Adventitious shoot organogenesis and encapsulation technology in hop (Humulus lupulus L.)

Adventitious shoot organogenesis and encapsulation technology in hop (*Humulus lupulus* L.)

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Abstract

Due to the increasing interest of growers in hop cultivation, there is a rising demand for hop plants that is not fully satisfied by nursery activity. Biotechnological methods and, specifically, *in vitro* propagation, could offer new opportunities to overcome these limitations, allowing the production of a great number of plantlets, in reduced space and independently of the season. In this research, organogenesis from two types of explant, petioles and internodes, and, for the first time to our knowledge, unipolar explants encapsulation have been studied to evaluate alternative methods for hop propagation and to try to satisfy hop nursery need. Our results confirmed the possibility of resorting to organogenesis as a propagation method, only if a precise and continuous check of somaclonal variation is carried out, due to the occurrence of a small percentage of tetraploids. Moreover, hop microcuttings were demonstrated to be a suitable starting material for encapsulation; indeed, in less than one month, it was possible to obtain a very high regrowth (up to 100%) and conversion (up to 82%). These results represent a first step towards the application of this technology to hop, also for other purposes, such as short and long term preservation.

Keywords: flow cytometry, hop, internodes, *in vitro* regeneration, petioles, synthetic seed.

1. Introduction

All over the world, the commercial value of hop (*Humulus lupulus* L.) is in its essential oils and resins that confer flavour, bitterness and aroma to beer (Zanoli and Zavatti, 2008).

In line with consumer growing interest for craft beers and for hops endowed with particular phytochemical profiles (Barth-Hass, 2016), recently, also in Italy, the culture of hop has attracted the interest of growers in increasing areas dedicated to hop. Unfortunately, the specialized nursery activity on the Italian territory is scarce, there is a lack of certified plants and, for this reason, hop growers resort to self-produced rhizome or purchase propagation material on parallel markets, regardless of genetic correspondence and phytosanitary quality (Carbone and Cherubini, 2016). Moreover, the problem of hop plant lack is, further, exacerbated by the plant growing slowness and

by the season-dependency of traditional propagation material, such as dormant rhizomes and soft wood cuttings (Neve, 1991). To support the nursery sector, micropropagation can represent a valuable alternative to hop traditional multiplication, allowing the obtainment of a high number of true-to-type plants out of the natural season, in a relatively short time and in limited space (Barlass and Skene, 1982). *In vitro* plant tissue culture can be performed starting from different types of explants: meristems, characterized by the presence of undifferentiated cells, and differentiated tissues, such as leaf portions, stems, petioles and buds (Smith, 2000; Thakur and Kanwar, 2018; Hesami et al., 2019).

Numerous are the published studies carried out by exploiting all the potentialities of hop *in vitro* tissue culture (Gurriarán et al., 1999; Faragò et al., 2009; Fortes et al., 2010; Roy et al., 2001); some of them report the use of micropropagation to eradicate viruses, resorting to meristem culture and cloning (Vine and Jones, 1969; Adams, 1975; Samyn and Welvaert 1983), others evaluated the organogenic ability of petioles and stems (Batista et al., 1996) or leaf portions (Liberatore, 2020) testing different basal media compositions.

Generally, micropropagation led to the obtainment of plants true to the starting material, but, sometimes, due to several conditions of *in vitro* tissue culture and, most of all, to the induction of de- and redifferentiation processes (Standardi and Piccioni, 1998), various type of changes, known as somaclonal variation, may occur (Larkin and Scowcroft, 1981). In hop, Šuštar-Vozlič et al. (1999) and Liberatore (2020) reported the obtainment of tetraploids, respectively from petiole and leaf portion culture.

To overcome this problem, it would be possible to resort, as starting material, to vegetative propagules (bulbs, tubers, corms) or shoot cuttings and buds, exploiting the natural ability of the plant species to produce already organized meristematic tissues (Standardi et al., 1999). Actually, in this way, the de- re-differentiation phase will be avoided, highly reducing the risks of somaclonal variation (Standardi and Piccioni, 1998). Encapsulation technology, when, as starting material, unipolar plant propagules are used, represents one of the solutions to overcome somaclonal

variation and, moreover, presents numerous advantages. Specifically, artificial seed or synthetic seed defined as "artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under *in vitro* or *ex vitro* conditions" (Aitken-Christie et al., 1995), combines the advantages of clonal propagation (high efficiency production, genetic uniformity of plant material, sanitary plant conditions, perfect reduced spaces requirements) with those of zygotic seeds (easy handling and transportability, storability, reduced dimensions, mechanization potentiality) (Standardi and Micheli, 2013). Moreover, encapsulation technology represents a valuable tool to facilitate the exchange of sterile plant material among different laboratories, thanks to the reduced dimensions of propagules and to the easiness of transportability (Redenbaugh, 1993; Gray et al., 1995). Several are the studies reporting the use of encapsulation technology for different plant species, resorting to *in vitro* derived unipolar explants, including ornamental plants (Lambardi et al., 2006; Benelli et al., 2017; Micheli and Benelli, 2019), *Morus indica* L. (Micheli et al., 2017), *Olea europaea* L. (Micheli et al., 1998; Micheli et al., 2007; Micheli et al., 2019), Carrizo citrange (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.) (Germanà et al., 2011) and *Vitis vinifera* L. (Benelli, 2016). In hop, this technique has been used to evaluate the response of several genotypes to cryopreservation, for germplasm storage (Reed et al., 2003; Reed and Hummer, 2013). However, to the best of our knowledge, nothing has been published about the use of encapsulation technology applied to hop.

