AO), with an overall average of 34.08% (Table 9). These results are similar to those reported by Kadi and Zirmi-Zembri (2016) for mastic tree from El Taref in Algeria. However, they are much lower than those of the tropical plants *Panicum maximum* and *Pennisetum purpureum* with respectively a content of 92.48% and 92.78% (Ettian, 2018).

Total oil content

Extraction of the oil contained in lentisk leaves with hexane for the eleven studied areas showed that the lowest content is obtained from the Azilal (AZ) population with a rate of 12.98%, and the higher content is recorded in the population of Modj (MO) by 32.67% with an overall

average of 21.40%. These values are higher than those found in tropical forage plants such as *Panicum maximum* and *Pennisetum purpureum* with 2.50% and 2.10% respectively (Ettian, 2018).

Crude fiber content

The crude fiber content has changed slightly between the different populations and ranged from 16.03% to 23.80%, with an overall average of 20.84% sTable 8). The lowest value was in the population of Ait Yahia Oussaad. These values are similar to those of Boudechiche (2014) who obtained 25.17%, lower than those reported by Emile (2018), 37.7%, on average for forest fodder species, and similar to those reported for English ryegrass fodder var Neuville with 23.0%, and higher than those of *Opuntia ficus indica* (9.4%; Amira, 2015).

Total nitrogen content and Crude protein content

The total nitrogen content (TNC) in the 11 populations ranged from 3.74% for the population Azilal to 6.54% for the population Mrirte (Mr), with an overall mean of 5.43%. We note that these levels are much lower compared to those obtained by Mebirouk-Boudechiche (2014) for the same species (*Pistacia lentiscus*). But by comparing it with other forage species such as *Robinia pseudoacacia* L. and alfalfa we find a resemblance with respectively 20.6% and 17.6% of total nitrogen content (Emile 2017). The determination of the protein content by the Kjeldhal method gave values which varied between 16.03% (Ait Yahia Oussaad population) and 23.80% (Immi Ouadar population) with an overall average of 20.84% and a coefficient of variation of 1.34%. In comparison to some wild forage species such as *Atriplex nummularia* and *Leucaena leucocephla*, we find almost the same content with respectively 20.40% and 20.30% of crude protein. But compared to *Opuntia ficus* indicas we find that the latter is much richer in crude protein with 31.10% (Le Houérou, 1980; Nefzaoui and Chermiti, 1991; Speedy and Pugliese, 1992; Carter, 1994; D'Mello and Devendra, 1995).

Total sugar content

The total sugar content in the 11 populations ranged from 2.83% in the provenance of Mrirte to 28% in the population of Isseksi (IS), with an overall average of 14.84% and a coefficient of variation of 53%. These results are lower than those reported for *Panicum maximum* and *Pennisetum purpureum* with 49.37% and 36.20% (Ettian, 2018), and they are higher than those reported for Orchard grass with 8.8%, as well as those of English ryegrass forage var Roskalia which have almost the same total sugar content with 15.4% (CTPS/GEVES 2021).

Energy value content

The energy value in kcal/100 g of dry lentisk leaves varied from 431.98 kcal/100 g in the origin of Azilal and 529.05 kcal/100 g in the origin of Modj, with a percentage variation of 91.12% (Table 2).

Table 2. Mean values of the primary biochemical parameters of *P. lentiscus* leaves in % DM. Results are presented as mean (n=3) ± SD. *– P< 0.05; **– P<0.01; ***– P<0.001

