SEQUENTIAL STUDY OF THE GENETIC STABILITY OF CALLUS AND REGENERATED SHOOTS IN CHRYSANTHEMUM

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Abstract

Callus and adventitious shoots were induced on chrysanthemum 'Red Reagan' leaves following a protocol with different steps in which several factors were studied: callus induction (two media and photoperiod/darkness), shoot regeneration (two media), and shoot elongation and rooting (three media). The influence of the different factors on the response observed and on the genetic stability was studied throughout the process. Genetic stability was assessed using RAPD markers. The identity of the samples was recorded throughout the entire process. The incubation conditions (photoperiod/darkness) did have a significant effect on the amount of callus produced. Shoot production in the regeneration phase was not influenced by the incubation conditions or media used in the previous step (callus induction) nor by the medium used to induce shoot development. Regarding the genetic stability study, all shoots regenerated showed identical RAPD marker profile to those of the pot mother plant (control) and to those of the *in vitro* shoots from which leaves were used as explants. However, 32% of the calli sampled after the callus induction step and 23% of those sampled after the shoot induction step were genetically different to the control. From the same explant, stable shoots and variant callus were obtained, which indicates a selection of cells that form organized growth during the process.

Keywords:, tissue culture, RAPD analysis, organogenesis, somaclonal variation.

Running title: Sequential analysis of genetic stability in regeneration

Introduction

Chrysanthemums are very popular cut flowers and ornamental pot plants of high economic value. *Chrysanthemum* ×*morifolium* Ramat (Asteraceae) has been bred for 3000 years in China and Japan. The species is ~~presents~~ self-incompatible and the current hybrids are not only heterozygous but also hexaploid~~ility problems~~; for this reason vegetative propagation is the main option. Additionally, obtaining new cultivars by crossing is difficult. New cultivars have traditionally been obtained from spontaneous mutations derived during ~~in~~ vegetative reproduction, sports, although in recent years, induced mutations and somaclonal variation derived from tissue culture process, alone or combined, have been employed as new variability sources (Broertjes et al. 1976, Khalid et al. 1989, Malaure et al. 1991, Schum 2003, Zalewska et al. 2007). Most recently, genetic transformation has contributed to obtain chrysanthemum plants containing genes of interest (Teixeira da Silva 2003).

Opmerking [SW1]: Such as…

~~Due to its self-incompatibility, different tissue culture protocols have been developed for the commercial production of chrysanthemum.~~ As a result of s~~S~~everal regeneration routes and source of explants ~~(stems, axillary buds, shoots, meristems, leaves, roots, anthers, florets, etc.) have been used in the development of new protocols (see reviews by Teixeira da Silva 2003; 2004). When tissue culture is employed, as it happens in commercial production of chrysanthemum,~~ genetic variation in the propagated plants may appear (Rout and Das 1997). In particular, tissue culture practices involving induction of callus cultures and subsequent regeneration of the whole plant have demonstrated a higher frequency of genetic variation than axillary shoot propagation ~~routes based on differentiated tissues~~ (Sharma et al. 2007, García et al. 2011, Takagi et al. 2011).

Chrysanthemum is generally considered as a crop with a typical ~~special~~ high frequency of somaclonal variation, due to the fact that many cultivars are periclinal chimeras. However, not all the cultivars are chimeras; furthermore, the origin and nature of the explant may also affect the occurrence of variation (Zalewska et al. 2007).

The importance of the genotype and the protocol used has been documented previously~~shown in previous works~~. The genetic analysis of commercial cultivars ('Red

Reagan' included) of chrysanthemum using RAPD markers has revealed a very high stability in the regenerated plants from shoot culture (Miñano et al. 2009). ; Hhowever, by using the same proliferation route and the same analysis method, very high variation frequencies were detected in other chrysanthemum cultivars (Martín et al. 2002). Likewise, genetic variation was detected in cv 'Pasodoble' regenerated plants derived from cryopreserved apices by an encapsulation-dehydration protocol (Martín and González-Benito 2005, Martín et al. 2011).

