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Research Article

Comparative Study of Three Varieties of *Cannabis sativa* L. Cultivate in Different Region of Morocco

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ABSTRACT

In this study, an experiment of hemp crops was conducted between in four different regions of the country, and concerned three varieties Santhica 27, Epsilon 68 and Futura 75. The tests were conducted in areas with geographical, climatic and soil characteristics different than European cultures, that's why it was necessary to verify the behavior of these plants by controlling their Δ-9-THC content. Sampling, drying and Δ-9-THC evaluation in these crops were performed according to procedure B of European Regulation, and using a simplified method based on the determination of the ratio α = Δ-9-THC/CBD. The best dry matter yields, plant height and density are obtained with the variety Futura 75 in the four sites, followed by Epsilon 68 and Santhica 27. The average contents of these crops in Δ-9-THC were ranged from 0.013% to 0.027% for the variety Epsilon 68, between 0.023% and 0.035% for the variety Futura 75. Analysis of this latter variety by GC / MS SIM mode and LC/MS/MS allowed the identification of traces of Δ-9-THC. In the other hand, the ratio α has been evaluated to 0.042 and 0.047 respectively for Epsilon 68 and Futura 75 varieties which is similar to those usually obtained for hemp fiber crops and stays much lower than 0.2. The Principal Component Analysis confirms the existence of possible correlations for each variety separately. Overall, the results reveal the existence of correlations between different parameters including the one between the dry matter yield, plant height and density.

Keywords: Industrial hemp, crops, Δ-9-tetrahydrocannabinol, Morocco.

INTRODUCTION

Cannabis is the most frequently used drug of abuse not only in Morocco but also in the whole world. Its use is increasing drastically every year **[1],** the plant has two main phenotypes:

Cannabis sativa L. subsp. indica or Indian hemp characterized mainly by the presence of its active ingredient the delta-9-tetrahydrocannabinol (THC) that has psychoactive properties.

Cannabis sativa L. subsp. sativa or industrial hemp, low in THC and very appreciated for its seeds, fibers and shives[2]. Planting industrial hemp can occupy yearly, an area of up to 18,000 ha/year[3].

In general, the cultivation of certain varieties of cannabis (rate Δ-9-THC less than 0.2%) is allowed primarily for controlled industrial uses (textile, plastics, building materials, cosmetics, food, bioenergy, Cigarette paper, Medical treatment etc.)[4, 5].

The cultivation of hemp fiber in the world is diversely authorized in accordance with the country. The research is oriented towards the selection of varieties more adapted and productive, which are able to meet the user requirements, e.g. the production of poor varieties of cannabinoids, etc.

The hemp is a polyvalent culture, capable of being cultivated in various climatic conditions. However, climatic conditions introduce effects very marked on the morphology of plants. The characteristics of the individuals of plants such as the length and the diameter of the stem depend considerably on meteorological conditions as well as on the density of the plant[6]**.** Indeed, the weak density of plants can draw away a reduction of output and a reduction of the quality of biomasses. The output of germination is directly linked to its morphological characteristics, so that the plant of hemp requires special care during its season of reproduction. The production of hemp of fiber depends widely on environmental conditions and the adopted plan of culture. The production of hemp was announced about 20 tones/hectare in the countries with temperate climatic

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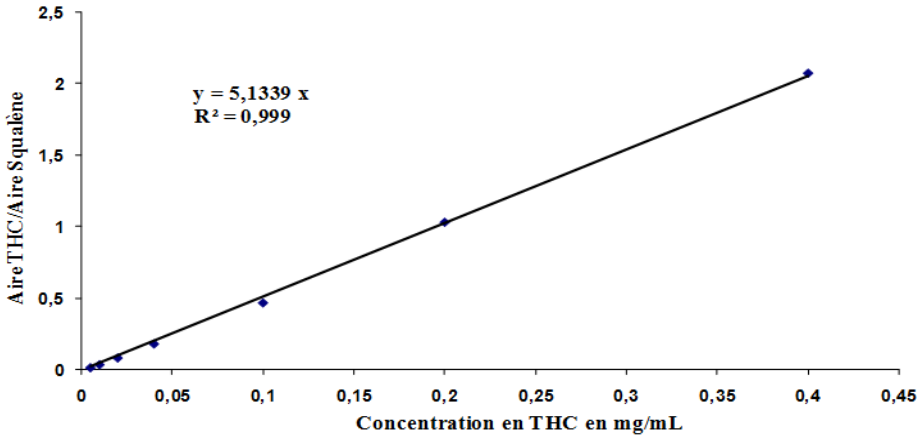

Figure 1: Calibration curve of Δ-9-THC.

Culture of hemp in Beni Mellal

Culture of industrial hemp in Sidi Allal

Figure 2: Experimental hemp plots in Beni Mellal and Sidi Allal

Figure 3: Plot of the Futura75 variety, cultivated in Afourer.

Table 1: Some agronomic characteristics of varieties of industrial hemp.

Varieties	Flowering	Registration year	Breeder	THC (%)	PMG	Fiber production (%)
Santhica 27	Average	2002	NFHP	<0.001	16.8	35.81
Epsilon 68	tardy	1996	NFHP	0.04	19.3	30.45
Futura 75	tardy	1998	NFHP	0.06	18.9	30.77

NFHP: National Federation of Hemp Producers; PMG: weight of thousand seeds; THC: Δ-9-trans-tetrahydrocannabinol.

Table 2: Characteristics of the region of experimentation.

Regions	Altitudes	Characteristics
Agadir region, the southwest Atlantic coast	1250 m	Sandy soil, average rainfall 250 mm / year
Sefrou region, the Middle Atlas	850 m	Stony soil, average rainfall of 640 mm / year
Allal Tazi region, in the northwest	40 m	Gravelly / sandy soil, average rainfall: 460 mm / year
Beni-Mellal region in the country center, door to the Middle Atlas	2000 m	Clayey soil, average rainfall of 600 to 800 mm / year

Table 3: Means and standard deviation for the obtained values of the physico-chemical parameters with the p-value of the two-way ANOVA carried out to evaluate the effect of the sampling site and the depth of each parameter.