The main aims of this ~~study~~ research ~~was~~ were the hop *in vitro* propagation improvement~~to evaluate the possibility of propagating hop~~, resorting to ~~*in vitro*~~ petiole and internode regeneration and, for the first time, ~~to study~~the study ~~, for the first time,~~ of the encapsulation technology in this species. With these~~is~~ ~~aim~~objectives, different types of growth regulators, at different concentrations, were applied, studying their effect on vegetative performance of the different explant types and methodologies tested. To verify the possible somaclonal variation occurrence among regenerants, a ploidy analysis was carried out within shoots obtained from petiole and internode *in vitro* culture.

2. Material and methods

2.1 Plant material

Petioles and internodes, for *in vitro* regeneration, and microcuttings, for encapsulation, were isolated from two-month-old *in vitro* cultured plantlets of hop, genotype "Gianni" (Mongelli et al., 2015). Plantlets were cultured in 500-ml glass jars containing 100 ml of **MS-HF** (Hormone Free) culture medium**:** Murashige and Skoog (MS) salt and vitamin mixture (1x) (Murashige and Skoog, 1962), 30 g L^{-1} of sucrose, 8 g L^{-1} of agar (pH 5.8); *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.

2.2 In vitro *petiole and internode regeneration*

Isolated petioles and internodes, deprived of the leaves, were cut in sections (0.5 mm) and put in culture, in sterile Petri dishes (ten explants per each Petri dish, ten Petri dishes per treatment). In order to evaluate the influence of type and concentration of growth regulators, added in the culture medium on hop petiole and internode regeneration, five culture media, with the following composition, were tested: **MS-BAP0:** MS-HF culture medium supplemented with 0.1 µM 1-Naphthaleneacetic acid (NAA); **MS-BAP2**: MS-BAP0 culture medium supplemented with 8.88 µM 6-Benzylaminopurine (BAP); **MS-BAP4**: MS-BAP0 culture medium supplemented with 17.77 µM BAP; **MS-BAP6**: MS-BAP0 culture medium supplemented with 26.66 µM BAP; **MS-BAP8**: MS-BAP0 culture medium supplemented with 35.55 µM BAP. All culture media, after adjusting the pH to 5.8, were sterilized in autoclave for 20 min at 121 °C. Petri dishes were then sealed and placed in growth chamber, at 25±1°C and light intensity of 20 µmol m^{-2} s^{-1}, under 16 h photoperiod.

2.2.1 Flow cytometry analysis of petiole and internode regenerants

The ploidy level of regenerants obtained from petiole and internode culture was evaluated using a NovoCyte (Acea Biociences) and compared with that of the mother plant. Each regenerant was analysed three times. About 0.5 cm^2 of sample (a young leaf from a developed shoot or from the

mother plant, genotype Gianni) was chopped using a razor blade in a Petri dish containing 0.5 mL of extraction buffer (Partec CyStain PI Absolute P Nuclei Extraction Buffer; Partec GMBH, Münster, Germany). The suspension was filtered 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, containing 12 µl of Propidium Iodide Solution and 6 µl RNase. Samples were maintained in the dark for 30 min and then analyzed 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.

2.3 Microcutting encapsulation

Uninodal microcuttings (3-4 mm long) from *in vitro* proliferated shoots, without leaves and with two axillary buds, were excised and, subsequently, subjected to encapsulation, using artificial endosperm (AE) with the following composition: half-strength MS medium, 50 g l^{-1} sucrose and 0.1 µM NAA (pH 5.8). Single microcuttings were immersed, firstly, in the encapsulating solution (AE enriched with sodium alginate - 2.5%, w/v - alginic acid sodium salt, medium viscosity; code 366551 Carlo Erba)); secondly, the alginate coated propagules were immersed in the complexing solution (AE enriched with $CaCl_2$ - 1.1% w/v) for 35 min. After complexation, the capsules were rinsed three times (15 min each time) with sterile AE (Micheli and Standardi, 2005; Standardi and Micheli, 2013).

In order to test the effect of type and concentration of growth regulators on hop encapsulated microcuttings vegetative parameters, the following thesis were assessed: **EMC-0**: microcuttings encapsulated in AE; **EMC-0.5**: microcuttings encapsulated in AE enriched with 2.22 µM BAP; **EMC-1.0**: microcuttings encapsulated in AE enriched with 4.44 µM BAP; **EMC-1.5**: microcuttings encapsulated in AE enriched with 6.66 µM with BAP; **EMC-2.0:** microcuttings encapsulated in AE enriched with 8.88 µM. Ten capsules were placed in each sterile Petri dish, containing **MS-HF** culture medium; ten Petri dishes were prepared per each treatment. As control, 100 not encapsulated

microcuttings (naked, **MC**) were put in culture on the same culture medium (ten microcuttings per Petri dish).

