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Heritability in Five Hemp Mother Plants on their Progeny from Clonal Tissue under Controlled Greenhouse Conditions

Roberto Santos[1], Ciro Rodriguez[2]
[1,2] Universidad Nacional Federico Villarreal, Lima, Perú,
[2]Universidad Nacional Mayor de San Marcos, Lima, Perú,

ABSTRACT

The purpose of the research is to evaluate five cannabinoid plants' heritability in the progeny generated from said mothers. The experiment was carried out in Jacksonville, Montevideo, Uruguay, during the agricultural year 2019-2020; for the analysis, forty-seven (47) clones were obtained from the five (05) plants of the cultivar Romalex were evaluated and which were sown at a density of 1.3 plants per m2. In the selection, the clones INVPS00006215 and INVPS00007157 were taken for having the highest quantitatively higher Total percentage values of Cannabidiol CBD and for statistically differentiating from the remaining ten clones of mother 256. The methods applied in this research work are analytical and statistical, corresponding to the experimental type; in the measurement, the High-Pressure Liquid Chromatographer HPLC method was used. The statistical analyzes presented include analysis of variance, Pearson's correlation, and regression. They are taking the information on regression coefficients, and it was interpreted for each unit in which the total CBD is increased, and the Tetrahydrocannabinol THC is increased by 12.81, starting from a minimum CBD was observed that the third week is the best results concerning the total percentage CBD. Plant 256 is the one with the lowest standard deviation, with a value of ± 0.57. Genetic variation (heritability) is 15.04% for CBD trait. For the case of total CBD and THC with a value of r = 0.96, that indicates there is a high and positive quantitative correlation.

Index Terms:
Heritability, Clones, Cannabinoids CBD, Hemp, High-Pressure Liquid Chromatographer HPLC, Tetrahydrocannabinol THC.

1.Introduction

THE selection or creation of monoecious cultivars has overcome the typical dimorphic character of Cannabis, with a different maturation time for each sex [1, 2].
The wide range of heritability and adaptability of 28 hemp plant traits for fiber production is statistically significant for environmental effects. Positive perspectives are prescribed with the heritability study to develop new hemp crops of excellent fiber quality [3].
Recent studies have shown some genes that regulate
phytohormones' production, such as gibberellic acid and auxins that affect sex determination in the hemp plant, testing new tools to develop the hemp crop [4].
The hemp plant's photosynthetic action in seven different environments was recently analyzed, analyzing the fluorescence kinetics of chlorophyll a, finding that extreme environments damage the photosynthetic machinery by producing free radicals, end up affecting the yield of the plant. The study determined a new detection method based on cannabinoid content [5].
In various types of traits maintained in populations in which evolutionarily [4], the quantitative characteristics vary; it is always a matter of debate. Hierarchy patterns for heritability values (h2) are proposed for traits related to life histories, physiography, behavior, and morphology [6,7].
Additive heritability is minimal when in populations, as alleles have been fixed over time, in which the traits that have been subject to selection strongly directed by biological adaptation in spaces in which they are found: on the contrary, high levels of heritability additive, in which the traits have been subject to weak selection [8].
We have another hypothesis regarding plasticity patterns; since phenotypic plasticity is high, physiological relationships can be observed. The heritability is inverse to the magnitudes of various environmental impacts. Highly plastic traits have less heritability [9].

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Genetic correlations allow considering the variation of quantitative traits over time linked to observed characteristics. Genetic correlation allows for integrating functionally related characters, allowing for better selection [10].
Unrelated independent traits will have a weak genetic correlation. [11]. They are analyzed via genetic correlations and heritability calculation [12].
Genotypic heritability is that which groups together all the genetic factors that affect the expression of a specific character; the genetic variance (σ2G) measures the variation due to genetic effects and is a necessary component to calculate the heritability of a particular trait such as the phenotypic variance (σ2F), which is the total phenotypic variation observed, whether due to genetic effects or environmental [13].

