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Assessments of somaclonal variation in micropropagated shoots of *Cedrus*: consequences of axillary bud breaking

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Abstract Reversed-phase HPLC analysis and random amplified polymorphic DNA (RAPD) markers were used to monitor DNA methylation status and genetic stability of *C. atlantica* and *C. libani* shoots generated through axillary bud proliferation. Average DNA methylation in *C. atlantica* or *C. libani* seedlings and mature 200-year-old trees of *C. libani* was 19.8, 19.5 and 22.3%, respectively. These global amounts showed no significant variation after the in vitro establishment of seedling-originated shoot stocks. In contrast, in vitro culture caused a significant decrease in the amount of 5-methylcytosine in genomic DNA of the tissue culture (TC) progenies of one of the adult *C. libani* genotypes. This DNA demethylation event accompanied an enhancement of the regrowth capacity of this genotype. Detected RAPD variation between mother plants and their TC progenies was species-related, with *C. libani* TC progenies being genetically more stable than those of *C. atlantica*. Nevertheless, similarity indices ranged from 0.97 to 1 among mother plants and their TC progenies. Furthermore, the analyses of molecular variance (AMOVA) suggest that RAPD variation among the mother plants and their TC progenies might be considered as not significant. The application of various statistical approaches, including cluster-based genetic distance methods and AMOVA, demonstrates that RAPD markers discriminate *C. atlantica* and *C. libani* appropriately.

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Introduction

In vitro propagation (micropropagation) of forest tree species is an effective way to capture genetic gain and produce large amounts of plant material (Bonga and Park 2003). Nevertheless, plant tissue cultures are especially prone to genome variation, a process generically known as somaclonal variation (Larkin and Scowcroft 1981).

Reports of somaclonal variation in woody plants as well as the possible causes and mechanisms implicated in their induction have been reviewed elsewhere (Ahuja 1998; Kaeppler et al. 2000). The complexity of somaclonal variation requires the use of several approaches so that plants can be correctly evaluated. This attains a special interest in species with extended growing periods, such as forest trees, where identifying variants as early as possible is essential to avoid the propagation of mutant plants (Olmos et al. 2002).

The use of molecular markers as a means of evaluating genetic stability of in vitro-grown plants is very frequent nowadays because these markers can characterize somaclonal variation with greater precision and less effort than cytological or morphological analyses (see Polanco and Ruiz 2002 and references therein). Among these, random amplified polymorphic DNA (RAPD) markers, despite their drawbacks (see Hedrick 1992), are an efficient technique to assess genetic stability of in vitro-regenerated conifers including *C. libani* (Isabel et al. 1996; De Verno et al. 1999; Piola et al. 1999; Tang et al. 2001). These markers have also been extensively used to evaluate natural genetic diversity in plant populations (Nybom and Bartish 2000).

The DNA of higher plants contains 5-methylcytosine (m^5C) as up to 30% of the total of cytosine residues (Finnegan et al. 1998). Both naturally occurring and induced differentiation/dedifferentiation processes, and several environmental stresses, initiate perturbations in the level and distribution of DNA methylation (Jaligot et al.

2002 and references therein). These same conditions, which favour epigenetic instability, also occur during micropropagation processes and often result in disruptions of clonal fidelity in the TC progenies (Lambé et al. 1997; Jaligot et al. 2000).

On the basis of a previously published method for micropropagation of juvenile *Cedrus libani* through axillary bud proliferation (Piola and Rohr 1996), we developed a protocol allowing the generation of shoots from cultured microcuttings (shoots 2–3 cm tall) of both juvenile (*C. atlantica* and *C. libani* seedlings) and adult (200-year-old *C. libani* trees) origin. In order to monitor possible variations in tissue culture (TC) progenies that validate the suitability of this propagation protocol, two complementary methodological approaches were undertaken: (1) HPLC quantification of the level of genomic DNA methylation; and (2) evaluation of the genetic fidelity using RAPD markers. The suitability of these markers to accurately resolve *C. atlantica* and *C. libani* seedlings used for in vitro culture establishment was also assessed.

