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Relationship between some Phenological Parameters and Somatic Embryogenesis in *Theobroma cacao* L.

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Abstract

The relationship between three phenological parameters and somatic embryogenesis was investigated during a two-year period. Staminodes and petals from six hybrids and two clones as controls were sowed on three distinct primary callus growth media. Flowering level, fructification level, and leaf thrusts rhythm as phenological parameters were measured simultaneously during the weekly harvest of flower buds. Mean and coefficient of variation of the measured parameters highlighted stable phenological phases. The relationship between phenological parameters and somatic embryogenesis was investigated first by comparing the variation of somatic embryogenesis and that of the phenological parameters, and second by using Pearson's linear correlation. Except for the fructification level in both control clones the first year, the other parameters recorded stable phenological phases, regardless of the genotype and year. Favorable and unfavorable phases for the somatic embryogenesis were identified. In hybrids, favorable phases included February, August, September, and October. In both control clones, time interval propitious to embryogenesis stretched from February to December. The significance of the coefficient of correlation seemed to establish a relationship between somatic embryogenesis and phenology. However, a causal link could not be established. Leaf thrusts rhythm was revealed to be the phenological parameter most linked to somatic embryogenesis. Attempts to optimize embryogenesis during unfavorable phases, showed that a correction of 2.4 D/TDZ concentration is not the solution.

Key words: Cocoa, floral explants, phenological phases, exogenous hormonal balance, Côte d'Ivoire

Introduction

Theobroma cacao L. is a perennial, allogamous, and diploid (2n = 20) plant. It provides a substantial income for the producer countries (Braudeau 1991; Mossu 1990). Its high-level of heterozygosity and especially the use of unselected plant material generates a strong heterogeneity of characteristics among progenies (Alemanno 1995; Braudeau 1991; Lopez-Baez et al. 1993). As a result, average yields in merchant cocoa are relatively weak (Alemanno 1995; Braudeau 1991). One of the processes able to increase these yields would be the diffusion by the producers of superior genotypes propagated vegetatively (Braudeau 1991; Li et al. 1998). Grafting and rooted cuttings used for this purpose are not efficient (Braudeau 1991; Li et al. 1998). Additionally, the dimorphism architecture of cocoa tree and the predominance of plagiotropic material in the field have made rooted cuttings inefficient and expensive (Figueira and Janick 1993; Maximova et al. 2002). In the case of grafting, the grafted plants give trees with an unbalanced habit. Such trees not develop a true crown (Bertrand and Dupois 1992). To palliate these insufficiencies, micropropagation by somatic embryogenesis has been considered as complementary solution to the techniques of vegetative propagation of superior genotypes. Moreover, the plantlets regenerated from somatic embryos behave as seedlings (Li et al. 1998; Santos et al. 2002 ; Tan and Furtek 2003), showing a good recovery in the field. Some research works allowed the production of somatic embryos from floral explants (Li et al. 1998; Söndahl 1993; Traoré et al. 2003). That of Li et al. (1998) marked a real turning point, by improving their maturation, their

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germination as well as their conversion in plantlets. Furthermore, in the course of the harvest of flower buds, cocoa tree presents a phenological state marked by a flowering level, fructification level, and leaf thrusts rhythm depending on the conditions of the environment. It has been noted that the variation of this phenological state during the year seems to influence the variation of the production of somatic embryos (Traoré, unpublished result). This variation could be one of the reasons of the weak production of somatic embryos. Moreover, ignorance of the 2.4 D/TDZ concentration able to promote somatic embryogenesis in unfavorable conditions constitutes a serious handicap.

This work reports not only the relationship between phenology and somatic embryogenesis, but also research regarding the 2.4 D/TDZ concentration able to support somatic embryogenesis in unfavorable conditions.

Materials and Methods

Plant Material and tissue culture

Six hybrids (L120-A2, L126-A3, L231-A4, L232-A9, L233-A4, and L330-A9) and two control clones (C151-61 and SCA6) were used (Table 1). The callogenic and embryogenic abilities of L232-A9 and L233-A4 were characterized as weak and fairly callogenic, respectively, while L231-A4, L120-A2, L330-A9, and L126-A3, as well as the both control clones C151-61 and SCA6 were classified as greatly callogenic. Regarding embryogenesis abilities, L232-A9 was marked weakly, while L330-A9, L233-A4, L126-A3, L231-A4, and L120-A2 were characterized as fairly embryogenic. Two control clones, C151-61 and SCA6, were found to be greatly embryogenic (Issali et al. 2008).

Table 1. Summary on the origin and the characteristics of each genotype (Lockwood and Gyamfi 1979).