2.4 Statistical analysis of data

2.4.1 *In vitro* petiole and internode regeneration

The following parameters were measured every week for 14 weeks, monitoring petioles and internodes in culture: viability (percentage of explants with a green or light brown appearance, without necrosis or yellowing), callogenesis (percentage of explants producing callus), rooting (percentage of explants developing roots), shooting (percentage of explants developing shoots), number and length of roots per each explant and number and length of shoots per each explant.

At the end of the experiment, Mean Regeneration Time (MRT) and Regeneration Energy (RE) were calculated (*formulae* below reported were adapted to organogenesis, respectively from Kader (2005) and from Paul (1972) who developed them for seed germination); the *formulae* used were the following: 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; peak= time at which regeneration is the highest; f_{tot}= number of all explants with shoots).

Two-way ANOVA (influence of "Type of explant" (TE) and of "Culture Medium Composition" (CMC) was used to calculate the differences between 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).

2.4.2 Microcutting encapsulation

The following parameters were measured every week for 4 weeks: viability (percentage of encapsulated explants with a green appearance, without necrosis or yellowing), regrowth

(percentage of encapsulated explants producing shoots >4 mm), conversion (percentage of explants with extrusion of shoots and 4 mm long roots), number and length of shoots per explant, and number and length of roots per explant.

At the end of the experiment, Mean Regrowth Time (MRET), Regrowth Energy (REE), Mean Conversion Time (MCT) and Conversion Energy (CE) were calculated (*formulae* below reported were adapted to organogenesis respectively from Kader (2005) (MRET and MCT) and from Paul (1972) (MRET and CE) who developed them for seed germination); the *formulae* used were the following: MRET = $\sum f*x/f_{tot}$ (f=number of microcuttings with shoots on a given day, x= days needed for explant to regenerate; f_{tot}= number of all microcuttings with shoots); REE = $\sum f*x$ before the peak*100)/f_{tot} (f=number of microcuttings with shoots on a given day, x= days needed for microcuttings to sproot; peak= time at which regeneration is the highest; f_{tot}= number of all microcuttings with shoots); MCT = $\sum f*x/f_{tot}$ x= days needed for explant to regenerate; f_{tot}= number of all microcuttings with shoots); CE = $\sum f*x$ before the peak*100)/f_{tot} (f=number of microcuttings with roots on a given day; x= days needed for microcuttings to root; peak= time at which regeneration is the highest; f_{tot}= number of all microcuttings with roots). Data were used to calculate means.

One-way ANOVA was used to calculate the differences for the factor "Culture Medium Composition", per each parameter considered; Tukey's test ($p \leq 0.05$) was used for mean separation; when data were not normally distributed, non-parametric tests (Kruskal-Wallis' test, $p \leq 0.05$) were carried out and Dwass-Steel-Critchlow-Fligner's test was used for mean separation (SYSTAT 13.1, Systat Software, Inc; Pint Richmond, CA).

3. Results

3.1 In vitro petiole and internode regeneration

In the course of the experiment, almost all explants remained viable, preserving their green colour for the first week, then turning light brown. Statistical analysis evidenced significant differences for

the factor “TE” (Table 1), with internodes statistically more viable than petioles (100% and 91.3%, respectively; data not shown).

Callus formation was the first response observed in the cultured explants, after about one week of culture; indeed, a yellowish/greenish spongy callus started to develop at the cut ends of explants, from which it increased rapidly, up to cover the entire surface of the explants (Fig. 1a). Statistical analysis carried out, at the end of the experiment, on the percentage of callus producing explants did not evidence a significant difference for both factors considered, TE and CMC (Table 1).

Rhizogenesis was observed, after one week of culture, from internodes (Fig. 1b) and, after two weeks of culture, from petioles. Statistical analysis evidenced a significant interaction between the two factors, TE and CMC (Table 1): considering the factor TE, t~~; t~~he significant highest percentage of explants with roots was recorded in the MS-BAP0 for petioles and in MS-BAP0 and MS-BAP2 for internodes; ~~.~~ whilst, considering the factor CMC~~Moreover~~, the percentage of explants with roots was statistically higher in internodes than in petioles, for both culture media MS-BAP0 (70.8% vs 40.0%) and MS-BAP2 (52.9% vs. 10.9%)~~,~~ (Table 1).

The number of roots produced per single explant was very variable, depending, mainly, on BAP concentration. Statistical analysis confirmed that CMC was the factor that significantly influenced the explant response (Table 1): indeed, both for petioles and internodes, in explants cultured on MS-BAP0 (3.6) a number of roots statistically higher than in those cultured on MS-BAP4 (1.6) and MS-BAP8 (1.3) was recorded (~~data not shown~~Fig. 2a).

Regarding the root length, statistical analysis evidenced a significant interaction between the two parameters; for both types of explant, petioles and internodes, culture media MS-BAP2 and MS-BAP4 induced roots to grow statistically more than MS-BAP0 (Table 1). Comparing the two types of explant, for every culture medium tested, it seems that internodes regenerated statistically longer roots than petioles (Table 1).

Together with the emergence of the first roots, in both types of explants, calli formed organogenic centers, some of which, after 2 weeks, started to turn into small shoots; for petioles, organogenesis

was observed in explants cultured in all culture media, while, for internodes, explants cultured in MS-BAP0 did not show any shoot regeneration. Mostly indirect organogenesis was observed (Fig. 1c); but, even though at very low rate (4.5%~~, data not shown~~), and only from petioles cultured in MS-BAP0, also direct shoot regeneration was observed (Fig 1d).

