In vitro leaf-derived organogenesis and somaclonal variant detection in Humulus lupulus L

In vitro leaf-derived organogenesis and somaclonal variant detection in *Humulus lupulus* L.

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Abstract

The exploitation of somaclonal variation potentially could be a valid strategy to overcome the depletion of hop intraspecific agrobiodiversity. To increase somaclonal variation induction, it is possible to resort to several strategies including a differentiated starting explant material such as leaves, roots and stems, an extended time in which cultures are maintained in vitro, and a well-balanced cytokinin/auxin ratio. In this research, firstly, the influence of growth regulator type and concentration and the effect of the period of *in vitro* hop leaf culture (6, 12 and 18 wk) were investigated. Secondly, cytofluorimetric and RAPD analysis were carried out to verify the occurrence of somaclonal variation. Adventitious shoots were obtained in all media containing 6-Benzylaminopurine (BAP) (except BAP at lowest concentration tested), with no influence detected by culture period. Mutants were detected among regenerants (16.8%) with more than half of the tetraploids obtained from medium containing the highest BAP concentration (35.55mM). Mutants detected by Randomly Amplified Polymorphic DNA (RAPD) analysis were independent of the medium composition and time in culture. A strong influence regarding explant was observed where nearly half of mutants obtained originated from cultured leaf tissues. Further studies are needed to characterize the field performance of mutants.

Keywords: Adventitious shoots regeneration, flow cytometry, hop, RAPD, somaclonal variation.

Introduction

Hop (*Humulus lupulus* L.) is a dioecious plant, cultivated mainly for its female inflorescences, used to add bitterness, aroma and flavo~~u~~r to beer. In recent years, the increasing interest on craft brewing and the widening of beer consumption in new markets has led to the research of hop genotypes characterized by peculiar phytochemical profiles. Unfortunately, during its domestication and breeding process, hop suffered from a shrinking of intraspecific agrobiodiversity that brought to an impoverishment genetic basis. Breeding is the solution to select hop genotypes endowed with

interesting phytochemical profiles, but also suitable for sustainable cultivation and adaptable to climate changes. A fundamental outcome for starting a breeding program is to increase genetic variability that can be reached through outcrossing or by manipulating the plant ploidy level. Plant biotechnology offer tools for accelerated breeding and exploiting the variation that can arise through the *in vitro* culture of plant cells, tissues and organs, referred to as 'somaclonal variation' (SV; Larkin and Scowcroft 1981). Somaclonal variation is triggered by several factors, among which the most studied are the auxin/cytokinin concentration and ratio, type of explant and the total time in culture across multiple subculture cycles (Cao *et al*., 2016; Krishna *et al*., 2016; Lestari 2006; Mahlanza *et al*., 2013; Novikova *et al.*, 2020; Pijut *et al.*, 2012; Ramírez-Mosqueda and Iglesias-Andreu 2015; Rival *et al.*, 2013; Smulders and de Klerk 2011). D'Amato (1985) reported that plant growth regulators can have mutagen-like effects where, in several species, it has been reported that somaclonal variation rate markedly increased when auxins and cytokinins were added to the culture medium (Matsuda *et al.*, 2014; Sales and Butardo 2014; Sun et al. 2013). The use of cytokinins to induce indirect organogenesis increases the occurrence of polyploid cells, while the presence of auxins can cause a higher DNA methylation rate and chromosomal instability (Bairu et al., 2011; Mançano *et al*., 2019). Bairu *et al*. (2006) reported a high rate of somaclonal variation in Cavendish banana (Musa AAA, cv. 'Zelig') because of the high concentration of 6-Benzylaminopurine (BAP) in the culture medium. In *Solanum melongena* L., the addition of BAP to the culture medium induced a high rate of polymorphism in adventitious shoots obtained through indirect organogenesis from leaf explants (Mançano *et al*., 2019).

In addition to type and concentration of plant growth regulators, the explant can influence the genetic stability of the *in vitro* cultured plant material. Generally, the use of more differentiated tissues, including roots, leaves and stems, gives rise to greater variation, especially, if indirect organogenesis occurs, where callus formation entails explant cellular de-differentiation and plant regeneration by organogenesis entails cellular redifferentiation resulting in genetic and epigenetic

changes (Leva *et al.*, 2012; Kaeppler *et al*., 2000; Grafi and Barak 2014). Zayova *et al*. (2010) reported somaclonal variants in *S. melongena* L. plants, regenerated through indirect organogenesis. Somaclonal variation, detected by ISSR markers, was also obtained from indirect organogenesis of *Arracacia xanthorrhiza* (Vitamvas *et al*., 2019). Furthermore, the longer the time in which a culture is maintained, the greater is the chance of obtaining novel genetic variability (Krishna *et al*., 2016). For example, it has been reported that with the increased culture age, the occurrence of callus variant karyotypes increases and therefore the possibilities of variation in regenerated plants (Zayova *et al*. 2010).

In hop, somaclonal variation can be a valid tool to recover polyploids, characterized by altered plant morphology, phenology and physiology (Levin 2002; Roy *et al.* 2001). Specifically, in traditional breeding programs, tetraploids play an important role in hop breeding, as they can be crossed with diploid plants to obtain triploids (Roy *et al.* 2001). Actually, hop producers and, most of all, brewers are particularly interested in triploid cultivars which are infertile and therefore seedless (Dhooghe *et al.* 2011). Hop seeds contain fatty acids and proteins that compromise beer fermentation (Hildebrand *et al.* 1975).

Unfortunately, traditional plant breeding methods require multiple generations taking years to produce a new variety. On the contrary, through tissue culture, tetraploids can be recovered in approximately six months. The mechanism that leads to tetraploid induction by indirect organogenesis is not well understood but could be induced using cytokinins and auxins that generate endomitosis phenomena. (Trojak-Goluch *et al.* 2015). It has been reported that aberration *in vitro* could be due to chromosomal damages and its consequences such as deletions, duplications, inversions, and translocations (Duncan 1997).

Adelberg and Rhodes (1994) found that the explant type is one of the most crucial factors affecting the occurrence of polyploidization. A large percentage of tetraploid regenerants were obtained from immature cotyledons of *Cucumis melon* L. compared with those obtained from apical meristem explants, both arising from the same seed.

In the past, to detect polyploids among all tissue culture regenerants, the primary method for detection was traditional cytological approaches based on chromosomes counts which is time consuming (Hamill *et al.*, 1992), while estimating ploidy based on guard cell stomatal characteristics (chloroplast number) is not always reliable due to environmental effects (Van Duren *et al*. 1996). Flow cytometric analysis of nuclear DNA content is being increasingly used for ploidy assessment. As previously cited, somaclonal variation not only induces polyploidization, it can cause a wide range of mutations. Different tools have been used to assess this variability based on the differences in morphological traits (Pérez *et al.*, 2009; 2011; Nhut *et al*., 2013), biochemical (Vujović *et al*., 2010; Kar *et al.*, 2014) and molecular DNA markers (Hossain *et al*., 2003; Krishna and Singh, 2007; Pathak and Dhawan, 2012; Bello-Bello *et al.*, 2014) or their combinations (Horáček *et al.*, 2013; Dey *et al*., 2015; Stanišić *et al.*, 2015). Specifically, since the probability of mutations is randomly distributed across~~long~~ the genome, an efficient way to analyze somaclonal variation are PCR based markers, such as random amplified DNA polymorphism (RAPD). RAPD has proved to be effective in several cases allowing for fast screening of the genome. In *Lolium* (Wang *et al*., 1993), *Triticum* (Brown *et al*., 1993), *Picea* (Isabel *et al*., 1993) and *Beta* (Munthali *et al*., 1996) changes in RAPD bands have been observed in somaclonal variants.