Genotypes		Origin	Characteristics
Hybrids	L120-A2	crossing descendent hybrid Pa13 x IMC67	Half sib of L232-A9, L126-A3, and L231-A4. Precocious and vigorous. Good shape and size of pods; good yield; good rate of fat.
	L126-A3	Crossing descendent hybrid Pa121 x IMC67	Full sib of L231-A4, half sib L233-A4, and L120-A2. Precocious and vigorous. Good shape and size of pods; good yield; good rate of fat.
	L231-A4	Hybrid descended of the crossing Pa121 x IMC67	Full sib of L126-A3, half sib of L233-A4, and L120-A2. Precocious and vigorous. Good shape and size of pods; good yield; good rate of fat.
	L232-A9	Crossing descendent hybrid Pa13 x Pa150	Half sib of L120-A2 and L330-A9. Precocious and vigorous. Good shape and size of pods; good yield; good rate of fat.
	L233-A4	Crossing descendent hybrid Pa121 x Pa150	Half sib of L231-A4, L126-A3, L330-A9, and L232-A9. Precocious and vigorous. Good shape and size of pods; good yield; good rate of fat.
	L330-A9	Crossing descendent hybrid P19A x Pa150	Half sib of L233-A4 and L232-A9. Precocious and vigorous. Good shape and size of pods; good yield; good rate of fat.
Control clones	C151-61	Clonal material come from Venezuela	Very elevated fruit set rate. More sensitive to pod rot, to Mirids and to malformations of pods due to wilt.
	SCAV-INA 6 (SCA 6)	Collected by Pound in upper Amazon close to the Sabina hacienda (Ecuador).	One of the ten best parents; very tolerant to witches' broom disease, resistant to Phytophthora, pod rot, but produces tiny beans; good yield; vigorous.

The first year of study stretched from September 2002 to August 2003, while the second year covered January to December 2004. Because of contamination recorded in the month of April 2003 in the first year of the study, its data were eliminated.

Flower buds measuring 4 to 5 mm in length were harvested once a week early in the morning and used as source of explants. Sterilization of buds, preparation of the culture media, and initiation of cultures were conducted based on the method of Li et al. (1998). Adaptation of the protocol concerned the hormonal concentrations of the primary callus growth media (Table 2). Seven flower buds were sowed in a single Petri dish in all experiments.

Table 2. Hormonal composition of the culture media used.

Culture media		Hormonal balances[a]
PCG	PCG3	[2,4 D] / [TDZ] : 4.52 µM / 11.35 nM
	PCG1	[2,4 D] / [TDZ] : 9.04 µM / 22.70 nM
	PCG4	[2,4 D] / [TDZ] : 18.08 µM / 45.40 nM
SCG		[2,4 D] / [Kinetin] : 9.04 µM / 1.394 µM
ED		Hormone free

[a]Hormonal balances: Concentration of PCG3 medium is the weakest. PCG1 medium hormonal concentration contained two-fold that of the PCG3 medium. In the same way, PCG4 medium hormonal concentration was four-fold that of the PCG3 medium. But the hormonal balance is the same.

A modified design in total randomization with factorial combination of factors variants was used. Modifications have concerned the association of staminodes and petals in co-cultivation. The genotype, explant, and medium are the factors used. The factorial combination was organized as follows: for each genotype (eight in total), two explants (staminodes and petals) were sowed in bulk on three distinct primary callus growth media (PCG1, PCG3, and PCG4). All the experiments were triplicated.

Measured phenological parameters

Flowering level (NIVFLO), fructification level (NIVFRU), and leaf thrusts rhythm (RYTHPF) as phenological parameters were measured simultaneously during the weekly harvest of flower buds (Table 3). NIVFLO and RYTHPF were estimated in percentage by visual appreciation from a scale of five percentages (0, 25, 50, 75, and 100%). These values corresponded to

Table 3. Phenological parameters, transformation applied, and symbol associated to each them.

Phenological parameters	Used transformation [a]	Symbol of the parameter
Flowering level	Angular (sine arc of the percentage square root)	NIVFLO
Fructification level	Square root	NIVFRU
Leaf thrusts rhythm	Angular (sine arc of the percentage square root)	RYTHPF

[a]: Square root and angular transformations were carried out to normalize the distributions and to equalize the variances.

the cover degree of the trunk and branches in flower buds and new leaf thrusts on cocoa tree. Appreciation of NIVFRU was performed by exhaustive numbering of cherelles, immature, and mature pods present on cocoa trees.

