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Ampelometry to Test for Genetic Diversity in Tunisian *Vitis sylvestris*

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ABSTRACT

An ampelometric method, based on the biometric study of parameters related to the adult leaves of the vine, was applied on 23 ecotypes of *Vitis vinifera sylvestris* collected in the Northwest area and the Cap-Bon peninsula in Tunisia. 33 foliar parameters were tested for 10 leaves representative of each ecotype. Direct measurements were used to calculate different indices and all the generated data was subject to principal component analysis (PCA). Our results showed that LN3 (distance between the lower side veins) and the leaf area are the most dispersed variables, their respective variation coefficients being 45 and 60%. The correlations of the 33 parameters studied for a coefficient higher than 0.70 and 21 degrees of freedom often appeared linear or polynomial. The study led to the identification of foliar parameters which are the most useful to differentiate ecotypes and the development of a phenotypic classification key. This key is determined by the matrix generated by PCA. This matrix constitutes a data-base for later phenotypical studies in the tested areas.

Keywords: adult leaf, ampelometry, classification, multivariate analysis, wild grapevine

INTRODUCTION

Identification of the wild ancestors of a crop is a prerequisite for reconstruction of its evolution under domestication. In the case of grapevine, both comparative morphological studies and tests of genetic affinities have lead to the identification of its wild progenitor with certainty (Zohary and Spiegel Roy 1975). *Sylvestris* grapes are considered as the wild race (subspecies) of the cultivated fruit-crop, and are botanically named *Vitis vinifera* L. subsp. *sylvestris* (Zohary 1996). The Mediterranean region overlaps with the area of distribution of *V. vinifera* subsp. *sylvestris*, the species from which the cultivated grapevine was domesticated (Zohary and Hopf 1993). The boundary between wild-types and cultivated varieties is frequently blurred by the occurrence of wild-looking escapees and by products of spontaneous hybridization between tame and wild.

In Tunisia, wild grapevines are known as Aneb El Jali or Hormos (Ben Slimane Harbi 1999) and wild populations have been reported along the seashores, as well as in the northwest region of the country (Levadoux 1956).

In Tunisia, the indigenous vine has a remarkable polymorphism but the inter-type variation of vines remains always weak. The spontaneous vine *V. vinifera sylvestris* could be used to enrich diversity within the germplasm of Tunisian local vines. The number of spontaneous vines remains very limited and especially threatened by various environmental and anthropogenic factors (Ben Slimane Harbi 2001a). Spontaneous ecotypes were found in forest sites of Northern Tunisia in isolated form or in groupings. The true *sylvestris* are often confused with abandoned types of vines, sub-spontaneous and some sub-spontaneous hybrids. They are ecotypes which have a great morphological variability (Harbi Ben Slimane 1999).

Morphological studies on wild grapevines were reported (de Toda and Sancha 1999; Ocete *et al.* 2008; Cunha *et al.* 2009). Advanced studies within *V. vinifera* species were achieved to characterize them and assess their genetic structure (Arroyo-Garcia *et al.* 2002, 2006). 222 cultivated (*V. vinifera*) and 22 wild (*V. vinifera* ssp. *sylvestris*) grape accessions were analyzed for genetic diversity and differentiation at eight microsatellite loci (Aradhya *et al.* 2003). Results revealed that French cultivars showed close affinity to the wild progenitor, ssp. *sylvestris* from Tunisia. More recently, 418 wild grapevine samples, belonging to 78 populations were collected in their main Mediterranean distribution areas and evaluated using nuclear and plastid microsatellite DNA polymorphism. Results of this study evinced that the distribution of all detected haplotypes suggests the Caucasian region as the possible centre of origin of *V. vinifera* ssp. *sylvestris* (Imazio *et al.* 2009). In Tunisia, the genetic diversity of the *sylvestris* of the Northwest and Cap Bon was evaluated during an ampelographic study according to IPGRI descriptors (Harbi Ben Slimane 2001b) and by nuclear and chloroplastic microsatellites (Snoussi *et al.* 2004).

In this study, an ampelometric study based on an examination of the biometric characteristics of the adult leaf was intended to bring additional information for the morphological characterization of the studied ecotypes. A classification key is proposed.