The objective of this research was to study indirect *in vitro* organogenesis from hop, genotype "Gianni", using leaf explants to determine the influence of type and concentration of growth regulators in the culture medium and the effect of the time in culture in which leaf explants were

maintained *in vitro*. Regenerants were characterized by cytofluorimetric analysis to determine their ploidy and evaluated by RAPD to verify any possible polymorphism.

Material and methods

Plant Material

Young, well expanded leaves of *Humulus lupulus* L.(hop), genotype "Gianni" (Mongelli *et al.*, 2015), were isolated from two-mo-old *in vitro* cultured plantlets, cultured in 500-mL glass jars containing 100 mL of Murashige and Skoog (MS) culture medium (Duchefa Biochemie, B.V., Haarlem, The Netherlands) (Murashige and Skoog, 1962) containing 30 g L^{-1} of sucrose (Duchefa Biochemie, B.V., Haarlem, The Netherlands), 8 g L^{-1} of agar (Duchefa Biochemie, B.V., Haarlem, The Netherlands) and pH adjusted to 5.8 prior to autoclaving for 20 min at 121 °C. *In vitro* cultures were maintained in a growth chamber, at 25±1°C and light intensity of 20 μmol m^{-2} s^{-1}, under 16 h photoperiod.

In Vitro Organogenesis from Leaf Portions

Isolated leaves were cut in sections (0.5 x 0.5 mm) and placed into culture, in sterile Petri dishes (ten explants for each Petri dish, ten Petri dishes per treatment), with the abaxial surface toward the culture medium. To facilitate medium uptake by tissue, several transverse cuts to the leaf segments were made before the culture initiation. In order to evaluate the influence of type and concentration of growth regulators on hop leaf explants, six culture media were tested: (i) **MS:** MS culture medium without growth regulators, used as control medium; (ii) **MS-BAP0:** MS culture medium supplemented with 0.1 μM of 1-Naphthaleneacetic acid (NAA) (Duchefa Biochemie, B.V., Haarlem, The Netherlands); (iii) **MS-BAP2**: MS-BAP0 culture medium supplemented with 8.88 μM of BAP (Duchefa Biochemie, B.V., Haarlem, The Netherlands); (iv) **MS-BAP4**: MS-BAP0 culture medium supplemented with 17.77 μM of BAP; (v) **MS-BAP6**: MS-BAP0 culture medium

supplemented with 26.66 µM of BAP; (vi) **MS-BAP8**: MS-BAP0 culture medium supplemented with 35.55 µM of BAP. Culture media was autoclaved and cultures incubated as described above.

Experimental Design and Analysis of Data

The experiment was carried out in a completely randomized design with ten replications and a total of 100 explants was used per each culture medium tested. Cultures were monitored every wk for 18 wk and, to evaluate plant regeneration potential and the effect of length of time length in which the explants were in culture, three specific time periods in culture (PC) were selected: first PC (**I PC**) after 6 wk of culture, second PC (**II PC**) after 12 wk of culture and third PC (**III PC**) after 18 wk of culture. For each PC, adventitious shoots were excised, and the following parameters were recorded: the number of explants producing callus, the number of explants producing roots, the number of explants producing shoots, the average number of roots per each explant (n° of roots), the average number of shoots per each explant (n° of shoots), the average length of roots and the average length of shoots. Data of explants with callus, roots and shoots were used to calculate percentages.

At the conclusion of the experiment, Mean Regeneration Time (MRT) and Regeneration Energy (RE) were calculated; *formulae* used were the following (*formulae* were adapted to organogenesis respectively from Kader (2005) and from Paul (1972) who developed them for seed germination): MRT = $\sum f$*x/f_{tot} (f=number of explants with shoots on a given day, x=days needed for explant to regenerate; f_{tot}=number of all explants with shoots); RE = $\sum f$*x before the peak*100)/f_{tot} (f=number of explants with shoots on a given day, x=days needed for explant to regenerate; *f*tot=number of all explants with shoots; “peak” indicated the specific time at which regeneration is the highest). Two-way ANOVA (influence of Culture Medium Composition, CMC, and influence of the PC) was used to calculate the differences among treatments per each parameter considered;

Tukey's test ($p \leq 0.05$) was used for mean separation (SYSTAT 13.1, Systat Software, Inc; Pint Richmond, CA).

Rooting and Acclimatization

After 6, 12 and 18 wk of culture, adventitious shoots, with the first leaflets fully expanded, were excised and cultured in 25 mL glass jars, on MS culture medium. When plantlets had well-formed roots and leaves, they were transferred from *in vitro* to *in vivo* conditions. Specifically, plantlets were extracted from the culture medium, the roots were washed with distilled water and plantlets transferred in 2x2 cm growing trays, containing sand and peat mixture (2:1 *w/w*).

Flow Cytometry Analysis of Regenerants

Flow cytometry analysis was performed to evaluate the ploidy of regenerated plants, using a NovoCyte (Acea Biociences, San Diego, CA, USA). Each regenerant was analysed three times. Approximately 0.5 cm^2 of leaf sample from regenerated plants and mother plant, genotype "Gianni" (control), were chopped with a razor blade for 30 to 90 sec, in a plastic Petri dish containing 0.5 mL of extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The resulting extract was passed through a 30 µm filter into a 3.5 mL plastic tube, to which was then added 2.0 mL of Partec CyStain PI Absolute P Staining Buffer (Partec GMBH, Münster, Germany), containing 12 µL of Propidium Iodide Solution and 6 µL RNase A. Samples were kept in darkness for 30 min before analysis by flow cytometry. At least 5000 nuclei were analysed in each sample (NovoCyte Flow Cytometer Operator's Guide), Acea NovoExpress v.1.25 software was used.

DNA Extraction and RAPD Analysis

To verify polymorphism, 83 regenerated plants, were analysed using RAPD molecular markers. Three leaflets were excised, stored at -80°C and used for molecular analysis from each regenerant

and mother plant. Genomic DNA was extracted following the CTAB (Cetyl Trimethyl Ammonium Bromide) procedure (Rodolfi et al., 2018) and quantified by spectrophotometric method (Spectrophotometer Uvikon 930, Kontron Instruments Inc., Boston, MA, USA). Genomic DNA was amplified using 16 decamer primers (Table 1). The amplification reaction was performed in a volume of 25 µL containing: 1X Reaction Buffer (KAPA Taq Buffer w/loading dye, KAPA Biosystems, Wilmington, Massachusetts, US), 1.5 mM $MgCl_2$, 2.5 µM dNTPs (Amersham Biosciences, Little Chalfont, UK), 0.4 µM of primer (Sigma-Genosys Ltd, Dorset, UK), 1 unit of taq DNA polymerase (KAPA Taq DNA Polymerase, KAPA Biosystems, Wilmington, Massachusetts, US), and 40 ng DNA. The amplification reaction conditions for the MJ PCT 100 thermo cycler (MJ Research, Watertown, Mass.) programming were a first step at 95°C for 5 min, followed by 40 cycles of 40 sec at 94°C, 40 s at 36°C, 2 min at 72°C, for denaturation, annealing, and primer extension. The last step included 10 min of incubation at 72°C. This reaction was performed three times for each oligonucleotide tested. The products of amplification were separated on a 2% agarose gel in TAE buffer and stained with ethidium bromide. Gels were photographed with a Canon PowerShot A720IS digital camera. DNA molecular weight marker VII (0.081-8.57kbp) (Roche Diagnostics GmbH, Mannheim, DE) was used to estimate the approximate molecular weight of the amplified products, with the aid of Kodak digital science 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY).