The goal of this study was to analyze the genetic stability of the regenerated plants obtained through a callogenesis process derived from a well known stable genotype of chrysanthemum, cv 'Red Reagan'. The effect of light conditions and plant growth regulators on the regeneration process and their possible implications in the genetic stability of the acclimatized plants were studied. Genetic stability was assessed using RAPD markers.

Materials and Methods

Plant material and culture conditions

In vitro-propagated shoots of *Chrysanthemum*–×*morifolium* Ramat, cultivar 'Red Reagan', were the starting material of the experiment. Those shoots had originally been initiated using buds excised from potted plants as explants, and micropropagated by monthly subcultures duringfor 13 months. The multiplication medium used was MS (Murashige and Skoog 1962) supplemented with 0.2 mgl^{-1} BA (6-benzylaminopurine) + 0.1 mgl^{-1} NAA (naphthalene acetic acid). Media were solidified with 7 gl^{-1} agar (Plant Agar, Duchefa Biochemie, The Netherlands) and 30 gl^{-1} sucrose were used as carbon source. The cultures were incubated at 25 ± 1 ºC with a 16 h photoperiod supplied by 50 µmol m^{-2} s^{-1} photon flux density fluorescent tubes.

Callus induction

Approximately 1 cm^2 of leaf explants from *in vitro* shoots were used for callus induction. The experimental design for this study was two factorial, with two levels for each factor: induction

media [1.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) + 0.23 mgl^{-1} BA, namely MC8 medium; or 0.5 mgl^{-1} 2,4-D + 0.23 mgl^{-1} BA + 2.0 mgl^{-1} indol acetic acid (IAA), MC9 medium] and light incubation conditions (16 h photoperiod with 50 µmol $m^{-2}s^{-1}$ irradiance or darkness). Both media had MS macro- and microelements and vitamins~~organic compounds~~. Explants were cultured in Petri dishes with the abaxial surface in contact with the medium. Sixteen explants were used per combination of medium and light condition. Percentage of survival and callus formation data were recorded after two weeks of culture. In order to quantify callus formation, explants were included in one of the following three categories: A, callus covering less than approximately half of the explant surface; B, callus covering approximately half of the explant surface; and, C, callus covering almost completely the explant surface. Samples were tracked and recorded throughout all the experiments.

Shoot regeneration

Following the callus induction period, explants were transferred to a regeneration medium in order to induce adventitious shoot formation. Explants were placed in Petri dishes containing MS medium supplemented with 2.0 mgl^{-1} IAA + 0.23 mgl^{-1} BA (medium MR1), or supplemented with 0.1 mgl^{-1} IAA + 0.23 mgl^{-1} BA (medium MR2). Eight explants cultured in each callus induction treatment were transferred to MR1 medium, and the other eight to MR2 medium. All cultures were maintained at 25 ± 1 ºC and 16 h photoperiod with 50 µmol $m^{-2}s^{-1}$ irradiance. After four weeks, regenerative response was evaluated according to the percentage of explants producing shoots, and the number of shoots per explant, which was evaluated according to these three categories: A, 1- 5 shoots per explant; B, 6 – 10 shoots per explant; C, more than 10 shoots per explant.

Shoot elongation, rooting and acclimatization to ex vitro conditions

Shoots with a length of at least 1.5 cm were isolated from the explants and transferred to glass jars containing 35 mL of one of the following elongation media: MS medium without growth regulators; MS supplemented with 2.0 mgl^{-1} kinetin (Kin) + 0.02 mgl^{-1} NAA + 10.0