Definition of the stable phenological phases

To establish the relationship between phenology and somatic embryogenesis, three steps were necessary: firstly, the monthly variations of phenological parameter were analyzed. For this purpose, two statistical tools were used: 1) comparison of monthly average to annual average of every phenological parameter, 2) comparison of coefficient of monthly variation at 30% level. Thus, two classes as time intervals were defined in relation to the annual average: Class of the months whose average values were superior to the annual average and time intervals of months whose average values were lower to the annual average. Inside every class, sub-groups were identified relative to their coefficient of variation. Thus, the months that recorded high fluctuation (CV > 30%) were separated from months which recorded low variation (CV < 30%). However, this structuring did not give full satisfaction, because of either plethoric number of time intervals generated or non-existence of them. In the second step, a comparison of the embryogenic averages allowed the clustering of these time intervals. In the third step, by averages separation, these times intervals were used to generate relevant time intervals from phenological parameters. Time intervals related to the variation of somatic embryogenesis were compared to those of each measured phenological parameter. When number of time intervals related to the variation of embryogenesis corresponded to that of phenological parameter, these time intervals were assimilated to phenological phases. Otherwise, these time intervals were considered as is and separately. Thus, a stable phenological phase was defined as a time interval characterized by a stability (CV < 30%) of both phenological parameter and embryogenesis. A favorable or unfavorable phase was defined as stable phenological phase characterized by a high or a weak average of somatic embryogenesis. In addition, the coefficient of correlation between phenological parameter and somatic embryogenesis must be significant.

Measured variables for tissue culture.

At the end of every culture cycle of three months, five variables were measured for each genotype: callogenic explants number, embryogenic explants number, embryos number per embryogenic explant, average number of embryos per embryogenic explant, and the percentage of embryogenesis. Square root transformation was applied to the first four variables, while arcsin$\sqrt{}$ transformation was applied to the percentage of embryogenesis. These transformed variables were used for statistical analysis.

Statistical analysis

All statistical analyses were performed using SPSS software, version 10.1.3. Average and coefficient of variation were calculated to appreciate the central trend and the dispersion. To study the variation within a factor consisting of more than two variants, the averages were separated by the Dunnett's T3 test. However, for a factor only consisting of two variants, the averages were separated by the Student-Fisher LSD test related to the contrast method (at 5% level). To investigate the relation between phenology and the somatic embryogenesis, their variation sense was first compared by averages analysis. Before researching this relationship, stability (CV < 30%) of responses was tested. Then Pearson's linear correlation allowed appreciating the significance of link (at either 5 or 1% level) between somatic embryogenesis and phenology. The relationship between 2.4D/TDZ concentration and somatic embryogenesis was investigated at favorable and unfavorable phases.

This relationship was used as a basis of comparison relative to the relationship media effect inside favorable and unfavorable phases of parameters significantly correlated with somatic embryogenesis. The test of media effect was performed by averages separation.

Results

Organization of the phenological parameters in stable phases in the hybrids

Stable phases were evidenced by analyzing embryogenesis and phenological parameters. After identification of these phases, a link was found between the phenology and the somatic embryogenesis.

Among the phenological parameters, only the fructification level (NIVFRU) of both the control clones the first year recorded a shifting between the number of the time intervals (two in number) and those of somatic embryogenesis (three in number). Furthermore, for a given phenological parameter and for a given genotype, the time intervals did not match from one year to another.

Stable phases of flowering level

In hybrids, three stable phases were identified the first year of study. However, the second year, two stable phases were evidenced. In the first year, the first phase (NO_1), including the months of May and June, was characterized by a weak flowering level. The second phase (NO_2), composed of the months of November, December, January, March, and July was marked by an elevated flowering level. The third phase (NO_3), constituting September, October, February, and August, was characterized by a mean flowering level. For the second year, the first phase (NO_1), regrouped the months from January to August and the month of December. It was represented by a raised flowering level. The second phase (NO_2), composed of the months of September, October, and November, was marked by a weak flowering level.

In contrast, in both control clones, whatever the year two stable phenological phases were identified. The first year, the first phase (NO_1), composed of the months of September, October, December, May, and June, was marked by a weak flowering

level. The second phase (NO_2), included the months of November, January, February, March, July, and August, was characterized by an increased flowering level. The second year, the first phase (NO_1), constituting the months of January and December, was represented by a weak flowering level. The second phase (NO_2), included the months from February to November, was characterized by an elevated flowering level (Table 4).

Table 4. Average values of flowering level as a function of phases, of genotypes and of years.

Year	Genotypes	NIVFLO phases	Transformed average	CV (%)	Untransformed average (%)
Year 1	Hybrids	NO_1	0.580	5.17	30.03
		NO_2	0.914	1.97	62.72
		NO_3	0.845	2.37	55.95
	Clones	NO_1	0.520	5.58	24.69
		NO_2	0.819	2.69	53.36
Year 2	Hybrids	NO_1	0.816	3.55	53.06
		NO_2	0.726	1.10	44.07
	Clones	NO_1	0.765	3.14	47.96
		NO_2	0.852	1.17	56.64

Stable phases of fructification level

In hybrids only the first year recorded a variation of fructification level. This year, the first phase (NU_1), composed of the months of May and June, was characterized by a high fructification level. The second phase (NU_2), composed of the months of September, October, November, January, February, March, July, August, and December, was characterized by a weak fructification level.

