CALLUS-DERIVED HOP PLANTS SHOW CORRELATION BETWEEN EPIGENETIC INSTABILITY AND TIME IN CULTURE

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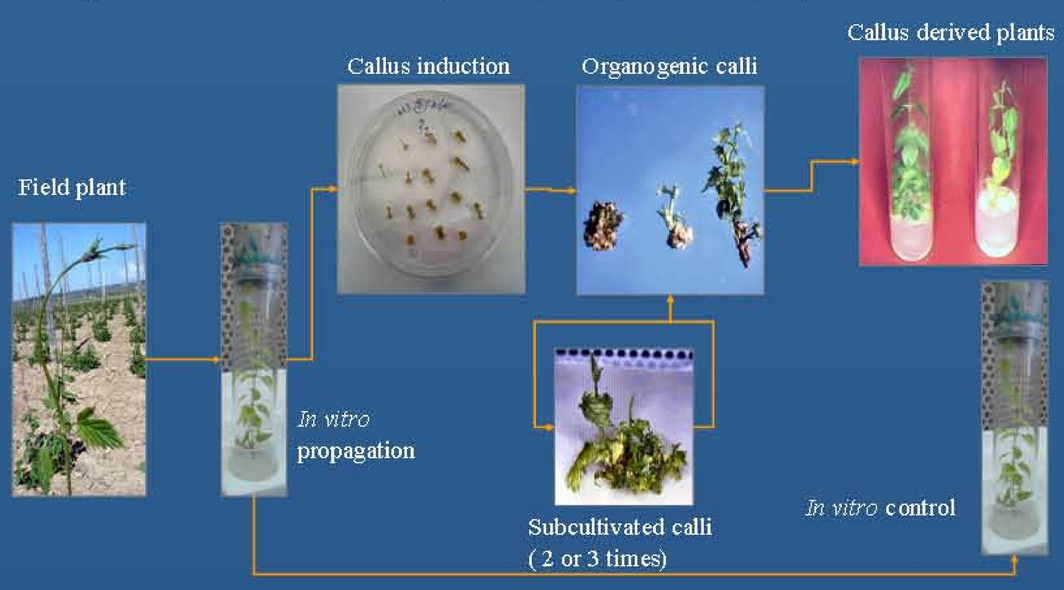
Our hop regeneration protocol commences with the induction of organogenic callus from an organized tissue which could be subcultured several times in order to increase the number of plants recovered. The indirect organogenesis and the long periods of culture could be related to an augment of the genetic instability. With the aim of testing the influence of our regeneration protocol and the period of culture in genetic variation, three sequential subcultures of calli-derived plants were grouped in pools and compared to control plants (field and *in vitro*). No major genetic rearranges were detected in the regenerated plants since none of the Amplified Fragment-Length Polymorphism (AFLP) loci were found to be polymorphic. However, slight epigenetic changes due to a demethylation process were detected by Methylation-Sensitive Amplified Polymorphism (MSAP). Moreover, a significant increase in the epigenetic instability could be related to the time in culture of the callus-derived plants.

MATERIAL AND METHODS

1. Shoot regeneration, Gurriarán *et al.*, 1999.

Plant material.
Obtained from S.A. Fomento del Lúpulo, (Villanueva de Carrizo, Leon, Spain).
The original individuals were field female plants *cv.* Chinook from clonal propagation.



2. DNA analysis

AFLP Vos *et al.* (1995) *Eco*RI and *Mse*I. 16 primer combinations.

MSAP Xiong *et al.* (1999) Cervera *et al.* (2002) *Eco*RI and *Hpa*II/*Msp*I 6 primer combinations.

3. Data analysis

Variation in presence/absence (1/0) of AFLP and MSAP bands
UPGMA cluster analysis performed on the matrix of similarity. GS(ij)= 2a/(2a+b+c) Dice.
Statistical program: NTSYS-pc 1.8.

