Efficient *in vitro* micropropagation and regeneration of *Humulus lupulus* on low sugar, starch-*Gelrite* media

I. SMÝKALOVÁ*, M. ORTOVÁ*, H. LIPAVSKÁ** and J. PATZAK***

*Laboratory of Tissue Culture, Faculty of Agronomy, Czech University of Agriculture, Kamýcká 129, CZ-165 21 Prague 6 - Suchdol, Czech Republic**
*Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, CZ-128 44 Prague 2, Czech Republic***
*Hop Research Institute Co., Ltd., Kadaňská 2525, CZ-438 46 Žatec, Czech Republic****

Abstract

Several Czech and foreign hop mericlones were tested *in vitro* for efficiency of green callus formation and plant regeneration from internodal or nodal explants. Modified MS media gelled either with agar, starch or a mixture of potato starch and *Gelrite*, supplemented with different concentrations of either glucose or maltose, were investigated. Two mericlones of Czech hop (Osvald 72 no. 5216 and Sládek no. 6908) were studied in more details because of their different regeneration capacities. The HPLC analysis of medium sugar concentrations after the explant cultivation has revealed slow uptake of sugar from the medium. Presence of glucose at concentration of 45 g dm^{-3} in agar medium resulted in a decreased number of nodes compared to the control with 30 g dm^{-3} of glucose. The use of a mixture of potato starch plus *Gelrite* instead of routinely used agar and decreasing the medium glucose concentration to 15 g dm^{-3} proved to be most efficient for multiplication rate. The use of this medium results in lower cost of micropropagation of healthy hop cultures without exhibition of vitrification.

Additional key words: gelling agents, glucose, hop, maltose, micropropagation.

Introduction

Agar is traditionally preferred gelling agent for tissue cultures at concentrations ranging between 5 and 10 g dm^{-3} (George 1993). Modified Murashige and Skoog (1962; MS) solid medium was succesfully used for *in vitro* node cultures of Czech hops by Svoboda (1991). The medium contained 7 g dm^{-3} of agar and 30 g dm^{-3} of glucose. For other genotypes, however, different gelling agents were successfully applied. *Gelrite*, another frequently used gelling agent, is a gellan gum produced by the bacterium *Pseudomonas elodea* (Kang *et al.* 1982). Nairn (1988) used agar-*Gelrite* medium for shoot culture of *Pinus radiata*. Henderson and Kinnersley (1988) have confirmed that starch can be used instead of agar for several species. Modified MS medium gelled with a mixture of maize starch and *Gelrite* was successfully used for micropropagation of some fruit crops (Zimmerman *et al.* 1995).

Glucose or sucrose are commonly added to the media for *in vitro* hop cultures in concentration ranging between 20 and 40 g dm^{-3} (George and Sherington 1984). Sucrose was used in hop cell suspension cultures (Langezaal and Scheffer 1992, Trevisan *et al.* 1997), glucose for shoot regeneration by direct organogenesis (Rakouský and Matoušek 1994), indirect organogenesis (Batista *et al.* 1996) and cultivation of callus or node cultures (Svoboda 1991, 1992a,b), and meristem culture of isolated apical shoot tips (Svoboda 1992c). The only protocol using maltose (40 g dm^{-3}) for callus induction in different hop cultivars was reported by Šustar-Vozlič *et al.* (1996). Maltose is reducing disaccharide capable of being broken down by autoclaving to glucose (Mathes *et al.* 1973).

The objectives of our study were to determine the

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** Author for correspondence; fax: (+420) 2 21953306, e-mail: lipavska@natur.cuni.cz

effect of starch-*Gelrite* gelled media in combination with different type and concentration of sugars (glucose or maltose) on multiplication rate of node culture, and induction of regenerative calli in different hop genotypes.

Materials and methods

Plants: Hop (*Humulus lupulus* L.) mericlones derived from Czech cultivars Bor (no. 11128, 11132), Osvald 72 (no. 6908, 10115, 10120), Sládek (no. 5216, 11100) or foreign cultivars Galena (no. 10047, 10052) and Eroica (no. 10061, 10066) from USA, Yeoman (10029, 10038) and Northern Brewer (no. 10095) from England, Southern Brewer from South Africa (no. 10074, 10082), and Taurus (no. 9998, 10006) from Germany were used for the experiments. The mericlones were obtained from the Collection of the Hop Research Institute, Žatec.