However, both control clones, the first year recorded a shifting between the time intervals of fructification level and the ones of somatic embryogenesis. In contrast, the second year, two concordant and stable phases were evidenced. Thus the first year, the first time interval (NU_1), constituting the months of January, February, March, May, June, July, September, October, November, and December, was marked by a weak fructification level. This interval of time (NU_1) has generated two ($NU_{1/1}$ and $NU_{1/2}$) time intervals of variation of somatic embryogenesis. The second phase (NU_2), composed only of the month of August, was characterized by an increased fructification level. This time interval (NU_2) corresponded to the time interval NU2 of somatic embryogenesis. The second year, the first phase (NU_1), included the months of January, February, May, June, July, August, September, October, November, and December, was characterized by a raised fructification level. The second phase (NU_2), composed of March and April, was represented by a weak fructification level (Table 5).

Stable phases of leaf thrusts rhythm

In the hybrids, two stable phases were identified each year. The first phase (R_1), composed of the months of November, December, January, February, March, May, June, July, and August, was characterized by a weak leaf thrusts rhythm. The second phase (R_2), constituted of month of September and October, was marked by an elevated leaf thrusts rhythm. The second year, the first phase (R_1) included the months of January, February, March, April, May, June, July, August, September, November, and December, was represented by a weak leaf thrusts rhythm. The second phase (R_2), composed only of the month of October, was characterized by a raised leaf thrusts rhythm.

Table 5. Average values of fructification level as a function of phases, of genotypes and of years.

Year	Genotypes	NIVFLO phases	Transformed average	RC (%)	Untransformed average (%)
Year 1	Hybrids	NU_1	3.957	3.87	15.65
		NU_2	2.918	2.33	8.514
	Clones	NU_1	3.757	3.01	14.115
		NU_2	5.038	5.26	25.381
Year 2	Hybrids	NU	3.904	1.74	15.241
		NU_1	5.255	1.26	27.615
	Clones	NU_2	4.647	2.73	21.594

In the same way, in the control clones, whatever the year, two stable phases were evidenced. The first phase (R_1), including the months of September, January, February, May, June, July, and August, was marked by a weak leaf thrusts rhythm. The second phase (R_2), constituted of the months of October, November, December, and March, was marked by a raised leaf thrusts rhythm. The second year, the first phase (R_1), composed of the months of January, February, March, April, May, June, July, August, and September was represented by a weak leaf thrust rhythm. The second phase (R_2), constituted of the months of October, November, and December was marked by an elevated leaf thrusts rhythm (Table 6).

Table 6. Average values of leaf thrusts rhythum as a function of phases, of genotypes and years.

Year	Genotypes	RYTHPF phases	Transformed average	CV (%)	Untransformed average (%)
Year 1	Hybrids	R_1	0.549	2.55	27.23
		R_2	0.772	3.63	48.66
	Clones	R_1	0.599	4.00	31.79
		R_2	1.294	2.62	92.53
Year 2	Hybrids	R_1	0.616	1.95	33.38
		R_2	0.891	4.15	60.48
	Clones	R_1	0.641	2.65	35.76
		R_2	0.839	3.81	55.35

Analysis of relationship between the phenology and the somatic embryogenesis in the hybrids

Relationship between the flowering level and the somatic embryogenesis

Analysis of coefficient of variation revealed only NO_1 as flowering level corresponding to an unstable embryogenesis (CV = 298.06%) the first year in hybrids.

In hybrids the first year, inside the NO_1 phase the sense of variation of the flowering level (weak) corresponded to the one

of the somatic embryogenesis (weak with MEXEMB = 0.103 i.e. 0.011 in untransformed value). However, NO_2 and NO_3 phases displayed a negative correlation between flowering level (high/mean) and somatic embryogenesis (mean with MEXEMB/NO_3 = 2.04 i.e. 4.162; high with MEXEMB/NO_2 = 0.907 i.e. 0.823). The NO_3 phase recorded highest value of somatic embryogenesis in hybrids for the two years of study. The second year the same opposition was observed inside NO_1 and NO_2 phases (weak with MEXEMB/NO_1 = 0.226 i.e. 0.051; high with MEXEMB/NO_2 = 1.089 i.e. 1.186). Concerning the control clones the first year, the NO_1 and NO_2 phases recorded the same correlation between flowering level and somatic embryogenesis (weak with MEXEMB/NO_1 = 0.819 i.e. 0.670; high with MEXEMB/NO_2 = 2.703 i.e. 7.30). The second year the same relationship between flowering level (weak/high) and embryogenesis (weak with MEXEMB/NO_1 = 2.872 i.e. 8.24; high with MEXEMB/NO_2 = 3.502 i.e. 12.26) was recorded (Figure 1).