113 mgl^{-1} gibberellic acid (GA_3); or MS supplemented with 0.1 mgl^{-1} BA + 2.0 mgl^{-1} IBA. Sucrose
114 concentration was reduced to 20 gl^{-1}, and incubation took place as indicated for shoot
115 regeneration, for a period of four weeks. Eighteen shoots were cultured per medium.
116 Percentage of rooted shoots on the three media was evaluated.
117 Shoot acclimatization was carried out according to a standard procedure established in our
118 laboratory for chrysanthemum: rooted plants were taken out of the culture jar, washed
119 carefully with running tap water to remove adhering medium and transferred to plastic pots
120 (4.5 cm diameter) containing vermiculite and commercial substrate (1:1). The plants were
121 kept in a mini-greenhouse for two weeks to maintain high humidity. After emergence of 2-4
122 new leaves, the plants were transplanted to potting mix and grown in a greenhouse to flower.
123 Survival percentages were evaluated after 15 and 35 days of *ex vitro* culture.
124
125 *Random amplification polymorphic DNA (RAPD) analysis*
126 Samples corresponding to the different steps of the regeneration process of *Chrysanthemum*
127 ×*morifolium* cultivar 'Red Reagan', from the callus induction to the regenerated plants in the
128 greenhouse, were evaluated using RAPD markers to assess their genetic stability.
129 For the evaluation during the callus induction phase, a small portion of callus was taken from
130 fourteen explants, from each of the four different treatments. They were stored at –80 ºC until
131 DNA isolation. Two different kinds of samples were collected from explants after the
132 regeneration step and stored at –80 ºC until DNA isolation: a portion of callus ('callus
133 sample') and a new leaf, or a portion of it, derived from a shoot produced in the callus ('shoot
134 sample'). Forty eight 'callus samples' were taken. Seventeen explants did not regenerate
135 shoots, but the other 31 explants did, and, therefore, their corresponding 'shoot samples'
136 were also included. The origin of all the samples (initial explant and treatments applied) was
137 recorded during the whole process. Additionally, leaf material from 26 acclimatized plants,
138 the pot mother plant and *in vitro* plantlets corresponding to the 13^{th} subculture (from which
139 callus induction was started) were collected for DNA extraction.

Total DNA was extracted from callus, shoots and regenerated plants after acclimatization as described by Gawel and Jarret (1991) with minor modifications. RAPD profiles were generated using four arbitrary primers from Operon Technologies: OPO-05 (5' CCCAGTCAC 3'), OPO-15 (5' TGGCGTCCTT 3'), OPO-18 (5' CTCGCTATCC 3'), and OPO-20 (5' ACACACGCTG 3'), as described in Miñano et al. (2009).

Data analysis

The statistical analyses were carried out using the GENMOD procedure of SAS programme (1990). Variables 'callus formation category' and 'shoot formation category ' were analysed using a proportional odds model for ordinal categorical data, and variables 'percentage of explants showing shoot formation', 'survival of acclimatized plants' and 'percentage of genetic variable samples' were analysed using a logistic regression method for binary data (Agresti 2002).

Amplified fragments from the RAPD analyses of the detected variable samples together with the mother plant (control) RAPD profile were scored as present (1) or absent (0). Genetic similarities were calculated using the Jaccard similarity coefficient (Jaccard 1908): $GS(ij) = a / (a + b + c)$; where a is the number of polymorphic DNA fragments common to both individuals, b is the number of fragments present in i and absent in j, and c is the number of fragments present in j and absent in i. The resultant matrix was subjected to cluster analysis by the unweighted pair-group method analysis (UPGMA) and a dendrogram was constructed from the clustering results with the TREE programme. These analyses were performed using the computer programme NTSYS-PC version 1.80 (Rohlf 1992).

Results

Callus induction

After seven days of culture, some initial calli could be observed in the four treatments studied and, after two weeks 100% survival rate was achieved. Green to pale-green morphogenetic

calli were induced on leaf explants within two weeks of culture on both tested media (MC8 and MC9; Fig. 1a). Callus formation was mostly medium to high (B and C responses) in all treatments; the combination of medium MC9 and darkness resulted in the highest callus production (Table 1). The most frequent category was the intermediate formation of callus (B), ranging from 75% of the explants (treatments MC8-photoperiod, and MC9-darkness), to 44% (treatment MC8-darkness). The analyses of medium and light effect on callus formation revealed that the media formulation did not have a significant effect (likelihood ratio statistic LR=0.03; degree of freedom df=1; p-value=0.857). However, the absence of light showed a significant effect (LR= 6.51; df=1; p-value=0.011) on the quantity of callus formed on the explants. Treatments under darkness showed a higher callus response: 94 % of the explants corresponded to categories B and C (Table 1). The interaction between media and light conditions was not significant (LR=0.3; df=1; p-value=0.584). At the end of the callus induction phase, every explant developed callus, however, only few of them showed clearly defined organized growth points, meristemoids (Fig. 1b).