Medium composition and culture conditions: *In vitro* nodal cultures derived from aseptic meristems were maintained by regular transfer of stem cuttings (4 or 5 middle nodal segments of donor plants) in 12 week intervals to fresh agar medium named CHO. This medium was based on half strength MS salts and vitamins. 0.1 g dm^{-3} of myo-inositol, 30 g dm^{-3} of glucose and 7 g dm^{-3} of agar (pH 5.7) were added according to Svoboda (1991). The medium was further modified by different concentrations of sugars, phytohormones and gelling agents. For micropropagation the plants were cultured on solid medium in 25 cm^3 glass tubes with aluminium covers and for regeneration in 100 cm^3 glass jars with aluminium foils or on pieces of filter paper being in contact with but not submerged in liquid medium (for sugar content determination in cultivation medium). All cultures were kept in cultivation chamber: 16 h-photoperiod, day/night temperature 23 ± 1/19 ± 1 °C, irradiance 360 - 540 µmol $m^{-2}s^{-1}$ (fluorescent tube type LT 36W/010 *daylight, Narva,* Germany).

The following characters were evaluated: the viability, chlorosis, necrosis of the plants, the spontaneous green calli formation, total number of nodes per plants, number of stems and growth of roots. Experiments were repeated 2 - 3 times.

Regeneration: Internodes (5 - 7 mm), leaf nodes and nodes without leaves (2 - 3 mm) of 10 - 12 weeks old plants were used for regeneration capacity evaluation of studied mericlones. Number of explants ranged between 24 and 142 for different mericlones and medium. The segments were excised and transferred to CHO media, modified with 20 and 40 g dm^{-3} of glucose or maltose, for induction of green calli and regeneration of new stems. Some explants were chilled for one week (4 °C) in the dark.

Sugar content determination: Samples of the media were stored at -18 °C. Content of extracted soluble non-structural saccharides was detected using high-pressure liquid chromatography (HPLC) with refractometric detection (*Spectra Physics*, Mountain View, USA; refractometer *Shodex RI-71*, pre-column *Hema-Bio 1000 Q + SB, Watrex*, Prague, Czech Republic, column *Hi Plex* Ca^{2+}, *Polymer Laboratories*, Church Stretton, Great Britain, eluent re-distilled H_2O, flow rate 0.5 cm^3 ~~min^{-1}~~, temperature 80 °C, volume of injected sample 0.01 cm^3).

Results and discussion

We tested 17 types of agar media for *in vitro* regeneration of 17 mericlones derived from Czech and foreign hops. The efficiency of green callus formation from internode, node and node with leaves was evaluated. The internode and node explants exhibited approximately the same efficiency (data not shown). The frequency of green callus formation on the nodes with leaves was very low. The Czech mericlones exhibited higher regeneration potential in comparison to foreign genotypes. The CHO agar medium containing 40 g dm^{-3} of maltose, 5 mg dm^{-3} of kinetin or 1 mg dm^{-3} of zeatin, and 0.1 mg dm^{-3} of 2,4-dichlorophenoxyacetic acid (2,4-D) was successfully used for most of them for proliferation of shoots from green calli. Mericlones required different medium composition for callus induction (Table 1). The cold or the dark pretreatment of explants had no effect on induction of green calli.

Mericlones derived from Czech hop cultivars were tested for micropropagation rate on agar media with different concentration of glucose or maltose (30, 60 and 90 g dm^{-3}). The higher concentrations (60 and 90 g dm^{-3}) of glucose or maltose significantly inhibited growth of node explants (data not shown). Two selected mericlones no. 6908 cv. Sládek and no. 5216, cv. Osvald 72 were used for further experiments, because of large difference in multiplication rates between these mericlones were found. The greater multiplication rate was observed for mericlone no. 6908 (Fig. 1). The glucose in concentration 45 g dm^{-3} caused the decrease in multiplication rate to 20 % of the same mericlon on 30 g dm^{-3} of glucose during the first period of cultivation (Fig. 2). The difference diminished during further cultivation. Transfer of node explants to the agar medium with 15 g dm^{-3} of glucose from 30 g dm^{-3} of glucose had stimulating effect on

rooting and multiplication of nodes, particularly during first 15 d of cultivation. Mean number of nodes per one shoot was significantly different in comparison to the control after 60 d of cultivation (Fig. 2). We found slight stimulation of multiplication of nodes on the media with low concentration of glucose (15 or 20 g dm^{-3}), while total inhibition or significant reduction of explant growth at higher concentrations of glucose or maltose. During 2 - 3 months 10 - 12 nodes were produced per one explant (node explant with two axilary buds).

