Variation of the *In Vitro* and Phenological Behaviours of 6 Cocoa Hybrids using the Discriminant Model in the Second Year of the Study

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ABSTRACT— *To analyse the variation of the in vitro and phenological behaviours, typological analyses were performed. Six newly promising cocoa hybrids known as L120-A2, L126-A3, L231-A4, L232-A9, L233-A4 and L330-A9 were used. Three culture media namely PCG1, PCG3 and PCG4, using DKW basal salt medium, only differing in hormonal concentration, were sown with staminodes and petals from these hybrids. SCA6 and C151-61 were included as controls. The 6 cocoa hybrids were monitored for 3 months at the end of which the callogenesis and embryogenesis variables were scored on each them. Collected data were processed via the Principal Component (PCA), Hierarchical Cluster (HCA) and Factorial Discriminant Analyses (FDA). Regarding the PCA, the flowering level, fructification level and leaves flush as well as number of embryogenic explants, embryos number yielded per embryogenic explant and embryogenesis percentage were found to be relevant. As for the FDA, only the flowering level, fructification level and number of callogenic explants were revealed to be pertinent. This postulates that the pertinentness seems to vary as a function of the analysis. Genotypes L120-A2 and L126-A3 expressed the highest callogenesis and flowering level compared to 4 others hybrids. They are half sibs with IMC67 as a common male parent. The line whose the equation is written Z = - 41.365 + 0.907*Nivflo discriminated the 3 clusters identified in the proportion of 100%. It allowed not only the describing of clusters, but also the predicting of the cluster of belonging of a new individual from its flowering level values. The aforementioned hybrids could be used to produce cocoa aroma, theobromin and cocoa butter from cell suspensions in bioreactors for biotechnology purposes. Such cultures should be achieved when flowering and fructification are high.*

Keywords— Promising hybrids, Culture media PCG, SCG and ED medium, Callogenesis, somatic embryogenesis, flowering level, Côte d'Ivoire.

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1. INTRODUCTION

Chocolate tree is a perennial, dicotyledonous and diploid plant of the Malvaceae [1]. It provides beans from which chocolate is manufactured. Côte d'Ivoire is the biggest producer of cocoa beans. Its production is about 44.25% of the world one [2]. In this country, cocoa area covers about 2,176,000 ha corresponding to 6% of national territory. Six millions of people depend directly or indirectly on cocoa. This accounts for 30% of working population [3]. Cocoa cultivation yields about 30% of global export revenue. Its incomes represent about 15% of gross domestic product [4].

Moreover, the average yieldings in beans, in cocoa farms, in the order of 400-800 kg / ha, are low. In contrast, in research station, these yieldings reach 2.5 tons / ha [5]. One of the means to improve it is the creation of new varieties. Thus, in 1988, 28 created hybrid progenies were planted in ancient field of the CNRA namely C2/3 located at Bingerville [6]. Thirty hybrid trees were preselected including 6 selected for tissue culture purposes. These are L120-A2, L126-A3, L231-A4, L232-A9, L233-A4 and L330-A9. They were tested *in vitro* for somatic embryogenesis purposes from calli. Three of them, notably L120-A2, L330-A9 and L231-A4, were included in the cycle of the reciprocal recurrent selection. Moreover, in the course of the floral buds collection, 3 phenological parameters were recorded. These are flowering and fructification levels as well as leaves flush. [7] showed that fluctuations of the endogenous plant growth regulator during

seasons trigger variations of phenological characters. [8] revealed, in cocoa tree, the influence of season on cocoa somatic embryogenesis. [9] evidenced the relationship between some phenological characters and somatic embryogenesis.

These cocoa hybrids were callogenically and embryogenically characterised in relation to their abilities, but after an univariate approach [10], [11], [12], [13]. This, in the objective to clone them. In the same vein, the relationship between phenology and somatic embryogenesis was also analysed, but always using only one embryogenesis parameter [14].