	Density	Dry matter yield	Plant height	Life time	Contents in Δ-9-THC	α = Δ-9-THC/CBD ratio
Sites						
Agadir	53.33±5.608	7.46±1.3	1.25±0.11	61.33±0.66	0.020±0.01	0.021±0.021
Allal Tazi	42.66±10.26	10.87±1.43	1.77±0.06	64.66±2.02	0.017±0.0085	0.035±0.01
Beni mellal	63±7.93	20.72±2.37	1.97±0.18	64±0	0.015±0.0075	0.036±0.018
Sefrou	38±2.02	3.13±0.33	1.45±0.04	68±0	0.012±0.0066	0.034±0.017
p(site)	0.027	0.0001	0.0024	0.015	0.2	0.49
Varieties						
Epsilon 68	46.25±7.87	11.17±3.58	1.57±0.2	64.75±1.25	0.022±0.0031	0.056±0.0026
Futura 75	61±6.89	13.32±4.04	1.79±0.18	65.5±1.5	0.026±0.026	0.039±0.013
Santhica 27	40.75±3.37	8.64±2.68	1.47±0.11	63.25±1.79	0	0
p(variety)	0.022	0.012	0.0395	0.23	0.0003	0

*** : Indicates significant results ($p<0.001$).

Table 4: Δ-9-THC content of Santhica 27, Epsilon 68 and Futura 75 varieties.

Variety	% of of Δ-9-THC Sefrou	Beni-Mellal	Allal Tazi	Agadir
Santhica 27	ND	ND	ND	ND
Epsilon 68	0.013	0.024	0.026	0027
Futura 75	0.023	0.021	0.025	0.035

conditions such as Italy, Netherlands and United Kingdom[7,8]. Regarding the production of industrial hemp, France and China are the largest producers in Europe and worldwide respectively.

In 2011, the main countries listed as a source country of hashish were Morocco, and Afghanistan and, to a lesser extent, India, Lebanon and Pakistan (Pakistan, it is estimated that the resin seized on its territory originated in Afghanistan) [1]. In Morocco, illicit cultivation of cannabis-type drugs dates back to the seventh century, still little is known about the hemp fiber. In 1961, the UN performed the very first tests before the adoption of the Single Convention on Narcotic Drugs. In the stated context, the main interest of this study was to test the three varieties of industrial hemp (Epsilon 68, Futura 75 and Santhica 27). For this purpose, tests were conducted in the experimental fields of the National Agronomical Research Institute (NARI) located in four regions of the country i.e. Agadir, Sefrou, Allal Tazi and Beni Mellal. Variance analyses were also tested to confirm the results

MATERIAL AND METHODS

Plant material

Three varieties of industrial hemp, monoecious type industrial hemp were chosen namely, Santhica 27, Futura 75 and Epsilon 68 listed in the French and European catalogs were received from the Company "The National Federation of Hemp Producers" (NFHP), France. Agronomic and industrial characteristics of the industrial hemp are presented in Table 1. The major criterion adopted in breeding is to achieve a very low THC contents (see the trace). This criterion cancels the drug effect of the plant.

Experimental plots

The three varieties were grown on plots of unit area of 2500 m^2. Normal seed dates spread from 20 April to late May 2010. Depending on the variety, the seed rate was determined on the one hand, as the thousand seed weight and on the other hand, depending on their germination capacity and for an average density of 300 plants/m^2. The varieties were grown in a randomized complete block device in three repetitions. The areas were chosen based on their altitudes; the basic plot was of 300 m^2, which makes nine plots having climatic and soil characteristics (Table 2).

Sampling

Control of these cultures was carried out by two deferent methods; first in accordance with procedure B of European Regulation Number EC 1177/2000 which recommends a sampling of upper third of 200 plants/plot for the evaluation their contents of Δ-9-THC by external calibration, and then using the method proposed by

Table 5: Summary table of the parameters for testing the Santhica 27 variety.

	THC contents	α = Δ-9-THC/CBD	Density	Plant height	RDT in dry matter	Cycle duration
Beni-Mellal	0	0	48	1.63	16.33	64
Agadir	0	0	45	1.14	5.45	60
Allal Tazi	0	0	35	1.66	8.33	61
Sefrou	0	0	35	1.45	4.47	68

Table 6: Component variability percentage related to Santhica 27 variety.

Component No.	*Appropriate value*	*Variance percentage*	*Accumulated percentage*
1	1.76031	44.008	44.008
2	1.53952	38.488	82.496
3	0.700171	17.504	100.000
4	2.66516E-16	0.000	100.000

Fournier et al[3]. The latter consists of determining the ratio α "Δ-9-THC / CBD" from a sampling of merely 200 sheets (which should not exceed 0.2). This procedure greatly minimizes the control by reducing the amount of plant material, and the chromatographic determination of the ratio 'α' from peak areas without recourse to the calibration range, which is less restrictive.

In both cases, the sampling is performed ten days after the end of flowering, during the day, following a systematic pattern to ensure a representative collection excluding the edges.

Sample processing is carried out by drying, grinding and sieving to obtain semi-fine powders (1 mm). The extraction of cannabinoids is carried out by maceration of 100 mg of powder in 5 ml of extraction solution (35 mg of the internal standard squalene (Sigma ref. S3626) in 100 ml of analytical hexane.

Chromatographic analysis

The Chromatographic analyses were conducted using a gas chromatograph coupled with a mass spectrometer (GC-FID) type Agilent 6890N equipped with a nonpolar column (HP-5, 5% phenyl-methyl-siloxane 30 m × 0.32 mm × 0.22 microns). The injections were performed in split mode 25/L, mass spectroscopy (GC-MS) under the following temperature conditions:

Oven temperature: 200°C - Ramp 10°C/min to 280°C isotherm 10 min , injector temperature: 280 °C detector temperature: 300 °C.