Materials and methods

Plant material and culture conditions

Cedar microcuttings (2–3 cm tall) collected from 3-month-old seedlings and adult plants were used as the primary explants in the experiments. Once the needles had been excised, the explants were surface-sterilized by immersion in 0.1% $HgCl_2$ for 3 min, followed by four 5-min washes in sterile distilled water, and then cultured individually in glass tubes (150×25 mm) on solidified MSBN/2 (Piola and Rohr 1996) medium without growth regulators. Microcuttings of adult origin were first immersed in 1% NaClO for 20 min and rinsed for 8 h in running tap water. They were then submerged for 15 min in an aqueous solution of 1% benomyl and kept at 4–6°C for 1–2 days. Mother plant microcuttings came from: (1) 16 *C. atlantica* seedlings (genotypes A1–A16); (2) 7 *C. libani* seedlings (genotypes L1–L7); and (3) 3 200-year-old trees of *C. libani* growing outdoor on the campus of the University of Reading, England (genotypes AL1–AL3). Axillary shoots formed on these explants were subcultured on the same medium to promote their elongation and the proliferation of new buds. This procedure was repeated, approximately every 2 months for 1 year. Cultures were kept in growth chambers at 26±2°C and a 16-h photoperiod with light supplied by Sylvania (GTE Gro-lux, F36W/Gro, Germany) fluorescent tubes (50 μmol m^{-2} s^{-1} irradiance at culture level).

Needles from microcuttings from mother plants and their TC progenies were used for DNA extraction. Microcuttings were sampled individually at the beginning of the experiments and after 6 and 12 months of culture. After each sampling period, needles were stored at −20°C until used. In the experiment with adult *C. libani* trees, sampling was carried out in nine of the clones obtained from each of the three genotypes established in vitro. In the remaining experiments, only one of the obtained clones from each mother plant was sampled. Plant material for the interspecific differentiation assay between *C. atlantica* and *C. libani* came from needles of the first sampling period.

DNA extraction and amplification

Genomic DNA from 0.1 g of needles was extracted using the DNeasy Plant Minikit supplied by Qiagen. Two samples of DNA were prepared for each individual.

The polymerase chain reaction (PCR) constituent concentrations and conditions were optimised for representative samples of *C. atlantica* and *C. libani* to give repeatable markers. DNA amplifications, band separations, and RAPD profile visualisations were performed as described in Nebauer et al. (2000). Primers were initially screened to identify well-amplified polymorphic bands among several *C. atlantica* an *C. libani* seedlings. Individual DNA samples were appropriately diluted and bulked by seedlings to screen 60 decamer primers (Series OPA, OPB, and OPC from Operon Technologies, Alameda, Calif., USA). Six primers from the initial screening process (OPA-9, OPA-18, OPA-20, OPB-8, OPB-12 and OPC-2) that exhibited a high polymorphism and showed the best readability were chosen for further study of the individual genotypes.

Duplicate reactions were run for all primers and all individuals. Only those bands consistently reproduced in different analyses were considered. Bands of similar molecular weight and migration distances across individuals were assumed to be homologous. Homology assessments were made across gels based on a standard individual amplified and run on each gel and a Gene Ruler DNA ladder mix (Fermentas, Vilnius, Lithuania). Control samples containing all the reaction material except DNA were used to test that no self-amplification or DNA contamination occurred. In order to test reproducibility and repeatability in the obtained RAPD profiles, the procedure was repeated twice.

RAPD markers analysis

Amplified fragments, named by the primer used and the molecular mass in base pairs (bp), were scored as presence (1) or absence (0) of homologous bands, and a binary matrix of the different RAPD phenotypes was assembled. Pairwise distance matrices were computed based on both Nei's coefficient of similarity (Nei and Li 1979) and the Euclidean metric distance (Excoffier et al. 1992), using the RAPDPLOT (Black 1998) and RAPDistance (Armstrong et al. 1996) programs respectively. The Nei's distance matrices were used to produce dendrograms using the unweighted pair-group method with arithmetical averages (UPGMA) as implemented in NEIGHBOR from the PHYLIP package (Felsenstein 1993). To give a measure of the variability in the data from the assessment of the genetic relationships between *C. atlantica* and *C. libani*, bootstrap analysis was conducted and 200 similarity matrices were produced using RAPDPLOT. The NEIGHBOR and CONSENSE programmes in PHYLIP were employed to generate the 200 trees that were then used to produce a consensus tree. When

appropriate, principal coordinate analysis (PCoA) was also performed, based on the same distance matrix, using different routines (DCENTER and EIGEN) available on the software package NTSYS-pc (Rohlf 1997).

