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Genetic stability of *in vitro* conserved germplasm of *Humulus lupulus* L.

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The genetic and epigenetic stability of hop accessions cryopreserved for one year or cold stored for three years was evaluated using several molecular markers (RAPD, AFLP, and MSAP). Clear, repetitive patterns were obtained among accessions and between control and treated samples. Although no genetic changes were detected among the control plants grown in the greenhouse and *in vitro* plants regenerated from slow-cooling cryopreserved shoot tips or cold stored *in vitro* shoots, MSAP analysis detected methylation changes in 36% of the *loci*. Nevertheless, only 2.6 to 9.8% of the detected changes could be ascribed to the conservation procedure and most of them seemed to be generated as a result of the *in vitro* introduction. Due to the number of accessions analysed (51) we can cautiously deduce that the genetic behaviour described in this work after cryopreservation or cold-storage protocols is common to most hop genotypes and these storage procedures are suitable for standard use. However, it is important to keep in mind the epigenetic changes produced, particularly during any *in vitro* processes.

Key-words: AFLP (Amplified Fragment Length Polymorphism), cold storage, cryopreservation, MSAP (Methylation Sensitive Amplified Polymorphism), RAPD (Random Amplified Polymorphic DNA), somaclonal variation.

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Introduction

Humulus lupulus L. is a dioecious climbing perennial of the Cannabaceae family indigenous to Europe, Asia, and North America. The female inflorescences (cones) are widely used to preserve beer and to give it a characteristic aroma and flavour. The appearance of downy mildew in the northern hemisphere has increased the need to breed new resistant varieties. Breeding programmes continue to focus on increasing hop alpha acid content and aroma profiles. Several cultivars were developed by crossing traditional European cultivars with wild plants from America, with the aim at combining the aroma qualities of the European varieties and the high yielding capacity of the American hops (Moir 2000).

It is extremely important to develop effective methods to store the many cultivars, as well as breeding lines and diverse wild material needed for developing new cultivars. Currently the most useful techniques to store plant material are refrigerated cold storage and cryopreservation at ultra-low temperatures, usually in liquid nitrogen. It is important to keep in mind the risk of genetic and epigenetic instabilities caused by any storage methods. Several factors associated with *in vitro* culture procedures, such as the medium composition (Viterbo et al. 1994), may result in somatic variation in cold stored plants. During cryopreservation, exposing tissues to physical, chemical and physiological stresses may result in cryoinjury, which ultimately may have effects at genome level (Harding 2004). Several molecular marker techniques are available to analyze genetic stability in plants. Two commonly used techniques are Amplified Fragment Length Polymorphism (AFLP) (Vos et al. 1995) and Random Amplified Polymorphic DNA (RAPD) (Williams et al. 1990). Epigenetic changes due to the stress generated during *in vitro* culture could possibly generate altered phenotypes in the recovered plants. This epigenetic instability is implicated in the timing of the DNA replication, in determination of chromatin structure, in increasing mutation frequency; as a causal agent for some human diseases; and as a basis for epigenetic phenomena (Finnegan et al. 1998). Methylation Sensitive Amplified Polymorphism technique (MSAP) can be used to identify methylation changes in anonymous CCGG regions using the isoschizomeric HpaII/MspI restriction enzymes.

The aim of this study was to evaluate the genetic stability of several hop accessions, representative of those growing around the world, in order to assess the efficacy of standard cryopreservation and/or cold storage protocols for the diverse cultivars and wild species in genebank collections.

Material and methods

Plant material

Hop accessions (51) representing wild hops, breeding lines, and cultivars supplied by the USDA, National Clonal Germplasm Repository (Corvallis, OR, USA) in February 2005 were analyzed. Each accession (cryopreserved or cold stored) included a control sample and at least two replicated sample treatments. Cold storage and cryopreservation were performed according Reed et al. (2003). Potted greenhouse-grown plants from the USDA core collection were used as control samples. Cold storage was applied for *in vitro* plants at 4 ºC with a 12-h photoperiod (10 $\mu molm^{-2}s^{-1}$) for one year. For cryopreservation, *in vitro* shoot tips were dissected from 2-week cold-acclimated plantlets and kept in liquid nitrogen (LN) for 3 years and regrown in the same *in vitro* conditions as the parent shoots for 4 months.