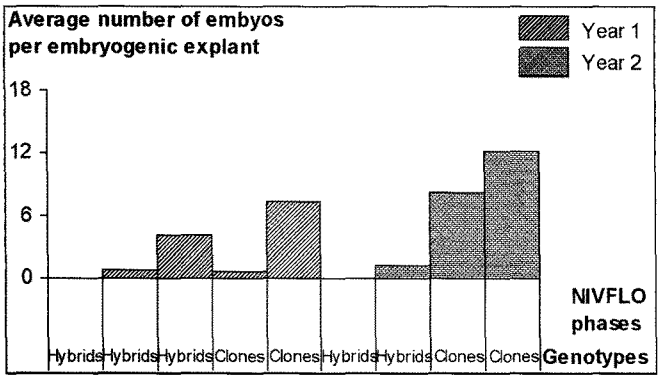

Fig. 1. Average number of embryos per embryogenic explant as a function of the stable flowering level phases of the two-year study in the hybrids.
- Three distinct phases (NO_1, NO_2, and NO_3) from the flowering level were identified in the hybrids the first year of study.
- Two distinct phases (NO_1 and NO_2) from the flowering level were identified in the control clones the first year of study.
- Two distinct phases (NO_1 and NO_2) from the flowering level were identified in the hybrids the second year of study.
- Two distinct phases (NO_1 and NO_2) from the flowering level were identified in the control clones the second year of study.

In hybrids evaluated during the first year, NO_2 and NO_3 phases previously negatively correlated showed a positive correlation. However, in the second year the correlation was negative. In the control clones irrespectively of the year, correlation was positive. The correlation between somatic embryogenesis and flowering level was significant in hybrids the first year and highly significant in control clones during both years (Table 7). Thus, NO_3 (for hybrids in the first year) and NO_2 (for control clones in both years) phase were positively associated with embryogenesis.

Table 7. Linear correlation between three phenological parameters and somatic embryogenesis during two years of study.

Genotypes		Linear correlation coefficient (r)[2]					
		Year 1			Year 2		
		NIVFLO	NIVFRU	RYTHPF	NIVFLO	NIVFRU	RYTHPF
Hybrids	Mexemb	+0.074*	-0.013	+0.114**	-0.007	+0.034	+0.087*
Clones	Mexemb	+0.246**	+0.129*	-0.026	+0.170**	+0.071	-0.085*

r: Correlation coefficients followed of one asterisk (*) mean that two parameters are correlated significantly at 5% level according to bilateral test of Pearson. Correlation coefficients followed by two asterisks (**) mean that two parameters are very significantly correlated at 1% level according Pearson's bilateral test.

Relationship between the fructification level and the somatic embryogenesis

Using the same approach as previously described, unstable embryogenesis was observed during the NU_1 phase in hybrids (CV = 301.94%) and the time interval NU_1 in control clones CV = 40.34%).

In hybrids, a significant variation in somatic embryogenesis and fructification level was recorded only the first year. Thus, during the NU_1 and NU_2 phases, the fructification level (respectively high and weak) was negatively correlated with somatic embryogenesis (respectively weak with MEXEMB/NU_1 = 0.103 i.e. 0.011 and high with MEXEMB/NU_2 = 1.400 i.e. 1.960). In control clones the first year, the correlation of NU_1 time interval of fructification level (weak) corresponded to a weak embryogenesis for $NU_{1/1}$ (with MEXEMB = 0.632 i.e. 0.399) and a mean embryogenesis for $NU_{1/2}$ (with MEXEMB = 1.927 i.e. 3.713). For NU_2 time interval, the correlation of fructification level (high) was similar to the one of somatic embryogenesis (high with MEXEMB/NU_2 = 4.195 i.e. 17.59). This NU_2 phase recorded the highest value of somatic embryogenesis in clones control during the both years of study. In contrast, the second year, inside NU_1 and NU_2 phases fructification level (respectively high and weak) was negatively correlated with somatic embryogenesis (respectively weak with MEXEMB/NU_1 = 3.228 i.e. 10.41 and high with MEXEMB/NU_2 = 4.098 i.e. 16.71) (Figure 2).