Shoot regeneration

After 10-12 days under the regeneration conditions, small leaves expanded from the meristemoids previously observed (Fig. 1c). The percentage of explants that showed shoot formation after four weeks was not significantly different between the two media tested (69% in medium MR1 and 62% in medium MR2, LR=0.609, df=1, p-value=0.609; Table 2). Within each regeneration medium, an evaluation of the conditions under which explants were cultivated in the previous phase was carried out. The callus induction medium did not have a significant effect on the percentage of explants developing shoots in the regeneration phase (LR=0.0, df=1, p-value= 0.971). Nor were there significant differences between explants developed under dark or light conditions in the induction callus phase (LR=1.13, df=1, p-value= 0.287). Similar results were found in both regeneration media (MR1 and MR2).

The average number of shoots per explant (Table 2) was statistically analyzed according to the following variables: regeneration media, callus induction media and light conditions.

196 Neither the main effects nor interactions between them were significant. However, some
197 combinations of these factors produced a higher percentage of explants showing more than
198 10 shoots (category C). Only explants cultured on MR1 and previously under darkness
199 (regardless of the induction callus media), and on MR2 and previously in the induction callus
200 medium MC9 (regardless of the light conditions) showed a considerable percentage of
201 explants included in category C (> 10 shoots/explant).
202
203 *Shoot elongation, rooting and acclimatization to ex vitro conditions*
204 Adventitious shoots 1.5 to 2.0 cm long were transferred to each of the three elongation
205 media tested. Most of the shoots elongated and rooted after 30 days of culture (Fig. 2a),
206 except those explants cultured on MS medium supplemented with 0.1 mgl^{-1} BA + 2.0 mgl^{-1}
207 IBA, which resulted in a high callus formation at the base of the shoot, followed by general
208 necrosis. Therefore, no survival was recorded for this treatment. On the other hand, explants
209 maintained in MS medium without growth regulators showed 100% elongation and rooting.
210 Explants transferred to MS supplemented with 2.0 mgl^{-1} Kin + 0.02 mgl^{-1} NAA + 10.0 mgl^{-1}
211 GA_3 also showed 100% shoot development but lower root formation (65% rooting). Thirty-
212 five of the rooted plantlets were removed from the *in vitro* containers and acclimatized to *ex*
213 *vitro* conditions (Fig. 2b). After two weeks, survival rate reached high values (88%), which
214 decreased to 74% at the end of the 35 day period (Fig. 2c). In order to evaluate the factors
215 affecting the survival rate after the whole process, survival data at 15 and 35 days were
216 studied according to callus induction medium, light conditions during this stage, regeneration
217 medium and elongation medium. None of these factors, except the light conditions, showed
218 a significant effect on the survival rate after acclimatization. The statistical analysis for this
219 particular variable revealed a significant reduction in the percentage of survived plants when
220 the initial explants were cultured under darkness during the callus induction stage, regardless
221 of the media used during this phase. The survival reduction was significant after 15 days of
222 acclimatization (LR=6.33, df=1, p-value=0.045), and the difference between the two
223 treatments (photoperiod/darkness) increased at the end of the acclimatization (LR=8.67,

df=1, p-value=0.007, Table 3). Surviving plants were satisfactorily grown in the greenhouse until flowering (Fig. 2d).

Random amplification polymorphic DNA (RAPD) analysis

DNA samples from the different steps of the regeneration process of chrysanthemum plants cv 'Red Reagan' were taken for their subsequent genetic analyses using RAPD markers. The origin and treatment of each explant during the different stages of the process were carefully anotated. A total number of 50 scorable markers were obtained from the RAPD amplifications carried out with the four primers selected for this analysis. The number of fragments per primer produced ranged from 10 to 15, and their size from 300 to 1500 bp. In all the amplifications carried out, the RAPD profile was identical for both controls, pot mother plant and *in vitro*-plants. Compared to the controls, seventeen of the RAPD fragments obtained resulted in a different pattern at least for one of the analyzed samples. Some variations corresponded to new markers that appeared in some samples and were not present in the controls, while other markers, detected in the controls, were not present in the variable samples (absent markers; Table 4).

