

Research article

AN EVALUATION OF A NEW APPROACH TO THE REGENERATION OF *Helichrysum italicum* (ROTH) G. DON, AND THE MOLECULAR CHARACTERIZATION OF THE VARIATION AMONG SETS OF DIFFERENTLY DERIVED REGENERANTS

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Abstract: A protocol for the induction of regeneration from leaves of *Helichrysum italicum* was established. Calli were found to form on the basal medium only when it was supplemented with thidiazuron (TDZ) alone or in combination with naphthalene acetic acid (NAA), with a percentage ranking of at least 80%. The hormone-free medium showed the highest percentage of shoot regeneration (62%) even though no callus formed. AFLP markers were employed to verify tissue culture-induced variation in the regenerated plantlets obtained by direct shoot regeneration or the indirect shoot regeneration process (callus formation). Seven out of the eleven AFLP primer pairs yielded polymorphic patterns. The average number of fragments per primer pair was 64.1. Singletons were represented by 12 (2.7%) fragments. Student's T-test was performed both on the average number of shared fragments and on the

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Abbreviations used: AFLP – amplified fragment length polymorphism; BM – basal medium; NAA – naphthalene acetic acid; RAPD – random amplified polymorphic DNA; RFLP – restriction fragment length polymorphism, SSR – simple sequence repeats; TDZ – thidiazuron

nucleotide diversity, and no significant statistical difference was observed between the two regeneration treatments.

Key words: *Helichrysum italicum*, *In vitro* culture, Tissue culture-induced variation, AFLP markers, Nucleotide diversity

INTRODUCTION

Creating genetic variability is vital to any plant breeding enterprise. Nowadays, genetic variability can be achieved not only via traditional breeding techniques, but also by means of several molecular and cellular laboratory techniques. For instance, plant cell culture is known to yield genetic variability, referred to as somaclonal variation [1, 2], and its potential has been employed to obtain valuable cultivars of different crop species [3]. However, when setting up a protocol to regenerate plantlets for industrial and commercial purposes, the primary goal is to keep specific desirable characteristics of the plant unaltered. Since tissue culture-induced variations can yield unwelcome phenomena, it is important to know the frequency, the genomic distribution, the mechanisms and the factors influencing those characteristics [4]. Somaclonal variation can be analysed at both the phenotypic and genotypic levels.

However, while phenotypic variations can easily be assessed using morphological characteristics or biochemical markers (e.g. storage protein levels in cereals and legumes), analyzing genotypic variations requires more appropriate tools, particularly considering that such variations do not necessarily lead to phenotype variations of agricultural value. The use of molecular markers can accomplish this task, and indeed they have been widely employed for the identification of somaclonal variants [5], with greater precision and less effort than karyological and phenotypic analyses [6, 7].

Several marker classes are employed, but each has some disadvantages. The RFLP technique requires large amounts of high quality DNA, and probe availability is also an issue. The RAPD technique has proven inconclusive in many cases due to its low reproducibility and its low potential of revealing tissue culture-induced variations [4]. By contrast, the AFLP technique allows the performance of reproducible analyses with reduced amounts of DNA, combining the peculiarities of RFLPs and PCR-based protocols and revealing a greater number of polymorphisms. This has made AFLP a powerful tool that has been employed in several studies of somaclonal variation: in oak [8], pecan trees [9], sugarcane [10], sunflower [11], and *Arabidopsis thaliana* [4]. Some authors [12, 13] compared the different types of molecular marker classes for discovering plant genetic variation, and all stated that AFLP consistently showed higher efficiency in detecting polymorphisms than RFLP, RAPD and SSR. Since SSR are not available for *H. italicum* and RFLPs have a low polymorphism level, AFLP is the best molecular tool for detecting tissue culture-induced variation.

The genus *Helichrysum* is a member of the family *Asteraceae*, and it includes over 500 species native to Africa, Asia, Australia and Europe. *H. italicum* (Roth)

G. Don is widespread in the Mediterranean area, where it grows as a small perennial shrub restricted to dry cliffs and open sandy soil habitats. The ornamental value conferred by its distinctive yellow scented flowers, and the properties of its essential oils contribute to its popularity. Many researchers have pointed out the anti-inflammatory, anti-histamine, antimicrobial and anti-viral properties of these essential oils, which are employed in various industrial sectors [14-18].