Shoot regeneration continued for all the period in which the explants were kept in culture and it was observed from the explants cultured in all culture media; a total of 90 shoots (67 regenerants obtained from petioles and 23 from internodes, data not shown) were obtained at the end of experiment; shoots carried on growing in length and forming well developed leaves (Fig. 1e).

Statistical analysis carried out on the percentage of shoot producing explants evidenced a significant difference only for the factor TE (Table 1). Indeed, within petioles, a statistically higher percentage of shoot regenerating explants was recorded (8.1% vs. 3.9%~~, data not shown~~) (Fig. 2b).

Regarding the parameter "number of shoots", no significant differences were detected; on the average, shoot number was 1.8 for petioles and 1.6 for internodes (data not shown).

Analyzing the shoot length, statistical analysis evidenced a significant interaction between the two factors: in petioles, significant differences were recorded between shoots obtained in MS-BAP8 (9.4 mm) and those in MS-BAP2 and MS-BAP4 (respectively 3.9 mm and 4.3 mm); whilst, in internodes, the statistically longest shoots were obtained from explants cultured in MS-BAP2 (13.5 mm). Moreover, significant differences were observed in the length of shoots obtained from explants cultured on MS-BAP2; indeed, from internodes, shoots statistically longer than those obtained from petioles were obtained (13.5 mm vs. 3.9 mm) (Table 1).

A statistically significant interaction was detected for the parameter MTR, calculated considering the explant response during the 14 weeks of culture. The analysis evidenced that petioles cultured on medium MS-BAP0 needed, to produce new shoots, statistically less time (9.5 dd) than those cultured on media with BAP; within explants in culture on BAP-containing culture media, the highest concentration of BAP (MS-BAP8) statistically slowed down the regeneration process (61.6 dd) (Table 1), mainly if compared to MS-BAP2 and MS-BAP6 (25.3 dd and 32.2 dd respectively);

regarding internodes, explants cultured on MS-BAP4 needed a statistically higher number of days (63.0 dd) to start organogenesis respect to MS-BAP6 (17.5 dd) and MS-BAP8 (14.0 dd). Moreover, internodes began to regenerate shoots statistically earlier than petioles, when cultured on media MS-BAP6 (17.5 dd vs 32.2 dd) and MS-BAP8 (14.0 dd vs. 61.6 dd). Considering RE, in petioles, MS-BAP0 induced explants to regenerate much more synchronously and faster than the other media considered; culturing petioles on a medium containing a high amount of BAP (MS-BAP8) determined the statistically lower RE (3.3%), an opposite trend was observed in internodes that gave better performances in terms of RE, when cultured MS-BAP8 (Table 1).

3.1.1 Flow cytometry analysis of petiole regenerants

Cytofluorimetric analysis, carried out on 90 regenerants, revealed that 89 of them were diploid, like the mother plant (Fig. ~~2a~~3a) and only one (regenerated from a petiole explant cultured on MS-BAP8) was tetraploid (Fig. ~~2b~~3b); whereas, other kind of ploidy variation, such as mixoploid or octoploid, were not recorded. Because of the limited number of obtained tetraploid, it was not possible to carry out a statistical analysis.

3.2 Microcutting encapsulation

In the thirty days of the experiment (at the end of this period, no more changes were observed in the cultured explants), all microcuttings, both naked and encapsulated, maintained their viability, showing a bright green color over the entire period in which they were kept in culture (Fig. 4a). Regrowth (Fig. 4b) varied from 74% to 100% and it was statistically lower for EMC-0 and EMC-2 encapsulated microcuttings (Table 2). Together with the MC, also the encapsulated ones produced shoots, demonstrating that hop microcuttings well respond to the encapsulation process (Table 2). Statistical analysis revealed that the parameter “number of shoots” was influenced by BAP concentration; indeed, by increasing the concentration of this growth regulator, the number of produced shoots decreased (Table 2). AE

containing 6.66 µM BAP (EMC-1.5) induced shoots to elongate statistically more than those encapsulated in others AEs (Table 2).

The analysis of MRET and REE, carried out considering the explant response during 30 days in culture, showed that 6.66 µM BAP containing AE (EMC-1.5) determined the statistically shortest MRET (10.8 dd) and the highest REE (9.6%) respect to EMC-0. It means that the best combination of MRET and REE is obtained with EMC-1.5, meaning that this artificial endosperm composition lead microcuttings to regrow faster and in a more synchronous way (Table 2).

Overall the conversion was high for all explants (Fig. ~~3c~~4c), except for EMC-0 explants which showed a conversion value significantly lower than the others (8.0%) (Table 2). Statistical analysis revealed that BAP concentration influenced the number of roots produced per single explant; indeed, the statistically highest number of roots was recorded in microcuttings encapsulated with AE containing 6.66 µM BAP (1.8), while the absence of BAP or its highest concentration in the AE seem to inhibit root formation. Moreover, it seems that BAP induces root elongation; as a matter of fact, the significantly shorter roots were observed in naked microcuttings (MC) and in those encapsulated in artificial endosperm without BAP (EMC-0) (Table 2).

Considering the combination of both parameters, MCT and CE, EMC-1.5 seems to reduce significantly the time needed for encapsulated microcuttings to produce roots, making the conversion a much more synchronous process.