A total number of 56 samples (14 from each treatment) of the first step of the process, callus induction, were analyzed. Between 28.6 and 35.7 % of these samples, within each of the four treatments, showed a different RAPD pattern compared to the controls (Table 5, Fig. 3). In those variable samples the number of polymorphic fragments ranged from 11 to 17. No significant differences were detected in the percentage of variation found for the different treatments. None of them (media composition or light conditions) significantly influenced the percentage of genetic instability in the callus induction process.

In the study of the genetic stability during shoot regeneration, two different kinds of samples were available: callus and shoots produced on the same explant. An analysis of the percentage of variation according to the regeneration medium used was carried out (Table 5). The analysis revealed that no significant differences in the degree of stability in the samples could be attributed to the media composition. Interestingly, the data on the genetic

252 stability of the shoot samples showed 100% fidelity (compared to the controls), while callus
253 samples from the same explants showed a considerable level of variation (20-26%; Table 5).
254 The statistical analysis did not show any effect of the media composition and the light
255 condition during the previous callus induction phase on the degree of stability after the shoot
256 regeneration step. Furthermore, as was expected, there was a significant association
257 between the variability found in the callus samples of the regeneration phase and the
258 variability in the previous step. Therefore, the instability detected in some calli during the
259 callus induction step remained in the calli obtained from the same explants when analyzed
260 after the shoot regeneration step; however, none of the shoots analyzed, developed from the
261 same explant, showed variation.
262 In order to find a pattern of the variability detected throughout the process, the variable
263 samples (all corresponding to callus tissue, from the callus induction phase or the
264 regeneration phase) were analyzed by a clustering method (UPGMA) and graphycally
265 presented in a dendrogram (Fig. 4). A control sample (MP) representing the RAPD profile of
266 the pot mother plant, the *in vitro* control and the genetic stable samples was included in the
267 analyses. The dendrogram shows that all the variable samples have a similarity coefficient of
268 72% with the control samples, and the minimum similarity between them was near 90%. Ten
269 different genotypes were discernable in the dendrogram, one of them being the stable
270 genotype (control). Thirteen of the variable samples shared the same RAPD profile, but no
271 relationship with the treatments or origin was found. However, most of the clusters contained
272 samples with a common origin (the same final code letter) which could indicate a variation
273 during the callus induction period that was maintained throughout the regeneration process.
274 When regenerated plants were acclimatized and established in a greenhouse, samples from
275 26 individuals were analyzed. All of them showed a 100% morphological and genetic fidelity
276 to controls.
277
278 **Discussion**

Chrysanthemum is one of the most relevant ornamental crops (cut flower and pot plant), which is produced mainly through vegetative propagation. In this context, tissue culture techniques play an important role and, therefore, different regeneration protocols (via direct or indirect organogenesis) have been developed for chrysanthemum production (Rout and Daas 1997).

Plant regeneration from callus culture in *Chrysanthemum ×morifolium* cv 'Red Reagan' has been evaluated in this work using different callus induction and regeneration media; dark and photoperiod conditions have also been studied during the callus induction phase. Implications of the different culture conditions on the final results have being considered, mainly from the genetic stability point of view.

Different molecular markers have been used to assess the genetic stability of many plant species regenerated from tissue culture , e.g. RAPD (Martín et al. 2011, Carra et al. 2012, Dafadar et al. 2012), AFLP (Martín et al. 2011) and SSR (Rodriguez-Lopez et al. 2010). Genetic instability (somaclonal variation) is a common phenomenon associated to tissue culture (Jain et al. 1998); however, the frequency of detected changes varies among species and culture conditions. Furthermore, the type of explants, age of the culture, genotype, culture conditions and method of plant regeneration are factors that influence the genetic stability of the regenerated plants (Rout et al. 2006).