	Mrirte (MR)	Tighssaline I (Ti)	Lkbab (LK)	Azilal (AZ°)	Moudj (MD)	Aite yahia oussad (AO)	Isseksi (IS)	Bien el ouidane (BO)	Tiferdine (TF)	Immi oudar (IO)	Tighssaline II(TGH)	Mean	F	CV
DM	41.73±3.06	37.63±3.47	43.63±3.93	37.25±0.32	33.3±2.44	29.3±4.15	37±1.92	41.8±3.69	38.37±3.38	47.53±1.05	44.27±2.00	39.25	16.50	7.30
H	58.27±3.06	62.37±3.47	56.37±0.39	62.75±0.32	66.7±2.44	70.7±4.15	63±1.92	58.2±3.69	61.63±3.38	52.47±1.05	55.73±2.00	60.74	16.50	11.29
MM	3.88±0.86	4.12±0.55	9.77±0.28	7.62±0.15	6.12±1.17	5.78±0.58	5.37±0.34	3.00±0.83	4.65±1.00	3.13±0.56	3.45±0.3	5.17	10.54*	2.34
OM	37.85±2.96	33.52±0.45	33.87±2.09	29.63±2.38	27.18±0.72	23.52±4.74	31.63±1.03	38.8±3.95	33.72±1.64	44.4±2.42	40.82±1.05	34.08	19.28	5.46
OC	24±4.36	20.17±3.15	17.5±2.05	12.98±1.03	32.67±3.40	18.75±7.11	29.18±8.20	23.45±2.56	20.33±2.57	15.6±1.53	20.77±2.04	21.40	5.90	3.29
FBR	23.5±2.36	16.2±2.65	20.9±5.60	20.17±3.32	23.67±6.81	16.03±2.86	22.8±4.54	22.33±8.25	20.13±4.48	23.8±4.44	19.73±1.65	20.84	1.04*	4.40
TSC	2.70±0.79	18.33±1.29	21.77±3.15	9.33±2.30	16.10±2.43	10.07±0.75	28.00±5.50	20.83±1.29	5.33±0.42	12.53±8.00	18.17±1.19	14.83	15.70	1.89
TNC	6.54±4.35	6.07±4.44	5.6±3.73	3.74±2.49	5.14±3.42	5.6±3.73	6.07±4.04	5.6±3.73	5.14±3.42	5.6±3.73	4.67±3.11	5.43	0.08***	1.34
Prot	40.86±6.16	37.94±12.38	35.03±6.19	23.35±6.19	32.11±0.00	35.03±6.19	37.94±0.00	35.03±6.19	32.11±12.38	35.03±6.19	29.19±6.19	33.96	0.08***	1.34
Carb	8.98±8.24	17.51±13.59	20.84±13.76	43.77±2.09	14.92±2.62	26.84±7.96	12.63±0.92	22.24±1.24	27.34±10.69	27.39±12.20	32.42±8.53	23.17	0.02***	1.38
Energy	510.6±13.19	491.65±77.78	446.5±54.34	431.98±46.17	529.05±0.93	449.35±59.79	500.05±33.59	499.13±11.60	482±60.46	467.3±81.85	493.8±84.32	481.94	0.57**	1.10

The Pearson correlation coefficient

The Pearson correlation coefficient was estimated between the different biochemical parameters studied and results are shown in Table 3. Positive correlations were obtained between nitrogen content, proteins and energy value (r = 0.962***), dry matter content and organic matter content (r = 0.938 ***), proteins, azote and carbohydrate content (r = 0.609***) and fiber content, nitrogen content and protein content (r = 0.281**). Similarly, negative and significant correlations were also observed between dry matter content and mineral matter content (r = -0.217**), moisture and oil content (r = -0.314 **), oil content and carbohydrate content (r = -0.397 **) as well as organic matter content and mineral matter content (r = -0.542 **). Our results are in agreement with the results obtained by Maamri (2015) who found a negative correlation between organic matter and mineral matter.

Principal component analysis

The principal component analysis (PCA) was carried out on the means of the 11 biochemical parameters. The first two axes explained 74.40% of the total variation, which demonstrated a great structuring of the observed variability. Axis 1, which explains 43.01% of the total diversity, is defined positively by the content of protein, total sugars, nitrogenous matter, energy value, fiber, organic matter and dry matter. Axis 2 (31.3%) is defined positively by carbohydrate content and organic matter and negatively by oil content (Fig. 2).

Table 3. Pearson correlation coefficient of all the biochemical parameters analyzed

	DM	H	MM	OM	Oil content	FBR	TSC	TNC	Prot	Energy
H	-1,000*	1								
MM	-0,217	0,217	1							
OM	0,938***	-,938***	-,542**	1						
Oil content	-0,19	0,19	-0,157	-0,108*	1					
FBR	0,314**	-0,314	-0,066	0,294	0,071	1				
TSC	0,081	-0,081	0,047	0,053	0,275	0,112	1			
TNC	-0,021	0,021	0,011	-0,022	-0,13	0,281	0,016	1		
Prot	-0,021	0,021	0,011	-0,022	-0,13	0,281	0,016	1,000**	1	
Energy	-0,012	0,012	0,02	-0,018	-0,104	0,313	0,026	,962***	,962***	1
Carb	0,028	-0,028	0,105	-0,013	-,397**	0,056	-0,076	,609**	,609**	,745**