Molecular techniques

Altogether 51 hop accessions were analysed, seven accessions were cryopreserved and 36 were kept under cold storage conditions. In addition, samples of four accessions were independently conserved under both treatments. For each accession one control plant and at least two controls were analysed.

In total 169 samples were analysed. The RAPD reactions were carried out according to Pillay and Kenny (1996) with slight modifications. Twelve annealing temperatures were tested for each primer and the most appropriate annealing temperature was selected to avoid repetition problems and to increase the number of easy-scored DNA fragments. In all the tested primers, the highest temperature which produced the most suitable band profile was selected (see Table 1 for list of primers and annealing temperatures). In the AFLP assay the genetic stability of five cold-stored and five cryopreserved accessions was tested. We consider that these 10 selected accessions (Table 2) are representative of the total variation within *Humulus lupulus* as they include wild hops, females, males, diploid and triploids. AFLP analysis was performed according to Cervera et al. (1998). The primers used for the selective amplification were Eco AGC, AGA, AAC, and Mse CAT, CTT. A standard silver staining protocol was used to reveal the bands.

Three hop accessions representative of commercially cultivated hops were selected for the evaluation of methylation stability (Table 3). The MSAP analysis was performed following the general steps according to Cervera et al. (2002). For the selective amplification fluorochrome-labelled primers were used, HpaII/MspI +AAC, +ACT, +ACG (Applied Biosystem, CA, USA) combined with EcoRI +ACT, and AAT. Samples were electrophoresed in an automatic sequencer ABI PRISM® 3100 Genetic Analyzer (Applied Biosystem, CA, USA). The *loci* were considered polymorphic when differences in the presence/absence of bands in the EcoRI/MspI and EcoRI/HpaII patterns among the control and the treated samples were detected.

Results

Eleven RAPD primers were selected for the assay. A total of 125 *loci* were detected with a mean of 11 *loci* per primer, ranging from 0.3 kb to 1.5 kb. Nearly 20,000 bands were scored in the 169 analysed plants. There were clear differences in the banding patterns among accessions but no differences were detected between the control and the treated samples from the same accession, no matter which storage protocol was used (Fig. 1).

Table 1. List of primers selected for the RAPD assay, annealing temperature selected for each RAPD primer, and number of RAPD *loci* detected in the 51 hop accessions analysed.

Primer	Sequence	Annealing T°	Detected bands
OPA 01	CAGGCCCTTC	42 °C	12
OPA 02	TGCCGAGCTG	42 °C	11
OPA 03	AGTCAGCCAC	51 °C	10
OPA 04	AATCGGGCTG	40 °C	13
OPA 05	AGGGGTCTTG	44 °C	10
OPA 06	GGTCCCTGAC	45 °C	9
OPA 07	GAAACGGGTG	39 °C	11
OPA 08	GTGACGTAGG	42 °C	13
OPA 09	GGGTAACGCC	42 °C	15
OPA 10	GTGATCGCAG	40 °C	12
OPA 11	CAATCGCCGT	42 °C	9
Total			125
Mean			11.36±1.85

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Table 2. List of accessions analysed using AFLP, primer combination assayed, and number of DNA fragments detected.