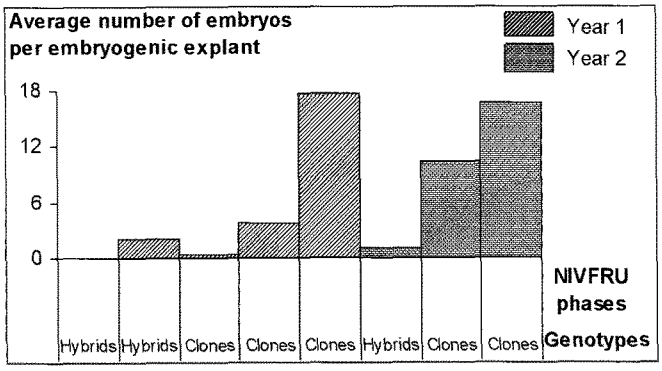

Fig. 2. Average number of embryos per embryogenic explant as a function of the stable fructification level phases of the two-year study in the hybrids.
- Two distinct phases (NU_1 and NU_2) from fructification level were identified in the hybrids the first year of study.
- Two distinct phases (NU_1 and NU_2) from fructification level were identified in the control clones the first year of study.
- Only one phase (NU) from the fructification level was identified in the hybrids the second year of study.
- Two distinct phases (NU_1 and NU_2) from the flowering level were identified in the clones the second year of study.

In hybrids evaluated the first year, NU_1 and NU_2 phases showing earlier opposite sense of variation recorded a negative

sign of coefficient of correlation. However, the control clones the first year, NU_2 expressing a similar sense of variation recorded a positive correlation. The second year the NU_1 and NU_2 presenting a negative correlation recorded a positive correlation. Link intensity between somatic embryogenesis and the flowering level was only significant in control clones the first year (Table 7). Hence only NU_2 phase was considered to be favorable to embryogenesis for control clones first year.

Relationship between the leaf thrusts rhythm and the somatic embryogenesis

Whatever the genotype and the year, all phases of the leaf thrusts rhythm recorded a stable somatic embryogenesis (CV < 30%).

In hybrids the first year, inside the R_1 and R_2 phases, the sense of variation of leaf thrusts rhythm (respectively weak and high) was similar to the one of somatic embryogenesis (respectively weak with MEXEMB = 0.991 i.e. 0.982 and high with MEXEMB = 2.010 i.e. 4.040). The same result was obtained the second year with respective embryogenesis average (weak MEXEMB = 0.913 i.e. 0.834; high MEXEMB = 1.994 i.e. 3.976). In control clones in contrast, the first year, inside the R_1 and R_2 phases the sense of variation of leaf thrusts rhythm (respectively weak and high) was opposite to the one of somatic embryogenesis (respectively high with MEXEMB = 2.215 i.e. 4.906 and weak with MEXEMB = 1.576 i.e. 2.484). In the second year the correlation of leaf thrusts rhythm was also negatively associated to that one of somatic embryogenesis (high with MEXEMB = 3.518 i.e. 12.376 and weak with MEXEMB = 3.043 i.e. 9.259) (Figure 3).

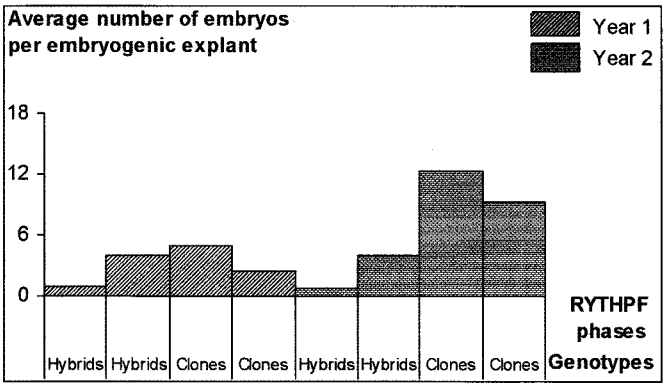

Fig. 3. Average number of embryos per embryogenic explant as a function of the stable leaf thrusts rhythm phases of the two-year study in the hybrids.
- Two distinct phases (R_1 and R_2) from the leaf thrusts rhythm were identified in the hybrids the first year of study.
- Two distinct phases (R_1 and R_2) from the leaf the thrusts rhythm were identified between the control clones the first year of study.
- Two distinct phases (R_1 and R_2) from the rhythm of the thrusts leafy were identified in the hybrids the second year of study.
- Only one phase (R) from the rhythm of the thrusts leafy was identified between the clones the second year of study.

The correlation previously observed was confirmed regarding the sign of coefficient of correlation (from one year to another positive in hybrids and negative in control clone). Link intensity between somatic embryogenesis and the leaf thrusts rhythm in hybrids was highly significant the first year and significant the second year. In control clones, this link intensity was significant only the second year (Table 7). Therefore R_2 (for hybrids both years) and R_1 (for control clones second year) phases were favorable to embryogenesis.