The calibration curve (Figure 1) is established using 6 concentrations 0.005, 0.01, 0.02, 0.04, 0.1, 0.2 and 0.4 mg/ml of Δ-9-THC standard (LGC Promochem ref. T-005) in the extraction of solution. Three tests per sample taken are considered and the results correspond to the average of a double analysis of these three trials taken.

Statistical Analysis

Two-factor ANOVA

The results were statistically evaluated by two way/two-factor analysis of variance (ANOVA) by considering the sampling sites and depths as a source of variation. This analysis is concerned with the investigation of the simultaneous effects of two nominal variables, say A and B, called factors. These factors can take different values known as levels. Each combination of a factor level of A and a factor level of B is a treatment**.**

Principal component analysis (PCA)

Principal components analysis (PCA) is one of the common techniques to summarize patterns among the variables in multivariate datasets. PCA is an efficient statistical tool for identifying patterns in variables, and demonstrating the data in such a way to highlight their analogies and differences. The principal merit of PCA is that, once the mentioned patterns have been found, data can be compressed reducing the number of dimensions without much loss of information.

RESULTS AND DISCUSSION

Agricultural aspects

Compared to European cultures, the plant growth cycle was faster in the four areas tested since end stage of flowering was achieved in 2 months in the areas of Agadir, Beni Mellal and Allal Tazi (Figures 2 and 3) and after 2.5 months for Sefrou region. This behavior can be explained on the basis of photoperiod characterization of these regions, particularly conducive to the development of the plant that seems not to be much affected by the intrinsic properties of these regions (altitude, soil type, rainfall, etc.).

Statistical study by two-factor variance analysis

Mean values with standard errors of the parameters assigned to the varieties grown in the four study sites are summarized in Table 3. This table also shows the p-values for the variance analysis tests conducted on two-factors of each parameter.

Study of the density effect

Average densities obtained in the different sites are between 38 and 63 plants / m^2 depending on the varieties and sites. Moreover, the variety Futura 75 is clearly

Table 7: Summary table of the parameters for testing the Epsilon 68 variety

	THC contents	α = Δ-9-THC/CBD	Density	Plant height	RDT in dry matter	Cycle duration
Beni-Mellal	0.024	0.058	57	2.03	21.33	64
Agadir	0.027	0.063	51	1.13	7.02	62
Allal Tazi	0.025	0.053	30	1.77	11	65
Sefrou	0.013	0.051	38	1.38	5.34	68

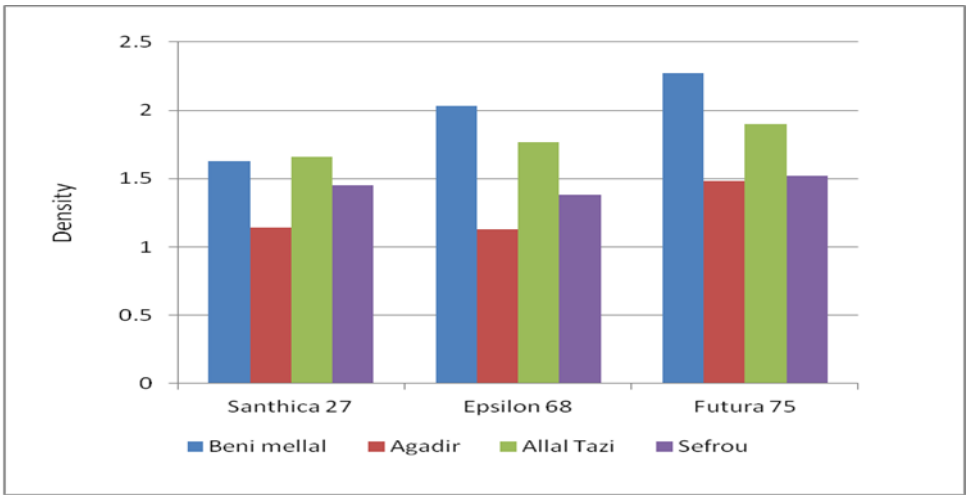

Figure 4: Densities of the varieties depending on the experimental areas.

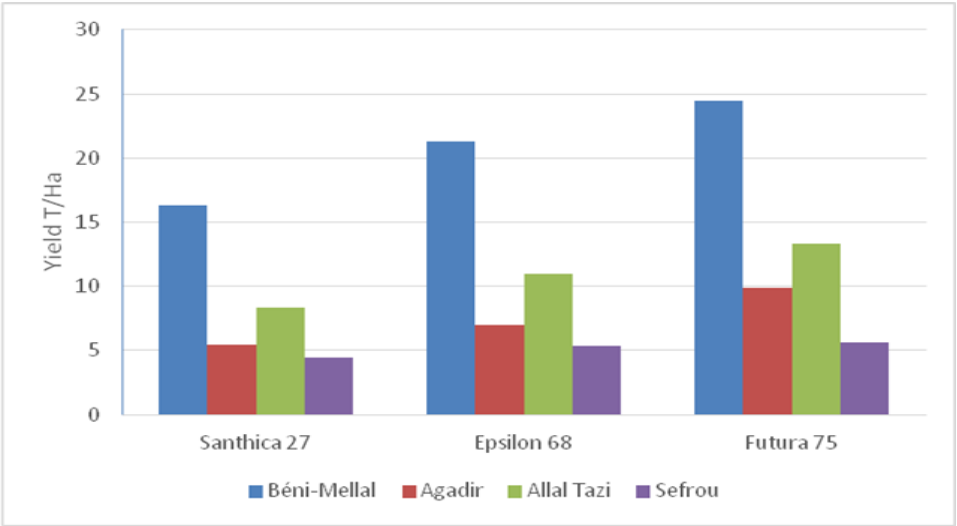

Figure 5: Dry matter yields of varieties depending on the experimental areas.