Overall, EMC-1.0 and EMC-1.5 had the best performance for most of the parameters considered, although by adding only 2.22 µM BAP (EMC-0.5) it was possible to obtain results almost comparable with those achieved with a much higher concentration of BAP, reducing significantly the production costs.

4. Discussion

As a rule, plant regeneration is influenced by several factors including culture medium composition, genotype and explant type (Ganeshan et al., 2002; Liu et al., 2010). In hop, numerous

are the studies reporting somatic regeneration, starting from different types of explants, such as leaf portions, internodes and petioles (Batista et al., 1996; Gurriarán et al., 1999; Motegi, 1979; Roy et al., 2001; Smýkalová et al., 2001; Peredo et al., 2006). In literature, many authors reported outstanding results in hop regeneration using internodal explants (Batista et al., 1996; Faragò et al., 2009; Gurriarán et al., 1999; Motegi, 1979). However, it is well known that in hop, as in other species, one of the main factors influencing regeneration response is the genotype (Batista et al., 1996; Gurriarán et al., 1999; Faragò et al., 2009).

Therefore, the purpose of this research was to study the regeneration ability from petioles and internodes of hop, genotype Gianni, evaluating the effect of the type and the concentration of growth regulators, added in the basal medium. In this study, callus initiation represented the first manifestation of the organogenic process. As reported by Batista et al. (1996), for stems and petioles of the cultivars Bragança and Brewer's Gold, also for the genotype Gianni, callus started to develop at the extremities of explants and increased notably up to cover the entire petiole and internode surface. The percentage of callus producing explants was very high, although there were no significant differences among considered media; this is in contrast with results obtained by Gurriarán et al. (1999), who reported that, when IAA was added in media containing BAP, callogenic response decreased for cv. Nugget explants and increased for cv. Brewers Gold explants, pointing out that, in hop, different genotypes have different reaction to regeneration inductive treatment.

Together with callus, explants produced roots, either directly or through callus formation. In hop, there is a lack in literature about root induction from petioles; however, even as petioles would seem to be less responsive than other explants sources (Škof et al., 2007), in this investigation no significant differences between the two types of explant have been recorded; indeed, a high rooting potential of petioles has been observed. Similar result was reported in a previous study, on regeneration from leaves of the same hop genotype (Liberatore, 2020).

Type and concentration of cytokinin represent a key factor affecting the organogenic ability in hop (Batista et al., 1996; Gurriarán et al., 1999; Šuštar-Vozlič et al., 1999); indeed, shoot regeneration has not been observed in internodal explants cultured in MS-BAP0. Moreover, in this study, only from petioles cultured in MS-BAP0, direct organogenesis has been observed; result comparable with those reported by previous studies, in which they describe how the presence of BAP in the culture medium promotes indirect organogenesis (Kazeroonian et al., 2018; Fujii and Shimizu 1990; Zayova et al., 2012).

However, it is also known that organogenic ability depends on the type of explants (Šuštar-Vozlič et al., 1999); actually, in this investigation shoot regeneration was detected in both types of explants tested, petiole and internodes, but with different response.

Since indirect organogenesis is well known to induce genetic variability (Larkin and Scowcroft, 1981), regenerated shoots were subjected to cytofluorimetric analysis to check their ploidy level. In this study only one regenerant, of 90 analyzed (1.1%), was tetraploid; a notably lower rate in comparison to that recorded in a previous study, in which 7.2% of regenerants obtained from genotype Gianni hop leaves was tetraploid (Liberatore, 2020); moreover, in this study, the only tetraploid regenerant was obtained from a petiole cultured on the culture medium containing the highest concentration of BAP (MS-BAP8); also in the above cited study on hop leaf regeneration, the highest percentage of tetraploids (66.7%) was obtained from leaf portions cultured on the same culture medium (Liberatore, 2020). Nevertheless, due to the low mutant rate, probably the induction of regeneration from petioles and internodes could represent a valid method to produce plants true to original starting material; but, anyway, before using petiole or internode regeneration as true-to-type propagation method, it will always be necessary to check the genetic correspondence of regenerants.

In order to avoid the occurrence of mutations in hop micropropagation process, resorting to unipolar propagules can be a valuable solution; indeed, skipping the de- and re-differentiation phase reduces significantly mutation occurrence (Standardi and Piccioni, 1998). A simple and efficient

method for i*n vitro* propagation is the synthetic seed technology, resorting to unipolar explants; as a matter of fact, several are the scientific studies, also in a specie strictly related to hop, such as *Cannabis sativa* L., reporting the obtainment of true-to type plantlets (Nyende et al., 2003; Lata et al., 2011; Chandrasekhara Reddy et al., 2012). Other than, encapsulation technology has other numerous advantages potential applications, among which the most interesting is its use as a valuable method for mass propagation of plant species; indeed, to date, numerous are the studies concerning the application of encapsulation technology to several plant species, including fruit tree crops, ornamentals, cereals and vegetables (Lambardi et al., 2006; Rai et al., 2009), but until now, this technology was never applied to hop propagation. Several are the factors that markedly influence *in vitro* encapsulated propagule behaviour, among which the most important are the initial choice of plant materials, both in terms of genotype and of type of explant, artificial endosperm and culture medium composition and growth conditions (Rai et al., 2009).