The Euclidean metric distances were used to perform analyses of molecular variance (AMOVA; Excoffier et al. 1992). The resulting variance components were used as estimates of the genetic divergence among species (*C. atlantica* vs *C. libani*) as well as mother plants and their TC progenies. Significance levels for variance-component estimates were computed by nonparametrical permutational procedures (5,000 permutations). The AMOVA was performed using the WINAMOVA 1.5 program (available from L. Excoffier, Genetics and Biometry Laboratory, University of Geneva, Switzerland).

DNA hydrolysis and quantification of methylation level by HPLC

Genomic DNA (approximately 20 μg) hydrolysis and HPLC base separations were performed as described in Demeulemeester et al. (1999), except that 15% acetonitrile was added to the original HPLC elution mixture. In the experiment with mature *C. libani* (genotypes AL1, AL2 and AL3), DNA hydrolysis was carried out in mixtures, appropriately diluted, of the sampled clones of each genotype. The efficiency of the hydrolysis was verified by electrophoresis in 0.5% agarose gels. The identity and relative amount of the bases were checked by co-chromatography with commercially available standards (Sigma): a mixture of adenine, thymine, guanine, cytosine and m^5C in a concentration of 2 mg/l each. The relative methylation of each DNA sample was quantified as m^5C as a proportion of total cytosine ©): $m^5C/[m^5C+C]$. All the analyses were carried out three times.

The effect of the different origins of the material (seedlings and mature trees) on DNA methylation was analysed by one-way analysis of variance (ANOVA). Where appropriate, a two-tail *t*-test with unequal sample size was also employed for comparisons. The effect of the number of subcultures on DNA methylation was analysed as a regression of the dependent variable (m^5C content) on the independent variable (number of subcultures). All the statistical analyses were performed using the STATGRAPHICS 4.1 computer program.

Results

Genetic relationships between *C. atlantica* and *C. libani* using RAPD markers

The six primers used to screen the 23 individual seedlings amplified a total of 183 well-amplified RAPD markers, ranging in size from 250 to 2,800 bp. The number of amplified bands per primer ranged from 25 (OPA-18) to 34 (OPB-8 and OPB-12). Most of these bands (86.3%) were polymorphic among the species (Table 1). Reflecting this high amount of genetic polymorphism, no individual had the same band pattern over all studied primers. Percentages of species-specific markers for *C. atlantica* and *C. libani* were 20 and 25%, respectively. Some of the RAPD markers were also genotype-specific: OPC2-1,030 bp (for genotype A1); OPA20-980 (A3); OPB8-620 (A8); OPA18-870 (A9); OPA18-1,190 and OPB8-740 (L1); OPB8-1,380 (L3); OPB12-810 and OPB12-1,000 (L6) and OPA20-600 (L7).

The 183 RAPD markers were first analysed using Nei's coefficient of similarity. The UPGMA dendrogram based on these data revealed the grouping of individuals within their own species (Fig. 1). In order to avoid any over-interpretation of the hierarchical clustering, a PCoA plot was also created. The general structure was similar to the one obtained with UPGMA and confirmed the distinction between *C. atlantica* and *C. libani* (Fig. 2).