	Primer combinations								
(*)Accessions	agc/cat	agc/ctt	aga/cat	aga/ctt	aac/ctt	acc/ cat	acc/ ctt	Total (1)	Mean (1)
USDA 21055 Cold (2)/Cryo (2)									
F 2n Oregon, USA -Breeding	74	72	60	70	78	70	74	498	71.1 ±5.6
USDA 62013 ×USDA 6616-35M									
USDA 21120 Cold (2)/Cryo (2)									
F 2n Oregon, USA -Breeding	69	67	62	72	86	70	74	500	71.4 ±7.5
19005 ×19046M									
Calicross Cold (2)/Cryo (2)									
F 2n New Zealand -Cultivar	71	75	65	78	86	70	74	519	74.1 ±6.7
California Cluster × Fuggle seedling									
Tardif de Bourgogne Cold (2)/Cryo (2)									
F 2n France Cultivar	62	71	61	80	73	70	74	491	70.1 ±6.7
Clonal selection landrace, Alsace									
Missouri 3 Cryo (4)									
F 2n Missouri, USA -Wild	70	70	66	79	74	70	74	503	71.8 ±4.2
Selected from wild in Missouri									
USDA 64033M Cold(2)									
M 2n Oregon, USA -Breeding	68	62	61	72	72	70	74	479	68.4 ±5.1
German female7k491 × OP									
Colorado 3-1 Cold (2)									
F 2n Colorado, USA -Wild	67	63	57	74	78	70	74	483	69 ±7.3
Selected from wild in Colorado									
Willamette Cold (2)									
F 3n Oregon, USA -Cultivar	64	68	61	71	82	70	74	490	70 ±6.8
USDA 21003 × Fuggle									
Vojvodina Cold (2)									
F 2n Yugoslavia -Cultivar	63	67	54	72	82	70	74	482	68.8 ±8.8
Northern Brewer × male									
Hallertauer Magnum Cold (2)									
F 2n Germany -Cultivar	65	65	59	74	83	70	74	490	70 ±7.9
Galena × German male 75/5/3									
Total (2)	673	680	606	742	794	700	740	4935	70.5 ±6.1
Mean (2)	67.3±3.8	68±4.1	60.6±3.5	74.2±3.5	79.4±5.2	70	74		

(1) Total and mean of bands per primer combination.
(2) Total and mean of bands per accession.
(*) Information supplied: Accession name. Treatment and number of treated samples (cold, cold storage; cryo, cryopreservation). Sex: F, female; M, male. Ploidy: 2n, diploid; 3n, triploid. Origin. State: breeding (line); wild; cultivar. Pedigree.

Table 3. Percentages of monomorphic and polymorphic MSAP *loci* detected in each cultivar. The polymorphic loci are arranged by behaviour category: variation present all analysed samples of both treatments, in all samples of one treatment (cryopreservation or cold storage), and present only in a single plant (no specific pattern of variation detected).

	Monomorphic *loci*	Polymorphic *loci*			
		In both treatments	Only in cryopreservated	Only cold stored	Singletons
USDA 21055	61.72	26.23	5.07	4.29	2.73
Calicross	63.3	26.59	2.66	2.66	4.78
Tardif de Bourgogne	52.6	23.7	9.82	8.67	5.19

In the AFLP analysis, clear and specific patterns were detected for each of the 10 accessions tested. The average number of *loci* per sample and primer combination was 70.5 ± 6.05 (Table 2), ranging in size from 130 bp to 450 bp. The maximum number of detected DNA fragments per accession and primer combination was 86 (EcoAAC/MseCTT, 'USDA 21120' and 'Calicross') and the minimum 54 (EcoAGA/MseCAT, 'Vojvodina'). Primer combinations EcoACC/MseCAT and ACC/CTT did not produce differing patterns among accessions. The most bands were detected in all the accessions with EcoAAC/Mse CTT (79.4±5.2) while the fewest was with AGA/ACT (60.0±3.5). Although nearly 19,000 bands were scored in total, no differences between the band patterns of the controls and the cryopreserved or cold stored samples were detected.