Differences in variation levels through time in MSAP
Tested with one-way ANOVA and Scheffé post-hoc comparison. Data transformed by square-root (x). Deviation from normality and homogeneity of variance tested with Shapiro-Wilk and Barlett test. Level of significance α=0.05.

Changes in methylation state estimated by analysis of whole band patterns of each pair of isoschizomeres. Differences in presence/absence or intensity between *Eco*RI/*Msp*I & *Eco*RI/*Hpa*II bands considered epigenetic changes. Variation scored as "loss" or "gain".

RESULTS

A total of 876 **AFLP** sites were detected. No differences were found among the 7000 fragments analysed.

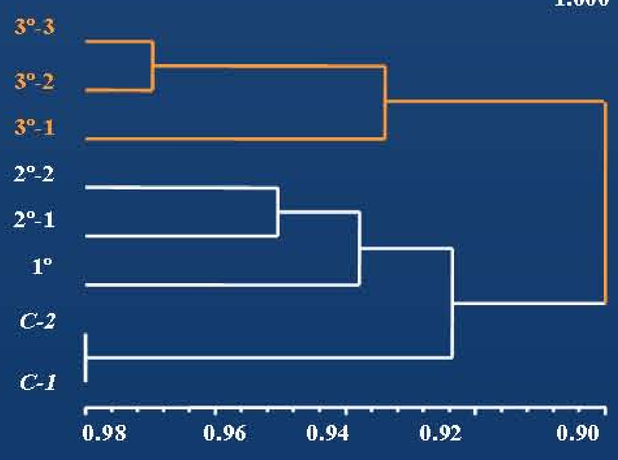
Only 172 out of a total of 566 loci were found to be polymorphic in **MSAP** analysis. All the analysed plants **shared a 69.61% of the detected bands. 13.37%** of the total variation is presented in all the callus-derived hop plants in contrast with the high percentage of variation assigned to **singletons (45.34%).**

The 3° subculture pools presented a significant augment in number of exclusive changes detected, up to **39.53%** of the total epigenetic variation is ascribed to plants grown in the 3° subculture.

Exclusively	AVERAGE	TOTAL
in vitro	**1.66[a]**	10
1° subculture	**1.0[a]**	6
2° subculture	**3.33[a]**	20
3° subculture	**10.66[b]**	64

[a,b] same letter, no differences in ANOVA (α=0.05) while different letters mean statistical significant differences.

The number of exclusive changes in each pools ranged from 0 up to 19, not uniformly distributed. The results obtained for the sum of exclusive changes of each sort of sample showed significant differences among number of subcultures (ANOVA, $F_{3,\ 20}$= 11,75; *P*<0.001). The **3° subculture** pools showed a **significant** increase in the variation levels (Scheffé test, control: p<0,001; 1° subc pools: p<0,001; 2° subc pools: p<0,05) while no differences were observed between the control and the first and second subcultures pools.



	C1	*C2*	1°	2°-1	2°-2	3°-1	3°-2	3°-3
C1	1.000	0.978	0.928	0.942	0.907	0.862	0.888	0.895
C2		1.000	0.933	0.939	0.904	0.859	0.887	0.897
1°			1.000	0.949	0.929	0.899	0.924	0.929
2°-1				1.000	0.951	0.899	0.932	0.931
2°-2					1.000	0.903	0.921	0.929
3°-1						1.000	0.927	0.943
3°-2							1.000	0.968
3°-3								1.000

UPGMA cluster analysis performed on the matrix of similarity coefficients showed that the callus-derived plants have a level of **similarity ranking between 86% and 94%** when comparing with controls. The plants regenerated from the first subculture share around a 93% of the fragments analysed with the *in vitro* controls. A similarity of 98% between the two *in vitro* controls was detected.