Relationship between 2.4D/TDZ concentration and somatic embryogenesis

General trend 2.4D / TDZ concentration - somatic embryogenesis

In the first year in control clones, PCG3 medium at weak 2.4 D/TDZ concentration promoted somatic embryogenesis, while PCG4 medium effect at high 2.4 D/TDZ concentration did not. In the second year in hybrids and in control clones, PCG4 medium at high 2.4 D/TDZ concentration increased somatic embryogenesis. On the contrary, media at weak 2.4 D/TDZ concentration (PCG3) and mean 2.4 D/TDZ concentration (PCG1) did not increase somatic embryogenesis (Table 8).

Table 8. Classification of embryogenesis averages as a function of PCG media effect, of genotypes and years.

Year	Genotypes	Media	Transformed averages [a]	RC (%)	Untransformed averages
Year 1	Hybrids	PCG3	0.93 a	23.50	0.86
		PCG4	1.23 a	17.57	1.52
		PCG1	1.40 a	15.85	1.96
	Clones	PCG4	1.52 a	14.84	2.32
		PCG1	1.99 ab	11.18	3.97
		PCG3	2.46 b	8.96	6.08
Year 2	Hybrids	PCG3	0.59 a	32.94	0.35
		PCG1	0.76 a	25.36	0.59
		PCG4	1.68 b	11.36	2.82
	Clones	PCG3	3.24 a	4.00	10.53
		PCG1	3.31 a	3.71	10.97
		PCG4	3.69 b	3.57	13.66

[a]: Values followed of the same letters for the three PCG media in a column are not different significantly at the 5% level by Dunnett's T3 test.

Relationship between 2.4D/TDZ concentration and somatic embryogenesis inside favorable and unfavorable phases

Previous general trend between 2.4 D/TDZ concentration and somatic embryogenesis was confirmed here. In fact, the first year in control clones, for the flowering and fructification levels, whatever the considered phenological phase, the PCG3 medium at weak 2.4 D/TDZ concentration favored somatic embryogenesis, while the PCG4 at strong hormonal concentration reduced somatic embryogenesis. In the second year regardless of the genotypes group for the favorable and unfavorable phases previously identified, the PCG3 and PCG1 medium at weak and mean 2.4 D/TDZ concentration decreased somatic embryogenesis. However, the PCG4 medium at strong hormonal concentration increased somatic embryogenesis (Table 9).

Table 9. Classification of embryogenesis averages as a function of PCG media effect, of favorable and unfavorable phenological phases of parameters significantly correlated with embryogenesis, of genotypes and years.

Years	Genotypes	F or UF Phases [a]	Media	Transformed averages [b]	RC (%)	Untransformed averages
Year 1	Hybrids	NO_3 F	PCG3	1.51 a	31.14	2.30
			PCG4	2.06 a	22.50	4.27
			PCG1	2.53 a	18.67	6.42
		NO_1-NO_2 UF	PCG1	0.74 a	28.17	0.55
			PCG3	0.60 a	34.00	0.36
			PCG4	0.75 a	26.95	0.57
	Clones	NO_2 F	PCG4	2.20 a	13.50	4.87
			PCG1	2.71 ab	10.75	7.37
			PCG3	3.16 b	9.22	10.03
		NO_1 UF	PCG4	0.37 a	69.89	0.13
			PCG1	0.73 ab	35.47	0.53
			PCG3	1.31 b	19.04	1.73
	Clones	NU_3 F	PCG1	4.04 a	13.66	16.33
			PCG4	4.19 a	14.27	17.59
			PCG3	4.35 a	13.01	18.97
		NU_1-NU_2 UF	PCG4	1.07 a	20.78	1.15
			PCG1	1.58 ab	14.03	2.52
			PCG3	2.12 b	10.31	4.51
	Hybrids	R_2 F	PCG1	1.66 a	41.82	2.78
			PCG3	1.43 a	49.16	2.06
			PCG4	2.82 a	23.46	7.96
		R_1 UF	PCG3	0.81 a	25.89	0.67
			PCG4	0.82 a	25.79	0.68
			PCG1	1.33 a	16.16	1.78
Year 2	Hybrids	R_2 F	PCG1	1.45 a	49.83	2.12
			PCG3	1.47 a	47.42	2.16
			PCG4	2.90 a	22.73	8.45
		R_1 UF	PCG3	0.49 a	40.77	0.24
			PCG1	0.69 a	28.84	0.48
			PCG4	1.53 b	12.94	2.34
	Clones	R_1 F	PCG3	3.39 a	4.39	11.52
			PCG1	3.49 a	3.98	12.18
			PCG4	3.67 a	4.13	13.53
		R_2 UF	PCG1	2.64 a	9.48	7.00
			PCG3	2.73 a	9.37	7.46
			PCG4	3.75 b	6.74	14.07
	Clones	NO_2 F	PCG3	3.32 a	4.24	11.08
			PCG1	3.34 a	3.95	11.15
			PCG4	3.86 b	3.70	14.96
		NO_1 UF	PCG4	2.72 a	11.47	7.40
			PCG3	2.76 a	11.30	7.62
			PCG1	3.13 a	9.96	9.82

[a]: Favorable (F) or unfavorable (UF) phases of phenological parameters significantly correlated with somatic embryogenesis.