The scarce availability of cultivated materials, lack of agronomic practices and extreme variability of spontaneous ecotypes justify the application of *in vitro* cultures to set up a platform for rapid multiplication in this plant species. Although plant propagation via shoot regeneration has been achieved in a vast array of plant species, studies on *H. italicum* tissue culture are rather limited [19]. Interest in this species has strongly increased for cosmetic and pharmaceutical uses, but there is a lack of basic knowledge on its genetics and on the effect of *in vitro* culture on the determination of tissue culture-induced variation in *Helichrysum*.

In this study, we established a protocol for inducing plantlet regeneration from leaves of *H. italicum*. We tested two different approaches to regenerate *H. italicum*, and performed an AFLP molecular characterization of the variation in sets of differently derived regenerants.

MATERIALS AND METHODS

Plant material

An ecotype of *H. italicum* subsp. *Microphyllum*, collected in Porto Vecchio (Corsica Island) was first subcultured three times, and a set of 50 micro-propagated plants was derived from it. From each plant of this set, leaf fragments about 10 mm in length were used as the explant source.

Cultural conditions and measurements

For shoot regeneration, the leaf explants were cultured with their abaxial surface in contact with the regeneration induction medium, which consisted of basal medium (BM) without growth regulators or supplemented with 0.1, 0.5, 1 or 2 mg l⁻¹ thidiazuron (TDZ) alone or in combination with 1 mg l⁻¹ naphthalene acetic acid (NAA). The BM consisted of macronutrients and micronutrients, Fe-EDTA (30 mg l⁻¹), thiamine HCl (0.4 mg l⁻¹), myo-inositol (100 mg l⁻¹) [20], sucrose (30 g l⁻¹), and agar (7 g l⁻¹). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Explants were cultured in sterile Petri plates containing 20 ml of medium and incubated at 23°C under a light intensity of 60 μEs⁻¹m⁻² with a 16-h photoperiod. Five explants per plate and five replicate plates per treatment were studied. The number of explants forming calli and the response of the explants to the different treatments were scored after 30 days. Adventitious shoots and calli were separated from the leaf explants and transferred to sterile vessels containing

50 ml of the original medium. The number of shoots formed per callus was determined after 8 weeks' culture. The adventitious shoots developed were transferred onto a hormone-free medium and maintained under the same conditions until root formation. The plantlet height, percentage of rooting, and number and length of roots were evaluated after 60 days' culture.

Statistical analysis

The plantlet regeneration experiments were conducted under a complete randomized block design. The data were subjected to analysis of variance (ANOVA) and comparisons within and between the mean values of treatments were made using the Student Newman Keuls (SNK) test calculated at a confidence level of $P \leq 0.01$.

DNA extraction and AFLP protocol

We analyzed 39 regenerated plantlets, 20 of them (scored 1-d to 20-d) obtained without callus formation (via direct shoot regeneration) and 19 (scored 1-i to 19-i) obtained with callus formation (via indirect shoot regeneration). As a control, the original ecotype of *H. italicum* subsp. *Microphyllum* was included in the molecular analysis.

DNA extraction was performed on a total of 100 mg of fresh leaves from each of the 39 plantlets with a Gene Elute Plant kit (Sigma).

The AFLP analysis was conducted essentially as described in [21], with some modifications. Genomic DNA (150 ng) was double digested for 1 h at 37°C in a final volume of 40 µl with 20 units of *Pst*I and 10 units of *Mse*I (New England Biolabs), and 5x R/L restriction/ligation buffer (10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate). To this mixture, 10 µl of ligation mix was added, containing 50 pmol of double-stranded adapters for *Mse*I and 5 pmol adapters for *Pst*I, 3.5 units T_4 ligase (Invitrogen) and 5x R/L restriction/ligation buffer, and the ligation reaction was performed overnight at 15°C. The use of *Pst*I was preferred to other restriction enzymes, such as *Eco*RI, for its specific C-methylation sensitiveness.

Twenty five microliters of the resulting digestion-ligation mixture was used without any dilution for PCR pre-amplification by adding 10x PCR buffer Fermentas (160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris HCl, pH 8.8, 0.1% Tween 20), 25 mM MgCl_2 , 50 ng of primer *Mse*I (+1N) and 250 ng of primer *Pst*I (+1N), 10 mM of each dNTP, and 1.5 units of Taq DNA polymerase (Fermentas), in a final volume of 45 µl. The PCR thermal conditions were: 3 min at 94°C, 21 cycles of 30 s at 94°C, 1 min at 56°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C. A thermal cycler I-Cycler (Biorad) was used.