Results

In Vitro Leaf Regeneration

Leaf portions cultured on all ~~culture~~ media tested maintained their green color for the first two wk, then turned light brown and started producing a yellowish spongy callus that increased rapidly, covering the entire surface of the explants. Explants continued producing callus for the first 12 wk, then stopped growing and the callus turned dark brown. Statistical analysis after 18 wk, on the percentage of explants producing callus, showed a significant interaction between the main factors

"Period in Culture" (PC) and "Culture Medium Composition" (CMC) and demonstrated that the factor that had the greatest influence on leaf *in vitro* culture response was "CMC". Callus regeneration was recorded only from leaves cultured on media containing BAP, independent of its concentration (Table 2). The first leaf explants regenerating roots was observed after two wk on culture medium without BAP (MS-BAP0); however, explants cultured on media containing BAP started regenerating roots after three wk. (Fig. 1a). The percentage of explants producing roots increased from the first "PC" to the second. Statistical analysis showed that both factors influenced significantly the response from leaf explants. Specifically, the percentage of explants with roots in the first PC (11.8%) was statistically lower than those observed in the second and third PC (both 23.3%). CMC differences were also statistically significant between media containing BAP and media without (MS and MS-BAP0); moreover, the medium with the highest BAP concentration (MS-BAP8) induced the statistically lowest percentage of roots from cultured explants (Table 2). The number of roots produced per single explant was variable, depending mainly on the medium composition with up to 10 roots per explant recorded from one explant cultured on MS-BAP2 medium (data not shown). A significant interaction between "PC" and "CMC" was observed where in the first PC the statistically greatest number of roots occurred from explants cultured on MS-BAP6 and the lowest on MS-BAP8, whereas in the other two PCs, increasing the concentration of BAP resulted in a statistically significant decrease in root number (Table 2).

Root length showed a significant interaction between factors, with a strong influence of CMC. The only statistically significant differences were observed in the first PC, in which statistically longer roots (12.4 mm) were observed from explants cultured on MS-BAP8 (Table 2).

Together with the emergence of the first roots, calli formed organogenic centers in explants cultured on MS-BAP4 (Fig. 1b). After 4 wk culture, some of the organogenic centers turned into small shoots with well-developed leaves (indirect organogenesis) (Fig. 1c). Direct organogenesis was not

observed. Except for the MS-BAP2 media treatment, adventitious shoot regeneration continued for all the PCs without transferring leaf segments to fresh medium.

Over 18 wk of explant culture, analysis of the Mean Regeneration Time (MRT) showed a statistically significant difference from the first to the third PC, showing a decreasing trend in the time needed to obtain shoot regeneration, However, an opposite trend was observed for the parameter Regeneration Energy (RE), which increased significantly from the first to the third PC (Fig. 2).

Statistical analysis on the percentage of explants with adventitious shoots showed a significant interaction between PC and CMC. Significant differences were observed only between the first and the other two PCs and only for explants cultured on MS-BAP8, where in the first PC, a significantly lower percentage of explants with shoots was observed.

Statistical analysis did not reveal any significant factor relating to the average number of shoots. However, a total of 138 adventitious shoots were obtained, independent of culture medium and PC, with~~and~~ up to 14 regenerants from one leaf explant cultured on MS-BAP4 (data not shown). The shoot length varied from 3 to 26 mm (data not shown) and statistical analysis showed a significant interaction between PC and CMC. Specifically, for the factor PC, significant differences among culture media were observed in the third PC in which MS-BAP6 and MS-BAP8 culture media induced the statistically longest shoot formation (respectively 16.5 and 9.1 mm). Regarding the factor CMC, ~~differences~~ statistically significant differences were observed only among explants cultured on MS-BAP4 culture medium; however~~indeed~~, in the second PC, the statistically longest shoots (11.0 mm) were observed (Table 2).

Rooting and Acclimatization

After approximately 14 d of culture on MS culture medium, 83 out of 138 shoots produced~~ing~~ roots. Root formation occurred directly from the shoot cut end, without a callus phase. Approximately 60% of cultured shoots developed a well differentiated root system within 30 d of culture (data not shown) and regenerated plants transferred to *in vivo* conditions with a 50% survival rate.

Flow Cytometry Analysis of Regenerants

All well-developed plantlets derived from adventitious shoots were subjected to flow cytometry to determine their ploidy. Cytofluorimetric analysis showed the presence of both diploid and tetraploid lines (Fig. 3a and b); other variations of ploidy, such as mixoploid or octoploid were not detected. Out of the 83 regenerants analyzed, six (7.2%) were tetraploids. It was not possible to carry on a statistical analysis because of the low number of tetraploids obtained. However, the efficiency of tetraploid recovery varied, mainly depending on the medium composition and MS-BAP8 induced the highest number of regenerated tetraploid plants (66.7%). The time in which leaf explants were in culture did not influence the ploidy variation because 83.3% of regenerated tetraploid plants were obtained during the first six weeks of culture (I PC). The starting explant seems to have a strong influence on the polyploidization process because ~~almost half~~3 out 6 ~~-~~of the tetraploids obtained were recovered from a single explant.

RAPD Analysis

A total of 183 bands were generated using 16 RAPD decamer primers. For all regenerated plants, the number of bands generated by each primer varied from 5 (primer 544) to 17 (primer AI12); moreover, primer AI055 generated 5 fragments only with regenerant 52. Each primer generated a set of fragments, ranging from 149 to 8570 bp. Results indicated that three of the sixteen primers tested revealed polymorphic DNA profiles: OPK16 for regenerant 134 (Fig. 1), AI12 for regenerant 19A, and AI05 for regenerated plants 7, 10, 48, 52, 78 and 112 (Table 3). Furthermore, OPK16

generated one RAPD amplification product (1625 bp) that was unique for regenerant 134, whereas AI05 generated amplification products present in regenerated plants 7, 10, and 48 and absent in regenerated plants 52, 78 and 112 (Table 3). The two amplification products (1650 bp and 1501 bp) from primer AI12 were absent in regenerant 19A.

RAPD analysis showed that 8 out of 83 regenerated plants (9.6%) showed scoreable polymorphisms. Due to the low number of mutants obtained, it was not possible to carry on a statistical analysis, but it was observed that mutants were recovered in equal number from MS-BAP4 and MS-BAP8 and none from MS-BAP6. Moreover, the time in which explants were in culture did not have a strong mutagenic effect for hop genotype "Gianni" leaf sections cultured *in vitro*. ——Five out of eight mutants were recovered during the first six wk of culture (I PC) independent of culture medium. It should be noted that 3 out of 8 mutants were regenerated by the same leaf section, during I PC, suggesting that the explant origin is also an important -factor to be considered.

Discussion

Medium composition and duration of culture are key factors influencing regeneration competence and the incidence of somaclonal variation. In hop, there are several studies reporting plant regeneration from different types of explants, such as internodes, petioles and leaf discs (Batista *et al.* 1996; Gurriarán *et al.* 1999; Motegi 1979; Peredo *et al.* 2006; Roy *et al.* 2001; Smýkalová et al. 2001). Many authors reported that the best plant regeneration in hop was from cultured internodal cuttings (Batista et al. 1996; Connell and Heale 1992; Gurriarán *et al*. 1999; Heale *et al*. 1989; Horlemann et al. 2003; Motegi 1979; Rakouský and Matoušek 1994; Šuštar-Vozlič *et al*. 1999), but it is also well known that the regeneration ability of hop is highly genotype dependent (Gurriarán *et al*. 1999). For this reason, the first aim of this study was the induction of organogenesis from hop

leaf segments of genotype “Gianni” by testing the type and concentration of growth regulators in the culture medium and the time in which explants remained in the culture.

The first sign of organogenesis was callus formation, starting from the edges and eventually covering all the leaf surface. The percent callus formation was greatest from leaf explants cultured on media containing BAP (91.5%) and agree with the findings of Rakouský and Matoušek (1994) who obtained similar results from in *in vitro* cultured hop leaf explants of two Czech clones. However, Skof *et al*. (2007) reported scarce callus formation from leaf discs cultured on media containing both BAP and NAA. The importance of auxin/cytokinin ratio in callogenesis is evidenced by Gurriarán *et al*. (1999) who obtained an increase in the number of callus cultures from explants of hop cultivar Brewers Gold internodal segments cultured on media containing both BAP and Indole-3-butyric acid (IBA).