[b]: Values followed of the same letters for three PCG media in a column are not significantly different at the 5% level by Dunnett's T3 test.

Discussion

A relationship between phenology and somatic embryogenesis has been investigated. 2.4 D/TDZ hormonal concentration able to support somatic embryogenesis in unfavorable conditions was researched as well.

The organization of parameters in phenological phases showed that, except for the fructification level of both control clones the first year, all other parameters showed stables phenological phases. Indeed in these control clones, the number time interval of fructification level (two in number) did not correspond to the one of variation of somatic embryogenesis (three in number). Such a lack of correspondence seemed to indicate that in control clones, especially this year; a weak variation of fructification level induced a strong amplification of variation of somatic embryogenesis. In addition, only the intensity of link between somatic embryogenesis and the fructification level of these two clones control was significant. No information relating to this amplification of somatic embryogenesis is available. It can be believed that the fructification level as phenological parameter has a strong influence on embryogenic expression.

Regarding the relationship between phenology and somatic embryogenesis, some phenological phases recorded strong dispersions of values of somatic embryogenesis, the first year (CV > 30%: NO_1/hybrids; NU_1/hybrids; NU_1/clones). Due to the instability of these phenological phases, they could not be chosen for optimization of somatic embryogenesis.

Our work showed that the correlation of somatic embryogenesis was opposite or similar to phenological parameters. This indicates the complexity of relations existing between somatic embryogenesis and phenology. This complexity is certainly responsible for the variation of influence of phenology on embryogenesis observed from one year to another. Likewise, some variations of embryogenesis related to seasons (Alemanno 1995; Antunez de Mayolo et al. 2003), genotypes (Lopez-Baez et al. 1993; Tan and Furtek 2003), explant (Esan 1975; Tan and Furtek 2003) and culture medium (Chatelet et al. 1992; Li et al. 1998) have been reported.

However, we identified some stable phases which allowed reliable analysis between phenology and embryogenesis. The favorable phenological phases of significantly correlated parameters were chosen to optimize somatic embryogenesis. It concerns NO_3 phase (flowering level) for hybrids the first year, R_2 phases (leaf thrusts rhythm) for hybrids the first and second year, and NO_2 phase (flowering level) for control clones the first and second year, NU_2 phase (flowering level) first year, R_1 phase (leaf thrusts rhythm) second year. In the NO_3 phase, a mean flowering level seems to trigger a high embryogenesis. In the NU_2 phase, a high fructification level seems to result in an increase of embryogenesis. These two phases appear to be the most embryogenic, suggesting that the flowering level and the fructification levels are liable to induce a high embryogenesis to certain periods of the year.

In spite of the plurality of factors affecting somatic embryogenesis (Guerra et al. 2001), the leaf thrusts rhythm appeared in our study as the most stable phenological parameter. It could be more suitable for studies of somatic embryogenesis, although the level of somatic embryogenesis was relatively low with this parameter (Figure 3). The flowering and fructification levels recorded variations from one year to another. This is probably due to a greater sensitivity of these phenological parameters to climatic variation. Cocoa tree is sensitive to variations of climate (Mossu 1990; Braudeau 1991).

The study of effect of hormonal concentration on embryogenic response indicates globally that the weak 2.4 D/TDZ concentration (PCG3 medium) was the most embryogenic the first year, while the highest 2.4 D/TDZ concentration (PCG4 medium) proved to be the best the second year. The hormonal concentration did not depend on genotypes (Table 6). This was confirmed by the detailed analysis which showed that hormonal concentration did not depend on phenological state nor genotype, but only the year (Table 6). Therefore, the response of embryogenesis to the 2.4 D/TDZ concentration seems to depend only on climatic variation occurring from one year to another. This shows the difficulty in identifying a specific hormonal balance able to support embryogenesis in unfavorable phases.

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Abbreviations

2,4-D - 2,4-dichlorophenoxyacetic acid; CV (%) - Coefficient of variation in percentage; LSD - Least significant difference of Student Fisher; L_xA_Y : X Line, Y Tree - Hybrid under assessment, but not yet certified, non registered into the catalogue, identified by its position on the experimental plot (x index) and by his rank on the line (y index), relating to the crossing from which it is descendant; NIVFLO - Flowering level; NIVFRU - Fructification level; PCG - Primary Callus Growth; RYTHPF - Leaf thrusts rhythm; TDZ - Thidiazuron - 1-phényl-3-(1,2,3 thiadiazol-5-yl) urea.

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