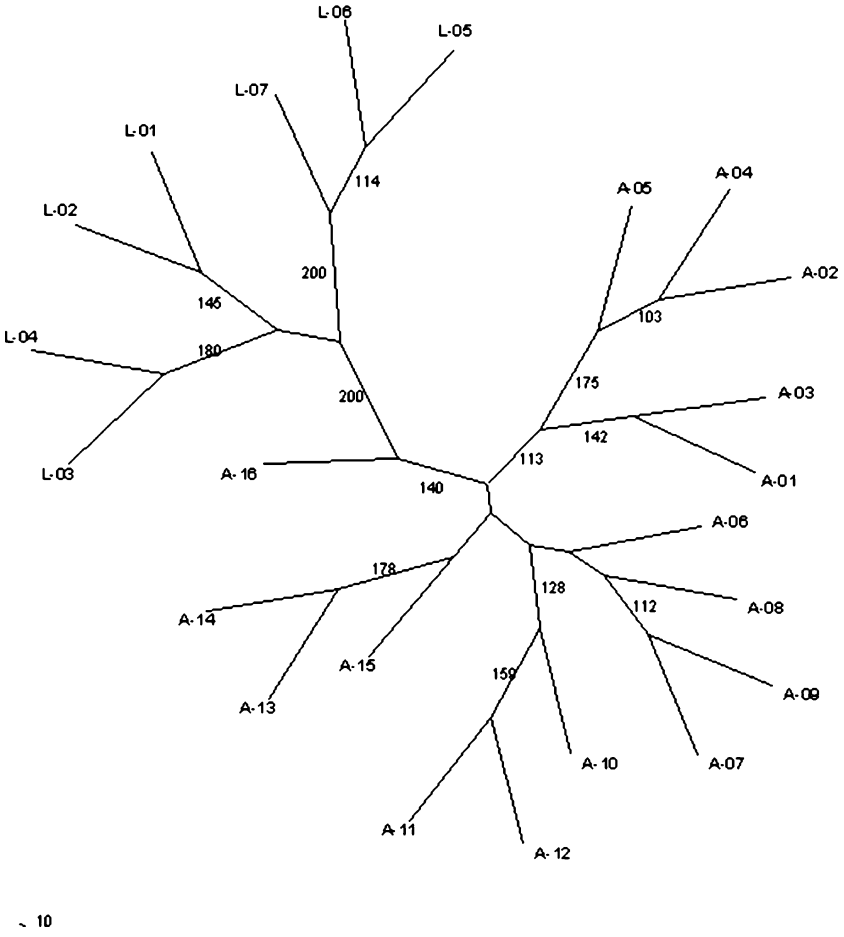
The AMOVA analysis transforms a phenotypic distance matrix into an equivalent analysis of variance (Excoffier et al. 1992), and has been previously used to optimise some aspects of the application of RAPDs in the assessment of genetic relationships among species (Nebauer et al. 2000). The AMOVA method was performed to determine the variance component accounted for in the among species variation. Of the total phenotypic diversity, 55% ($P<0.001$) was attributable to differences between *C. atlantica* and *C.libani*.

Characterisation of the genetic stability of micropropagated *C. atlantica* and *C. libani* shoots using RAPD markers

No differences were found in the morphological characters of TC progenies when compared to their respective micro-cutting mother plants. A summary of the RAPD markers

Table 1 Primers employed, sequence and RAPD markers obtained from seedlings of *C. atlantica* and *C. libani*

Primers	Sequence	Size (bp)	Number of bands		
			Polymorphic	Monomorphic	Total
OPA-9	GGGTAACGCC	420–2,500	28	1	29
OPA-18	AGGTGACCGT	380–2,300	20	5	25
OPA-20	GTTGCGATCC	350–2,200	20	8	28
OPB-8	GTCCACACGG	250–2,800	30	4	34
OPB-12	CCTTGACGCA	280–2,300	27	7	34
OPC-2	GTGAGGCGTC	450–2,020	33	0	33
		Total	158	25	183



generated by the six selected primers used to evaluate genetic stability in cedar cultures of juvenile and adult origin is given in Table 2. All these primers detected polymorphism between *C. atlantica* seedlings and their respective TC progenies. Significantly lower variability was observed in either juvenile or adult *C. libani* cultures. In the former, only primer OPB12 generated polymorphism, while in the latter, two primers (OPA20 and OPB8) detected polymorphism among mother plants and their TC progenies.