The pre-amplification products were first checked on 1% agarose gel. Subsequently, the samples were diluted 1:10 or 1:20 based on their initial concentration, in order to equalize all the concentrations. 4 µl of pre-amplification products were used as a template for selective amplification by adding 10 x PCR buffer (160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris HCl, pH 8.8, 0.1% Tween 20, Fermentas), 25 mM MgCl_2 , 96 ng primer *Mse*I (5'-GATGAGTCCT

GAG-TAA-3'+3N) and 16 ng primer *Pst*I (5'-AGACTGCGTACATGCAG-3'+3N), 10 mM dNTP each, 0.4 units Taq DNA polymerase (Fermentas), in a final volume of 10 μ l. *Pst*I primers were radiolabelled with γ -[33P]-ATP, and the following PCR conditions were used: 94°C for 3 min, 12 cycles of 30 s at 94°C, 30 s at 65°C (the annealing temperature was reduced every cycle by 0.7°C) and 1 min at 72°C. 23 additional cycles were then done at: 30 s at 94°C, 30 s at 56°C and 1 min at 72°C.

Selective PCR was performed in an MJR-PTC100 thermal cycler. As suggested in [4], all 64 selective primer pairs available from SIGMA Genosys were tested in order to select the 5 *Pst*I primers and 11 *Mse*I primers that gave the more informative set of primer pairs. The criteria for the selection of primers was to use the primer combinations that produced the maximum number of markers in order to facilitate the detection of marker losses. For this goal, 11 primer combinations with 3 selective nucleotides were used. Amplified fragments were separated by denaturing 5% (w/v) polyacrylamide gel electrophoresis (PAGE; OWL Separation System). Each AFLP procedure was reproduced at least twice for each DNA sample. The bands were visualised by autoradiography and scored manually for their presence or absence with two different operators. Only clear and unambiguous bands were considered, and weak or high and very low molecular weight bands were excluded from the analysis. According to the criteria of Bagley *et al.* [22], we applied the exclusion of almost 50% of the ambiguous polymorphic bands, obtaining 100% reproducibility.

AFLP data analysis

In order to verify the reproducibility of a selected amplification, we repeated the reaction twice for each primer combination. When no parameter was varied (PCR conditions, thermal cycler, modalities of DNA extraction), the profiles obtained were highly reproducible with all the primer combinations.

Selectively amplified DNA polymorphic amplicons from all the regenerated plantlets were used to create 7 matrices, one for each primer pair, where fragments were scored as either present (1) or absent (0). Finally, the 7 matrices were grouped in one single matrix.

Genetic similarity was calculated using the Jaccard index [23] and the Unweighted Pair Group Method using Arithmetic Averages (UPGMA) procedure was used for cluster analysis. A dendrogram was generated to present the phenetic relationships between the genotypes.

The nucleotide diversity between the regenerated plantlets was estimated using the method for AFLP data [24], and the average proportion of shared fragments was calculated by averaging pairwise comparisons between the regenerated plants according to the procedure in [25] and following the methodology described in [24].

RESULTS

Callus induction and shoot regeneration

The screened hormone combinations were fully effective for inducing callus formation in eight out of the nine experiments. Calli were produced on BM supplemented with TDZ at 0.1, 0.5, 1 or 2 mg l⁻¹ alone or in combination with NAA, and a percentage ranking of at least 80% was obtained (Fig. 1). No callus formation was observed on BM without growth regulators, but all the media supplemented with growth regulators induced callus production (Fig. 2).