Table 1. The relative frequency of green callus forming of hop genotypes mericlones of Czech cultivars (6908, 5216, 10120, 10115, 11132) and mericlones of foreign cultivars (10052, 10038, 10029, 9998, 10006, 10095, 10066) and the composition of the medium with the highest callus formation frequency [20 or 40 g dm^{-3} of glucose (G20 and G40), 40 g dm^{-3} of maltose (M40), cytokinins kinetin, zeatin, zeatin riboside (zeatin-r), thidiazuron (TDZ), purine, and auxin 2,4-D].

Mericlone	Callus frequency [%]	Sugar	Cytokinin	[mg dm^{-3}]	2,4 D [mg dm^{-3}]
6908	10.3	M40	zeatin	1	0.1
5216	68.9	M40	kinetin	5	0.1
10120	70.3	G20	kinetin	5	10
10115	70.8	M40	zeatin	1	0.1
11132	40.5	G20	TDZ	2	0
10052	6.3	G20	zeatin-r	5	0.1
10038	1.2	G20	TDZ	5	0.1
10029	10.0	G20	zeatin	2	0.1
9998	40.4	G20	purine	2	0.1
10006	32.1	G40	kinetin	10	0.1
10095	25.0	M40	zeatin	1	0.1
10066	16.7	M40	zeatin	1	0.1

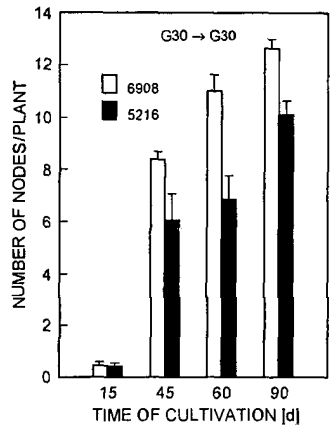

Fig. 1. The multiplication rate of two selected mericlones no. 6908, cv. Sládek and no. 5216, cv. Osvald 72 in agar medium. The mother plants were grown in the same medium with 30 g dm^{-3} glucose (G30). Means ± SE, $n = 132$.

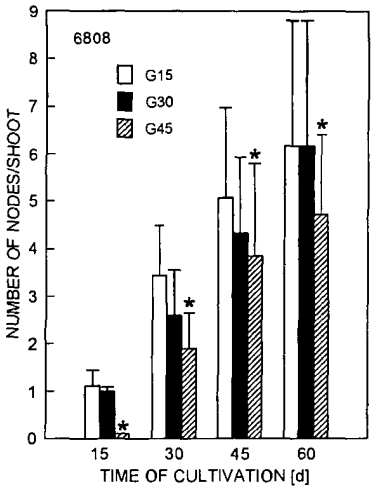

Fig. 2. The effect of glucose concentration (15, 30 and 45 g dm^{-3}; G15, G30, G45) in agar medium on the multiplication rate of mericlone 6908, cv. Sládek. Means ± SE; 44 segments per treatment.

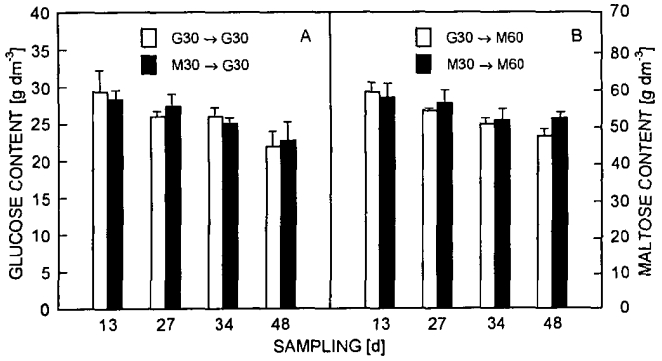

Fig. 3. The liquid medium glucose (*A*) or maltose (*B*) concentration during growth of node cultures of mericlone no. 5216, cv. Osvald 72. The mother plants were cultivated on the agar medium with 30 g dm^{-3} of glucose or maltose and node explant were cultivated on liquid media with 30 g dm^{-3} of glucose or 60 g dm^{-3} of maltose (G30 → G30, G30 → M60, M30 → G30; M30 → M60). Means ± SE; 22 segments per treatment.