Cluster analyses were performed to confirm the genetic stability of TC progenies. Figure 3 shows the phenetic dendrograms based on Nei's coefficient of similarity. It is evident from the dendrograms that, irrespective of the microcutting origin (juvenile or adult), mother microcuttings and their putative clones are closely related, sharing values of genetic similarity higher than 0.97. The highest genetic stability was found in juvenile *C. libani* cultures, where 86% of the initially cultured microcuttings shared the max-

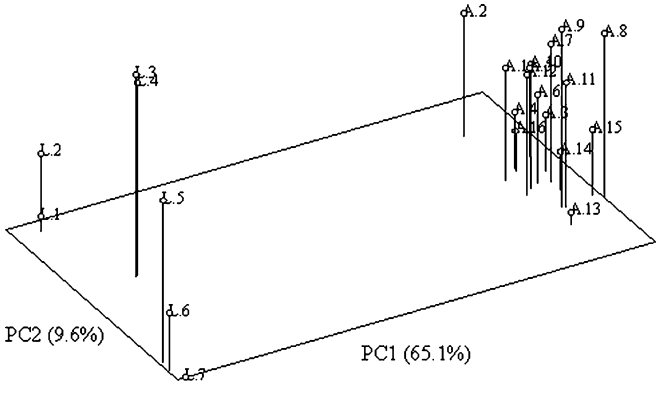


Fig. 2 Plot of the first two principal coordinates for each *C. atlantica* (A1–A16) and *C. libani* (L1–L7) individual. Eigenvalues for each principal coordinate are listed in *parentheses*

Table 2 Primers employed and RAPD markers obtained in cultured microcuttings of *C. atlantica* and *C. libani*. *T* total number of bands, *P* number of polymorphic bands among mother plants and their tissue culture progenies

Primer	Size (bp)	Juvenile *C. atlantica*		Juvenile *C. libani*		Adult *C. libani*	
		T	P	T	P	T	P
OPA-9	420–3,600	18	7	24	0	29	0
OPA-18	380–2,400	19	5	24	0	22	0
OPA-20	350–2,500	20	5	23	0	25	5
OPB-8	250–2,300	29	4	19	0	27	7
OPB-12	280–2,300	24	2	29	1	27	0
OPC-2	420–2,200	26	4	32	0	27	0
	Total	136	27	151	1	157	12