Following the same idea, the analysis of data from the first year of the study showed that 4 hybrids namely L120-A2, L126-A3, L231-A4, L330-A9 expressed the highest number of callogenic explants and leaves flush. The flowers collection for culture purposes from these hybrids should take place when the leaves flush is high. The line $Z1 = -29.123 + 0.201*Flush + 1.71*Ncal$ allowed the complete discriminating of 2 clusters identified. It also allowed the predicting of cluster of belonging of a new observation from these leaf flush and callogenesis values (Issali *et al*., submitted for publication).

To date, the structuring of theses 6 cocoa hybrids, during the second year of the study using simultaneously callogenesis, somatic embryogenesis and phenology parameters has remained badly known. The simultaneous analysis of these individuals from 8 parameters from callogenesis, somatic embryogenesis and phenology will first allow to better know them and describe them correctly. Then, it will allow the identifying of the phenological phase which optimises callogenesis and somatic embryogenesis. Thus, collecting of floral buds and initiating of staminodes and petals culture from these buds could take place at this phase basing on only the visual observation. Finally, it also allows the predicting of the cluster of belonging of a new individual based on values of better predictors identified. Likewise, we did not know the degree of suitability relatively to structuring of hybrids during the first and second year.

By reason of the identity of cocoa hybrids, they could be structured in 3 clusters as in [10].

The objective of this work was to structure the *in vitro* and phenological variabilities of 6 cocoa hybrids using the discriminant model.

2. MATERIALS AND METHODS

2.1. Plant materials, experimental sites and design

Plant materials consisted of 6 promising hybrids preselected for yielding and resistance to Phytophthora pod rot [14]. These are L120-A2, L126-A3, L231-A4, L232-A9, L233-A4 and L330-A9. These hybrids were obtained from crosses between upper amazon parental clones, namely Pa13, Pa121, IMC67, Pa150 and P19A. The first 3 hybrids are half sibs with IMC67 as common male parent, whereas the last 3 are half sibs with Pa150 as common male parent. Trees were planted in a completely randomised design in three geographical areas namely C2/1, C2/2 and C2/3 of the ancient station of CNRA (Centre National de Recherche Agronomique). The 6 hybrid trees were planted in field C2/3. Clones SCA6 and C151-61 were used as controls. The former was identified like very embryogenic [15], while the latter descends from back cross ICS1 x (ICS1 x SCA6). The former was planted in field B10, while the latter was it in field C2/1. All of these fields were located at Bingerville research Station, Côte d'Ivoire. Forty trees were planted per progeny. A border composed of 138 trees was associated with the design. The gap among trees on the same row was 2.5 m, while the one among rows, was 3 m. This corresponds to density of 1333 trees / ha. The experiment was carried for 2 years. Nevertheless, only the data collected in the second year of the study were analysed here. In this second year, the experiment especially the flowers collection for tissue culture ranged from January to December 2004.

Experimental sites were two. These are floral bud collection and tissue culture sites. The floral bud collection site was represented by the ancient station of CNRA situated at Bingerville. This site was located at 3°52'59" West and 5°21'42" North. During the second year of the study, the weekly pluviometric total, weekly average maximum temperature, weekly average minimum temperature, sunshine average relative humidity were 2179.30 mm, 30.96°C, 21.15°C, 38826.70 hours, 82.14%, respectively.

The second site for tissue culture was represented by the Central Biotechnology Laboratory (CBL). Cultures initiation and monitoring as well as scoring of calli and somatic embryos were performed there. Floral buds of 4-5 mm in length were collected on the 6 cocoa hybrids once a week, early in the morning. They were used as a source of explants. Primary somatic embryos were obtained as described in [16] by culturing of staminode and petal explants onto 3 primary callogenesis media known as PCG1, PCG3 and PCG4. Fourteen days later the culture onto PCG, the callogenic explants were subcultured onto Secondary Callus Growth (SCG). Fourteen days later, callogenic explants were again subcultured onto hormone free Embryos Development medium (ED). Onto the latter, callogenic explants were subcultured thrice every 21 days.