MATERIALS AND METHODS

Plant material

23 ecotypes of *V. vinifera sylvestris* originating from the Northwest and from the Cap Bon Peninsula were used in this study. The locations of the sites are indicated in **Fig. 1** and **Table 1**.

The spontaneous ecotypes were often found along the continuous streams.

Methods

The studied samples correspond to adult leaves representative of each ecotype. These adult leaves were taken at the ripening stage either facing the inflorescence for the male ecotypes, or facing the bunches for hermaphrodite and female ecotypes. Leaves were collected from the median third of the branch of the year. Measurements were applied to a total of 230 adult leaves with 10 leaves per ecotype. The considered phyllometric parameters correspond to rough or combined measurements in the form of calculated indices (**Fig. 2**) (Harbi Ben Slimane 2001b).

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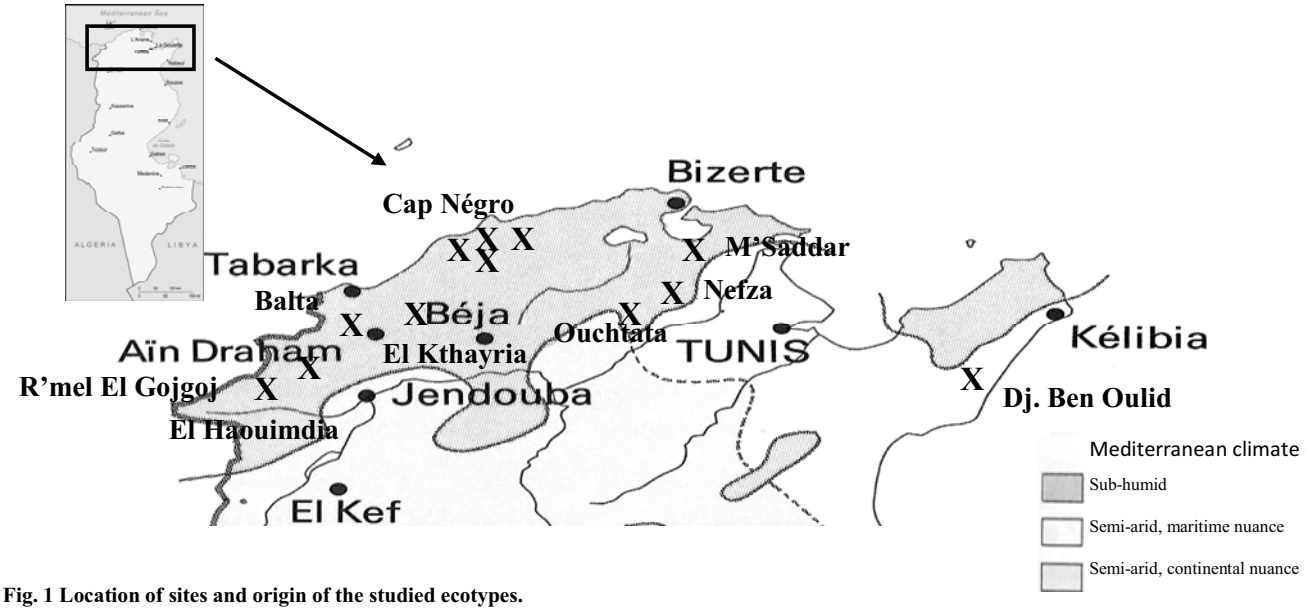

Fig. 1 Location of sites and origin of the studied ecotypes.

Table 1 Origin of the 23 studied ecotypes (Harbi *et al.* 2005).