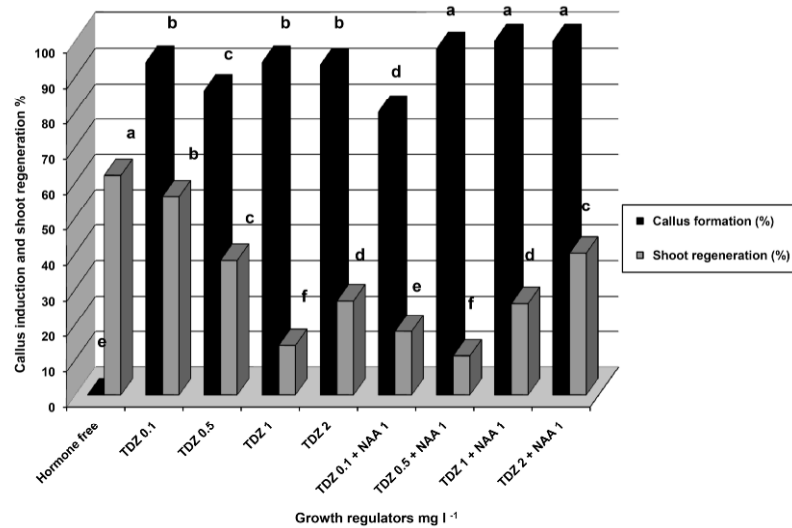


Fig. 1. The percentage of explants forming callus (callus production) and explants forming shoots (shoot regeneration) after 30 days of *in vitro* culture in *H. italicum*. Means followed by the same letters are not significantly different at the P = 0.01 level (SNK test).



Fig. 2. The direct (left) and indirect (right) regeneration on the leaves of *H. italicum* 90 days after induction on culture medium enriched with 0.1 mg l⁻¹ TDZ.

Furthermore, in three hormone combinations (TDZ 0.5 mg l⁻¹ + NAA 1 mg l⁻¹; TDZ 1 mg l⁻¹ + NAA 1 mg l⁻¹; TDZ 2 mg l⁻¹ + NAA 1 mg l⁻¹), the percentage of callus production differed significantly from that in the other combinations, with a 100% callus formation. Despite the absence of callus formation, the hormone-free medium showed the highest percentage of shoot regeneration (62%). The percentage of regeneration in the presence of growth regulators reached acceptable values only with the medium supplemented with the lowest concentration of TDZ. In general, the presence of TDZ alone inhibited shoot regeneration, but the simultaneous presence of NAA had an influence on the effect of the TDZ. If NAA was added to a low concentration of TDZ (0.1 or 0.5 mg l⁻¹), it had a negative regulatory effect, while if the same concentration of NAA was added to a high concentration of TDZ (1 or 2 mg l⁻¹), an opposite effect, like a strengthening of the TDZ efficiency, was observed.

Regeneration induction and adventitious shoot growth

The highest culture response in terms of the induction of regeneration occurred on the medium without growth regulators. An average of 2.73 shoots were produced per leaf explant at the end of the subculture (after 90 days) on the hormone-free medium through direct regeneration. The presence of TDZ or the combination of TDZ with NAA induced the regeneration of adventitious shoots from callus, but the shoot number was significantly lower (from 2.15 to 0 shoots/leaf explant; Fig. 3).

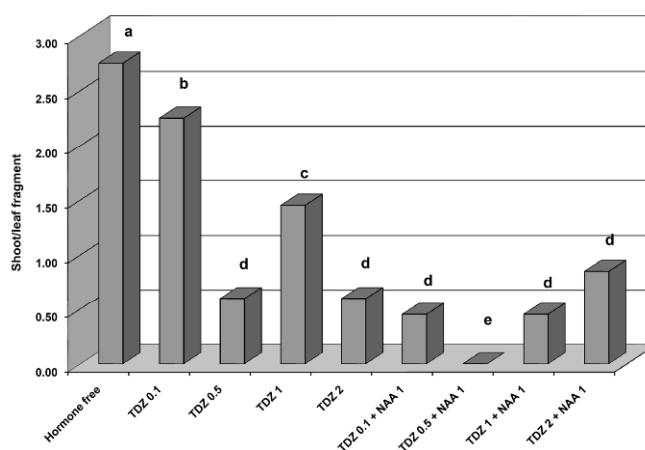


Fig. 3. The number of shoots per explant 90 days after regeneration induction in *H. italicum*. Means followed by the same letters are not significantly different at the P = 0.01 level (SNK Test).

When the calli were separated from primary leaf explants and transferred onto BM, the small green meristems developed into shoot buds. Leaf formation and shoot elongation occurred in the following 4 weeks and plantlets with an efficient root system were obtained by the end of 8 weeks of culture.

Shoots harvested directly from the leaf explants grew to an average height of 4.70 cm. Those obtained from calli reached heights from 2.44 cm (with 0.1 mg l⁻¹ TDZ) to 0.58 cm (with the combination of 0.1 mg l⁻¹ TDZ and 1 mg l⁻¹ NAA; Tab. 1). No significant differences were found between the treatments with growth regulators. The elongated shoots appeared normal and healthy, and no hyperhydricity was observed.