At CBL, a 8 x 2 x 3 factorial scheme in a modified completely randomised design was used. Thus, 8 genotypes providing each 2 explants were cultured onto 3 primary callogenesis media. Modifications concerned explants, namely

staminodes and petals, of a same treatment which were cultured in the same Petri dish onto the same medium. Each treatment was prepared in triplicate. In all, 72 treatments were obtained at the end of a weekly culture initiation.

2.2. Measurement of variables

Concerning tissue culture, at the end of 3 months, the number of callogenic explants, number of embryogenic explants, embryos number yielded per embryogenic explant were scored. From these, the average embryos number yielded per embryogenic explant as well as embryogenesis percentage were calculated. In the analyses, means from these 8 variables were used. Regarding the phenological characters, they were observed during flowers collecting. The fructification level was scored. In contrast, the flowering level and leaves flush were estimated according to a visual notation scale of 5 percentages, namely 0, 25, 50, 75 and 100.

2.3. Methods of data analysis

SPSS and Xlstat, versions 16.0 and 2007, respectively were used for statistical analyses. The Principal Component (PCA), Hierarchical Cluster (HCA) and Factorial Discriminant Analyses (FDA) were performed. In the first method, the Kaiser's normalisation was performed. In the second one, the euclidian distance was achieved for distances calculation, while the Ward method was used as an aggregation option. Regarding the third one, Wilks' statistics was used.

3. RESULTS AND ANALYSIS

3.1. Measure of the data compressibility

The measure of Kaiser-Meyer-Olkin (KMO) was 0.603. Such a value is considered to be average, according to Kaiser's scale, because it is between 0.6 and 0.7. The Bartlett's sphericity, indicating the existence significant correlation between at least 2 variables, was significant (approximate χ^2 = 58.042; p-value = 0.000). The meeting these two conditions allowed the achieving of the PCA. Issali et *al.*, (submitted for publication) reporting the analysis of data from the first year of the study also provided an average KMO.

The number of callogenic explants and average embryos number per embryogenic explant made negative the Pearson's linear correlation matrix. Consequently, they were dropped from the study. Thus, the flowering level, fructification level, leaves flush, number of embrogenic callus, embryos number yielded per embryogenic explant and embryogenesis percentage were used in the rest of the study.
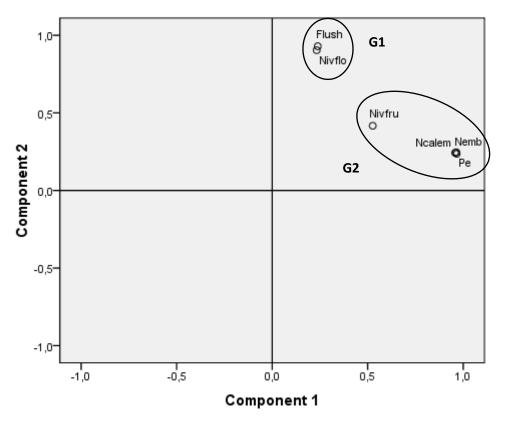
From 6 principal components extracted from the PCA, sole the first two showed eigenvalues higher than 1. Thus, they met the Kaiser's criterion. They were used in the rest of the study to analyse the variation displayed by the 6 cocoa hybrids (Table 1).

The principal plane constituted of the first 2 components accounted for 86.63% total variation. The first one, namely F1, accounted for 67.73% total variation. It is determined by the fructification level, number of embryogenic explants, embryos number yielded per embryogenic explant and embryogenesis percentage. It described hybrids expressing high ability for somatic embryogenesis (Table 1).

The second one, noted F2, revealed 18.90% of the unexplained variation by the axis F1. It is defined by the flowering and fructification levels as well as leaves flush. It was formed by hybrids showing a good phenological expression (Table 1).