Many chrysanthemum cultivars are periclinal chimeras, and chimerism has been related to the generation of genetic instability (Stewart and Dermen 1970, Bush et al. 1976). For this reason, high frequency of somaclonal variation in chrysanthemum is not a rare phenomenon. However, not all the cultivars are periclinal chimeras, and some of them show a very high genetic stability in tissue culture processes, as is the case of cultivar 'Red Reagan'. Regenerants of cv 'Red Reagan' obtained from shoot micropropagation showed a high genetic stability throughout several culture cycles, since no variation was detected using RAPD markers (Miñano et al. 2009).

Not only the genotype but the explant source may play an important role in the final genetic stability obtained through the regeneration process. This stability may be lost when

307 regeneration is obtained through a callus phase. It is a well-known fact that callus
308 regenerants are prone to genetic instability due to dedifferentiation followed by a
309 redifferentiation process during which genetic modifications are frequent (Munthali et al.
310 1996, Al-Zahim et al. 1999, Salvi et al. 2001).
311 In this work, we have studied the relationship of callus induction conditions (darkness /plant
312 growth regulators) with the regeneration capability and the genetic stability. A high
313 regeneration rate may be originated by a rapid cell division cycle which could be the cause of
314 genetic variations. According to Yeoman (1970), the initiation and development of calli
315 involve vigorous cell division, and different plant growth regulators can be used to control the
316 process.
317 In relation to the effect of light, we have observed that darkness significantly improved the
318 quantity of callus during the callus induction phase, although it had no significant effect in the
319 morphogenesis or the genetic stability of cultures. The stimulation of callus formation and
320 growth in darkness is a frequent procedure in tissue culture (George and Sherrington 1984),
321 but may not necessarily affect the morphogenesis process. Kulpa (2011) studied different
322 culture conditions (type of explant, growth regulators and light) on the organogenesis of
323 chrysanthemum inflorescences, finding that light/darkness conditions had no significant
324 effect on the course of morphogenesis. On the other hand, other previous studies support
325 the idea that photoperiod might affect the regeneration frequency or regeneration pattern
326 (direct or indirect). Additionally, Almeida et al. (2003) working with *Citrus sinensis* found that
327 direct organogenesis was achieved only when cultures were maintained under photoperiod
328 conditions; under darkness the response obtained was callus formation and indirect
329 organogenesis. Also working with *C. sinensis*, Khan et al. (2009) did not find any effect of the
330 light conditions on the regeneration response in direct organogenesis. Likewise, these
331 authors carried out a genetic stability analysis using RAPD markers, and, similar to our
332 findings, none of the regenerants showed variation.
333 Genetic stability analyses usually focus on the final product, i.e. the regenerated plants, since
334 obtaining true-to-type plants is the main objective. Some studies address the genetic stability

analyses of different type of tissues when diverse regeneration routes are to be tested. The analyses of calli and adventitious shoots, from different organs, and regenerated plants were carried on in *Saussurea involucrate* using RAPD and ISSR markers (Yuan et al. 2009). Significant differences were detected in calli samples and adventitious shoots when compared to the original material. The interpretation of these results is difficult since DNA samples were obtained from a pool of different cultures. Calli samples showed the highest differences from all the material analyzed, and the level of difference was slightly higher than the values obtained in our work. Contrary to our results, they observed differences in the adventitious shoots, although the variation found in shoots was not as high as in the calli from which they derived. As has been mentioned, these differences could be partly explained by the fact that the analyzed samples were a pool of cultures.