Other than callus, leaf explants regenerated roots if cultured on media containing NAA. No reports are available, to our knowledge, about root induction from hop leaf explants. However, Liberatore *et al.* (2020) reported thea ~~a~~ percentage of explants with roots was greater ~~higher~~ from internodal segments than that obtained from leaves in the present study.

Adventitious shoot regeneration was observed in all media containing BAP, except from MS-BAP2, with an average percentage of 8.3%. This agrees with the report by Šuštar-Vozlič *et al*. (1999) where the choice and concentration of cytokinin was found to be essential for organogenic capacity. There are few reports in which leaf explants are used as for induction of organogenesis. Skof *et al*. (2007) reported that leaf discs producing shoots are highly genotype dependent, ranging from 5.1% in Savinjski to 20.0% in Tettnanger. In “Gianni”, adventitious shoots appeared after approximately 4 wk of culture, requiring two-fold longer incubation time than what was reported by Skof *et al*. (2007), for the cultivars Aurora, Savinjski golding and Tettnanger.

Cassells (1979) and Gurriarán *et al*. (1999) observed that due to apical dominance the first shoot produces auxins that may inhibit regeneration of new shoots. This is the likely reason why organogenic centers ~~keep~~ develop~~ing~~ new buds only following shoot excision. In order to avoid this phenomenon~~, in this study~~, every 6 wk, shoots were excised and cultured on MS medium. The percentage of explants producing adventitious shoots increased significantly between the first and the other two periods of culture, specifically for explants cultured on MS-BAP6 and MS-BAP8, demonstrating that the adventitious shoot regeneration capacity of "Gianni" leaf explants was affected by the time in culture. Similar results were obtained in cultivars Brewers Gold and Nugget with an increase in regeneration rate from the first to the third subcultures (Gurriarán *et al.* 1999).

Considering that plants regenerated from *in vitro* cultures can exhibit an array of genetic and epigenetic changes, known as 'somaclonal variation', shoots regenerated from leaf explants were analyzed using flow cytometry and RAPD molecular markers to evaluate their variability. There are three main factors giving rise to somaclonal variation: i) explant source, ii) callus formation and iii) organogenic process (Benzion and Phillips 1988). Among these factors, it has been demonstrated that most abnormalities are accumulated if the organogenic process is indirect (Phillips *et al.* 1994; Roy *et al*. 2001). In hop varieties, Skof et al. (2007) demonstrated that indirect organogenesis could be considered a successful method to obtain polyploids. In this study, tetraploid hop, "Gianni", plantlets were obtained through regeneration from *in vitro* cultured leaf explants. Trojak-Goluch and Skomra (2013) compared diploid and tetraploid hop plants, assessing the effect of polyploidization on the morphological and chemical characteristics and observed an increase in flower size, delayed flowering time, greater vigor and higher yield. Moreover, tetraploids play an important role in hop breeding as they can be crossed with diploid plants in order to obtain triploid progeny, considered to be superior to both diploids and tetraploids (Roy *et al*. 2001), mostly because of their lack of seed production~~lessness~~. In this study, some of the regenerants analyz~~s~~ed by

RAPD molecular markers maintained their diploid chromosomic set but showed a different DNA pattern when compared to the mother plant. *In vitro* culture can result in genomic alternations, other than polyploidy, such as deletion/insertion between priming sites or in restriction sites (De Verno et al., 1999).

Genetic variation was detected in 16.8% of mutated regenerants (tetraploids and –RAPD) hop variants, confirming what reported by several authors (Jain, 2001; Patzak, 2003; Hashmi *et al*., 1997; Al-Zahim *et al*., 1999; Rout, 2002). *In vitro* culture is a mutagenic system with a mechanism comparable to what happens in nature (Linacero *et al*., 2000).

Conclusion**s**

In this study, indirect organogenesis from leaf explants of hop, genotype "Gianni", was induced following culture on MS medium containing 6-Benzylaminopurine and 1-Naphtalenacetic acid. The percentage of leaf explants producing adventitious shoots increased with the period in which explants were kept in culture. Moreover, the longer the period of culture, the shorter the time needed for regeneration. The *in vitro* culture conditions tested in this study determined a polymorphism rate of 16.8%. This result was independent of the BAP concentration and not influenced by the time in culture. Mutants obtained will be further characterized to evaluate their field performance.

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References

Adelberg JW, Rhodes BB (1994) Explant origin affects the frequency of tetraploid plants from tissue culture of melon. Sci 29:689–692 https://doi.org/10.21273/HORTSCI.29.6.689

Al-Zahim MA, Ford-Lloyd BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. Plant Cell Rep18:473-47 https://doi.org/10.1007/s002990050606

Bairu MW, Aremu AO, Staden JV (2011) Somaclonal variation in plants: causes and detection methods. Plant Growth Regul 63: 147-173; doi: 10.1007/s10725-010-9554-x

Bairu MW, Fennell CW, van Staden J (2006) The effect of plant growth regulators on somaclonal variation in Cavendish banana (Musa AAA cv. 'Zelig'). Sci Hortic 108:347–351

Batista D, Sousa MJ, Pais MS (1996) Plant regeneration from stem and petiole-derived callus of *Humulus lupulus* L. (hop) clone Bragança and var. Brewers's Gold. In Vitro Cell Dev Biol - Plant 32:37–41 https://doi.org/10.1007/BF02823011

Bello-Bello JJ, Iglesias-Andreu LG, Avilés-Vinãs SA, Gómez-Uc E, Canto-Flick A, Santana-Buzzy N (2014) Somaclonal variation in habanero pepper (*Capsicum chinense* Jacq.) as assessed ISSR molecular markers. Hort Sci 49:481–485 https://doi.org/10.21273/HORTSCI.49.4.481

Benzion G, Phillips RL (1988) Cytogenetic stability of maize tissue cultures: a cell line pedigree analysis. Genome 30:318–325 https://doi.org/10.1139/g88-056

Brown PTH, Lange FD, Krang E, Lorz H (1993) Analysis of single protoplasts and regenerated plants by PCR and RAPD technology. Gen Genet 237:311–317 https://doi.org/10.1007/BF00279433

Cao Z, Sui S, Cai X, Yang Q, Denget Z (2016) Somaclonal variation in 'Red Flash' caladium: morphological, cytological and molecular characterization. Plant Cell Tiss Org Cult 126, 269–279 https://doi.org/10.1007/s11240-016-0996-3

Cassells AC (1979) The effect of 2,3,5-triiodobenzoic acid on caulogenesis in callus cultures of tomato and *Pelargonium*. Physiol Plant 46:159–64 https://doi.org/10.1111/j.1399-3054.1979.tb06550.x

Connell SA, Heale JB (1992) Sensitivity of cells and protoplasts of hop cultivars to cytotoxic components of culture filtrates of *Verticillium albo-atrum* isolates from hop. Plant Pathol 39:92–101 https://doi.org/10.1111/j.1365-3059.1990.tb02479.x

D'Amato F (1985) Cytogenetics of plant cell and tissue cultures and their regenerants. CRC Crit Rev Plant Sci 3:73–112 https://doi.org/10.1080/07352688509382204

De Verno LL, Park YS, Bonga JM, Barret JD (1999) Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss.]. Plant Cell Rep 18:948–95 https://doi.org/10.1007/s002990050689

Dey T, Saha S, Ghosh PD (2015) Somaclonal variation among somatic embryo derived plants—evaluation of agronomically important somaclones and detection of genetic changes by RAPD in *Cymbopogon winterianus*. S Afr J Bot 96:112–121 https://doi.org/10.1016/j.sajb.2014.10.010