Reference	Ecotype	Origin
A	Cap Négro I	Cap Négro
B	Cap Négro I-89	Cap Négro
C	Gpt Cap Négro I	Cap Négro
D	Cap Négro II-89	Cap Négro
E	Gpt Cap Négro II	Cap Négro
F	Cap Négro III	Cap Négro
G	Gpt Cap Négro III	Cap Négro
H	Cap Négro III bis	Cap Négro
I	Cap Négro 5/2000	Cap Négro
J	Cap Négro 6/2000	Cap Négro
K	Tabarka G1	Tabarka
L	Tabarka D2/2000	Tabarka
M	Tabarka D3/2000	Tabarka
N	GPT Ouchtata II	Ouchtata
O	Ouchtata 16	Ouchtata
P	Ouchtata 17/2000	Ouchtata
Q	V.S.Balta	Balta
R	El Kthayria I	El Kthayria
S	R'Mel El Gojgoj	Aïn Draham
T	M'Saddar II	M'Saddar
U	Nefza 1	Nefza
V	El Haouimdia 2/2000	El Haouimdia
W	Ben Oulid	Djebel Ben Oulid

Statistical analyses

The results of various measurements were the subject of a multivariate statistical synthesis by the method of principal components analysis. SAS software (1998) was used.

RESULTS AND DISCUSSION

The adult leaf is considered as an important organ for varietal and even clonal recognition in vine (Cid-Alvarez *et al.* 1994). A strong morphological variability was shown among the studied spontaneous ecotypes (**Fig. 3**).

Elementary statistics of the results

Variation coefficients of certain parameters such as the leaf surface area and the distance between the ends of the lower side veins are more dispersed than other parameters (60 and 45% respectively, **Table 2**).These variations might be due to the environmental characteristics of the origin site for each ecotype and/or to the particular genetic characteristics of the ecotypes.

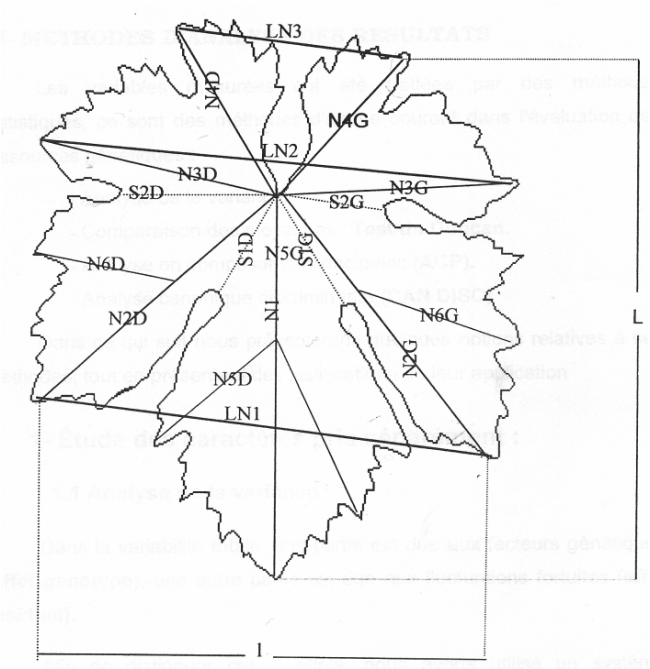

Fig. 2 Diagram of measurements carried out. 20 rough measurements: N1= Length of the median principal vein, N2G and. N3G = Length of the side principal veins on the left side. N2D and N3D = Length of the side principal veins on the right side, N4G.N6G and N5G = Length of the secondary veins on the left side, N4D.N6D and N5D = Length of the secondary veins on the right side, S1G and S1D = Depth of the higher side sinus left and right, S2G and S2D = Depth of the lower side sinus left and right, LN1, LN2, LN3 = Distance between the higher side (1), medians (2) and lower (3) veins, L = Length of the leaf, l = width of the leaf. **13 calculated phyllo-metric indices:** N1/N2G+N2D = Coefficient of lengthening of the principal vein, N2G+N2D/2N1 = standard higher vein, SF= N2G+N2D with the square = Leaf surface area, N3G+N3D/2N1 = median standard vein, N4G+N4D/2N1 = lower standard vein, N4G+N4D/N3G+N3D = Relationship between N4 and N3 on both right and left sides, N4G+N4D/N2G+N2D = Relationship between N4 and N2 on both right and left sides, N5G+N5D/2N1 = tertiary higher standard vein, N6G+N6D/2N1 = tertiary lower standard vein, S1G+S1D/N2G+N2D = Coefficient of cutting out of the higher side sine, S2G+S2D/N3G+N3D = Coefficient of cutting out of the lower side sine, LN1/N2G+N2D = Coefficient of extension of the higher lobe, LN3/N4G+N4D N2D = Coefficient of extension of the lower lobe.