The casting of the retained parameters on the principal 1-2 from the PCA displayed 2 clusters. The first one was constituted of the leaves flush and flowering level. The second one was composed of the fructification level, number of embryogenic explants, embryos number yielded per embryogenic explant, embryogenesis percentage. To summarise, the fructification level explained the number of embryogenic explants, embryos number yielded per embryogenic explant as well as embryogenesis percentage (Figure 1).

In brief, the number of embryogenic explants, embryos number yielded per embryogenic explants, embryogenesis percentage, flowering level, fructification level and leaves flush were found to be relevant for the performing of the PCA (Figure 1; Table 1). Consequently, the number of callogenic explants and average embryos number yielded per embryogenic explants were dropped from the analysis. Indeed, the latter make negative the Pearson's correlation matrix and blocked the outputting of some results. In the same vein, in Issali (a, submitted for publication), the same parameters were found to be relevant. The structuring of the parameters on the plane 1-2 produced 2 clusters in the first and second year. However, their composition was not the same. Sure enough, in the first year, leaves flush and fructification level explained embryogenesis parameters (Issali a, submitted for publication). Nonetheless, in the second year, only the fructification level explained the same embryogenesis parameters (Figure 1). Therefore, fructification level exerts an influence on the somatic embryogenesis expression. Indeed, endogenous indol-3-acetic acid and gibberellic acid induce the proliferation of pericarp tissues, in the fructification process [17]. Yet, these 2 exogenous plant growth regulators act in the somatic embryogenesis processes [18]. The internal fluctuations of the plant growth regulators induce those of the phenological characters which, in turn, favour the somatic embryogenesis [7]. Moreover, such variations in structuring, could find an explanation through differences between climatic parameters recorded in the first and second year.

Component Plot in Rotated Space

Figure 1: Structuring of the used parameters on the plane 1-2 from the PCA

Table 1 : Summary for eigenvalue, variability and parameter values on each of the components from the PCA.

	F1	F2
Eigenvalue	4.064	1.134
Variability (%)	67.73	18.90
Cumulative %	67.73	86.63
Nivflo	0.233	**0.904**
Nivfru	**0.527**	0.417
Flush	0.239	**0.929**
Ncalem	**0.958**	0.243
Nemb	**0.966**	0.236
Pe	**0.964**	0.247

3.2. Typology of the in vitro and phenological behaviours of 6 cocoa hybrids revealed through the HCA

The total number of individuals implied in the analysis was equal to 8. Such a number, smaller than 100, authorised the choice of the Hierarchical Cluster Analysis (HCA) instead k-means method (http://www.lemoal.org/spss/). These individuals were partitioned into 3 clusters, consisted of 2, 4 and 2 individuals each, respectively. They accounted for 25, 50 and 25%, respectively. Such percentages, widely higher than 10% level, allowed the validation of the analysis carried out. At the truncation 10 from the dissimilarity scale of the dendrogramme, the partitioning was done. Such differences were revealed through the Manova (p-value / Pillai's Trace = 0.017; p-value / Wilks' Lambda = 0.020; p-value / Hotelling's Trace = 0.000; p-value / Roy's Largest Root = 0.001). Further examination of data showed that these differences derived from 5 out of 8 parameters used. Among them, four discriminated partially the 3 clusters. It concerns the number of embryogenic explants, embryos number yielded per embryogenic explant, average number of embryos per embryogenic explants and embryogenesis percentage. However, only one completely discriminated the 3 clusters. It is about the flowering level (Table 2; Figure 2).

Cluster C1, consisted of 2 individuals, namely L120-A2 and L126-A3. It was characterised by high flowering level, high leaves flush, high number of callogenic explants, but by low fructification level, low number of embryogenic explants, low average number per embryogenic explant and low embryogenesis percentage (Table 2).