2. Methods and Materials

The methods applied in this research work are of the Analytical and Statistical type, corresponding to the Experimental type.
The experiment was conducted in Jacksonville, Montevideo, Uruguay, during the 2019-2020 agricultural year. The cannabinoid concentration evaluation was carried out in greenhouse conditions with a controlled environment. For the experiment, cuttings of the cultivar Romalex [2]. Installed with a density of 1.3 plants per m2 were used. For the cannabinoid analysis, the HPLC (High Pressure Liquid Chromatographer) method was used, installed in the analytical laboratory in Montevideo. THC and THCA, CBD, CBDA, CBN, and CBG levels were measured. On the other hand, the measurements were started 30 days after the flower bud of 47 clones extracted from five (05) selected mothers. It starts from a simple database, where cannabinoid levels are evaluated on 47 clones. Pearson, ANOVA, Heritability, and regression analyzes are presented.

Sampling

For the analysis, Cannabis plant samples were collected under controlled greenhouse conditions.
The 47 plants were sampled in each of its three parts for six consecutive weeks, making a total of 846 analyzes, and were collected and from the selected flowers from each of the three parts of the plants chosen for the present experiment. The samples were sampled separately.
The samples were labeled, taking into account each plant's code, part of the sampled plant, cultivar, date of collection, place of collection, name of the person responsible for taking samples, and observations.
The samples were protected to avoid possible damages during transport, such as exposure to excessive heat (> 40ºC) and humidity (> 10%). The samples were placed in paper envelopes kept at room temperature until they were sent to the laboratory. The samples were delivered to the laboratory on the same day of sampling.

Sample preparation and extraction method

When the samples were received in the laboratory, it was verified that they contain a label with all the information necessary for the analysis. The plant samples' preparation included drying at 35 Celsius for 24 hours to prepare the respective solutions.
The solutions obtained were analyzed, obtaining the qualitative identification of THC and THCA, CBD, CBDA, CBN, and CBG; and, having as identification criteria: (i) the relative retention time; and (ii) mass spectrum analysis.
CBN, THC, and CBD standards were used; and, results were stated on a dry basis. The validation of the quantitative method included chromatographic parameters, such as (i) selectivity, (ii)

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linearity, (iii) precision, (iv) accuracy, (v) linear range; and (vi) the limits of detection and quantification.

For the quantification of the mentioned cannabinoids, the following materials were used:

--Agilent 1100 HPLC / DAD
--Crumbling
--Automatic pipette
--Personal security elements
--HPLC quality solvents (Methanol, Acetonitrile, Purified Water)
--Formic acid
--Filters and syringes
--Agilent HPLC vials
--Analytical balance
--Sonicator
--50mL dispenser
--Spatulas
--20mL Glass Jars

Cannabinoiddosageinplantsamples

Once the samples were dry, they proceeded to homogenize them with the crumbler. A dry 20 mL glass tube proceeded to weigh approximately 0.1 g of the homogenized sample on the analytical balance (in triplicate) and then recorded the respective formats' weight.

Then proceed to add 20mL of Methanol and sonicate for 11 minutes at 60-65 ° C. When the samples were removed from the sonicator, they were shaken and filtered with a syringe and a 0.22 µm nylon filter and then placed in the 2 mL vial for later analysis.

With the weighing and dilution carried out, he proceeded to calculate the Multiplying Factor; this is the value placed in the HPLC Sequence to obtain the final results of the direct dosage of the Software need to perform mathematical calculations of concentration of the different Cannabinoids. It is calculated as follows:

$$Concentration = \frac{Dilution\ \ (mL)}{Sample\ \ (mg)} \quad (1)$$

Once the samples were dry, they proceeded to homogenize them with the crumbler.

With a dry 20 mL glass tube, we proceeded to weigh approximately 0.1 g of the homogenized sample on the analytical balance (in triplicate) and then record the weight in the respective formats.

Then proceed to add 20mL of Methanol and sonicate for 11 minutes at 60-65 ° C.

When the samples were removed from the sonicator, they were shaken and filtered with a syringe and a 0.22 µm nylon filter and then placed in the 2 mL vial for later analysis.

The result obtained was recorded in the respective format.

The last step is to place the vial on the HPLC and proceed using the analytical technique.

Once the analysis was completed, the results obtained were recorded in the corresponding records.

Analytictechnique

The analytical technique used in the present experiment is described below:

1) Preparation of the mobile phase

Aqueous phase: water / formic acid (pH≈3.0)

--1mL Formic acid
--Csp 1000mL Water quality HPLC

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Organic phase: ACN / formic acid
--0.8mL Formic Acid
--Csp 1000mL Acetonitrile HPLC quality
2) HPLC method conditions
--Flow: 0.8mL / min
--Injection volume: 1µL
--Temperature: 50 ° C
--Wavelength: 220nm
--Gradient

TABLE I.TIME GRADIENT CONTROL CONSIDERING STOP TIME: 4.5 MIN AND POST TIME: 2.2 MIN.