The important requirement is also the ability of explant to metabolize the used type of sugars (Welander and Pawlicki 1994). The sugars remaining in the liquid medium analysed using HPLC showed slow uptake of glucose or maltose (Fig. *3A,B*). Approximately 10 g dm^{-3} of glucose or maltose was the highest amount depleted from the medium during 48 d of cultivation. These results indicate that plants were not able to use the substantial part of the sugar when the media with 30 g dm^{-3} of glucose or 60 g dm^{-3} of maltose were used. The medium used for pre-cultivation of mother plants had no effect on the utilisation of the sugar from liquid medium by node explants (Fig. 3). Requirement of carbon source could be different for a particular genotype or cultivar. Also it is difficult to decide, if the effect of sugar on the growth is achieved through its utilization as a source of carbon and energy or its indirect effect as osmoticum (Welander and Pawlicki 1994). Glucose is partially taken up by explants from the media, but the greater part of carbon and energy demand the plants probably acquire through photosynthesis. Solárová *et al.* (1989) in their experiments grew tabacco plants under full autotrophic conditions on medium without sugars. From the comparison between photosynthesis rate in the presence and in the absence of sugar in media, the authors concluded, that the development of photosynthetic apparatus was only slightly affected by sugar present in the medium. Also Tichá (1996) did not observe any inhibition of photosynthesis in tobacco cultures growing on the medium with sucrose in comparison to full autotrophic culture. Sheen (1990) found, however, that high level of sugars inhibited expression of genes, which play role in photosynthesis. In some cases the presence of sugar in medium increases the efficiency of photosynthesis (Lee *et al.* 1985, Galzy and Compan 1992, Paul and Stitt 1993).

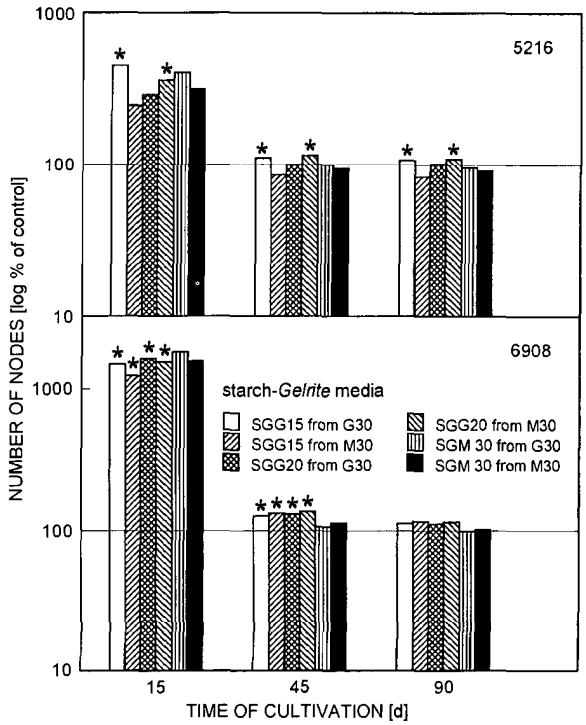

Fig. 4. The effect of different concentration of glucose and maltose in starch-*Gelrite* medium on the multiplication rate. mericlones no. 5216, cv. Osvald 72 and no. 6908, cv. Sládek. The mother plants from agar medium with 30 g dm^{-3} of glucose or maltose (G30 or M30, respectively) were passaged to starch-*Gelrite* medium with 15, 30 g dm^{-3} of glucose or 30 g dm^{-3} of maltose (SGG15, SGG20 and SGM30); 22 nodes per culture; * - statistically significant differences at $P \leq 0.05$.

We tested alternative gelling agents for reduction of cost of *in vitro* micropropagation of hop cultures. Agar as gelling agent was replaced by combination of starch and *Gelrite* (*Sigma Aldrich*, Germany) at concentrations 50 and 0.5 g dm^{-3} as reported by Zimmerman *et al.* (1995). They used maize starch-*Gelrite* medium to produce more apple and raspberry shoots of suitable appearance. A major advantage of the starch-*Gelrite*, used in our experiments, is the reduced cost of the medium. Using starch alone lead to vitrification of culture (data not shown). However, the addition of *Gelrite* to that gelling agent suppressed the symptoms: proliferation of new shoots from axillary buds of main shoot(s), deformation of shoots (thickened internode), reduction of leaf area, vitrification of leaves. The most plants growing on SG media were healthy without colour and morphological changes. Testing of starch-*Gelrite* media was performed with different concentrations of glucose