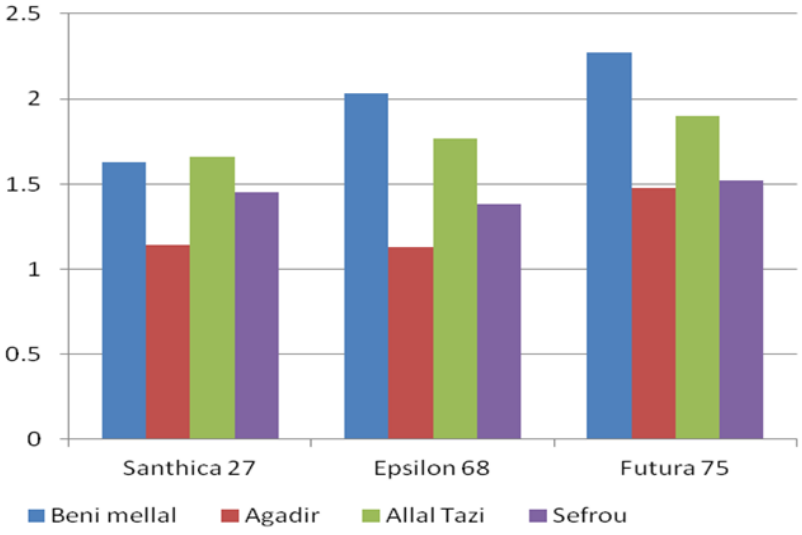

Figure 6: Average heights of the plants.

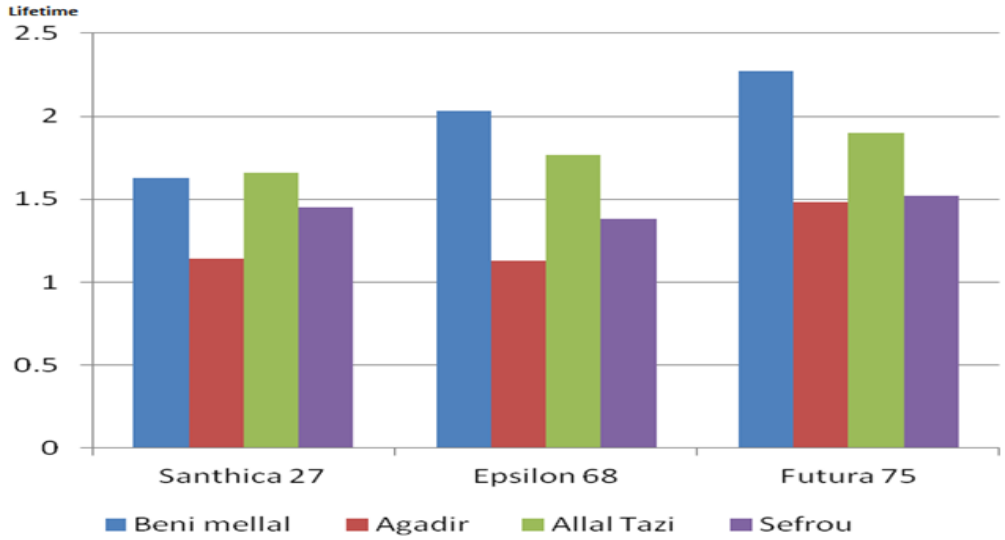

Figure 7: Life time duration of the three varieties in the different study sites.

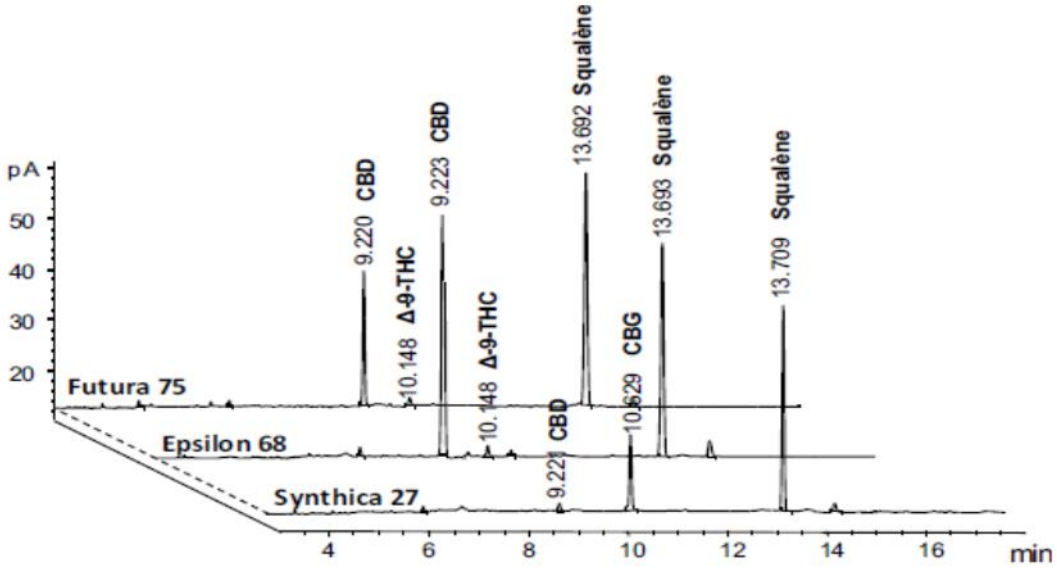

Figure 8: Chromatographic profile of Futura 75, Santhica 27 and Synthioca 27 varieties.