In this study, in which hop microcuttings were encapsulated, different artificial endosperm compositions were tested, in order to evaluate, first of all, the suitability of hop, Gianni, microcuttings to be encapsulated, then to study how their vegetative performances are influenced by the artificial endosperm composition, in terms of BAP concentration. All the encapsulated explants, independently on artificial endosperm composition, showed a high viability (100%), throughout the experiment, demonstrating that hop microcuttings could absorb the water and nutrients they need from the capsule; results reported in this study are in line with those obtained in other species, such as *Actinidia deliciosa*, *Malus domestica*, *Olea europaea*, Carrizo citrange (Gardi et al., 1999; Micheli and Standardi, 2005; Germanà et al., 2011; Micheli et al., 2019).

Researchers working on synthetic seed technology (Adriani et al., 2000; Micheli et al., 2019) agree on the importance of regrowth, but most of all, of conversion in making this technology really valuable. Our results showed that hop microcutting regrowth and conversion were strongly influenced by the presence of growth regulators. Being this the first study on hop synthetic seed technology, there is a complete lack of literature to compare our results with, the only study that

could be useful is the one about the nodal segment encapsulation of *Cannabis sativa*, a plant species genetically close to hop, both species belonging to *Cannabaceae* family (Lata et al., 2009). In the study on encapsulation of *Cannabis sativa*, in which the cytokinin (Thidiazuron) was added to artificial endosperm, at a concentration 10 fold higher than that used in this study (BAP), up to 77% of conversion was obtained; moreover, in *Cannabis*, around 21 days were needed to capsules to convert (Lata et al., 2009); both results are similar to those reported in this research; indeed, in hop, up to 82% of conversion was obtained in 20 dd, on the average, but, adding in the artificial endosperm a concentration 10 fold lower of cytokinin. Other than in *Cannabis sativa*, the importance of type, concentration and ratio of auxins and cytokinins is ~~highlithed~~highlighted for several species, as reported by Lambardi et al. (2006).

In this study, BAP was added at different concentrations to induce regrowth and conversion in encapsulated hop, cv. Gianni, microcuttings. After one month of culture, the highest BAP concentration used (8.88 µmol) appears to inhibit the regrowth process in hop, as reported by Badr-Elden (2013) in strawberry capsules; on the contrary, in *Mimosa pudica* L., Banu at al. (2014) observed the best results, in terms of regrowth, from microcuttings encapsulated with artificial endosperm containing the same BAP concentration (8.88 µmol). Conversion of encapsulated hop, ~~cv.~~genotype Gianni, was highly stimulated by the presence of BAP in the artificial endosperm, independently on its concentration; results in contrast with those reported in this study are those reported in *Celastrus paniculatus* (Fonseka et al., 2019) and in Prata-anã' banana's microshoots, clone Gorutuba, (Pereira et al., 2017) which the best performance, in terms of conversion, was obtained from explants encapsulated in 8.88µmol BAP enriched artificial endosperm.

As above reported in some examples, consulting the literature about the influence of artificial endosperm composition on vegetative parameters of several plant species encapsulated microcuttings, results are extremely different, demonstrating, once more, the strong influence of the genotype on their *in vitro* response.

5. Conclusion

A biotechnological approach to propagate *Humulus lupulus* L. represents a valid instrument to obtain a relevant number of plants in a relatively short time. In this work, two methods such as organogenesis from petioles and internodes and, for the first time to our knowledge, encapsulation technology were described. With~~T~~ the first one, it was possible to ~~confirmed the possibility of using~~use petioles and internodes as starting material for hop, genotype Gianni, *in vitro* propagation; however, since ploidy analysis detected the presence of a certain, even though low, percentage of tetraploids, there is the need of a continuous check of genetic correspondence, due to the possibility of the occurrence of genetic mutations.

Encapsulation technology, by resorting to non-embryogenic (unipolar) plant propagules that allow to by-pass the de-re-differentiation phase, represents an alternative to traditional methods, exploiting the regeneration process from meristematic centres.

In less than one month, a very high regrowth (up to 100%) and conversion (up to 82%). was obtained, proving that hop microcuttings are suitable for encapsulation. This result represents a first step towards the application of the encapsulation technology for other purposes, such as hop germplasm conservation. However, further investigations and insights are required to assess the practical applicability of these techniques to different hop genotypes.

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Table 1. Influence of "Type of explant" and "Culture medium composition" on several vegetative parameters of *in vitro* cultured hop, cv. Gianni, petioles and internodes, after 14 weeks of culture