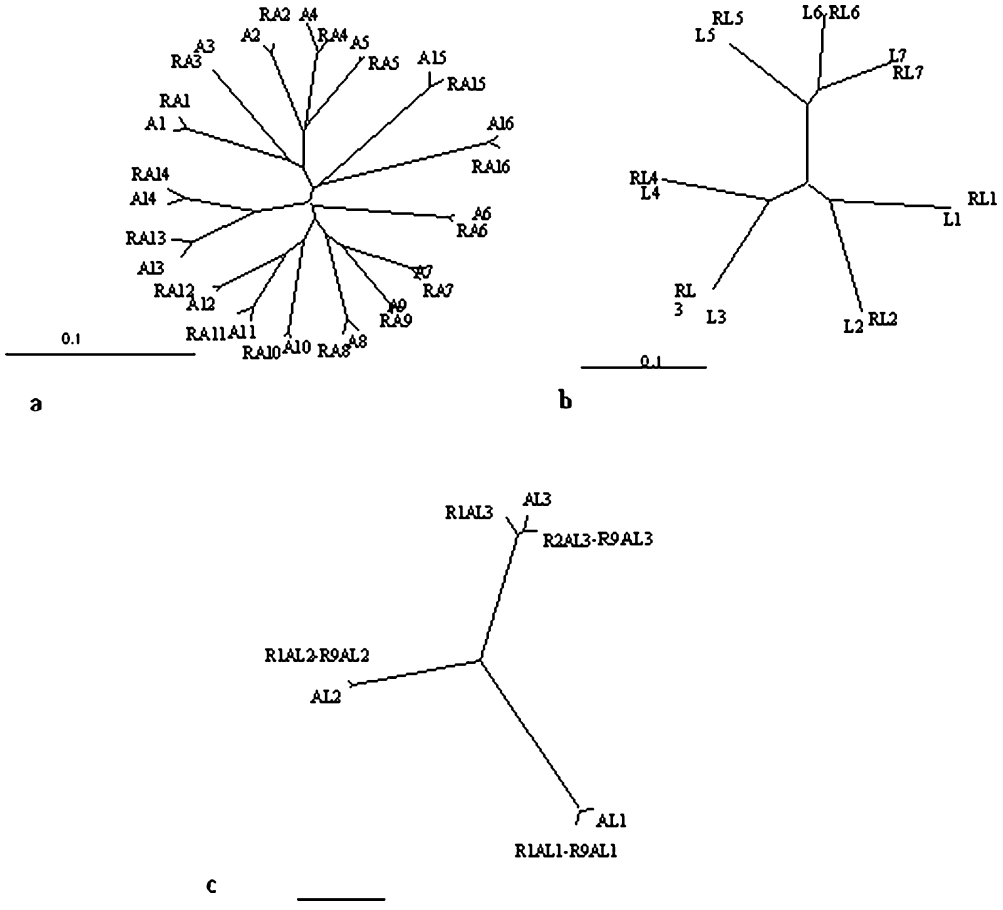


Fig. 3 UPGMA dendrograms based on Nei's coefficient. **a** Juvenile *C. atlantica* microcuttings (parentals, A1–A16; TC progenies, RA1–RA16); **b** juvenile *C. libani* microcuttings (parentals, L1–L16; TC progenies, RL1–RL16); and **c** adult *C. libani* microcuttings (parentals, AL1–AL3; TC progenies, R1AL1–R9AL1, R1AL2–R9AL2, R1AL3–R9AL3)

imum value of the coefficient of similarity with their respective TC progenies (Fig. 3b).

Molecular marker fingerprints are commonly not identical for two samples of the same clone (Douhovnikoff and Dodd 2003 and references therein). Thus, quantitative methods have been developed to determine the threshold of similarity that allows the identification of the clones (Douhovnikoff and Dodd 2003). In our case, we evaluated global genetic differentiation among mother plants and their TC progenies by performing AMOVAs based on the phenotypic distance matrices among RAPD markers. These analyses calculate significance levels for the components of genetic variance within and among populations (mother plants and their TC progenies in our case). An insignificant component of the variance among populations would indicate the absence of population differentiation. As shown in Table 3, variance components among populations were not significant, suggesting that total genetic RAPD differentiation between cedar mother plants and their TC progenies can be considered, therefore, as not significant.

Table 3 Summary of the AMOVA analyses for the differentiation among populations (mother plants and their TC progenies) in *C. libani* and *C. atlantica* cultures of different origin. Statistics include degrees of freedom (*df*), sum of squares (SSD), variance-component estimates (CV) and percentages of total variance (% Total) contributed by each component

Explant origin	Source of variation	*df*	SSD	CV	% Total
C. libani juvenile	Among populations	1	0.07	−3.3[a]	0.0
	Within populations	12	276.29	23.0	100.0
C. libani adult	Among populations	1	5.48	−2.5[b]	0.0
	Within populations	11	185.13	16.8	100.0
C. atlantica juvenile	Among populations	1	1.66	−0.8[c]	0.0
	Within populations	30	446.06	14.9	100.0

[a] P=0.91 (significance tests after 5,000 permutations)
[b] P=0.93 (significance tests after 5,000 permutations)
[c] P=0.99 (significance tests after 5,000 permutations)

Table 4 Methylcytosine content (mean±standard error) in microcuttings of *C. atlantica* and *C. libani* of different origin at the beginning of the experiment (T0) and after 12 months (T12) of culture. The Student's *t*-test was used to compare mean values of m^5C at T0 and T12. Data are mean values of 9–48 replications.