The percentage of rooting of elongated shoots varied significantly with regard to the composition of the induction medium. The maximum frequency (100%) of rooting, and the production of normal roots (21.79 roots/shoot with a mean length of 1.52 cm), was observed in the hormone-free medium. Although all the regenerated shoots yielded a complete plantlet ready for acclimatization, the hormone-free medium allowed better responses to be obtained both for shoot growth, root formation and root number.

Tab. 1. Adventitious shoot growth of *H. italicum* after 60 days in BM* culture.

Regeneration induction medium (mg l ⁻¹)	Shoot height (cm)	Root formation (%)	Root/shoot (n.)	Root length (cm)
Hormone free	4.7 a	100 a	21.8 a	1.5 bc
TDZ 0.1	2.4 b	46.1 d	10.2 b	2.0 b
TDZ 0.5	1.8 bc	56.3 c	11.6 b	1.8 bc
TDZ 1	0.7 c	30.7 e	4.9 b	1.7 bc
TDZ 2	0.5 c	29.4 e	4.4 b	1.5 bc
TDZ 0.1 + NAA 1	0.6 c	25.3 f	4.0 b	1.0 c
TDZ 1 + NAA 1	1.5 bc	23.5 f	8.7 b	3.0 a
TDZ 2 + NAA 1	1.1 bc	71.9 b	9.9 b	1.2 c

Means followed by the same letters are not significantly different at the P = 0.01 level (SNK test).

Genetic analysis

A total of 39 regenerated plantlets were divided into two groups depending on the regeneration method used to obtain them. Twenty plantlets were procured by direct regeneration, and 19 by indirect regeneration on an induction medium containing 0.1 mg l⁻¹ TDZ.

Seven out of the eleven primer pairs used for the tissue culture-induced variation analysis gave polymorphic patterns (Tab. 2). A total of 449 fragments were detected by AFLP in the 39 regenerated plantlets. The average number of fragments per primer pair was 64.1. The *Pst*I/AGG and *Mse*I/AAG combination gave the lowest number of fragments (45), while the highest number of fragments (81) was given by the *Pst*I/ACC and *Mse*I/ATG combination (Fig. 4). Considering only unambiguous and clear bands, 6.2% of the total scored

Tab. 2. Summary of the AFLP fragment variation in the two groups of regenerated plants analysed. The control plant fragments are comprised.

Primer pair	Number of scored fragments		Average number fragments per plant		Number of polymorphic fragments *			
	Total	Group	Group	Group	Total	Group		
<i>stI/MseI</i>		Direct	Indirect	Direct	Indirect	Direct	Indirect	
ACC/ATG	81	79	81	72.2	73.8	12	10 (0)	11 (2)
ACC/ATT	79	77	76	68.2	67.4	11	8 (5)	6 (2)
AGA/ATA	54	54	54	53.9	54.0	1	1 (1)	0 (0)
AGA/ATC	47	47	47	46.1	46.2	1	1 (0)	1 (0)
AGG/AAG	45	45	45	44.3	44.4	1	1 (0)	1 (0)
AGG/AGA	75	75	74	74.1	74.0	1	1 (1)	0
AGG/AGC	68	67	68	67.0	67.1	1	0	1 (1)
Total	449	444	445	60.8	61.0	28	22 (7)	20 (5)