The parameters with high variation coefficients are of great importance for the constitution of the axes of principal

Table 2 Elementary statistics relating to the studied parameters.

Ref variable	Variable	Average	Standard deviation	Cœfficient of variation	Ref variable	Variable	Average	Standard deviation	Cœfficient of variation
Z1	N1	8.71	2.67	30.61	Z21	N1/N2G+N2D	0.59	0.03	4.27
Z2	N2G	7.39	2.42	32.76	Z22	N2G+N2D/N1	0.85	0.04	4.20
Z3	N2D	7.47	2.43	32.59	Z23	SF= $(N2G+N2D)^2$	244.33	146.38	59.91
Z4	N3G	5.04	1.77	35.04	Z24	N3G+N3D/N1	0.58	0.04	7.55
Z5	N3D	5.13	1.78	34.80	Z25	N4G+N4D/N1	0.33	0.05	15.45
Z6	N4G	2.94	1.27	43.18	Z26	N4G+N4D/N3G+N3D	0.56	0.06	9.68
Z7	N4D	2.95	1.25	42.32	Z27	N4G+N4D/N2G+N2D	0.38	0.05	12.80
Z8	N5G	5.02	1.71	34.05	Z28	N5G+N5D/N1	0.57	0.04	6.77
Z9	N5D	5.05	1.69	33.38	Z29	N6G+N6D/N1	0.48	0.04	8.08
Z10	N6G	4.23	1.51	35.74	Z30	S1G+S1D/N2G+N2D	0.59	0.14	23.11
Z11	N6D	4.26	1.52	35.80	Z31	S2G+S2D/N3G+N3D	0.79	0.13	15.75
Z12	S1G	4.23	1.33	31.39	Z32	LN1/N2G+N2D	0.71	0.06	7.94
Z13	S1D	4.20	1.36	32.29	Z33	LN3/N4G+N4D	0.83	0.23	27.13
Z14	S2G	3.88	1.21	31.11					
Z15	S2D	3.92	1.19	30.28					
Z16	LN1	10.45	3.29	31.45					
Z17	LN2	9.83	3.42	34.82					
Z18	LN3	4.70	2.13	45.36					
Z19	L	8.68	2.67	30.80					
Z20	I	10.49	3.31	31.59					

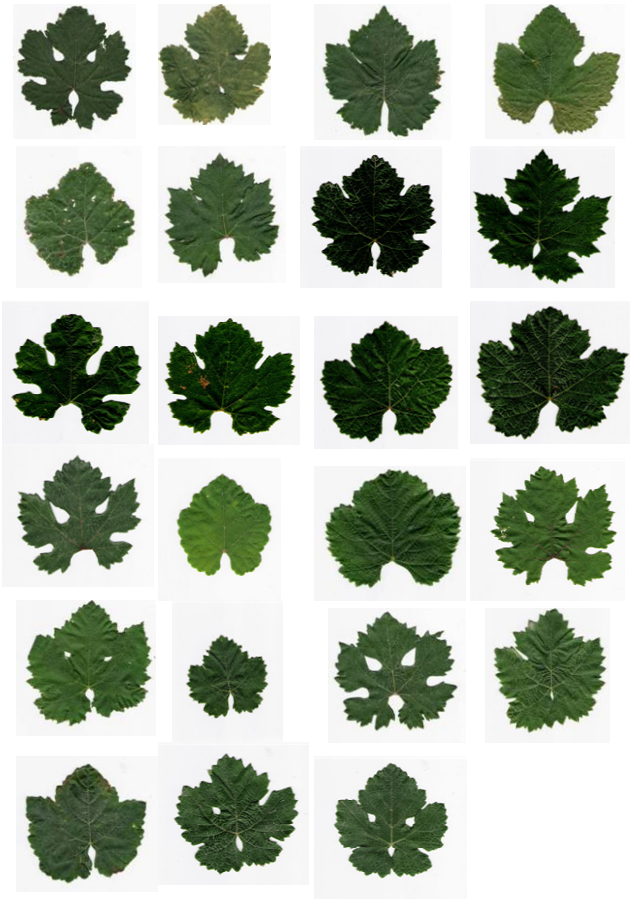

Fig. 3 Adult leaves phenotypical variability of the 23 ecotypes of *Vitis sylvestris* studied.

component analysis (PCA) and the identification of parameters which will the most suitable to differentiate the ecotypes.