The sequential analysis of the genetic stability of chrysanthemum cv 'Red Reagan' throughout the indirect organogenesis process (callus induction, shoot regeneration, acclimatization) has established that the genetic instability detected in this work is not related to the different conditions (darkness and media composition) tested, but instead depends on the type of tissue analyzed. Variant samples appeared in some calli during the callus induction phase (28.6 – 35.7 % depending on the treatment) and in callus tissue of the same samples in the next step of the process, shoot regeneration (20 – 26 %). However, no variable samples were found in the organised tissue of shoots obtained from those calli. This response could be due to the formation of a mixture of stable and variant cells during the callus formation in some samples. In the next step of the organogenesis process, when shoots are formed from the callus, only those cells without variation seem to be able to regenerate organised growth. Those variable cells remained as callus or died. In his review, Yeoman (1970) reported different examples of heterogeneous calli like mixture of cells of different ploidies which, adjusting the composition of the medium, was possible to drive to the proliferation of cells with one ploidy. In chrysanthemum, Rout and Das (1997) also reported a mixed organogenic response that was explained as a cellular heterogeneity present in the initial explant. This initial heterogeneity does not seem to be the origin of callus variation in

the highly stable genotype cv ‘Red Reagan’, since all the controls and the previous work (Miñano et al. 2009) revealed great stability.

Since only a sample of the callus is taken for the genetic analysis together with the dominant character of the molecular markers employed, RAPDs, it does not seem possible to obtain a complete representation of the cell mixture originating the callus. Therefore, depending on the proportion of variable cells contained in the sample it should be possible to detect the variation. The fact that shoots and acclimatized samples showed a stable profile could be explained by an origin of these structures from stable cells contained in the callus instead of from the variable ones.

Further studies are necessary to formulate an explanation on the possible mechanism involved in this selective response. Changes affecting the loss of organogenesis potential and acquisition of dedifferentiation have been related to DNA methylation variations in sugar beet (Maury et al. 2012). Epigenetic changes could be implicated in the development of only stable shoots observed in chrysanthemum cv ‘Red Reagan’, and, according to Kaeppler et al. (2000) the epigenetic changes should be associated to genetic alterations, as it has been detected in this work in callus tissue. Additional analyses to understand the mechanisms that could be blocking the organogenesis from variable cells of the callus, including epigenetic studies, are needed.

In conclusion, the results obtained in this work show that the regeneration of stable plants from indirect organogenesis in chrysanthemum is possible when stable cultivars as ‘Red Reagan’ are used, even when the calli, from which those shoots were obtained, were genetically instable.

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Table 1. Evaluation of callus formation in chrysanthemum leaf explants, cultivar 'Red Reagan', after a two-week culture period, and significance of the effects according to a proportional odds model for ordinal categorical data. Quantity of callus has been evaluated in three categories according to the explant surface covered by callus (A, B, and C, see text). (MC8: MS + 0.5 mgl^{-1} 2,4-D and 2.0 mgl^{-1} IAA; MC9: MS + 0.5 mgl^{-1} 2,4-D + 0.23 mgl^{-1} BA + 2.0 mgl^{-1} IAA).

		Callus formation categories (number and percentage of explants)		
		A	**B**	**C**
Treatment	**MC8- photoperiod**	3 (19 %)	12 (75 %)	1 (6 %)
	MC8- darkness	2 (12 %)	7 (44 %)	7 (44 %)
	MC9- photoperiod	4 (25 %)	9 (56 %)	3 (19%)
	MC9- darkness	0	12 (75 %)	4 (25%)

Effect	***Likelihood ratio (LR)***	***df***	***p value***
Induction medium	*0.03*	*1*	*0.857*
Light incubation	*6.51*	*1*	*0.011*
Induction medium x light incubation	*0.3*	*1*	*0.584*

503 **Table 2.** Effect of the regeneration medium (MR1 and MR2) on the percentage of explants
504 producing shoots, and the average number of shoots per explant after 30 days in culture.
505 (MR1: MS + 2.0 mgl^{-1} IAA + 0.23 mgl^{-1} BA; MR2: MS + 0.1 mgl^{-1} IAA + 0.23 mgl^{-1} BA).
506 Categories for classification of number of shoots per explant: A (1-5 shoots/explant), B (6-10
507 shoots/explant), C (>10 shoots/explant).
508