Type of explant	Culture medium composition	Viability (%)	Explants with callus (%)	Explants with roots (%)	Explants with shoots (%)	Roots (n°)	Root length (mm)	Shoots (n°)	Shoots lenght (mm)	MRT (dd)	RE (%)
Petioles	MS-BAP0	84.1	90.7	40.0	3.0	3.2	5.3	2	5.0	9.5	11.3
	MS-BAP2	94.9	94.9	10.9	7.1	2.5	11.7	2.8	3.9	25.3	7.1
	MS-BAP4	95.7	95.7	7.3	7.1	1.4	10.8	2.6	4.3	57.3	6.0
	MS-BAP6	87.3	96.4	5.8	8.1	3.0	7.3	2.4	5.1	32.2	5.8
	MS-BAP8	94.5	91.5	2.7	15.0	1.7	8.4	2.4	9.4	61.6	3.3
Internodes	MS-BAP0	100.0	89.6	70.8	0.0	4.1	7.4	-	-	-	-
	MS-BAP2	100.0	98.3	52.9	10.4	2.7	17.2	2.2	13.5	40.8	5.5
	MS-BAP4	100.0	100.0	20.9	2.1	1.7	18.2	1.0	1.0	63.0	1.6
	MS-BAP6	100.0	93.2	6.7	3.5	1.3	11.4	2.0	6.6	17.5	6.0
	MS-BAP8	100.0	100.0	3.3	3.5	1.0	11.3	2.5	7.3	14.0	7.1
Statistical analysis of factors											
TE		0.016	0.424	0.000	0.027	0.632	0.000	0.363	0.393	0.279	0.510
CMC		0.777	0.526	0.000	0.103	0.000	0.000	0.892	0.062	0.076	0.174
TE*CMC		0.777	0.741	0.002	0.249	0.390	0.036	0.959	0.005	0.047	0.040

Two-way analysis of variance (ANOVA), followed by Tukey's test; $p \leq 0.05$.

TE: Type of explant**; CMC:** Culture Medium; **MS-BAP0:** MS-HF culture medium supplemented with 0.1 µM of NAA; **MS-BAP2:** MS-BAP0 culture medium supplemented with 8.88 µM of BAP; **MS-BAP4:** MS-BAP0 culture medium supplemented with 17.77 µM of BAP; **MS-BAP6:** MS-BAP0 culture medium supplemented with 26.66 µM of BAP**. MS-BAP8:** MS-BAP0 culture medium supplemented with 35.55 µM of BAP; **MRT**: Mean Regeneration Time. **RE**: Regeneration Energy.

Table 2. Influence of encapsulation and artificial endosperm composition on several vegetative parameters of hop, cv. Gianni, microcuttings, after 4 weeks of culture

Thesis	Viability (%)	Regrowth (%)	Conversion (%)	N° of shoots (n°)	L. of shoots (mm)	N° of roots (n°)	L of roots (mm)	MRET (dd)	REE (%)	MCT (dd)	CE (%)
MC-HF	100	94.0 a	72.0 a	1.3 a	5.5 c	1.0 d	5.8 b	15.0 ab	6.4 ab	17.1 b	5.9 ab
EMC-0	100	74.0 b	8.0 b	1.2 a	2.5 d	1.2 c	2.3 b	18.1 a	4.5 b	28.0 a	3.6 b
EMC-0.5	100	98.0 a	78.0 a	1.1 ab	13.4 b	1.3 b	9.5 a	6.4 b	7.6 ab	24.0 a	4.8 b
EMC-1.0	100	100.0 a	68.0 a	1.1 ab	11.2 b	1.3 b	9.9 a	13.4 ab	8.9 ab	18.3 b	6.6 ab
EMC-1.5	100	96.0 a	82.0 a	1.0 b	19.3 a	1.8 a	13.2 a	10.8 b	9.6 a	15.8 b	7.2 a
EMC-2.0	100	90.0 b	67.5 a	1.0 b	14.9 b	1.0 c	11.1 a	12.1 ab	8.2 ab	16.0 b	6.7 ab

One-way analysis of variance (ANOVA), followed by Tukey's test; $p \leq 0.05$. Per each column, values followed by different letters are statistically different.

MC-HF: naked microcuttings, cultured on Hormon Free (HF) culture medium; **EMC-0**: microcuttings encapsulated with Artificial Endosperm (AE); **EMC-0.5**: microcuttings encapsulated in Artificial Endosperm (AE) with 2.22 µM BAP; **EMC-1.0**: microcuttings encapsulated AE in 4.44 µM BAP; **EMC-1.5**: microcuttings encapsulated in AE with 6.66 µM with BAP; **EMC-2.0:** microcuttings encapsulated in AE with 8.88 µM BAP. **MRET**: Mean Regrowth Time. **REE**: Regrowth Energy; **MCT**: Mean Regrowth Time. **CE**: Conversion Energy.