Study of the correlations between the measured variables

The coefficients of correlation between the variables taken in pairs, compared with those provided by the statistical tables (Dagnelie 1986) with a degree of freedom (ddl= 21), are in most cases, significant at a threshold of probability of 5% (data not shown).

As representatives of the axes one can retain for axis 1 variables Z29 and Z5 according to the function:

Z29 = 0.0174 Z5 + 0.3895 (ddl of 21 and Coefficient of determination $R^2 = 0.54$).

Axis 2 would be best represented by the variables Z32 and Z18, according to the function:

Z32 = -0.0194 Z18 + 0.8 (ddl 21 and coefficient of determination $R^2 = 0.54$).

The adjustment by the trend curve for Z29 (standard lower tertiary vein) according to Z5 (principal vein side lower) is shown in **Fig. 4A**.

The adjustment by the trend curve for Z32 (coefficient of extension of the higher lobe) according to Z18 (distance between the lower side veins) is shown in **Fig. 4B**.

Principal component analysis

The PCA method is based on the research of correlated variables and the selection of a more reduced number of independent synthetic or not correlated variables. These selected variables which correspond to the axes of the PCA are linear combinations of the measurements described by their eigenvalue and their percentage of inertia.

This analysis led to the eigenvalues of the axes reported in **Table 3**.

It appears that the first three axes of the PCA explain 90.7% of inertia. Axes 1 and 2 only explain 83.6% of this inertia and 74.4% by the axis 1. Therefore, results can be explained by the two first axes.

The representation of the 33 variables according to the two first axes (**Fig. 5**) shows that:

Axis 1 is best correlated with the variables relating to foliar dimension such that: (Z4 = N3G), (Z5 = N3D), Â (Z10 = N6G), (Z2 = N2G), (Z11 = N6D), (Z8 = N5G), (Z7 = N4D), (Z3 = N2D), (Z6 = N4G), (Z17 = LN2), (Z9 = N5D), (Z23 = SF), (Z1 = N1), (Z19 = L), (Z20 = I), (Z16 = LN1), this axis would be a dimension axis.

Axis 2 is correlated best with the variables relating to the shape of the limb such as Z30 = (S1G+S1D/N2G+N2D), Z31 = (S2G+S2D/N3G+N3D), Z12 = S1G, Z13 = S1D, Z28

Table 3 Eigenvalue of the axes.

Axis	1	2	3
Eigenvalue	24.5469	3.0461	2.3446
% of inertia	74.4	9.2	7.1
% of cumulated inertia	74.4	83.6	90.7