T(minutes)	Acetonitrile / Formic Acid	Water/ Formic Acid
0	75	25
4.50	100	0
4.51	75	25
5.50	75	25

Source: Authors

TABLE II ELUTION ORDER WITH A RELATIVE RETENTION TIME OF CANNABINOID.

Relative retention time	Cannabinoid
1	CBDa
1.08	CBG
1.14	CBD
1.46	CBN
1.69	THC
1.96	THCa

Source: Authors

3. Results

Tables III and IV and Fig. 1 show the analysis of the HPLC measurement results of the progenies corresponding to five dams selected for the present document, from which 47 clones were obtained in total.

TABLE III. SUMMARY OF ANALYSIS OF THE AVERAGE CBD CONTENT, ACCORDING TO THE MOTHERS OF THE INDIVIDUALS STUDIED.

Mother	% total CBD	Standard deviation
254	9.59	± 1.12
256	9.61	± 0.57
271	8.77	± 0.72
114A	8.45	± 0.75
26A	8.07	± 1.08

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Source: Authors

TABLE IVSUMMARY OF ANALYSIS OF THE AVERAGE CBD CONTENT, ACCORDING TO THE MOTHERS OF THE INDIVIDUALS STUDIED.

Statistic	254	256	271	114A	26A
N	11.00	8.00	10.00	9.00	9.00
Average	9.59	9.61	8.77	8.45	8.07
Stand. Dev.	1.12	0.57	0.72	0.75	1.08
Var (1-n)	1.26	0.33	0.53	0.57	1.16
Var (n)	1.15	0.28	0.47	0.51	1.03
Min	8.15	8.58	7.36	7.02	6.34
Max	11.54	10.31	9.59	9.32	9.80
Median	9.36	9.77	8.98	8.59	8.12
Square Sum	1023.5 3	741.4 8	773.5 0	647.5 2	595.25

Source: Authors

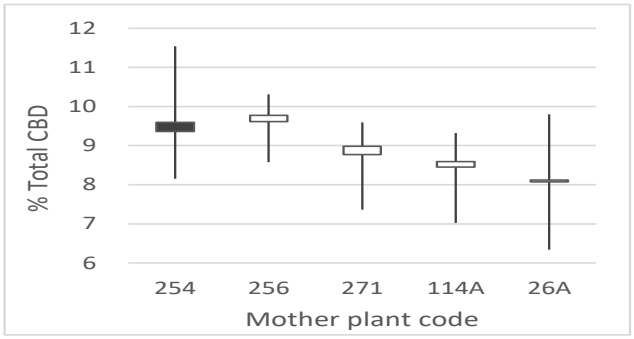

Fig. 1. A positive relationship between total CBD and total THC. Source: Authors

Table V shows that the Friedman test result determines if any of the medians' differences are statistically significant and compares the p-value concerning the significance level to evaluate the null hypothesis. Mother 254 is the one with the highest chi-square sum, and the youngest corresponds to mother 26A. There is no statistical difference between mothers 26A, 114A, and 271. There is a statistical difference between mothers 256 and 254 concerning mothers 26A, 114A, and 271.

Table V Friedman test for selected mothers

254	256	271	114A	26A	T	P
4.50	4.17	2,83	1.83	1.67	10,52	<0,0001

The least significant difference between the sum of ranks = 71.05

Treatment	Sum (Rank)	Average (ranks)	n				
26A	131,00	2,79	6	A			
114A	137,00	2,91	6	A	B		
271	145,00	3,09	6	A	B	C	
256	177,50	3,78	6				D
254	180,00	3,83	6				D

Means with a common letter are not significantly different ($p > 0.05$)

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From the standard deviation analysis of the analyzed populations, we can mention that plant 256 is the one with the lowest deviation with a value of ± 0.57, followed by plant 271 with a value of ± 0.72, and the third plant within 1 sigma is the plant 114A with a standard deviation ± 0.75.
With these data, we proceed to analyze the plant with the lowest standard deviation to determine: (i) ANOVA to analyze if there are differences between the evaluated materials; and, in turn, determine the proportion of genetic variability generated by the mother 256, (ii) Pearson's Correlation Analysis to determine the linear association between the variables Total CBD and Total THC; and, finally, regression analysis to study how changes in the predictor variable affect the response variable.