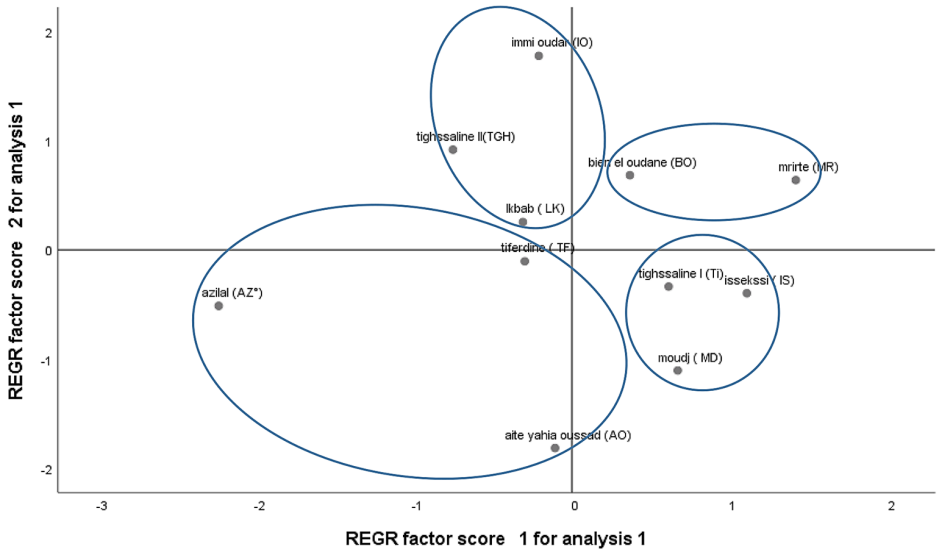

Fig. 2. Principal component analysis of the eleven Moroccan mastic populations according to the different biochemical parameters

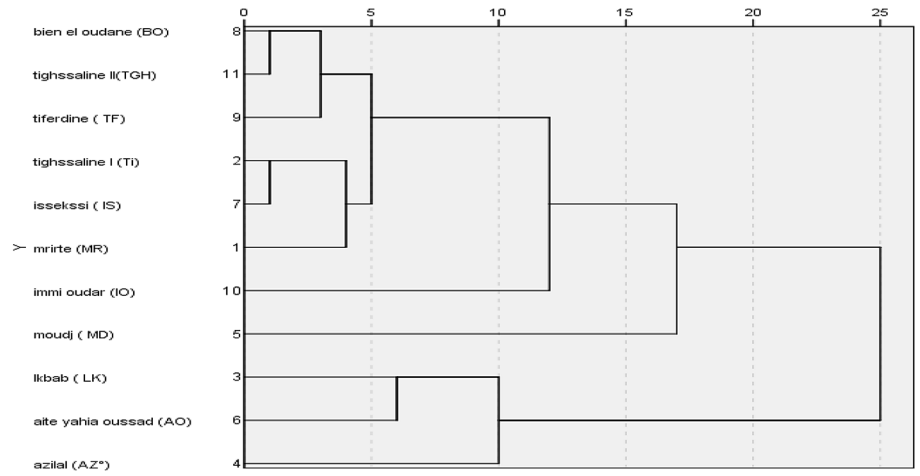

Fig. 3. Dendrogram of the biochemical analysis of the eleven Moroccan lentisk populations

Analysis of this figure shows the presence of four groups. The first group (G.1) composed of two populations (Bin el Ouidane and Mrirt), characterized by high values of dry matter, proteins and fibers. The second group (G.2) is made up of three populations (Tighssaline II, Immi Oudar and Lkbab). This group is distinguished by high values of ash, organic matter and total sugars. The third group (G.3) consisted of three populations (Azilal, Ait Yahia Oussad and Tifrdine) characterized by a low quantity of nitrogenous matter. Group (G.4) is composed of three populations (Tighssaline I, Issekssi and Modj) having a low organic matter content. This analysis clearly shows that the structuring of Moroccan mastic populations into four groups operates independently of their geographical origins. This result is in agreement with that obtained in the first part of this work using pomological and morphological parameters (Bouta et al. 2020).

Ascending hierarchical classification

The analysis of all the average biochemical data measured in the eleven populations made it possible to obtain a dendrogram according to Ward's method and the City-block genetic distance. This dendrogram led to the identification of two large groups (Figure 3). The first group is divided into two subgroups, one (I.1) is composed of the Azilal population, and the other (I.2) formed of Ikbab and Ait Yahia Oussad populations. The second group was also bifurcated into two subgroups. The first subgroup (II.1) is made up of the modj population and the second subgroup (II.2) consisted of Immi oudar, Mrirt, Issekssi, Tighssaline I, Tifrdine, Tighssaline II and Bin el oudane populations.

CONCLUSION

This first investigation confirms the results of conventional methods on a new plant material, very little studied and coming from an environment subjected to ecological constraints under the effect of climate change and desertification. It must, however, be reinforced by testing on a range of larger samples at different latitudes of the country, so this work must be continued by a more exhaustive study, including populations from other regions in Morocco, to better understand the range of variability of this species. This would make it possible to create a database on the fodder composition of the *Pistacia lentiscus* adapted to the Moroccan context and thus contribute to providing solutions to the many problems which oppose the improvement of the performance of the species and consequently the socioeconomic development of the habitat of the species. as well as the scientific valorization of the country's phytogenic heritage.

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