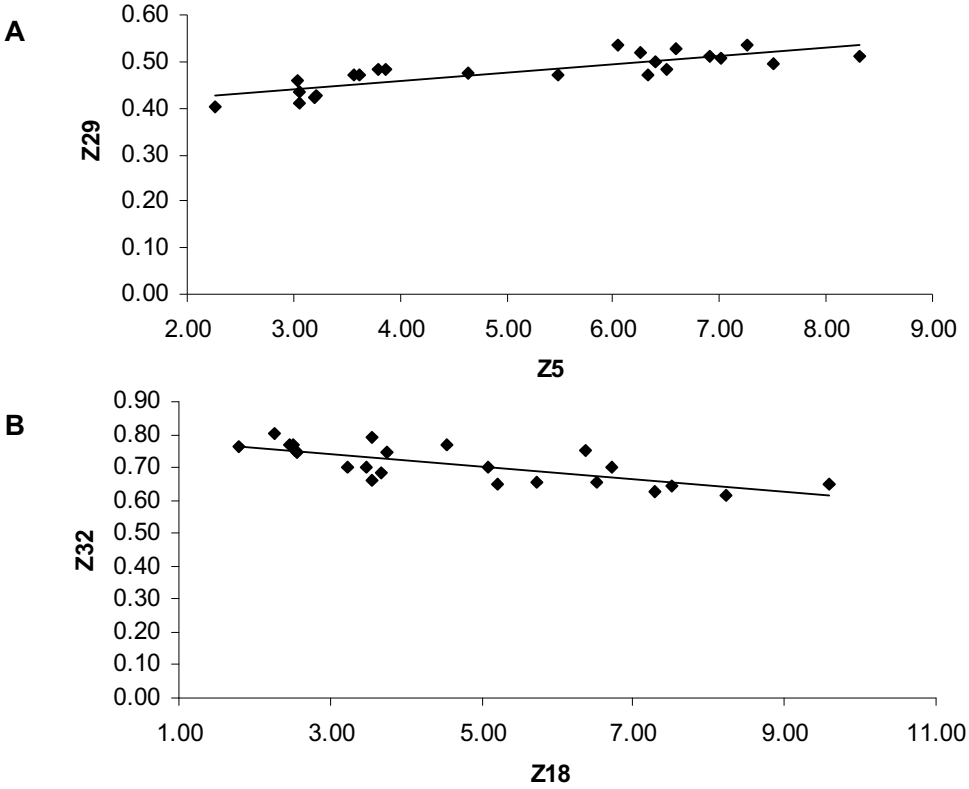

Fig. 4 (**A**) Graphic representation of the functions Z29 = 0.0174 Z5 + 0.3895 (ddl = 21 and R^2 = 0.67) and (**B**) Z32 = -0.0194 Z18 + 0.8 (ddl = 21 and R^2 = 0.67).