*The number of singletons in parenthesis

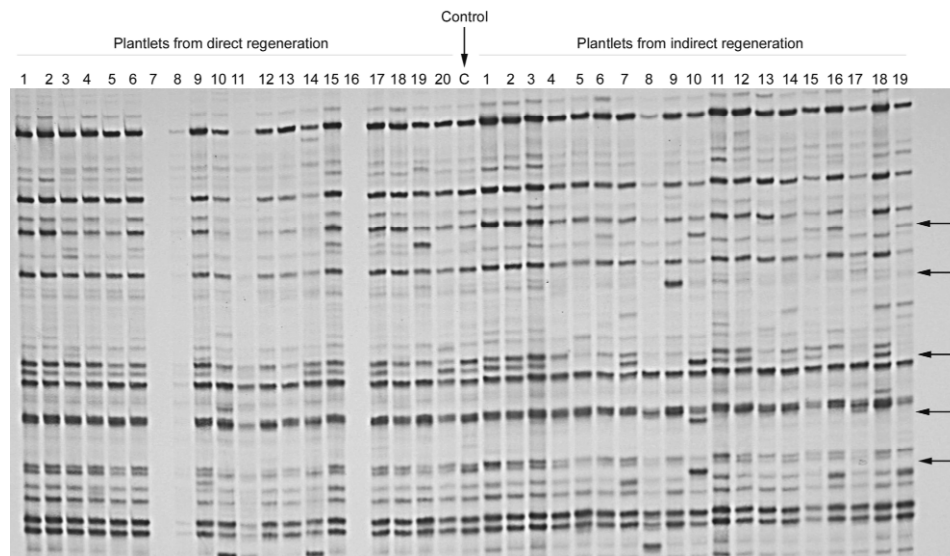


Fig. 4. The electrophoretic pattern of the AFLP primer pair ACC/ATG. The arrows indicate some polymorphic sequences.

fragments (28) were polymorphic among all the plantlets. Consequently, 93.8% of the total scored fragments were shared by all the regenerated plantlets, for a total of 421 bands. In particular, the variability between the two groups of regenerated plantlets was revealed by 14 of the 28 fragments, while the remaining 14 polymorphic fragments described the variability existing among the plantlets of the same group. 16 out of 28 polymorphic fragments (3.6% of

the total) were scored in more than one plant, while the remaining 12 fragments (2.7%) were singletons, fragments present or absent in a single plant from a single group: 9 (2.0%) presence singletons and 3 (0.7%) absence singletons. Two classes, each composed of 9 plantlets, were the most represented: the class with 3 changes (5 plantlets from direct and 4 from indirect regeneration), and the class with 4 changes (3 plantlets from direct and 6 from indirect regeneration) to a total of 46.2%. Seven was the maximum amount of changes recorded on 7.7% of the total number of plantlets (1 plant obtained via direct shoot regeneration and 2 via indirect shoot regeneration), while plantlet 6-d, obtained from direct regeneration, did not show any change relative to the control plant (Fig. 5).

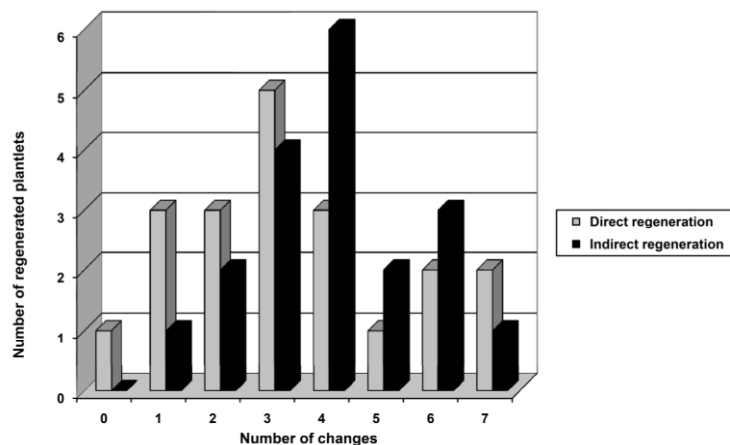


Fig. 5. The distribution of changes in the AFLP pattern among the 39 analysed regenerated plantlets.

The dendrogram obtained with AFLP data revealed an average genetic similarity of 0.55. (Fig. 6). 31 plantlets out of the entire set were unequivocally distinguished. Among the 20 plantlets obtained by direct regeneration, 3-d, 7-d and 15-d were indistinguishable from each other, similar to 5-d and 8-d. 6-d was identical to the control plant, and showed the same AFLP electrophoretic pattern as the control, for the primer pairs examined.

No variations in the banding pattern were observed between 24-i and 34-i, while all the other plantlets obtained by indirect regeneration emerged clearly distinguishable. A large cluster appeared at a similarity level of 0.12, with the exception of sample 16-d, which was apart from the group. At 0.43, a small cluster of two plantlets (24-i and 25-i) was parted from the rest of the samples. At 0.49, another cluster of 3 plantlets (10-d, 11-d, 27-i) was found, while at 0.55, a large cluster, including all the remaining samples, was found in the middle part of the figure, except for 39-i. The main cluster was divided into two sub-groups,