A. Anova

We want to decompose the population's total variability or sample into mean squares (CM), each associated with a known variation source with the present analysis. The main objective is to identify if there are significant differences between the evaluated materials.
Determination of variables:
--Dependent variables: The variable to be examined (response variable) is the percentage of total CBD.
--Classification variables: corresponds to the variables that represent factors or sources of variation that allow the observations to be separated or classified; for our case, we have chosen the clones evaluated in groups.
ANOVA is a linear model that only confirms whether or not there is significant genetic variability in the starting material.

TableVI
Analysis of variance (SC type III) of the population derived from plant 256

Variable	N	R	R=Aj	CV
% Total CBD	48	0.78	0.70	9.38

FV	SC	gl	CM	F	p-value
Model	85.81	12	7.15	10.17	<0.0001
Clon	51.34	7	7.33	10.43	<0.0001
Repetition	34.47	5	6.89	9.80	<0.0001
Error	24.61	35	0.70		
Total	110.42	47			

Table VII. LSD Fisher test corresponding to the 8 clones of plant 256
Alpha = 0.05, DMS = 098279, Error = 0.7031, gl = 35

Clon	Average	n	E.E.			
INVPS00006298	6.94	6	0.34	A		
INVPS0006302	7.82	6	0.34	A		
INVPS00007095	8.82	6	0.34		B	
INVPS00007039	8.91	6	0.34		B	
INVPS00007055	9.34	6	0.34		B	C
INVPS00007009	9.40	6	0.34		B	C
INVPS00006215	10.03	6	0.34			C
INVPS00007157	10.26	6	0.34			C

Means with a common letter are not significantly different (p> 0.05)

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Table VIII. LSD Fisher test for the number of repetitions
Alpha = 0.05, DMS = 085112, Error = 0.7031, gl = 35

Repetition	Average	N	E.E.			
2	8.11	8	0.30	A		
6	8.14	8	0.30	A		
1	8.18	8	0.30	A	B	
5	9.34	8	0.30		B	
4	9.60	8	0.30			C
3	10.28	8	0.30			C

Means with a common letter are not significantly different (p> 0.05)

1) The value of R^2 is 0.78 indicates reliable values. The CM Error is 0.70.
2) From the analysis of variance (SC type III), it can be observed:

i. The p value <0.0001 of the model, suggests that the linear model of the ANOVA (Yij = μ + τi + εij) is significant (p value ≤ 0.05)
ii. The value p = <0.0001 of the classification variable Clones suggests the rejection of the hypothesis of equality of treatment means, that is, there are statistically significant differences between the clones considering the variable percentage CBD.

3) According to the Test: LSD Fisher of clones, it is observed:

i. A ranking of mean values of% CBD for all clones from lowest to highest values, accompanied by a letter.
ii. The same letter does not show statistically significant differences between them according to the proposed level of significance (α = 0.05) and the MSD = 1.06288 (Minimum Significant Difference).
iii. Four groups of clones are observed where, statistically, there are no significant differences.
iv. At the time of selection, the INVPS00006215 and INVPS00007157 clones are recommended because they have the quantitatively highest percentage Total CBD values and because they differ statistically from the remaining 10.

4) The repetition test per weeks analyzed with LSD Fisher, it is observed:

i. There are no statistically significant differences when displaying different letters.
ii. Repeat 3 is the one with the best results for the percentage of Total CBD.

B. Estimation of Genetic Variation - Heritability (h2) in the Broad Sense from ANOVA

The ANOVA allows partitioning of the total variability into genetic variance (VG), environmental variance (VE), and phenotypic variance (VP). As in table VI, the variance analysis of the evaluation of 10 clones in 6 repetitions was considered in (1) and (2).

$VP = VG + VE(2)$

$\%VG = \frac{VG}{VP} * 100(3)$

The variance analysis of the 10 clones in 6 repetitions was carried out from the data collection.

The genetic parameters (variances and statistics (MC) were calculated with the ANOVA table's data.