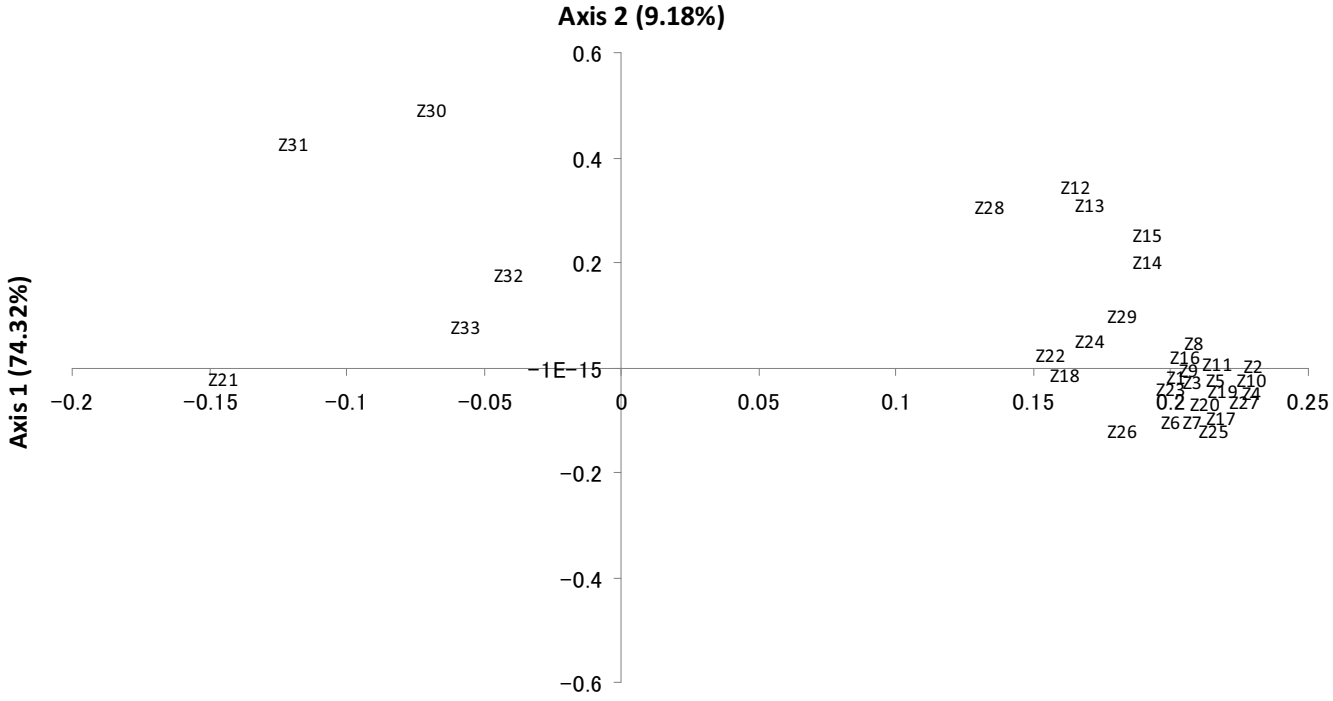

Fig. 5 Dispersion of the 33 variables according to the two first principal axes of PCA. Axis 1 (horizontal) and Axis 2 (vertical).

= (N5G+N5D/N1), this axis would be a form axis.

The dispersion of the ecotypes according to axes 1 and 2 (**Fig. 6**) emphasized three groups:

The first group (G1) is characterized by: (Z4 = N3G), (Z5 = N3D), (Z10 = N6G), (Z2 = N2G), (Z11 = N6D), (Z8 = N5G), (Z7 = N4D), (Z3 = N2D), (Z6 = N4G), (Z17 = LN2), (Z9 = N5D), (Z23 = SF), (Z1 = N1), (Z19 = L), (Z20 = I), (Z16 = LN1) with high values. They are whole and large-sized leaves.

The second group (G2) is characterized by the same parameters which characterize the G1 group but with low values. They are whole and small-size leaves.

The third group (G3) comprises two ecotypes characterrized by Z30= (S1G+S1D/N2G+N2D), Z31 = (S2G+S2D/N3G+N3D), Z12 = S1G, Z13 = S1D, Z28 = (N5G+N5D/N1) presenting high values which provide information about cut leaves of large or small dimensions.

The 33 tested parameters allowed a classification of 14 ecotypes according to the two first axes of the PCA in groups G1, G2 and G3 (**Table 4**); the 9 other ecotypes proved to be dispersed and could not be classified with certainty in any of these groups. The introduction of new parameters