(15, 20 and 30 g dm^{-3}) or maltose (30 g dm^{-3}) for *in vitro* micropropagation of mericlone no. 5216, cv. Osvald clone 72 and mericlone no. 6908, cv. Sládek (Fig. 4). For both mericlones the final yields of nodes per one culture increased on the media with 15 and 20 g dm^{-3} of glucose, particularly after 15 d of cultivation of both mericlones. Although significant effect of sugar type in pre-cultivation media on multiplication rate of both mericlones was observed for agar solidified medium (Fig. 5), the result was not exactly proved for starch-*Gelrite* media. From visual evaluation of nodal culture we concluded that the segments grown on all types of agar media had normal rooting as control. Opaque grey-white colour of starch or starch-*Gelrite* media make impossible to observe rooting during cultivation. Rooting evaluation made at the time of plant harvest revealed, however, that gelling agents had no adverse effect on root development. We could frequently observe green callus, which was formed on the base of the node explants growing on SG media (Table 2). Both axillary buds on stem segments proliferated in both mericlones grown on starch-*Gelrite* medium with 15 g dm^{-3} of glucose. The greatest induction and proliferation of new shoots were found in SGG15 medium for mericlone no. 5216 and in M30 agar medium for mericlone no. 6908. Induction of green calli was much more effective by mericlone no.5216 than 6908.

Adverse effect of higher sugar concentration on growth and a low utilisation of the added glucose or

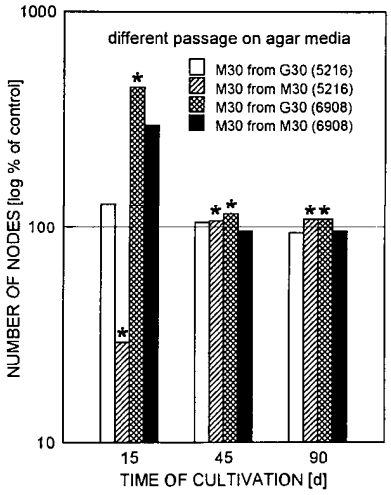

Fig. 5. The effect of maltose in agar medium on the multiplication rate. The mother plants (mericlones no. 5216, cv. Osvald 72 and no. 6908, cv. Sládek) from agar medium with either 30 g dm^{-3} of glucose or 30 g dm^{-3} of maltose (G30 or M30) were passaged to the same medium or on the agar medium with the second type of sugar; 22 plant per culture; * - statistical significant differences at $P \leq 0.05$.

Table 2. The effect of medium composition on of the development of axillary buds and formation of regenerative calli in two mericlones no. 6908, cv. Sládek and no. 5216, cv. Osvald 72 (gelling agents: 7 g dm^{-3} of agar, 50 + 0.5 g dm^{-3} of starch and *Gelrite* (SG); sugars: 15, 20 or 30 g .dm^{-3} of glucose or 30 g dm^{-3} of maltose; each nodal explant had two axillary buds and two leaves; 90 d of cultivation).

Mericlone	Medium		Tested cultures	Cultures [%] forming 0 shoots	1 shoot	2 shoots	callus	Calli [%] forming shoots
6908	G30	agar	132	0.8	6.8	92.4	14.4	2.3
	M30	agar	110	2.4	22.6	75.0	24.5	13.6
	G15	SG	44	0	0	100.0	11.4	6.8
	G20	SG	44	0	6.8	93.2	22.7	2.3
	M30	SG	44	0	9.1	90.9	11.4	11.4
5216	G30	agar	132	3.9	17.5	78.6	8.3	3.8
	M30	agar	110	6.1	16.2	77.7	18.2	10.9
	G15	SG	44	0	0	100.0	20.5	18.1
	G20	SG	88	0	8.0	92.0	6.8	2.3
	G30	SG	44	0	8.3	91.7	13.6	2.3
	M30	SG	88	1.1	2.3	96.6	9.1	5.7

maltose by the explants is documented in our results. We propose, that the effect of sugar present in the medium is preferentially of osmotic nature and to less degree it is caused by changes in the availability of a source of carbon and energy. We can conclude that the use of the starch-*Gelrite* medium with 15 g dm^{-3} of glucose increase yield of regenerants in hops culture via micropropagation or *in vitro* regeneration.

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