Cluster C2, consisted of 4 individuals, namely L231-A4, L232-A9, L233-A4 as well as L330-A9. It was distinguished by low flowering level, low leaves flush, low number of callogenic explants, low fructification level, low number of embryogenic explants, low average number per embryogenic explant and low embryogenesis percentage (Table 2).

Cluster C3, comprised the 2 controls, namely C151-61 and SCA6. It differed from the 2 previous by high flowering level, high leaves flush, high number of callogenic explants, high fructification level, high number of embryogenic explants, high average number per embryogenic explant and high embryogenesis percentage (Table 2).

Consequently, cluster C1 consisted of hybrids L120-A2 and L126-A3 recorded the highest flowering level and number of callogenic explants compared with C2 hybrid cluster (Figure 2). The callogenic and flowering behaviours of these 2 hybrids are alike. The 2 hybrids are half sibs with IMC67 as a common male parent (Issali et *al*., 2008; Issali, 2012). Thus, their similar behaviour finds an explanation through the blood that they have in common. In contrast, in Issali et *al*., (a, submitted for publication) these were hybrids L120-A2, L126-A3, L231-A4 and L330-A9 which displayed the best callogenesis and leaf flush values. Floral buds from which staminodes and petals are extracted for culture purposes must be harvested when the flowering and fructification will be high (Figure 1). Therefore, these 2 genotype hybrids could be used to produce chocolate aroma, cocoa butter and theobromin from the calli suspensions in bioreactors for biotechnology purposes.
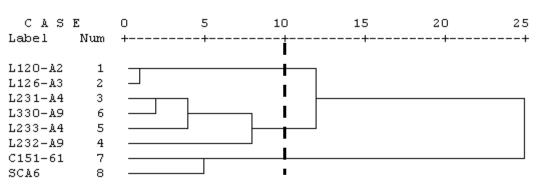

Figure 2 : Hierarchical tree showing the structuring of 6 cocoa hybrids by means of callogenesis, embryogenesis and phenology parameters.

Table 2 : Classification of the class means provided by the HCA using the 8 parameters after Student-Newman-Keuls' test.

	Cluster			**Mean***	**F**	**p-value**
Parameter	**C1**	**C2**	**C3**			
Nivflo	58.586a	34.898b	54.011c	49.165	385.526	< 0.0001
Nivflru	18.642a	16.790a	28.952a	21.461	1.111	0.399
Flush	42.212a	30.902a	41.295a	38.136	4.847	0.068
Ncal	19.255a	16.840a	20.577a	18.891	0.984	0.436
Ncalem	0.110a	0.047a	1.887b	0.681	178.377	< 0.0001
Nemb	0.319a	0.138a	9.056b	3.171	1342.716	< 0.0001
Mece	0.256a	0.112a	2.650b	1.006	851.973	< 0.0001
Pe	0.564a	0.340a	9.752b	3.552	773.666	< 0.0001

Legend: Mean* : It was calculated from values of the clusters C1, C2 and C3.

3.3. Link among the 3 clusters and parameters via the FDA
This link was evidenced via the investigating of : 1) differences among clusters, 2) validation of the Wilks' Lambda procedure, 3) the estimate of coefficients of the discriminant function and 4) the analysis of the representation quality. Nevertheless, the relevant parameters for this analysis were first looked for. Thus, the leaves flush, number of

embryogenic explants, embryos number per embryogenic explant, average number of embryos per embryogenic explant as well as embryogenesis percentage expressed Variance Inflation Factors (VIF) greater than 10 (VIF / Flush = 20.539; VIF / Ncalem = 439.938; VIF / Nemb = 1314.431; VIF / Mece = 15610.933; VIF / Pe = 2155.879). They were dropped from the analysis. In contrast, that of the flowering level, fructification level and number of callogenic explants was lower than 10 (VIF / Nivflo = 7.873; VIF / Nivfru = 2.944; VIF / Ncal = 8.746). Consequently, they were used in the rest of the study (Table 3).