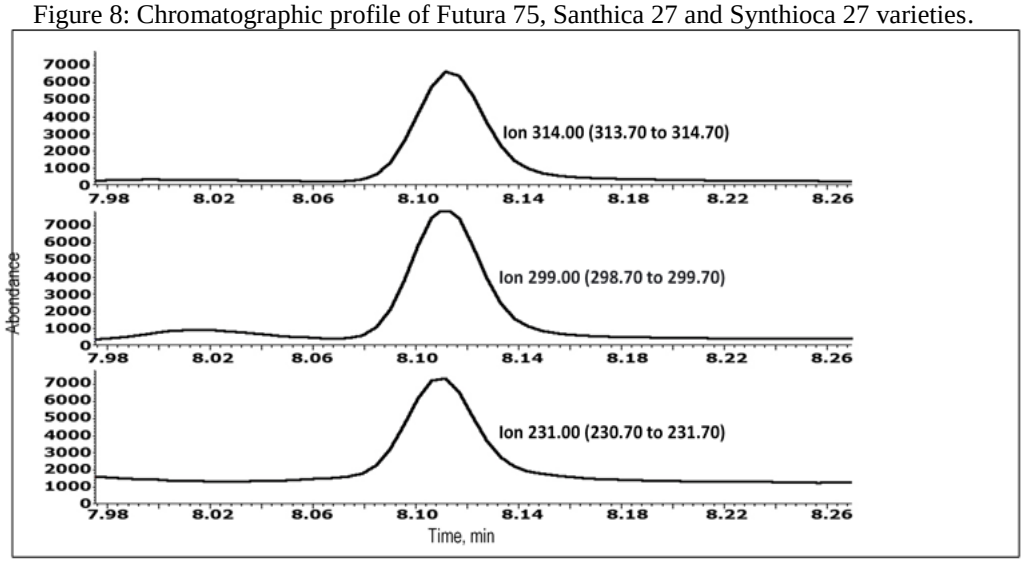

Figure 9: Demonstration of traces of THC in the variety Santhica 27 by GC/MS analysis in SIM mode.

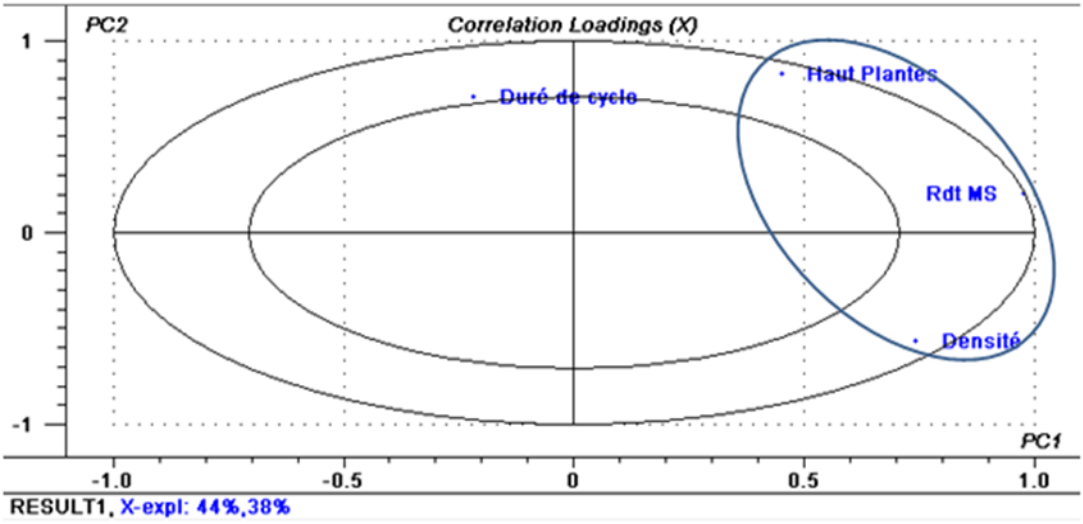

Figure 10: Graph of the correlations among the parameters.
Rdt MS = Yield in the dry matter; Haut Plantes = Plant height.

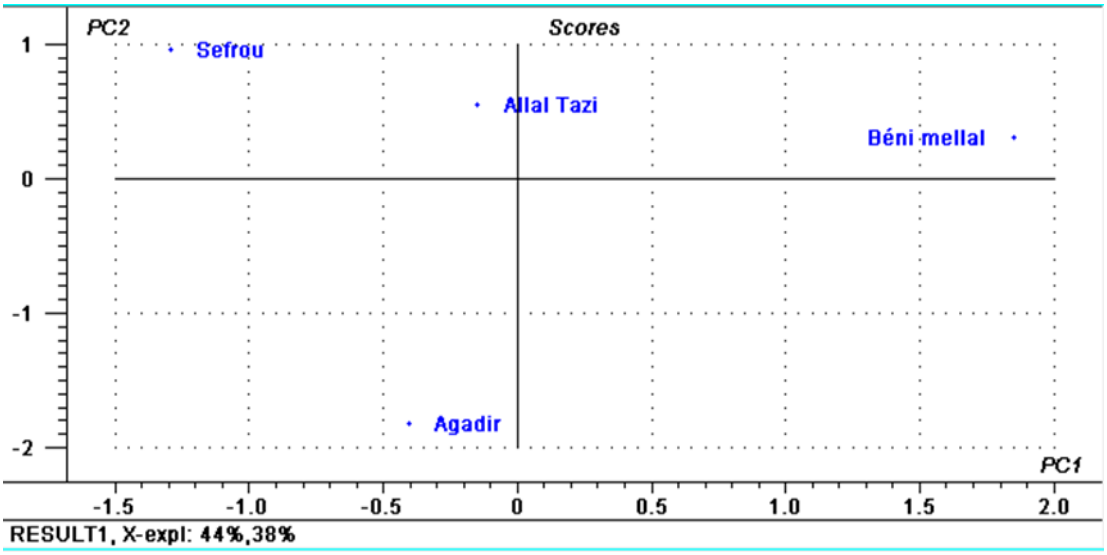

Figure 11: Graph of the correlations among the parameters.