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissue *in vitro*. Plant Cell Tiss Org Cult 104:359–373 https://doi.org/10.1007/s11240-010-9786-5

Duncan RR (1997) Tissue culture-induced variation in crop improvement. Adv Agron 58:201–240 https://doi.org/10.1016/S0065-2113(08)60256-4

Farahani F, Yari R, Masoud S (2011) Somaclonal variation in Dezful cultivar of olive (*Olea europaea* subsp. *europaea*). Gene Conserve 10:216–221

Gao X, Yang D, Cao D, Ao M, Sui X, Wang Q, Kimatu JN, Wang L (2010) *In vitro* micropropagation of *Freesia hybrid* and the assessment of genetic and epigenetic stability in regenerated plantlets. J Plant Growth Regul 29:257–267 https://doi.org/10.1007/s00344-009-9133-4

Grafi G, Barak S (2014) Stress induces cell dedifferentiation in plants. Biochim Biophys Acta https://doi:10.1016/j.bbagrm.2014.07.015

Gurriarán MJ, Revilla MA, Tamés RS (1999) Adventitious shoot regeneration in cultures of *Humulus lupulus* L. (hop) cvs. Brewers Gold and Nugget. Plant Cell Rep 18:1007–11 https://doi.org/10.1007/s002990050699

Hamill SD, Smith MK, Dodd WA (1992) *In vitro* induction of banana autotetraploidy by colchicine treatment of micropropagated diploids. Aust J Bot 40:887–896 http://dx.doi.org/10.1071/BT9920887

Hashmi G, Huettel R, Meyer R, Krusberg L, Hammerschlag F (1997) RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Rep 16:624–627 https://doi.org/10.1007/BF01275503

Heale JB, Legg T, Connell S (1989) *Humulus lupulus* L. (Hop): *In vitro* culture; attempted production of bittering components and novel disease resistance. In: Bajaj Y.P.S. (eds) Medicinal and Aromatic Plants II. Biotechnology in Agriculture and Forestry, vol 7. Springer, Berlin, Heidelberg, pp 264-285. https://doi.org/10.1007/978-3-642-73617-9_15

Hildebrand RP, Kavanagh TE, Clarke BJ (1975) Hop lipids and beer quality. Brewers Dig 50: 58–60

Horáček J, Švábová L, ŠarhanováP, Lebeda A (2013) Variability for resistance to *Fusarium solani* culture filtrate and fusaric acid among somaclones in pea. Biol Plant 57:133–138 https://doi.org/10.1007/s10535-012-0131-1

Horlemann C, Schwekendiek A, Höhnle M, Weber G (2003) Regeneration and *Agrobacterium*-mediated transformation of hop (*Humulus lupulus* L.). Plant Cell Rep 22:210–217 https://doi.org/10.1007/s00299-003-0676-8

Hossain MA, Konisho K, Minami M, Nemoto K (2003) Somaclonal variation of regenerated plants in chili pepper (*Capsicum annuum* L.). Euphytica 130:233–239 https://doi.org/10.1023/A:1022856725794

Isabel N, Tremblay L, Michand M, Tremblay FM, Bousquet J (1993) RAPD as aids to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill.). Theor Appl Genet 86:81–87 https://doi.org/10.1007/BF00223811

Jain SM (2001) Tissue culture-derived variation in crop improvement. Euphytica 118:153–166 https://doi.org/10.1023/A:1004124519479

Jevremović S, Subotić A, Miljković D, Trifunović M, Petrić M, Cingel A (2012) Clonal fidelity of *Chrysanthemum* cultivars after long term micropropagation by stem segment culture. Acta Hortic 961:211–216 https://10.17660/ActaHortic.2012.961.25

Kader M (2001) Varying temperature regimes affect osmotically primed sorghum seeds and seedlings. Int. Sorghum Millets Newslett. 42:39-40

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179–188

Kar B, Kuanar A, Singh S, Mohanty S, Joshi RK, Subudhi E, Nayak S (2014) *In vitro* induction, screening and detection of high essential oil yielding somaclones in turmeric (*Curcuma longa* L.). Plant Growth Regul 72:59–66 https://doi.org/10.1007/s10725-013-9836-1

Krishna H, Alizadeh M, Singh D, Singh U, Chauhan N, Eftekhari M, Sadh RK (2016). Somaclonal variations and their applications in horticultural crops improvement. 3 Biotech, 6:1-18https://doi.org/10.1007/s13205-016-0389-7

Krishna H, Singh SK (2007) Biotechnological advances in mango (*Mangifera indica* L.) and their future implication in crop improvement—a review. Biotechnol Adv 25:223–243 https://10.1016/j.biotechadv.2007.01.001

Kuznetsova OI, Ash OA, Gostimsky SA (2006) The effect of the duration of callus culture on the accumulation of genetic alternations in pea *Pisum sativum* L. Russ J Genet 42:555–562 https://doi.org/10.1134/S1022795406050139

Larkin PJ, Scowcroft WR (1981) Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214 https://doi.org/10.1007/BF02342540

Lestari EG (2006) *In vitro* selection and somaclonal variation for biotic and abiotic stress tolerance. Biodiversitas 7:297–300 https://doi.org/10.13057/biodiv/d070320

Leva AR, Petruccelli R, Rinaldi LMR (2012) Somaclonal variation in tissue culture: a case study with olive. In: Leva AR, Rinaldi LMR (eds) Recent advances in plant *in vitro* culture. INTECH Open Access Publisher, Croatia, pp 123–150

Levin DA (2002) The role of chromosomal change in plant evolution. Oxford: Oxford University Press https://doi.org/10.1600/036364404774195656

Liberatore CM, Rodolfi M, Beghè D, Fabbri A, Ganino T, Chiancone B (2020) Adventitious shoot organogenesis and encapsulation technology in hop (*Humulus lupulus* L.). published online Sci Hort https://doi.org/10.1016/j.scienta.2020.109416

Linacero R, Freitas Alves E, Vázquez AM (2000) Hot spots of DNA instability revealed through the study of somaclonal variation in rye. Theor Appl Genet 100:506–511 https://doi.org/10.1007/s001220050066

Mançano AP, de Oliveira Soares B, Rodrigues de Oliveira Garcia R, Atalla Mansur E, Gagliardi RF (2019) Genetic stability among *in vitro* eggplant clones induced by different plant growth regulators. Biotecnología Vegetal 19:75-83.

Mahlanza TR, Rutherford S, Snyman SJ, Watt MP (2013) *In vitro* generation of somaclonal variant plants of sugarcane for tolerance to *Fusarium sacchari*. Plant Cell Rep 32:249–262. https://doi.org/10.1007/s00299-012-1359-0

Matsuda S, Sato M, Ohno S. (2014) Cutting leaves and plant growth regulator application enhance somaclonal variation induced by transposition of VGs1 of *Saintpaulia*. J Jpn Soc Hortic Sci 83:308–316 doi: 10.2503/jjshs1.MI-009

Mongelli A, Rodolfi M, Ganino T, Marieschi M, Dall'Asta C, Bruni R (2015) Italian hop germplasm: characterization of wild *Humulus lupulus* L. genotypes from Northern Italy by means of phytochemical, morphological traits and multivariate data analysis. Ind Crops Prod 70:16-27 http://dx.doi.org/10.1016/j.indcrop.2015.02.036

Motegi T (1979) Differentiation of shoots from hop stem callus culture. Kyoyubokenkyu Neupo Iwate Ika Daigaku 14:15–17

Munthali MT, Newbury HJ, Ford-Lloyd BV (1996) The detection of somaclonal variants of beet using RAPD. Plant Cell Rep 15:474–478 -https://doi.org/10.1007/BF00232977

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479 http://dx.doi.org/10.1111/ j.1399-3054.1962.tb08052.x

Nhut DT, Hai NT, Thu PTM, Thi NN, Hien TTD, Tuan TT, Nam NB, Huy NP, Chien HX, Jain SM (2013) Protocol for inducing flower color somaclonal variation in Torenia (*Torenia fournieri* Lind.). Methods Mol Biol 11013:455–462 doi: 10.1007/978-1-62703-074-8_34

Novikova TI, Asbaganov SV, Ambros EV, Zaytseva YG (2020) TDZ-induced axillary shoot proliferation of *Rhododendron mucronulatum* Turcz and assessment of clonal fidelity using DNA-based markers and flow cytometry. In Vitro Cell Dev Biol-Plant. https://doi.org/10.1007/s11627-019-10049-9

Paul A (1972) A guide to forest seed handling (with special reference to the tropics). FAO Forestry, 20:387.