With respect to the differences among the 3 evidenced clusters, the means or variances, on the one hand, as well as the analysis of Fisher-Snedecor' F statistics, on the other hand, were analysed. Concerning means, they appeared to discriminate the 3 groups identified (Nivflo = 45.60; Nivfru = 20.29; Ncal = 18.38). As for the Fisher-Snedecor' F statistics, they were high (Nivflo = 385.526; Nivfru = 1.111; Ncal = 0.984). For the Wilks' Lambda, those from 3 identified parameters were lower or equal to 0.9 (Nivflo = 0.006; Nivfru = 0.692; Ncal = 0.718). These showed the existence of differences among the clusters identified.

Regarding the validation of the Wilks' Lambda approach, the Box's M statistics, global correlation as well as Wilks' Lambda were analysed. The Box's M statistics was high (M = 5.254). The variance-covariance matrixes were equal (p-value = 0.138) justifying the choice of the linear FDA. The global correlation tended towards 1 (r = 0.997). The Wilks' Lambda from the stepwise method was both low and significant (Wilks' λ = 0.006; p-value = 0.000) during the introduction in the analysis of the only parameter, namely the flowering level.

The stepwise approach showed that it was possible to extract from 3 previously identified parameters, only one parameter which completely discriminated the 3 clusters. It is about the flowering level. The linear discriminant function 1, containing the flowering level, discriminated in proportion of 100% the 3 clusters identified. These allowed the validation of the study.

The Clusters C1 was placed in the negative part of axis F1, while C2 was placed in the positive one. Cluster C3 straddled this function 1 (Figure 3). The C1 recorded the highest flowering level. However, the C2 and C3 recorded the lowest and average flowering levels (Table 2).

The linear discriminant model is spelt : Z = - 41.365 + 0.907*Nivflo. This equation corresponded to model Z = Z0 + W1X1. In this equation, Z is the discrimination score; Z0 is the intercept for the disciminant function. It is equal to – 41.365. W1 is the discrimination weight and is equal to 0.907. X1 is the predictor. It is represented here by Nivflo. Z0 and W1 are the estimates of coefficients of the discriminant function.

The appreciation of the representation quality was performed through the confusion matrix. This revealed that in clusters C1, C2 and C3, 100% individuals were well-reclassified, in each them, thanks to the discriminant function Z. This represented 100% of success (Table 4).

The Mahalanobis' distances among the 3 clusters were calculated (distance C1-C2= 1058.234; distance C1-C3= 55.696; distance C2-C3= 635.016). The Wilks' Lambda test associated with the hypothesis of the equality of average vectors was significant (Lambda = 0.002; p-value = 0.001). Therefore, at least 2 clusters are significantly different. Further examination of data, via the Hotelling T² test, showed that the 3 identified clusters are significantly different from each other (p-value / C1-C2= 0.019; p-value / C1-C3= 0.010; p-value / C2-C3= 0.006). In Issali et *al*., (a, submitted for publication), pairwise distances were calculated and their significance was assessed using Fisher-Snedecor' F statistics. Out of 3 clusters, 2 only were significantly different, and thus constituted 2 morphologically distinct entities. The 2 approaches are comparable and give the same results (F C1-C2 = 615.728, p-value = 0.000; F C1-C3 = 17.226, p-value = 0.000; F C2-C3 = 400.856, p-value = 0.000). Here, the 3 identified clusters represented 3 groups of morphological differentiation (Tables 5 and 6; Figure 3).

In short, regarding the FDA, in the second year of the study, the flowering level, fructification level and number of callogenic explants were found to be relevant (Table 3). Nevertheless, in the first year, the flowering level, leaves flush and number of callogenic explants were revealed pertinent (Issali, a, submitted for publication). Thus, the relevant parameters identified for the achieving of the FDA varied from year to year. In sum, sole the flowering level and number of callogenic explants are common to 2 years, taken individually. Here also, the differences in the response could be due to variations in the climatic parameters expression from year to year.