-CME (Mean Square Between - Clones): estimates the phenotypic variance VP, it is observed in the CM of Clone = 7.33

--CMD (Mean Square Inside - ERROR): estimates the environmental variance VE, can be observed in the CM of ERROR = 0.70. The ERROR involves environmental influence (between

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repetitions) and all those variations due to non-genetic causes related to the analysis themselves, data collection, instrumental error, observations, and others. Genetic variation 0 (zero) is considered within the same hybrid's repeats because these materials are genetically uniform; that is, each plant belonging to each Clone has the same genotype.

--The VG is calculated by solving the first equation:

VG = (VP - VE) / Repetitions = 1.1050

The value shows the genetic variability between the different clones analyzed.

Heritability (h2) is calculated from the variance data obtained:

h^2 = VG / VP * 100 = 15.4

The calculation of h^2 for plant 256 was 15.04% for the character% Total CBD.

This proportion of Genetic Variability was calculated to know how much total genetic variation existed among the evaluated clones. We analyzed with genetic material that is not already stabilized; and which continues with a genetic improvement selection process. The only objective is to recommend one or the other Clone based on their differences at the CBD% level.

C. Correlation Analysis

Analyzing the association between variables, we observe that:

--The number of rows = number of columns = selected variables.

--The main diagonal elements are equal to 1 since they represent the correlation of a variable with itself.

--Below the main diagonal and in each position between the variables, we see the correlation data. (corresponding row and column). Table IX shows the associated probability is displayed above the diagonal (p-value).

Table IX Pearson Test for CBD and THC

Pearson's correlation: Coefficients / Probabilities

FV	T CBD	T THC	CBD	CBDa	CBG
Model	1.00	0.00	3.0E-04	0.00	2.7E-05
Clon	0.96	1.00	3.2E-04	0.00	4.5E-06
Repetition	0.50	0.50	1.00	0.01	0.01
Error	0.99	0.94	0.37	1.00	6.5E-05
Total	0.57	0.61	0.40	0.55	1.00

Source: Authors

Interpretation of Pearson's Phenotypic Correlation Analysis

1) With the assumption of a confidence level of 5%, it is assumed that the error for this analysis is $\alpha = 0.05$.

2) Above the diagonal (values 1.00), the significant p-values are observed and identified at the corresponding confidence level. That is all p-values $\leq \alpha$ 0.05.

3) The significant p-values were found to correspond to a specific association of 2 variables. Therefore, that same association must be found in the "r" values located below the mainline.

4) Each correlation coefficient “r” (red) has an absolute value and a sign (+/-). The “r” value is analyzed considering both connotations. The positive indicates that the variables decrease or increase in the same direction; On the contrary, the negative sign refers to the increase of one, the other decreases [2].

5) The case of Total CBD and Total THC with a value of r = 0.96 indicates a quantitatively high correlation (according to its absolute value) and positive; that is, as total CBD increases, Total THC also increases. Therefore if individuals are selected for their Total CBD content, it increases for the Total THC concentration.

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D. Linear regression

This analysis allows studying the functional relationship between a response variable Y (dependent variable - Total CBD (%)) and a regressor variable X (independent or predictor variable - Total THC (%)).

Using regression is possible to study how changes in the predictor variable affect the response variable. Linear regression prediction is useful for uncorrelated variables.

We use least squares as a method to obtain the coefficients of each equation that explain related variables. The prediction equation is constructed from these coefficients that allow knowing the value and the regressor variable / s. The statistical model used to analyze the linear regression is $Y = X\beta + \varepsilon$, the variable Y must be placed as a Dependent variable, and the variable X is assigned as Regressive variables. These respond to the linear function (mathematical model) $y = a + bX$.

Table X. Regression coefficients, associated statistics, and analysis of variance (SC type III)

Coef	Stad	E.E	LI 95%	LS 95%	T	p-value	CpMallows	VIF
Const	3.59	0.25	3.09	4.09	14.53	<0.0001	502.84	1.00
T THC	12.81	0.57	11.66	13.96	22.42	<0.0001		

Table XI Analysis of variance (SC type III)

FV	SC	gl	CM	F	p-value
Model	101.16	1	101.16	502.84	<0.0001
T THC	101.16	1	101.16	502.84	<0.0001
Error	9.25	46	0.20		
Total	110.42	47			

Through the Analysis of Variance Table (SC type III), it is possible to know how much of the data's variation is explained by regression and how much should be considered unexplained or residual (error). If the explained variation is substantially more significant than the unexplained, the proposed model will be good for predictive purposes. Thus, its application in Plant Genetic Improvement is related to RPPC ("Response to Average Selection per Cycle"), Heritability Estimation (Father - Progeny regression), and Analysis of phenotypic stability of yields.