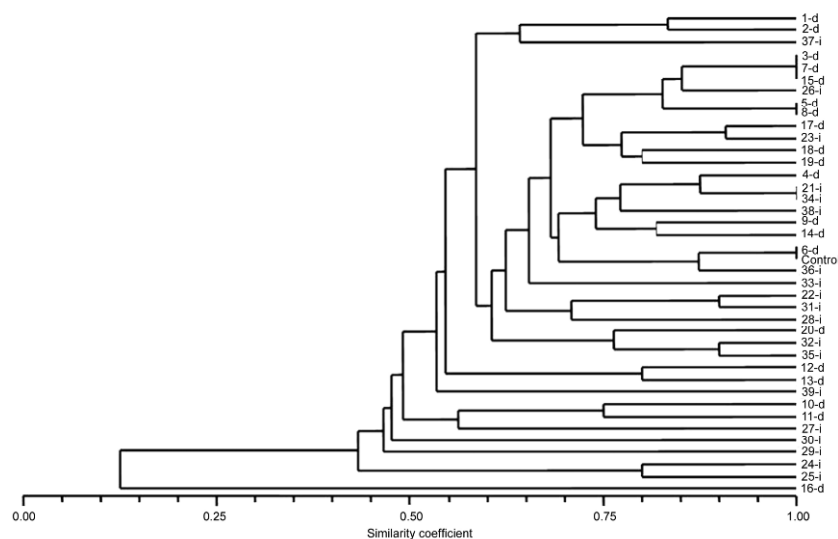


Fig. 6. The dendrogram obtained using the Jaccard index and UPGMA cluster analysis for the 39 regenerated plantlets with respect to the control plant.

Tab. 3. The average proportion of shared AFLP fragments and nucleotide diversity for the two groups of regenerated plants analysed.

Primer pair	Average proportion of shared fragments		Nucleotide diversity (x 1000)	
	Group		Group	
<i>PstI/MseI</i>	Direct	Indirect	Direct	Indirect
ACC/ATG	0.97635	0.97680	1.197	1.174
ACC/ATT	0.99394	0.99467	0.304	0.267
AGA/ATA	0.99866	1	0.067	0
AGA/ATC	0.99887	0.99668	0.057	0.166
AGG/AAG	0.99529	0.99444	0.236	0.279
AGG/AGA	0.99929	1	0.036	0
AGG/AGC	1	0.99918	0	0.041
Total	0.99450	0.99312	0.276	0.345

one of which included 1-d, 2-d and 37-i. The dendrogram evidenced the absence of a clear distinction between the two types of regeneration treatment. Finally, the dendrogram showed no particular clustering related to the type of regeneration method tested, so that the samples obtained from direct regeneration were mixed with those derived by indirect regeneration.

The *in vitro* culture itself was the leading factor that, in all samples, except in the case of plantlet 6-d, was responsible for the detected polymorphisms. Moreover, a genome-wide estimate of nucleotide diversity was obtained for each group of

regenerated plantlets (Tab. 3) using the method presented in [24]. The recorded values varied for the different primer pairs proportionally to the polymorphism detected: primer pairs showing a higher number of polymorphic fragments (i.e. *Pst*I/ACC and *Mse*I/ATG) also revealed higher nucleotide diversity values (0.001197 and 0.001174).

Although from the SNK test, the hormone-free medium and 0.1 mg/l BM treatments gave statistically different results, they are the nearest relative to the rest of the treatments performed. This consideration is probably reflected in the absence of a clear distinction between the two regeneration treatments in the dendrogram. Moreover, this aspect is confirmed by the non-significant result of the Student's T-test performed. Indeed, comparing the Student's T-test values of the two groups of regenerated plantlets, performed both on the average number of shared fragments and on the nucleotide diversity, the calculated values, respectively 1.14 and 0.17, were lower than the tabular value.

DISCUSSION

In this study, we investigated the *in vitro* culture system via direct and indirect shoot regeneration and the effects on tissue culture-induced variation in *H. italicum*. Don. Our results indicate that the composition of the medium culture is the key factor influencing direct shoot regeneration, and suggest that a hormone-free medium can lead to shoot regeneration without callus production. Therefore, growth regulators can be considered as not decisive for the regeneration of *H. italicum* plantlets. In any case, the presence of thidiazuron (TDZ) causes the callus production in *Helichrysum* as well as in other species [26, 27]. However, high TDZ concentrations tend to inhibit regeneration in *H. italicum* as previously reported by several authors [28-30]. The effect of TDZ on regeneration depends on the concentration and composition of the medium: the presence of NAA in the substrate inhibits the effect of low concentrations (0.1 or 0.5 mg l⁻¹) of TDZ, and has an opposite effect on higher concentrations (1 or 2 mg l⁻¹) of TDZ [31-33]. These results indicate that NAA increases callus formation in the presence of higher concentrations of TDZ. Increased efficiency in terms of the percentage of regeneration and the number of produced shoot explants was observed on BM without growth regulators. This result can be explained with the presence of predetermined cells which first dedifferentiate themselves, subsequently form new meristematic centres, and finally differentiate again producing new organs [34-39]. This process, due to cell totipotency, has been observed in several species [30, 40-47] and is very efficient for regeneration.