Microcutting origin	$M^5C/(m^5C+C)$		*t*-Student	
	T0	T12	*t*-Statistic	*P* value
Seedlings of *C. atlantica*	0.198±0.007	0.198±0.007	0.05	0.957NS
Seedlings of *C. libani*	0.195±0.013	0.172±0.009	1.75	0.088 NS
Adult trees of *C. libani*	0.223±0.010	0.167±0.013	3.30	0.002**

NS not significant for a confidence interval of 95%
**Significant for a confidence interval of 95%

Effect of in vitro shoot multiplication on 5-methylcytosine content in DNA of *C. atlantica* and *C. libani*

Table 4 summarises mean DNA methylation levels in microcutting from mother plants and their TC progenies. No significant differences in m^5C content were found among the three origins employed for culture establishment (ANOVA *F* value 1.94, *P*=0.15). The relative amounts of m^5C remained stable after the in vitro establishment of seedling-originated shoot stocks; in contrast, in vitro culture caused a significant hypomethylation in TC progenies from adult *C. libani* trees (Table 2).

We also studied changes in DNA methylation along with subcultures for each individual. Globally, there was no significant effect of the number of subcultures on the m^5C content in shoots of juvenile origin of either *C. atlantica* (*r*=−0.003, *P*=0.97) or *C. libani* (*r*=−0.15, *P*=0.20). Note, however, that the behaviour of some individual TC progenies was not homogeneous since subculture induced significant hypermethylation (progenies of genotypes L2, A1, A6, A9, A12 and A13) or hypomethylation (L1, L4, L5, A2–A5, A11 and A15).

The effect of subculture on DNA methylation in TC progenies of mature *C. libani* was clearly genotype dependent. As shown in Fig. 4, the m^5C content of TC progenies from genotypes AL1 and AL3 did not suffer significant fluctuations. In contrast, the subculture caused a significant DNA hypomethylation in TC progenies from genotype AL2. It is worth noting that the initial multiplication efficiency of genotype AL2 (as measured by the frequency of axillary bud breaking after 2 months of culture) was smaller than that of the genotypes AL1 and AL3 (sprouting percentages of 3.0, 7.6 and 9.3%, respectively). Subculture favoured the multiplication efficiency of the three genotypes. Within 6 months, sprouting percentages from microcuttings of genotype AL2 equalled those of genotypes AL1 and AL3 (70%), suggesting that DNA hypomethylation accompanied enhancement of the capacity of regrowth of this genotype.

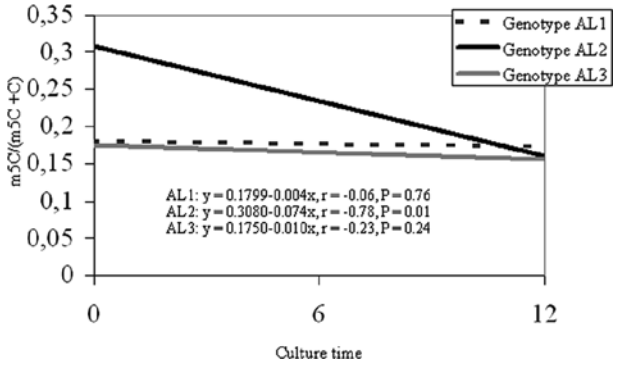


Fig. 4 Effect of the number of subcultures (culture time) on DNA methylation in adult *C. libani* microcuttings

Discussion

The incidence of somaclonal variation depends on the explant origin and regeneration method as well as the regenerant source (Ahuja 1998). It is generally assumed that micropropagation through axillary bud proliferation is the method that offers least risk of genetic instability since meristems are more resistant to genetic changes than disorganized tissues (Ahuja 1998; Rani and Raina 2000). Corroborating this, axillary bud breaking from juvenile *C. libani* microcuttings has demonstrated to be a good micropropagation system ensuring genetic stability of the regenerants (Piola et al. 1999). In this work, we extend this system to both juvenile *C. atlantica* and adult *C. libani* with the aim of defining rapid and accurate molecular techniques to detect possible somaclonal variation in their TC progenies. Due to the close genetic relationship between *C. atlantica* and *C. libani* (Fady et al. 2003), we first examined whether RAPD fingerprints could appropriately discriminate seedlings from both species. The high level of agreement among the different statistical approaches used for our RAPD data analysis demonstrates the suitability of these DNA markers to accurately discriminate *C. atlantica* and *C. libani*. Also, the capacity to fingerprint individuals within each species is particularly valuable for ecological studies involving the identification and characterisation of genotypes adapted to the environmental conditions of a particular habitat, an important strategy for the use of natural genetic diversity.