Six primer combinations were used in the MSAP analysis which produced a total of 617 clearly detected *loci*. Clear and repetitive peaks were detected with the automatic sequencer (Fig. 2). The mean number of observed *loci* per primer combination and cultivar was 34.94 and over 6200 bands were scored. For each accession, and in all the primer combinations tested, variations in the epigenetic profiles were detected between the treated samples and the control. The percentage of monomorphic *loci* detected in each accession was 61.7% ('USDA 21055'), 63.3% ('Calicross'), and 52.6% ('Tardif de Bourgogne') (Table 3). Each polymorphic *locus* was assigned to one of the following categories: polymorphism present in both treatments, exclusively in the cryopreserved samples, only in cold stored samples, and singleton (change present in just one plant under the same

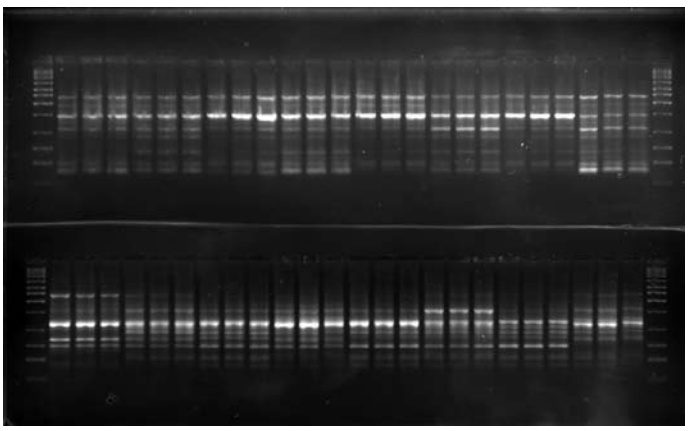

Fig. 1. Examples of RADP patterns detected in several hop accession with RAPD primer OPA 01. For each accession no differences were found between the control and treated samples (cold stored or cryopreserved). In each group of three: control, and two treated samples.

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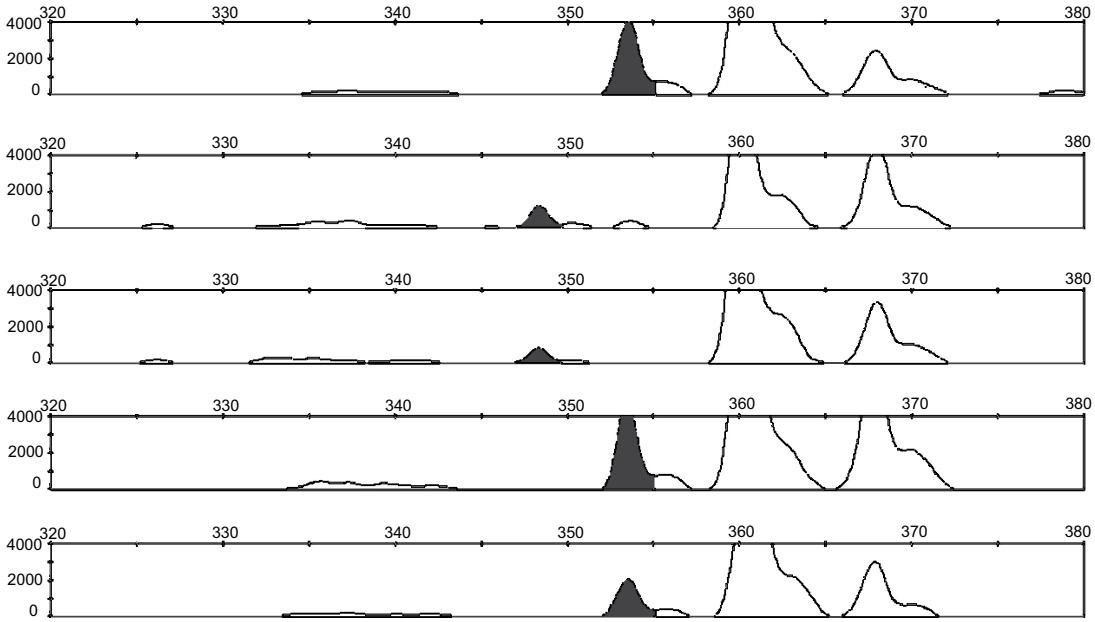