Our results showed that AFLP is a very sensitive and reliable molecular marker technique for revealing specific genomic alterations induced by tissue culture and for identifying slightly different genotypes. In addition, no differences were observed between the direct and the indirect *in vitro* regeneration treatments in terms of obtained plantlets and nucleotide diversity in *H. italicum*. This analysis

proved to be an efficient method for the detection of tissue culture-induced variation in regenerated plantlets of *H. italicum*.

AFLP analyses can be considered a powerful and reliable tool for the investigation of genetic polymorphisms in the genome of a species, especially if performed on an automatic sequencer with fluorescent-labelled primers. This could reduce the drawbacks due to the technical complexities of silver staining or the hazardousness of the radiolabelled substances.

Moreover, AFLP molecular markers can be successfully employed in plant species for which there is genetic knowledge, such as *H. italicum*. AFLP currently applied for molecular characterisation and genetic mapping strategies showed their potential in discovering tissue culture-induced variations in several species like *Quercus* spp. [8], *Carya illinoensis* [9, 48], *Syngonium podophyllum* [49], *Agave fourcroydes* Lem. [50], *Elaeis guineensis* [51] *Actinidia deliciosa* [52], and *Phoenix dactylifera* [53]. In *Arabidopsis*, 66.7% of the analysed regenerated plants showed at least one polymorphism [4], while for barley, the literature data is controversial, since Ruiz *et al.* [6] found that somaclonal variation did not appear to be a very frequent event, while Bednarek *et al.* [54] reported that all the regenerated plants showed at least one polymorphism with respect to the control plant. By contrast, our research revealed a high rate of changes among the plantlets, since 97.4% of them scored at least one polymorphic band relative to the control plant, even though the regenerated *H. italicum* plantlets showed a high identity, sharing on average more than 99.3% of the AFLP fragments. It can be argued that 12 out of the 28 polymorphic fragments were singletons, and that they contributed significantly to determining these values. Indeed, the percentage of singletons revealed by AFLP analysis in *H. italicum* was lower than in *Arabidopsis* [4], in which the percentage of presence singletons was 9.5% and absence singletons 1.3%. In addition to singletons, we observed several changes (3.6%) that affected the same locus in more than one regenerated plant. The presence of loci with a high rate of changes promoted by the *in vitro* conditions (the so-called "hypervariable sequences") has been reported for some species [55-57]. These authors hypothesised that independent events can occur in exactly the same sequences, and that hypervariable bands or their flanking sequences, represent hot spots of DNA instability. It is well known that changes caused by *in vitro* propagation could be represented by several events like single base changes, but also by alterations in the DNA methylation pattern [2]. As suggested by Matthes *et al.* [51], the AFLP protocol was modified by substituting the standard methylation-insensitive enzyme (*EcoRI* and *MseI*) with methylation-sensitive enzymes, such as *PstI*. This choice allowed us to analyse a wide sampling of the genome whilst targeting different polymorphisms, including those resulting from changes in methylation status. Hence, the differences revealed in this study could be due to methylation events rather than structural changes, which similarly can represent a source of instability in the genome of *Helichrysum italicum*.

To date, hardly any knowledge is available on the genomic structure of *H. italicum*, so an in-depth investigation on how AFLP markers are distributed along the genome would be of general interest. This analysis could give rise to the possibility of using these results on tissue culture-induced variation revealed in *H. italicum* to better focus on genomic peculiarities, similarly to what can currently be done for those species for which linkage maps or sequenced regions are available. In [4], it was reported that the estimated values of nucleotide diversity due to somaclonal variation were from a hundred to a thousand times smaller than the values obtained for natural variation between *A. thaliana* ecotypes. Therefore, future perspectives will focus on the application of AFLP markers to assess the level of nucleotide variation in *H. italicum* ecotypes in order to compare it with the one observed in regenerated plantlets.

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