Pathak H, Dhawan V (2012) ISSR assay for ascertaining genetic fidelity of micropropagated plants of apple rootstock Merton 793. In Vitro Cell Dev Biol - Plant 48:137–143 https://doi.org/10.1007/s11627-011-9385-0

Patzak J (2003). Assessment of somaclonal variability in hop (*Humulus lupulus* L.) *in vitro* meristem cultures and clones by molecular methods. Euphytica 131:343-350 https://doi.org/10.1023/A:1024096401424

Peredo EL, Ángeles-Revilla M, Arroyo-García R (2006) Assessment of genetic and epigenetic variation in hop plants regenerated from sequential subcultures of organogenic calli. J Plant Physiol 163:1071–1079

Pérez G, Yanez E, Isidròn M, Lorenzo JC (2009) Phenotypic and AFLP characterization of two new pineapple somaclones derived from *in vitro* culture. Plant Cell Tiss Org Cult 96:113–116 https://doi.org/10.1007/s11240-008-9463-0

Pérez G, Mbogholi A, Sagarra F, Aragòn C, Gonzàlez J, Isidròn M, Lorenzo JC (2011) Morphological and physiological characterization of two new pineapple somaclones derived from

in vitro culture. In Vitro Cell Dev Biol - Plant 47:428–433 https://doi.org/10.1007/s11627-011-9342-y

Phillips RL, Kappler SM, Olhof R (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. Proc Natl Acad Sci USA 91:5222–5226 https://doi.org/10.1073/pnas.91.12.5222

Pijut PM, Beasley RR, Lawson SS, Palla KJ, Stevens ME, Wang Y (2012) *In vitro* propagation of tropical hardwood tree species—a review (2001–2011). Propag Ornam Plants 12:25–51

Rakouský S, Matoušek J (1994) Direct organogenesis in hop - a prerequisite for the application of *A. tumefaciens*-mediated transformation. Biol Plant 36:191–200 https://doi.org/10.1007/BF02921085

Ramírez-Mosqueda MA, Iglesias-Andreu LG (2015) Indirect organogenesis and assessment of somaclonal variation in plantlets of *Vanilla planifolia* Jacks. Plant Cell Tiss Org Cult 123:657–664. https://doi.org/10.1007/s11240-015-0868-2

Rival A, Ilbert P, Labeyrie A, Torres E, Doulbeau S, Personne A, Dussert S, Beulé T, Durand-Gasselin T, Tregear JW, Jaligot E. (2013) Variations in genomic DNA methylation during the long-term *in vitro* proliferation of oil palm embryogenic suspension cultures. Plant Cell Rep. 2:359–368 doi: 10.1007/s00299-012-1369-y.

Rodolfi M, Silvanini A, Chiancone B, Marieschi M, Fabbri A, Bruni B, Ganino T (2018). Identification and genetic structure of wild Italian *Humulus lupulus* L. and comparison with European and American hop cultivars using nuclear microsatellite markers. Genet Resour Crop Evol 65:1405–1422 doi: https://doi.org/10.1007/s10722-018-0622-9

Rout G.R. (2002). Direct plant regeneration from leaf explant of *Plumbago* species and its genetic fidelity through RAPD markers. Ann Appl Biol 140:305-313 DOI: https://doi.org/10.1111/j.1744-7348.2002.tb00186.x

Roy AT, Leggett G, Koutoulis A (2001) *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). Plant Cell Rep 20:489–495 https://doi.org/10.1007/s002990100364

Sales EK, Butardo NG (2014) Molecular analysis of somaclonal variation in tissue culture derived bananas using MSAP and SSR markers. Int J Biol Vet Agric Food Eng. 8:63–610 https://doi.org/10.13140/RG.2.1.4897.4564

Skof S, Bohanec B, Kastelec D, Luthar Z (2007) Spontaneous induction of tetraploidy in hop using adventitious shoot regeneration method. Plant Breed 126:416–421 https://doi.org/10.1111/j.1439-0523.2007.01378.x

Smulders M, de Klerk G (2011) Epigenetics in plant tissue culture. Plant Growth Regul 63:137–146 doi: 10.1007/s10725-010-9531-4

Smýkalová I, Ortová M, Lipavská H, Patzak J. (2001) Efficient *in vitro* micropropagation and regeneration of *Humulus lupulus* on low sugar, starch-gelrite media. Biol Plant 44:7-12

Sun S, Zhong J, Li S, Wang X (2013) Tissue culture-induced somaclonal variation of decreased pollen viability in torenia (*Torenia fournieri* Lind.). Bot Stud 54:36-42 https://doi.org/10.1186/1999-3110-54-36

Stanišić M, Raspor M, Ninković S, Milošević S, Ćalić D, Bohanec B, Trifunovića M, Petrića M, Subotić A, Jevremović S (2015) Clonal fidelity of *Iris sibirica* plants regenerated by somatic embryogenesis and organogenesis in leaf-base culture—RAPD and flow cytometer analyses. S Afr J Bot 96:42–52 https://doi.org/10.1016/j.sajb.2014.10.014

Šuštar-Vozlič J, Javornik B, Bohanec B (1999) Studies of somaclonal variation in hop (*Humulus lupulus* L.). Phyton – Ann Rei Bot 39:283–287

Trojak-Goluch A, Kawka M, Czarnecka D (2015) The effects of explant source and hormone content on plant regeneration and induction of tetraploids in *Humulus lupulus* L. In Vitro Cell Dev Biol - Plant 51:152-159 https://doi.org/10.1007/s11627-014-9661-x

Trojak-Goluch A, Skomra U (2013) Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. Breed Sci 63:393–399 https://doi:10.1270/jsbbs.63.393

Van Duren M, Mopurgo R, Dolezel J, Afza R (1996) Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. Euphytica 88:25–34 https://doi.org/10.1007/BF00029262

Vitamvas J, Viehmannova I, Cepkova PH, Mrhalova H, Eliasova K (2019) Assessment of somaclonal variation in indirect morphogenesis-derived plants of *Arracacia xanthorrhiza*. Pesquisa Agropecuária Brasileira, v.54, e00301. https://doi.org/10.1590/S1678-3921.