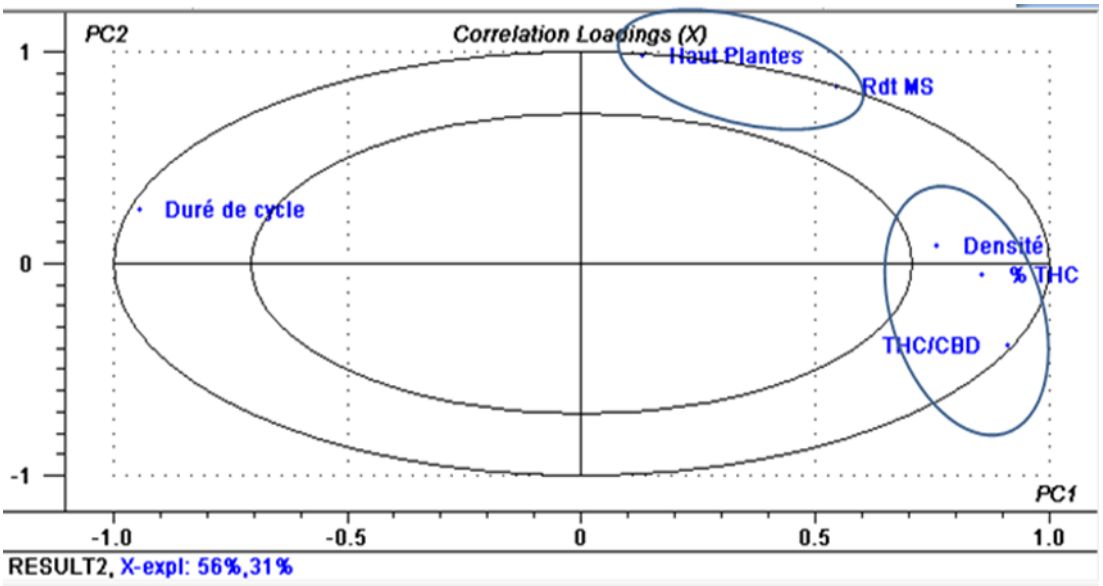

Figure 12: Correlation between the parameters for the variety Epsilon 68.

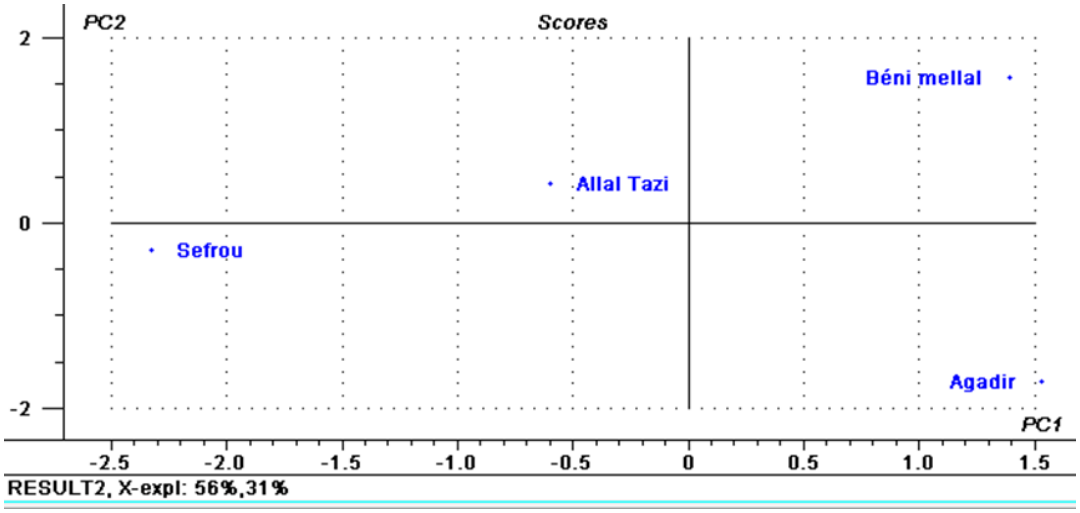

Figure 13: Study of the probable correlations among the sites.

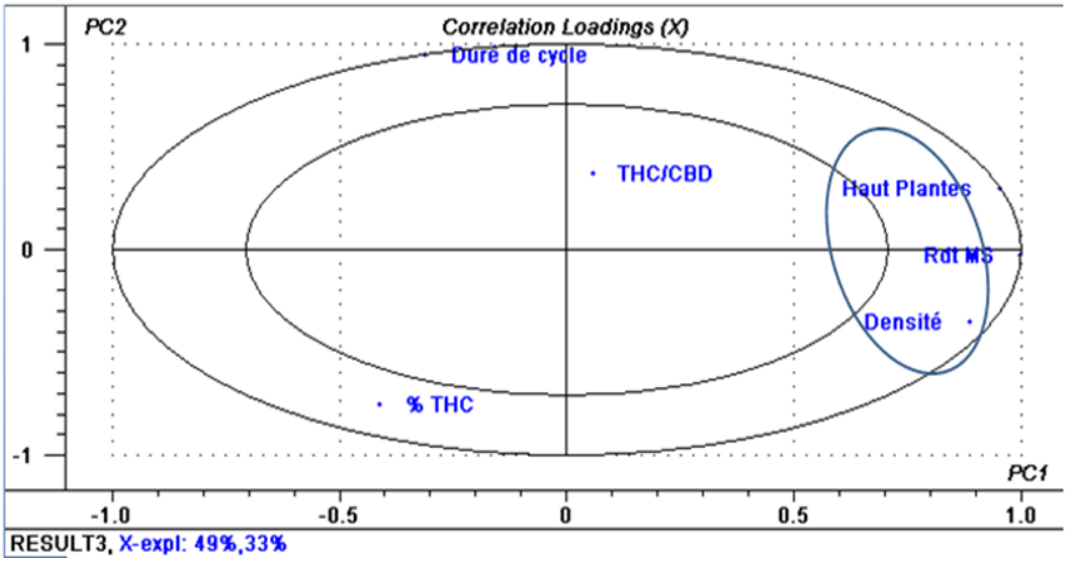

Figure 14: Correlation graph of parameters in relation to the Futura 75 variety.