Figures

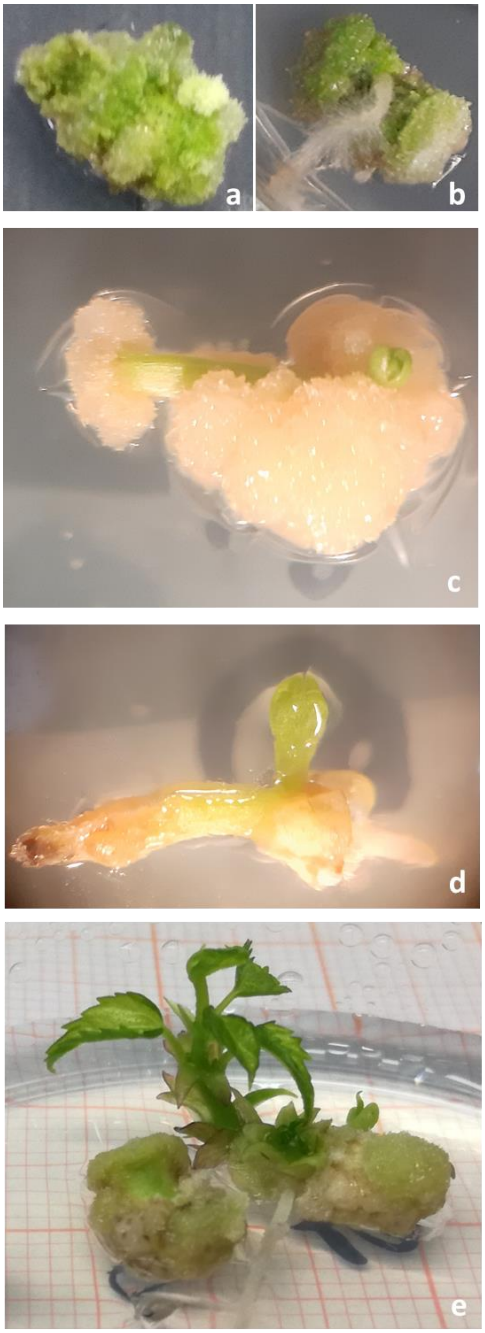

Figure 1. Organogenesis from hop, cv. Gianni, petioles and internodes: **a)** callus covering the petiole surface; **b)** first root regeneration from an internode; **c)** indirect organogenesis from a petiole; **d)** direct organogenesis from a petiole; **e)** shoot development from an internode.

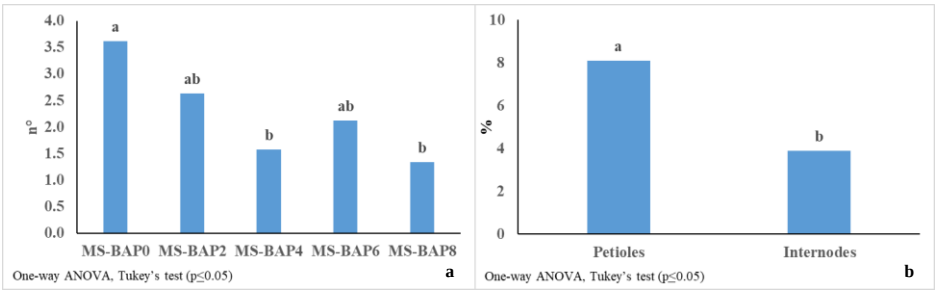

Figure 2. a) One-way ANOVA of the parameter "n° of roots" for the factor "Culture Medium Composition"; b) One-way ANOVA of the parameter "Percentage of explants with shoots" for the factor "Type of Explant".

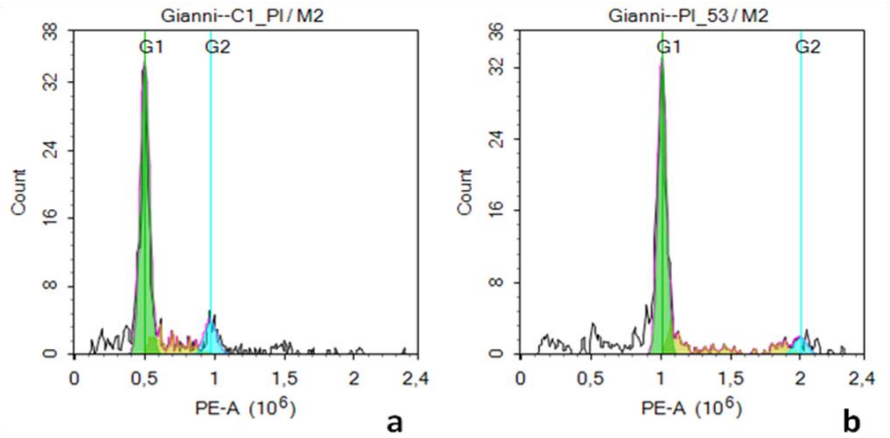

Figure ~~2~~3. Cytofluorimetric analysis: histograms of fluorescence intensity of nuclei from diploid leaf tissue of genotype Gianni **a)** mother plant **b)** tetraploid regenerant from a petiole.

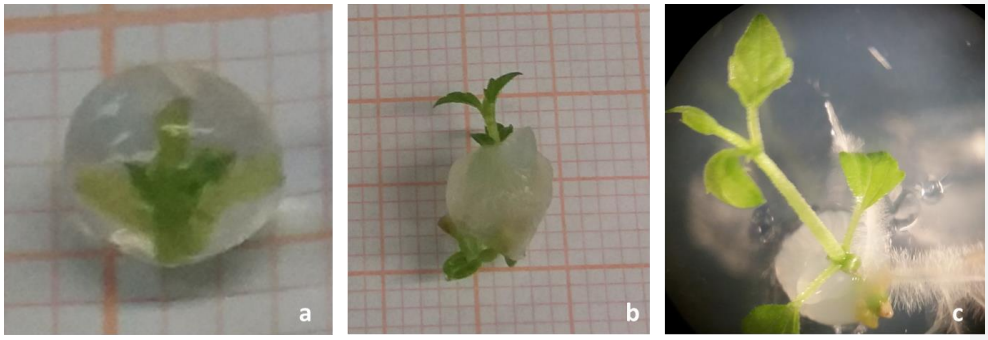

Figure ~~3~~4. Hop, cv. Gianni, encapsulated microcutting development: **a)** viable encapsulated microcutting; **b)** microcutting regrowth; **c)** microcutting conversion.