In the **UPGMA tree**, regenerated pools of plants obtained from the same subculture clustered together, showing a close relationship among each other. 3° subculture callus-derived pools of hop plants are grouped apart from the controls, 1° and 2° subculture pools. Moreover, the most variable pools which accumulate the **highest degree of variation (86-90%)** are those corresponding to 3° subculture and the longest time in culture.

MSAP allows to identify the possible mechanism of change in methylation state. 3 isoschizomere combinations generated a total of 298 loci.

6.71% appeared specifically in *Eco*RI-*Msp*I digest (internal cytosine of the recognition site might be methylated). **5.03%** (15) of the detected bands were *Eco*RI-*Hpa*II exclusives suggesting the methylation of the external cytosine in one of the strands at the recognition sites.
A total of 11 changes in the methylation pattern were ascribed to *de novo* methylation process, representing a **9.4%** of the total variation detected. However, the most common methylation change observed was the **demethylation** of the tagged sites **(83.76%).**

Change types	Change patterns[a]
Methylation	M-H-/M'+H'+, M+H-/M'+H'+, M-H+/M'+H'+
***de novo* methylation**	M+H+/M'-H'-, M+H+/M'+H'-, M+H+/M'-H'+
Others	M+H-/M'-H'-, M-H+/M'-H'-, M-H-/M'-H'+, M-H-/M'+H'-

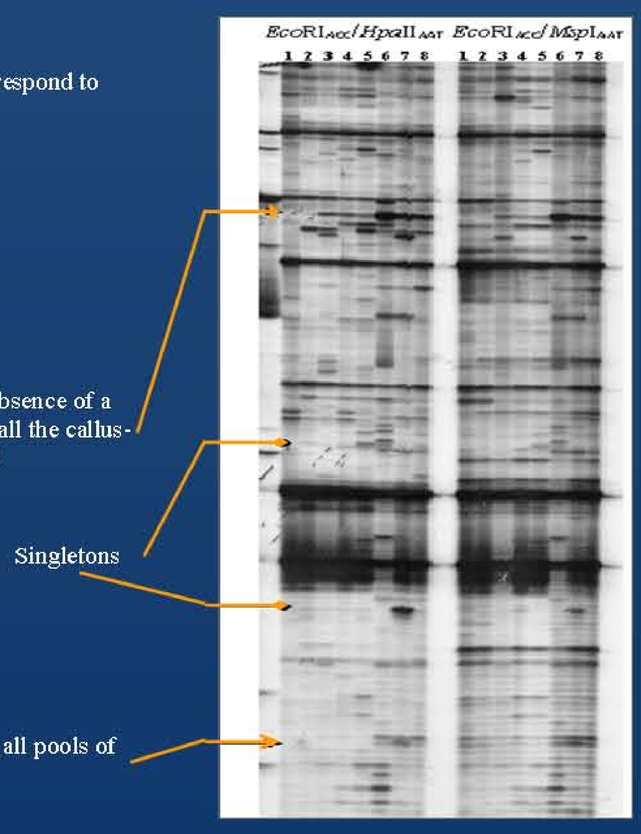
[a]M and H bands specific to EcoRI-MspI & EcoRI-HpaII digest, ;respectively, in the controls. M' and H' represent those in the callus-derived pools. + and – symbolize the presence and absence of DNA fragment, respectively.

MSAP patterns

All the differences signaled in the figure correspond to demethylation process.

Lane 1-2: *in vitro* material

Lane 3-8: plants regenerated from callus:
3 ⇒ 1° subculture,
4-5 ⇒ 2° subculture,
6-8 ⇒ 3° subculture.



CONCLUSION

The data presented here show that the regeneration of hops through a calli state does not produce significant alteration of the genetic and epigenetic state of the genetic material.

AFLP does not detect any change allowing us to conclude that no gross rearrangements were produced during the regeneration process.
However, slight variation in the methylation pattern is detected by MSAP analysis, these changes significant increase as the plants were maintained a longer period in culture.

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