Results herein also demonstrate the suitability of RAPD markers to characterise and to determine the genetic stability of *C. atlantica* and *C. libani* TC progenies obtained through axillary bud proliferation. The detected RAPD variation was species-specific, with *C. libani* clones being more stable than those of *C. atlantica*. The slight variability

detected in our *C. libani* cultures of juvenile origin contrasts with the results reported by Piola et al. (1999) who did not find RAPD variation in 20 clones of *C. libani* coming from four seedlings. It should be mentioned, however, that these authors used different primers.

Irrespective of the microcutting origin, RAPD variation detected in our experiments was very low and the AMOVA results suggest that global genetic differentiation between mother plants and their TC progenies were not significant. Although the complexity of the biological systems makes any statistical treatment of somaclonal variation difficult (Côte et al. 2001), AMOVA is a sufficiently sensitive method to estimate the genetic differentiation among populations using RAPD markers (Nybom and Bartish 2000). Note, however, that an absence of RAPD polymorphism does not ensure the genetic stability of the in vitro regenerated plants (Fourrè et al. 1997).

The mean DNA methylation degree in *C. atlantica* seedlings (19.8%) and *C. libani* (19.5% in seedlings and 22.3% in mature trees) was comparable to that observed in other plant species, including conifers (Greenwood et al. 1989; Finnegan et al. 1998; Demeulemeester et al. 1999), but significantly lower than in *P. radiata*, where the average m^5C content in juvenile and mature needles was 35 and 60% respectively (Fraga et al. 2002).

Our in vitro culture system did not induce significant variations in global m^5C content in TC progenies of juvenile origin, but caused a significant hypomethylation in TC progenies of an adult *C. libani* genotype. This DNA demethylation event seems to be related to an enhancement of the regrowth capacity of this genotype. The relationship between the DNA methylation level and the in vitro morphogenetic capacity of the explants has also been observed in *P. radiata* (Fraga et al. 1999, 2002). In this species, all tissues, from juvenile or adult trees, which showed DNA methylation percentages above 60%, could not be adapted to in vitro conditions unless subjected to reinvigoration. It is well known that DNA methylation plays an important role in regulating plant development and organ or tissue differentiation (Lambé et al. 1997). Thus, DNA methylation changes associated with in vitro culture may be able to affect the expression of axillary branching genes. Nevertheless, further investigation is required to verify a direct relationship between DNA demethylation and the expression of these genes. In any case, our results suggest that m^5C content of adult *C. libani* may be a useful marker to estimate the in vitro performance of the species.

It has been suggested that DNA methylation may contribute to the generation of RAPD polymorphisms (Olmos et al. 2002). Although the m^5C content in genomic DNA of cedar TC progenies was genotype-dependent, it was not possible to establish a correlation between a particular hypomethylation (or hypermethylation) event and the detected RAPD variability. Also, neither RAPD nor m^5C variations were related to any detectable phenotypic differences between mother plants and their TC progenies.

To summarise, molecular and biochemical examinations were carried out to assess genetic variation and to detect possible changes in the DNA methylation status during in vitro culture of *C. atlantica* and *C. libani*. Excepting a hypomethylation in TC progenies of an adult *C. libani* genotype, the culture conditions used in our experiments did not induce remarkable effects on both RAPD variability and average content in m^5C in the species. Axillary bud proliferation seems to be a good micropropagation system for cedar ensuring the genetic stability of the regenerants. Finally, our results demonstrate that RAPD markers are very useful for discriminating *C. atlantica* and *C. libani* and identifying slightly different genotypes.

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