Regression coefficients and associated statistics:

--The statistic value for the constant is 3.59 and corresponds to the ordinate at the linear model's origin.

--The statistic for total coefficient THC is 12.81 and corresponds to the linear model's slope (regression coefficient).

--The standard error of the estimate (S.E.).

--The 95% confidence limits (LI and LS).

--The T statistic value to test the hypothesis that the parameter (constant or coefficient) is zero.

--The significance value p to test the hypothesis based on T and the Mallows Cp index.

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To study the relationship between Total CBD (%) and Total THC (%), Total CBD was taken as a dependent variable and Total THC as a regressive variable [13].
Fig. 2 indicates that there is a positive relationship between Total CBD and Total THC.

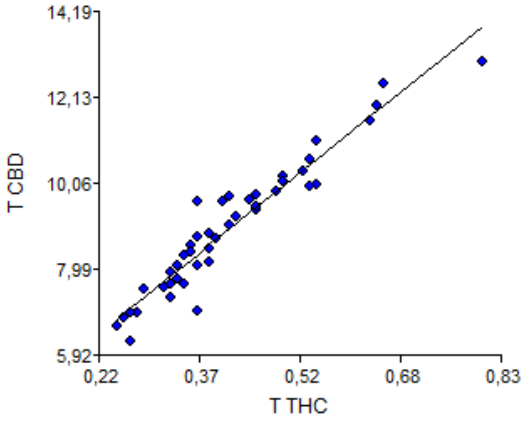

Fig. 2. A positive relationship between total CBD and total THC. Source: Authors

It is also observed that the proposed model does not present a lack of fit (p = 0.0001). Taking the information about the regression coefficients, the equation of the fitted model can be written as in (4):
$y = 3{,}59 + 12{,}81x(4)$
It is interpreted that for each unit in which Total CBD is increased, Total THC increases 12.81, starting from a minimum Total CBD of 3.59.

4. Discussion

The article shows a statistical difference in specific mother plants' progeny, even though they are asexual material (clones), as presented by [1,2]. The results also show the heritability of the character % total of CBD of the best mother plant, was calculated in 15.04%, which is also contemplated in the proposal of [3], with the difference of the repetition of three weeks in which it was determined that the highest % total CBD concentration was obtained. According to [4,5], the research also shows that a high correlation was identified between THC and CBD content with an r2 = 0.96. The research's novelty is that it was possible to determine a method to select the best mother plants in terms of progeny variability and high CBD content.

5. Conclusion

Mother 254 has a mean of 9.59% Total CBD with a standard deviation ± 1.12. Mother 256 has a mean of 9.61% total CBD with a standard deviation ± 0.57. Mother 271 has a mean of 8.77% total CBD with a standard deviation ± 0.72. Mother 114A has a mean of 8.45% total CBD with a standard deviation ± 0.75. Mother 26A has a mean of 8.07% total CBD with a standard deviation ± 1.08.
From the standard deviation analysis of the analyzed populations, we can mention that plant 256 is the one with the lowest deviation with a value of ± 0.57, followed by plant 271 with a value of ± 0.72, and the third plant within 1 sigma is the plant 114A with a standard deviation ± 0.75.
At the time of selection, the INVPS00006215 and INVPS00007157 Clones are recommended because they have the quantitatively highest% Total CBD values and because they differ

statistically from the remaining 10.
According to the Test: LSD Fisher of Repetitions (weeks), it is observed that repetition 3 is the one that obtains the best results concerning the% Total CBD ([14,15].
The genetic variation (% VG) or heritability is, for the 256 plants, 15.04% for the% CBD character.
For the case of Total CBD and Total THC with a value of r = 0.96, it indicates that there is a quantitatively high correlation (according to its absolute value) and positive, that is when Total CBD is increased, Total THC is also increased; therefore, if individuals are selected for their Total CBD content, take into account the Total THC concentration [2,16,17].
It is also observed that the proposed model does not present a lack of fit (p = 0.0001). The fitted model equation can be written using the information on regression: y = 3.59 + 12.81x. It is interpreted that, for each unit in which the Total CBD is increased, the Total THC is increased by 12.81, starting from a minimum Total CBD of 3.59 [2].

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