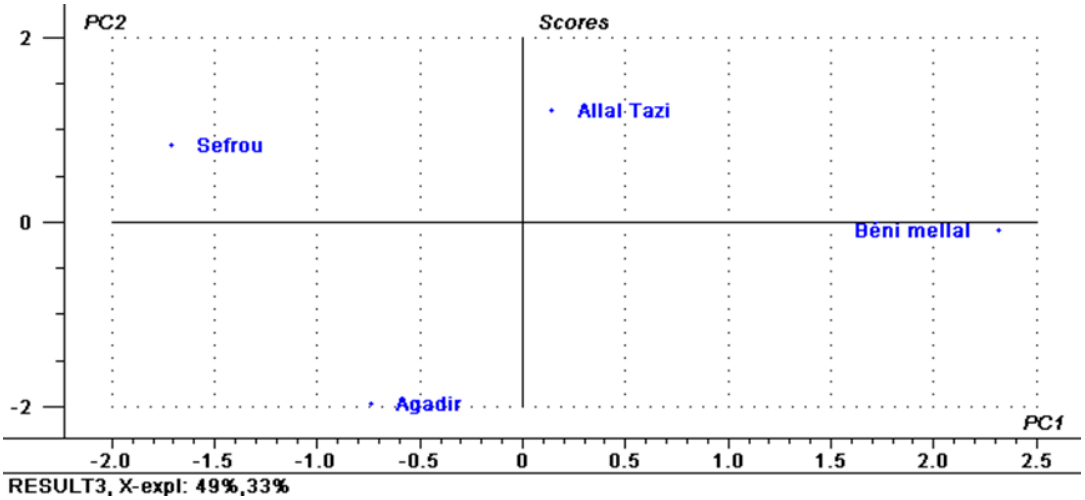

Figure 15: Correlation graph of test sites.

distinguished by its best densities (Table 3, Figure 4). In the soil and climatic conditions, density can be revised increased by covering the corresponding needs for water and nutrients to ensure higher yield.

The results of the two-factor ANOVA adversely affect the variability of density parameter due to the contribution of

Table 8: Variability between the different parameters in connection with the variety Epsilon 68.

Component No.	*Appropriate value*	*Variance percentage*	*Accumulated percentage*
1	3.34862	55.810	55.810
2	1.88671	31.445	87.255
3	0.764672	12.745	100.000
4	2.72218E-16	0.000	100.000
5	5.33817E-17	0.000	100.000
6	0,0	0.000	100.000

Table 9: Summary table of parameters for testing the Futura 75 variety.

	Density	Yield in dry matter	Plant height	Lifetime	Δ-9-THC contents	α = Δ-9-THC/CBD ratio
Sefrou	42	5.58	1.52	68	0.023	0.052
Beni mellal	75	24.5	2.27	64	0.021	0.052
Allal Tazi	63	13.3	1.9	68	0.026	0.053
Agadir	64	9.92	1.48	62	0.035	0

Table 10: Variability among the different parameters by ration parameter in the Futura 75 variety

Component No.	*Appropriate value*	*Variance percentage*	*Accumulated percentage*
1	2.95848	49.308	49.308
2	2.00529	33.422	82.730
3	1.03623	17.270	100.000
4	3.07889E-16	0.000	100.000
5	1.85982E-16	0.000	100.000
6	0.0	0.000	100.000

various factors. As the probability values are less than 0.05 for the two-factors and up variety, these factors have a statistically significant influence on the density with 95% confidence.

Yield in dry matter

The dry matter yields (t / ha) in three varieties are highly variable depending on the site. The best production (24.5 t/ha) was obtained by the variety Futura 75, Beni Mellal, while the lowest production (4.47 t / ha) was obtained by Santhica 27 Sefrou (Table 3, Figure 5). The analysis results show that the performance varies depending on the varieties and the locations of sites.

In general, if all varieties are adapted to the soil and climatic conditions of the experimental areas, "Futura 75" variety is the most effective, followed by the variety "Epsilon 68" and last the variety "Santhica 27". Regardless of soil type, variety "Futura 75" seeds better than others do. These results demonstrate perfectly the behavior of varieties in experiments. Indeed, the site of Beni Mellal is the most favorable of the four sites, with an average production of all varieties of 20.72 t/ha. Allal Tazi follows it, where the average production of all varieties is 10.87 t/ha; environmental conditions Beni Mellal therefore seem the most appropriate. The yields achieved in Beni Mellal is 85% of the potential yields determined in France (NFFC, 2008) for the variety Futura 75 (28.5 t / ha), 63% for the variety Santhica 27 (25.75 t/ha) and 80% for the variety Epsilon 68 (26.70 t/ha).

The ANOVA results show that both factors site and variety have a statistically significant effect on dry matter yield with 95% confidence.

Plant height

The plant height is a very parameter for the selection of varieties more adapted and productive, which are able to meet the raw material requirements and ensure good productivity. Table 4 and Figure 6 show that the plant height varies depending on the varieties and site.

The heights of the plants vary from 1.14 to 2.27m, with the best performance recorded in the Beni Mellal region (2.27 m). The variety "Futura 75" generates the tallest plants in the four studied regions (Figure 6).

The results of the analysis of variance have shown that both the site and variety factors induce statistically significant effects on plant height with 95% confidence.

Life time parameter

Table 3 and Figure 7 group the obtained lifetime parameter results. The results of the two-factor ANOVA adversely affect the variability of density parameter due to the contribution of various factors. As the probability value is less than 0.05 for the site factor, this factor has a statistically significant effect on the time cycle with 95.0% confidence level. However, the variety factor cannot be considered statistically significant on this parameter since the value of the probability is greater than 0.05.

It can be deduced that for the cycle time parameter, there is no significant difference between the studied varieties; though, the difference was statistically significant for the sites.

Delta-9-Tetrahydrocannabinol contents parameter

Among the cannabinoids present in Cannabis ssp, delta-9-tetrahydrocannabinol (THC) is the main psychoactive. The presence of this product must meet the applicable standard as for its content, which must not exceed 0.2%. Indeed, this standard is essential to guide its exploitability in the industrial fields related to the fibers of this plant, and hence the importance of this study.

The mean levels of THC determined for 12 samples from powder varieties Epsilon 68, Futura 75, and Santhica 27 are 0.022%, 0.026%, and <0.005% respectively. These levels clearly meet the European stipulated standards (mean contents of less than 0.2%).