Fig. 2. Example of methylation changes detected with MSAP in accession 'Tardif de bourgone'. From top to bottom: control plant, two cryopreseved plants, two cold stored plants.

treatment). Most of the detected variation was shared by both treatments, ranging from 23.7% to 26.7% depending on the accession. The amount of variation that could be specifically related to either of the treatments was relatively low ranging from 2.6 to 9.8% of the total detected MSAP *loci* for the cryopreserved plants, and from 2.6 to 8.6% in cold-stored plants. The variation that could not be attributed to either or both of the treatments ranged from 2.7 to 5.19%.

Discussion

Cryopreservation and cold storage are two of the most appropriate techniques to conserve large collections of plants (Ashmore 1997). Both techniques involve *in vitro* culture manipulation of the stored tissues, that could be considered a potential risk for the generation of genetic instability (Brar and Jain 1998). An increasing number of studies indicate that plants recovered from cold storage or slow growth have no genetic alterations (Hao et al. 2004, Renau-Morata et al. 2006). This is also the case observed for these cold-stored hops; no RAPD or AFLP variation could be detected in any of analysed accessions. Similar data were obtained for cryopreserved hop accessions. No changes attributed to somaclonal variation were detected in the eleven accessions subjected to cryopreservation when analysed either with RAPD or AFLP. Based on these observations, it is reasonable to deduce that both cold storage and cryopreservation can be used for the routine storage of hops. Similar results are reported for other species in the current literature: no somaclonal variation was observed in cryopreserved apple (Hao et al. 2001, Lui et al. 2004), or grape and kiwi (Zhaj et al. 2003). However, it is important to note that somaclonal variation was reported in *Dendrathema grandiflora* (Martín and Gonzalez-Benito 2005), and *Hypericum perforatum* (Urbanová *et al*. 2006)

Epigenetic changes are a common cause for somaclonal variation, due to the stresses generated

during *in vitro* culture, and these changes could possibly generate altered phenotypes in the recovered plants. In our study, clear epigenetic changes were detected in each accession when compared to the potted plants used as controls. Over 26% of the detected MSAP *loci* shared some sort of modification after cold storage or cryopreservation. In any of the cultivars, the variation explained by the storage method itself was higher than the amount of variation shared by both treatments. This might be explained by the epigenetic changes related to physiological alterations produced by *in vitro* establishment. DNA methylation is a dynamic mechanism by means of which plasticity is induced by environmental and/or ontogenic signals (Ramchandani et al. 1999). Therefore, it is not surprising that there is a correlation between the physiological changes produced due to *in vitro* growth and epigenetic alterations detected in all the *in vitro* plants. Similar results were found in previous studies of *in vitro* establishment in hop in which nearly 30% of the detected MSAP *loci* were polymorphic (Peredo et al. 2009). However, as there are exclusive methylation changes in the cold-stored and cryopreserved plants, we can assume that each protocol is an additional source of epigenetic variation. Methylation changes were also reported in cryopreserved apple and strawberries (Hao et al. 2001 and 2002) and citrus callus under slow growth (Hao et al. 2004). Few studies on the genetic stability of hop plants are available. Patzak (2003) described an increased frequency of genetic changes after thermotherapy of *in vitro* hop meristems. MSAP polymorphisms were detected in hop plants regenerated from callus (Peredo et al. 2006).

In summary, methylation changes were detected in both cold stored and cryopreserved plants, although no genetic changes were identified using RAPD and AFLP. We can assume that the genotypes behave similarly when cryopreserved or cold stored as no major differences were found in a range of hop accessions. Both protocols are suitable for use as standard storage methods, but it is important to take into account that a considerable amount of epigenetic change can be induced, particularly during the *in vitro* process.

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