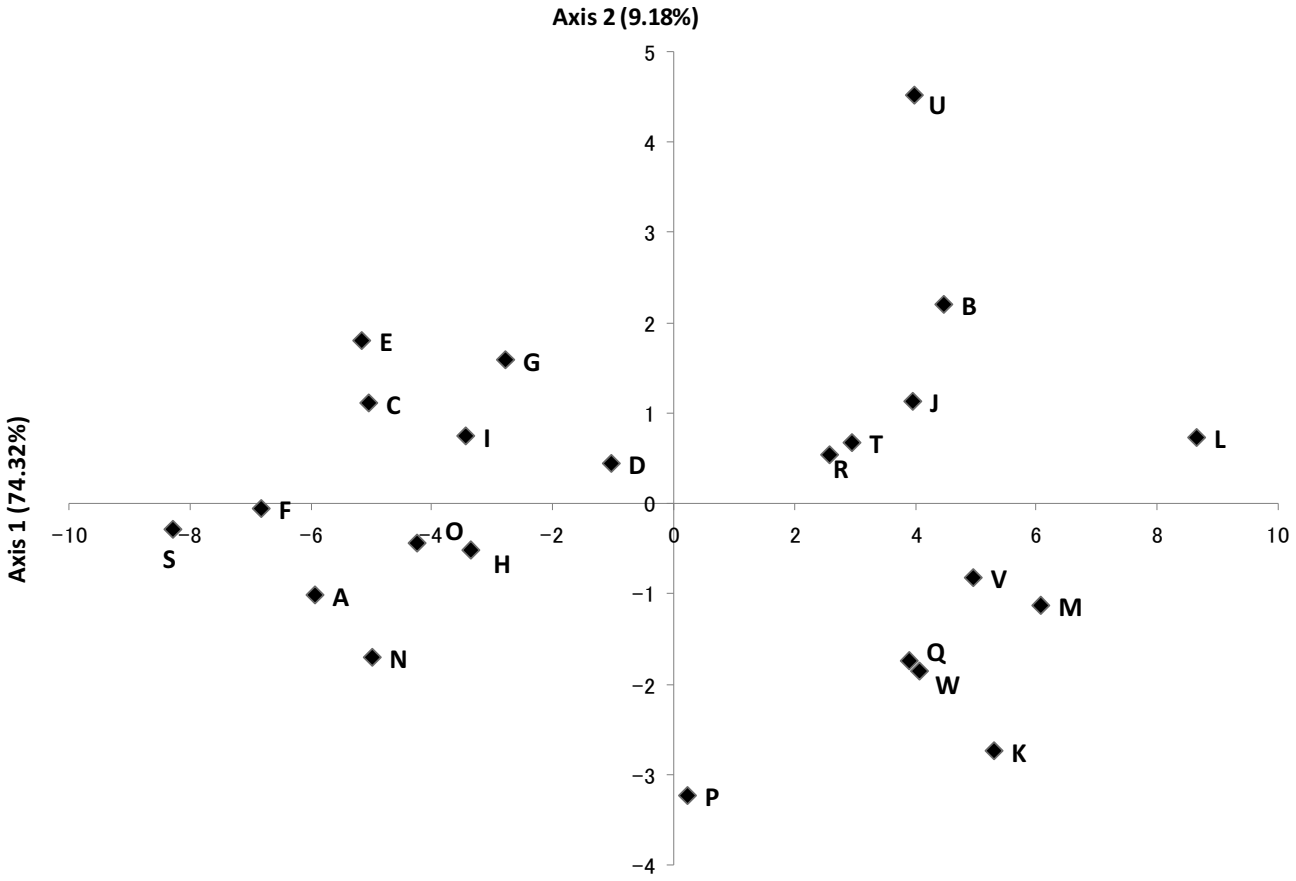

Fig. 6 Representation of the ecotypes according to the two first principal axes of the PCA. Axis 1 (horizontal) and Axis 2 (vertical).

Table 4 Distribution of the ecotypes in the three groups defined by the two first axes of the PCA.

G1		G2		G3	
Ref. ecotype	**Ecotype**	**Ref. ecotype**	**Ecotype**	**Ref. ecotype**	**Ecotype**
S	R'Mel El Gojgoj	L	Tabarka d2/2000	P	Ouchtata 17/2000
A	Cap Négro I	M	Tabarka D3	U	Nefza 1
C	Gpt Cap Négro I	V	El Haouimdia 2/2000		
F	Cap Négro III	B	Cap Négro I-89		
E	Gpt Cap Négro II	T	M'Saddar II		
O	Ouchtata 16				
N	Gpt Ouchtata II				

derived from the foliar measurements could allow a better characterization for a classification of the specific ecotypes.

CONCLUSIONS

The method of classification of the collected local ecotypes of *V. vinifera* ssp. *sylvestris* by PCA reinforced their characterization and allowed the recognition of ecotypes of spontaneous vines originating in Tunisia.

The percentages of inertia (variance) cumulated from two axes, reached 83.6%. They are largely sufficient for the interpretation of this PCA STATE. Axis 1 is mainly correlated with the variables related to foliar dimension, while axis 2 is correlated with the variables related to the limb shape. The results of the method of the PCA STATE provided elementary statistics characterized by the averages and the coefficients of variation for each studied parameter. Leaf surface area and LN3 (distance between the lower side veins) are more variable than other parameters among the studied ecotypes. The study of the correlations between the 33 measured and calculated parameters revealed coefficients of correlation significant to the threshold of probability of 5%; these connections are often linear.

The study led to the determination of the best foliar parameters which differentiate between the ecotypes and the identification of a phenotypical key of classification. This key is constituted by the matrix analyzed by the PCA STATE. Indeed, we consider that this matrix constitutes a data base that can be used for subsequent phenotypical studies. When measurements of the same type (using these 33 studied parameters) are carried out on a new ecotype, it will be introduced as an additional individual. In the case of a new measurement on the leaves or of a new calculated parameter for 23 ecotypes, it will be also introduced as an additional parameter. However, this key has its limits and can only be tested in the studied areas.

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