Thus, for the four sites, variety Santhica 27 is considered free of THC (<0.001%), and has significant levels of CBG (Figures 8 and 9).

However, analysis by GC/MS SIM mode (314, 299, 231) or LC/MS/MS (MRM 193.0, 123.0, 107.2, 93.0) has detected small traces of Δ-9 THC in this variety. The other two varieties, Epsilon 68 and Futura 75, possess levels of Δ-9-THC located respectively in the ranges (0.013 to 0.027%) and (0.021 to 0.035) next to larger proportions

CBD.

In general, it was noted that the levels of Δ-9-THC recorded for Agadir site are slightly higher compared to other areas, particularly for the variety Futura 75 (Table 4).

The results of the two-factor ANOVA adversely affect the variability of the parameter "contents of Δ-9-THC" in relation to various factors. As the probability value is less than 0.05 for the variety factor, this factor has a statistically significant effect on the contents of Δ-9-THC with a confidence level of 95.0%. However, the site factor cannot be considered statistically significant since the probability value is greater than 0.05. This allows us to conclude that, contrary to the effect of the variety, the experimental sites have not given rise to significant differences in the parameter Δ-9-THC.

Paramter of ratio α = Δ-9-THC/CBD

The variety Santhica 27 being devoid of Δ-9-THC (<0.005%), the corresponding α ratio is zero. For Futura 75 Epsilon 68 varieties, their ratios is estimated respectively 0.052 and 0.056, which is the same order of magnitude as usually obtained for fiber hemp crops, and remains well below 0.2.

The results of the two-factor ANOVA adversely affect the variability of the parameter α (Δ-9-THC / CBD) concerning the contribution due to various factors. As the value of the probability is less than 0.05 for the variety factor, this factor has a statistically significant effect on the ratio α = Δ-9-THC / CBD with a confidence level of 95.0%. Although, site factor cannot be considered statistically significant on this parameter. In addition, since the probability value is greater than 0.05, therefore it can be deduced that for the ratio α = Δ-9-THC / CBD, there is no significant difference between the site cultures; the difference is nevertheless statistically significant in terms of the variety studied.

Principal Component Analysis (PCA)

Santhica 27 variety

For this variety we will not consider the first two parameters namely THC and α = Δ-9-THC / CBD in the study of the PCA as they attain zero values (Table 5).

Explantation of the variability

To determine the number of components to retain, we adopted the Kaiser criterion, which states that the components whose values are greater than 1 are retained in a normalized PCA. Table 6 shows that only the first two components have Eigen values greater than 1. It means that these two components may be considered for the explanation of variability in the data. This table also shows the percentages variability explained by each component and the cumulative percentages.

Study of the paramters

The graph of parameters (Figure 10) reveals the existence of correlations between different parameters including the one between the dry matter yield and density. This correlation confirms the results obtained previously.

Study of the sampling sites

At the end of this study, no correlation exists between the different sampling sites for Santhica 27 variety (Figure 11) which confirms the obtained results.

Epsilon 68 variety

The results of the various parameters related to the variety Epsilon 68 are summarized in Table 7. The purpose of this section is to reveal the different correlations that may exist regarding to that variety.

Explantation of the Variability

Table 8 of the explained variability shows that only the first two components have Eigen values greater than 1. This implies that these two components can be considered in explaining the variability of the data. This table also shows the percentage of variability explained by each component and the cumulative percentages.

Study of the parameters

The graph of parameters (Figure 12) reveals the existence of correlations among various parameters including the one between the dry matter yield and plant height, and between the density and THC & α = Δ-9-THC / CBD. no correlation exists between the different sampling sites for Epsilon 68 variety (Figure 13).

Futura 75 variety

The results of various parameters related to the variety Futura 75 are summarized in Table 9. The purpose of this section is to reveal the different correlations that may exist in relation to that variety.

Table 10 shows that the first five components have eigen values greater than 1. In addition, we will keep the first three components that explain 100% of the data variability. This table also elucidates the percentages of the variability explained by each component and the cumulative percentages.

Study of the parameters

The graph parameters reveal correlations between certain parameters including the one between the dry matter yield, the plant height and the density (Figure 14).

Concerning the effect of sampling site, the graph does not show any correlation between the sampling sites for the variety Futura 75 (Figure 15**).**

CONCLUSION

This statistical study included three varieties of Moroccan hemp fibers i.e. Santhica 27, Epsilon 68 and Futura 75. The parameters studied in this study are the duration of the lifetime, dry matter yield, density, contents THC, α ratio (Δ-9-THC / CBD) and the experimental sites. The best results have been obtained with the variety Futura 75 in four sites, followed by Epsilon 68 and Santhica 27. In general, control of the contents of Δ-9-THC according to procedure B of the regulation showed that all varieties with values below 0.2% for the 4 sites of experimentation. In regard to the results of the ANOVA two-factors, it can be concluded that the 4 test sites are perfectly homogeneous. However, it demonstrates that the two varieties namely Futura 75 and Epsilon 68 are mutually identical, but different from the variety Santhica 27. The density parameter, plant height and dry matter yield show different dependence on the site and varieties. The lifetime duration parameter has been found site dependent.

The results obtained by both methods reveal no exceeded values however the culture conditions are not exactly the same as those adopted in Europe. In addition, photoperiod

characteristic of these south Mediterranean regions are very favorable, thereby resulting in a fast maturing crops which have all reached the late stage of flowering after two and half months.

The Principal Component Analysis (PCA) confirms and affirms the existence of possible correlations for each variety separately. Overall, the results reveal the existence of correlations between different parameters including the one between the dry matter yield, plant height and density. However, no correlation between the experimental sites was detected.

The behavior of the three varieties tested in four regions of Morocco that are characterized by differences in climates, soil conditions, different geographical situations, and soil characteristics, is quite promising. These varieties can be an alternative to drug, and will eventually lead to the establishment of new fiber hemp industries.

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