Regeneration medium	***Callus Induction conditions***	***% explants with shoots[a]***	***Shoot formation categories (%)[b]***		
			A	**B**	**C**
MR1	MC8 Light	62	40	60	-
	MC8 Dark	75	50	17	33
	MC9 Light	62	100	-	-
	MC9 Dark	75	50	33	17
MR2	MC8 Light	62	40	60	-
	MC8 Dark	62	60	40	-
	MC9 Light	50	50	25	25
	MC9 Dark	75	33	50	17

509
510 [a]: *No significant differences according to the logistic regression method for binary data.*
511 [b]: *No significant differences according to a proportional odds model for ordinal categorical data.*
512
513
514 **Table 3.** Survival percentages of chrysanthemum regenerated plants after 15 and 35 days of
515 acclimatization according to the illumination conditions during the callus induction phase.
516 Means with the same letter are not significantly different ($P > 0.05$) according to the logistic
517 regression method for binary data, within each culture time.
518

Survival (%)			
15 days		35 days	
Light	Darkness	Light	Darkness
100.0 a	76.5 b	94.4 a	52.9 b

519
520
521

Table 4. Variable markers obtained in at least one sample of the amplifications with the four primers tested. Markers are named with the name of the primer followed by the size of the amplified fragment in base pairs (bp).

Primer	New markers	Absent markers
OPO-05	O5-1150 bp	O5-1200 bp
		O5-1100 bp
OPO-15	O15-920 bp	O15-900 bp
	O15-880 bp	
OPO-18	O18- 980bp	O18-950 bp
	O18-850 bp	O18-800 bp
		O18-500 bp
OPO-20	O20-1150 bp	O20-1200 bp
	O20-980 bp	O20-900 bp
	O20-850 bp	O20-820 bp
Total variable markers	8	9

Table 5. Number of samples analyzed through molecular markers (RAPD) during the regeneration protocol of chrysanthemum cv 'Red Reagan' and percentage of stable samples. For media composition see text; L, photoperiod; D, darkness.

	Callus induction				Shoot regeneration			
					callus sample		shoot sample	
Medium/ incubation conditions	$MC8_L$	$MC8_D$	$MC9_L$	$MC9_D$	MR1	MR2	MR1	MR2
Nº analyzed samples	14	14	14	14	25	23	17	14
% stable samples[a]	71.4	64.3	64.3	71.4	80	74	100	100

[a]*: No significant differences according to the logistic regression method for binary data within each propagation phase and type of sample.*

Legends

Fig. 1.- Different stages of the organogenesis process in *Chrysanthemum ×morifolium* 'Red Reagan': **a)** morphogenic callus formation on leaf explant on MC9 medium after 2-week culture period in darkness; **b)** meristemoids formation on MC8 medium after 2-week culture period in light; **c)** adventitious shoots regenerated after a 15 day culture on MR2 medium; **d)** elongated shoots developed on MR1 medium after four-week culture period. Line: 2 mm.

Fig. 2.- Elongation and acclimatization of 'Red Reagan' chrysanthemum plants: **a**) shoot elongated and rooted on MS medium containing 2.0 mgl^{-1} Kin, 0.02 mgl^{-1} NAA and 10.0 mgl^{-1} GA_3; **b**) shoots elongated and rooted on MS medium ready to be transferred to *ex vitro* conditions; **c)** plants after 30 day acclimation; **d**) flowering plants after 6 months.

Fig. 3.- RAPD profile obtained with the primer OPO-05 in samples corresponding to callus (from the callus induction period) and callus tissue from explants in regeneration cultures. M= DNA molecular size marker; MP = control mother plant; iv-13 = sample of the explant corresponding to the 13^{th} subculture control; MC8, MC9: callus induction media; MR1, MR2: shoot regeneration media; l: photoperiod; d: darkness; g-p: sample code. Arrows: new and absent markers.

Fig. 4.- Dendrogram generated by the UPGMA method using Jaccard's similarity coefficient, based on RAPD markers of the samples from the regeneration process in different culture conditions of *Chrysanthemum ×morifolium* 'Red Reagan'. MP: mother plant; MC8, MC9: callus induction media; MR1, MR2: shoot regeneration media; l: photoperiod; d: darkness; g-p: sample code.