Vujović T, Ružić Đ, Cerović R, Momirović GŠ (2010) Adventitious regeneration in blackberry (*Rubus fruticosus* L.) and assessment of genetic stability in regenerants. Plant Growth Regul 61:265–275 https://doi.org/10.1007/s10725-010-9474-9

Wang ZY, Nagel J, Potrykus I, Spangenberg G (1993) Plants from cell suspension-derived protoplasts of *Lolium* species. Plant Sci 94:179–193 https://doi.org/10.1016/0168-9452(93)90019-V

Zayova E, Vassilevska IR, Kraptchev B, Stoeva D (2010) Somaclonal variations through indirect organogenesis in eggplant (*Solanum melongena* L.). Biol Divers Conserv 3:1–5

Tables

Table 1. Sequences of 16 decamer Randomly Amplified Polymorphic DNA (RAPD) primers used for identifying unique

Name	Sequence (5'a 3')	Name	Sequence (5'a 3')
OPA10	GTGATCGCAG	AI05	GTCGTAGCGG
OPK16	GAGCGTCGAA	AI08	AAGCCCCCCA
OPC16	CACACTCCAG	AI11	ACGGCGATGA
544	TAGAGACTCC	AI12	GACGCGAACC
AH01	TCGGCAACCA	AI14	TGGTGCACTC
AH09	AGAACCGAGG	AI16	AAGGCACGAG
AH12	TCCAACGGCT	OPB10	CTGCTGGGAC
AH18	GGGCTAGTCA	OPB20	GGACCCTTAC

Table 2: Effect of period of culture and of culture medium composition on callus, root and shoot formation from *Humulus lupulus* L., genotype "Gianni", leaf portions, after 18 wk of culture

PC	Culture Medium Composition	Explants with callus	Explants with roots	Explants with shoots	Roots	Shoots	Root length	Shoot length
		%±SE	%±SE	%±SE	n°±SE	n°±SE	mm±SE	mm±SE
I	MS	0.00±0.00	0.00±0.00	0.00±0.00	-	-	-	-
I	MS-BAP0	0.00±0.00	2.00±2.00	0.00±0.00	-	-	-	-
I	MS-BAP2	94.00±6.00	31.00±8.88	0.00±0.00	2.09±0.23	-	6.60±0.59	-
I	MS-BAP4	93.00±7.00	17.00±4.73	5.00±1.67	1.88±0.42	3.80±2.06	8.52±1.21	5.41±1.21
I	MS-BAP6	99.00±1.00	11.00±3.48	4.00±1.63	2.31±0.29	1.50±0.29	6.00±0.59	7.13±1.54
I	MS-BAP8	80.00±11.83	10.00±4.47	2.00±2.00	1.10±0.10	2.50±1.50	12.40±2.17	6.00±1.23
II	MS	0.00±0.00	0.00±0.00	0.00±0.00	-	-	-	-
II	MS-BAP0	0.00±0.00	2.00±2.00	0.00±0.00	-	-	-	-
II	MS-BAP2	94.00±6.00	50.00±9.66	0.00±0.00	2.10±0.22	-	6.58±0.46	-
II	MS-BAP4	93.00±7.00	39.00±8.88	11.00±4.07	1.69±0.21	5.33±1.78	7.82±0.93	11.00±2.79
II	MS-BAP6	99.00±1.00	42.00±10.31	13.00±9.52	1.70±0.17	2.73±0.51	6.39±0.49	13.08±2.53
II	MS-BAP8	80.00±11.83	15.00±6.37	14.00±3.71	1.27±0.27	3.22±0.62	9.58±1.47	11.60±2.16
III	MS	0.00±0.00	0.00±0.00	0.00±0.00	-	-	-	-
III	MS-BAP0	0.00±0.00	2.00±2.00	0.00±0.00	-	-	-	-
III	MS-BAP2	94.00±6.00	50.00±9.66	0.00±0.00	2.10±0.22	-	6.58±0.46	-
III	MS-BAP4	93.00±7.00	41.00±9.60	6.00±2.21	1.66±0.21	2.50±0.67	7.82±0.93	6.60±1.12
III	MS-BAP6	99.00±1.00	47.00±10.55	9.00±6.52	1.94±0.21	2.79±0.53	6.12±0.40	16.50±3.02
III	MS-BAP8	80.00±11.83	19.00±7.22	10.00±2.98	1.63±0.37	3.13±0.63	8.39±0.99	9.14±2.48
[a]Statistical analysis of factors								
PC		0.633	0.042	0.030	0.535	0.182	0.900	0.000
CMC		0.000	0.000	0.000	0.000	0.439	0.004	0.650
PC*CMC		0.000	0.000	0.000	0.000	0.521	0.000	0.000

[a]Two-way analysis of variance (ANOVA), followed by Tukey's test ($p \leq 0.05$). **PC:** Period of Culture; **CMC:** Culture Medium Composition. **I**: 6 weeks of culture; **II**: 12 weeks of culture; **III**: 18 weeks of culture. **MS**: 0 NAA and 0 BAP; **MS-BAP0**: 0.1mM NAA and 0 BAP; **MS-BAP2**: 0.1mM NAA and 8.88mM BAP; **MS-BAP4**: 0.1mM NAA and 17.77mM BAP; **MS-BAP6**: 0.1mM NAA and 26.66mM BAP; **MS-BAP8:** 0.1mM NAA and 35.55mM BAP. **MS:** Murashige and Skoog medium. **n° of roots:** average number of roots regenerated from one explant; **n° of shoots:** average number of shoots regenerated from one explant; **SE:** Standard Error.

Table 3. Presence of polymorphic Randomly Amplified Polymorphic DNA (RAPD) fragments in regenerants obtained from leaf portions of *Humulus lupulus* L., "Gianni", and in the mother plant (MP). The symbols + and – indicate, respectively, the presence or the absence of a RAPD marker.

RAPD bands	**Regenerated Plant (number) and Mother Plant (MP)**								
	7	10	19A	48	52	78	112	134	MP
OPK16-1625	-	-	-	-	-	-	-	+	-
AI12-1650	+	+	-	+	+	+	+	+	+
AI12-1501	+	+	-	+	+	+	+	+	+
AI05-1110	+	+	+	-	+	+	-	+	+
AI05-910	-	+	-	-	-	-	-	-	-
AI05-866	+	+	+	+	-	+	+	+	+
AI05-800	+	+	-	-	-	-	-	-	-
AI05-760	+	-	-	-	-	-	-	-	-
AI05-710	+	-	+	+	-	-	-	+	+
AI05-630	+	-	+	+	-	-	+	+	+
AI05-570	+	-	-	-	-	-	-	-	-
AI05-515	+	+	-	+	+	+	+	-	-
AI05-470	+	+	+	+	-	+	-	+	+

Figure legends

Fig. 1 *In vitro* organogenesis from *Humulus lupulus* L., genotype "Gianni", leaf explants. a) indirect root regeneration; b) organogenic centers coming from undifferentiated callus; c) adventitious shoot regenerated from undifferentiated callus.

Fig. 2 Mean Regeneration Time (MRT) (a) and Regeneration Energy (RE) calculated after 18 wk of culture. One-way ANOVA, Tukey's test ($p \leq 0.05$).

Fig. 3 Cytofluorimetric analysis: histograms of fluorescence intensity of nuclei from diploid *Humulus lupulus* L. leaf tissue of genotype "Gianni" a) mother plant b) tetraploid regenerant from leaf portion.

Fig. 4 OPK16 RAPD marker showing differences in DNA profile amplification in mother plant, *Humulus lupulus* L. "Gianni", and one regenerant from hop, "Gianni", leaf. Lane R134 = leaf regenerant; Lane MP = hop, genotype "Gianni", mother plant; Lane M = DNA molecular weight marker VII (M) (Roche Diagnostics GmbH, Mannheim, DE). Arrow indicates the differences in DNA profiles.