The only function completely discriminating the 3 clusters is written Z = - 41.365 + 0.907*Nivflo (Table 5; Figure 3). This equation showed that, for genotypes expressing higher flowering level, the discrimination scores Z range from 7.576 to 12.171. It is mainly about individuals belonging from the clusters C1 and C3. However, for those expressing a low flowering level, the discrimination scores stretch out from -10.815 to -8.015. It essentially concerns individuals belonging to cluster C2. This linear equation will allow the predicting of cluster of belonging of a new individual from its values from the flowering level.

Table 3 : Multicolinearity statistics of the 8 parameters measured.

Statistics	Nivflo	Nivfru	Flush	Ncal	Ncalem	Nemb	Mece	Pe
Tolerance	0.127	0.340	0.049	0.114	0.002	0.001	0.000	0.000
VIF	7.873	2.944	20.539	8.746	439.938	1314.431	15610.933	2155.879

Legend : VIF* : Variance Inflation Factor calculated from formula 1 / Tolerance. The latter itself is calculated from formula 1 – R², where R² represents the coefficient of determination expressing the fit degree of the data to model.

Table 4 : Evaluation of the representation quality through the confusion matrix

		Ward Method	Predicted Group Membership			Total
			C1	C2	C3	
Original	**Count**	C1	2	0	0	2
		C2	0	4	0	4
		C3	0	0	2	2
	%	C1	100	0	0	100
		C2	0	100	0	100
		C3	0	0	100	100

Table 5. Mahalanobis' distances calculated among the 3 clusters identified.

	C1	**C2**	**C3**
C1	0	1058.234	55.696
C2	1058.234	0	635.016
C3	55.696	635.016	0

Table 6. Significance of the Mahalanobis' distances based on Hotelling T^2 test.

Distance between clusters	Hotelling's T² statistics	F	df1	df2	p-value
C1-C2	31.504	12.602	2	4	0.019
C1-C3	296.063	98.688	2	2	0.010
C2-C3	59.665	23.866	2	4	0.006

Legend : df* : Degree of freedom. **p-value* :** Calculated probability to compare to 5% likelihood.
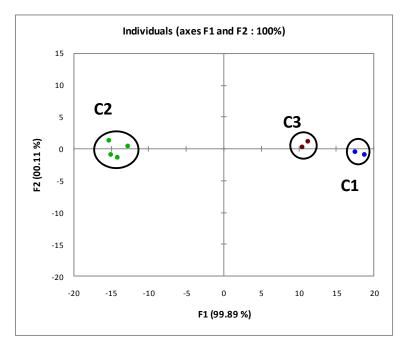

Figure 3: Factorial map showing the 3 clusters formed from the 6 hybrids using the FDA during the second year of the study.

4. CONCLUSION

We made the assumption that, on account of the highlighting of 3 clusters after classification of 6 cocoa hybrids by an univariate approach, the 8 callogenesis, somatic embryogenesis and phenological parameters would also discriminate them into 3 clusters using a multivariate approach. Effectively, the 6 analysed hybrids were discriminated into 3 clusters. Cluster C1 consisting of hybrids L120-A2 and L126-A3 expressed the highest callogenesis and flowering level values relatively to cluster C2. Fructification level explained somatic embryogenesis postulating that the floral buds harvest providing staminodes and petals which are cultured *in vitro*, should take place when flowering and fructification will be high. Moreover, the line $Z = -41.365 + 0.907*Nivflo$ completely discriminated the 3 clusters. It also showed that the flowering level is the only parameter containing the sufficient information allowing the discrimination of 3 clusters. This equation allowed the predicting of cluster of belonging of a new individual from its flowering level values. Thus, individuals whose the discrimination scores will vary from 7.576 to 12.171 will belong to clusters C1 and C3. However, those which will have the discrimination scores spreading out from -10.815 to -8.015 will belong to cluster C2.

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