Figures

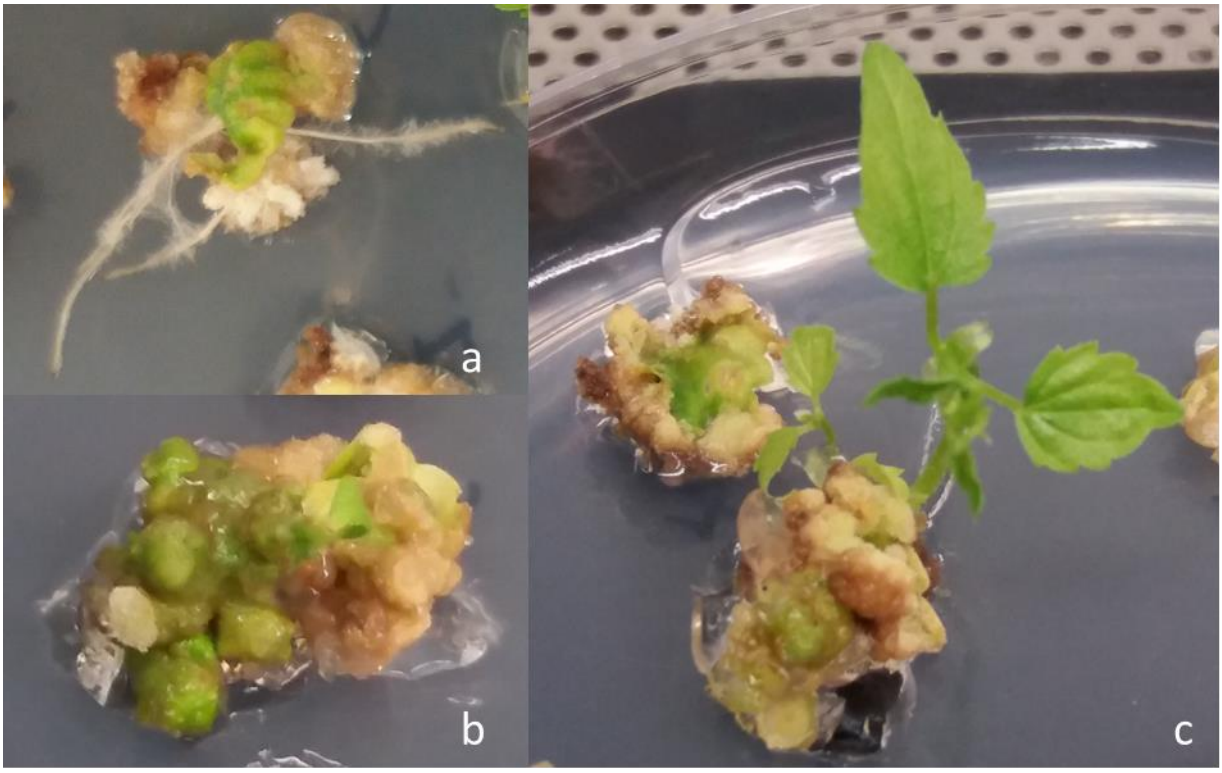
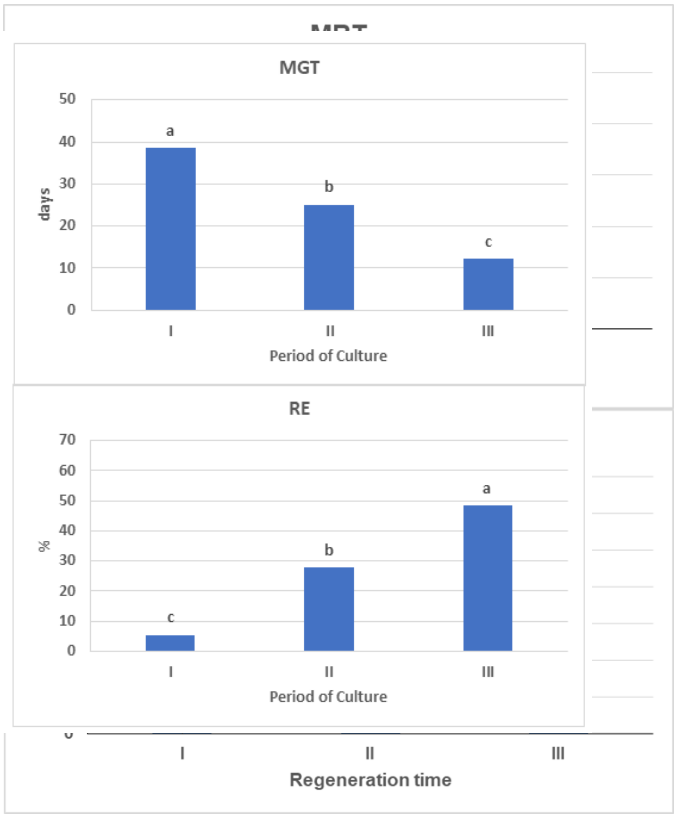


Fig. 1



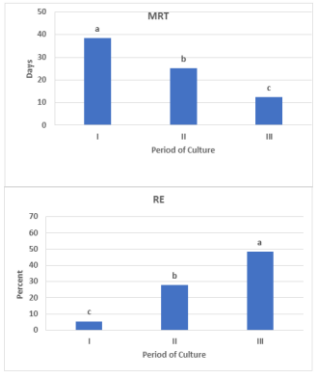


Fig. 2

Fig. 2

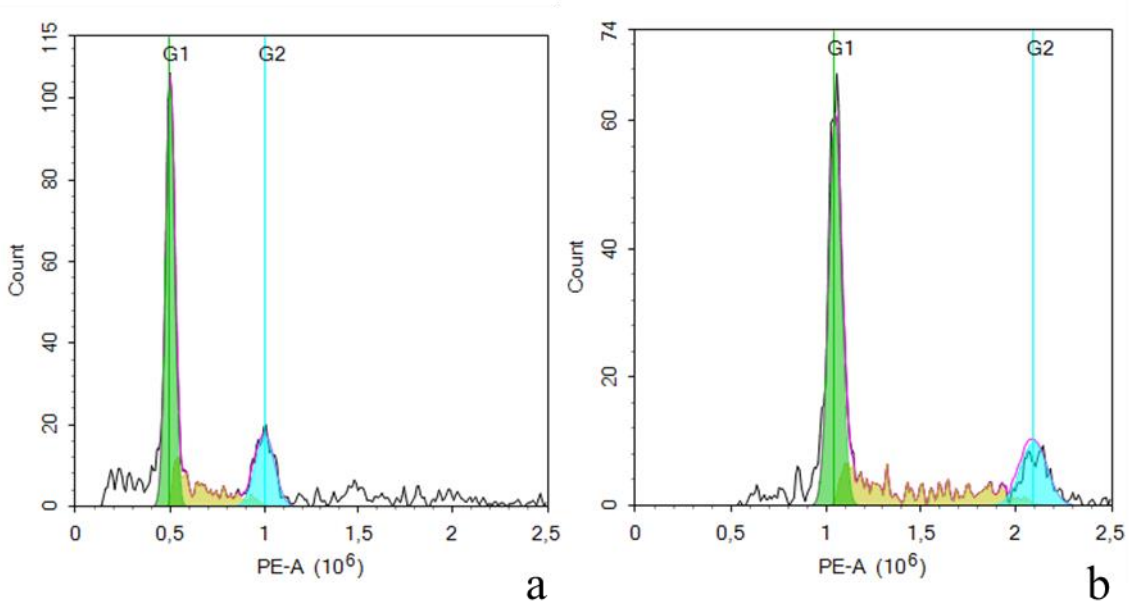


Fig. 3

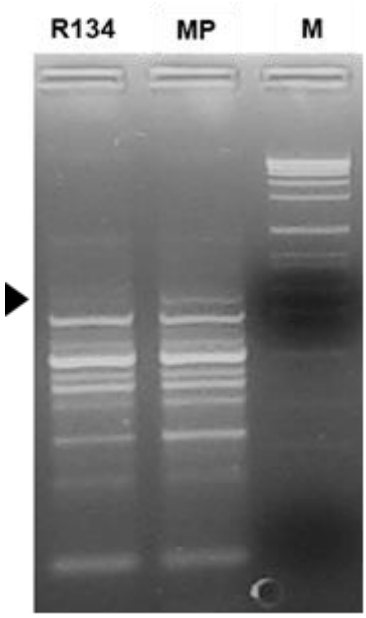


Fig. 4