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The propagation of chestnut (*Castanea sativa* Mill.) nodal explants

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ABSTRACT

Chestnut is a woody species, which is difficult to propagate either generatively by seed or vegetatively by grafting or cuttings. The experiment of vegetative propagation included *in vitro* propagated shoots of the chestnut clone 'Sobota' from the northeast area of Slovenia. Propagation MS-½NO_3 medium contained basic (macro- and microelements) MS medium with a half concentration of two nitrates (KNO_3 and NH_4NO_3) and 100 mg/l inositol, 1 mg/l thiamine, 30 g/l sucrose, and 8 g/l agar. The propagation BW medium consisted of half strength broad leaved tree BT medium and half strength woody plant WP medium. Both propagation media contained 1 mg/l BAP (6-benzylaminopurine) or 1 mg/l zeatin. The *in vitro* shoots developed much better when propagated on medium containing BAP. With BAP irrespective of the medium, clusters developed significantly more shoots (average 2.1 per cluster) and showed a trend of more vigorous growth with the inclusion of BAP. Shoots grown on BW medium were of poorer quality, with high browning or necrosis and extremely highly intensive hyperhydration ratios.

Key words: chestnut, shoot propagation, propagation media, cytokinin

IZVLEČEK

RAZMNOŽEVANJE KOSTANJA (*Castanea sativa* Mill.) IZ NODIJSKIH IZSEČKOV

Pravi kostanj (*Castanea sativa* Mill.) je lesnata rastlina, ki se težje razmnožuje tako generativno s semenom, kot tudi vegetativno s cepljenjem in potaknjenci. V poskus vegetativnega razmnoževanja so bili vključeni *in vitro* razmnoženi poganjki oz. nodiji pravega kostanja klon 'Sobota' iz območja severovzhodne Slovenije. Razmnoževalno MS-½NO_3 gojišče je vsebovalo bazalno (makro- in mikroelemente) MS gojišče s polovično koncentracijo dveh nitratov (KNO_3 in NH_4NO_3) ter 100 mg/l inozitola, 1 mg/l tiamina, 30 g/l saharoze in 8 g/l agarja. Razmnoževalno BW gojišče je vsebovalo polovični koncentaciji gojišč za listavce BT in lesnate rastline WP. Obe gojišči sta vsebovali 1 mg/l BAP (6-

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benzilaminopurin) oz. 1 mg/l zeatina. Poganjki *in vitro* so se razvijali veliko boljše, če je gojišče vsebovalo citokinin BAP. Ne glede na vrsto gojišča se je ob prisotnosti BAP razvilo statistično značilno več poganjkov (v povprečju 2,1 v skupku), ki so kazali trend močnejše rasti. Poganjki, ki so rasli na obeh BW gojiščih so bili slabše kvalitete, zaradi večjega odstotka porjavenja oz. pojavljanja nekroz in zelo močne vitrifikacije.

Ključne besede: kostanj, razmnoževanje poganjkov, razmnoževalno gojišče, citokinin

1 INTRODUCTION

Chestnut propagation is very difficult, as is also the propagation of other species such as oak or beech from the same plant family *Fagaceae*. The leading propagation method for chestnut is grafting, although several problems are associated with this method. The wound made during the grafting procedure can serve as an entrance point for several pathogens, such as chestnut canker (*Cryphonectria parasitica*). Additionally, incompatibility problems between the rootstock and the scion are known in relation to grafting chestnut trees (Osterc *et al*., 2001).

The improvement of various *in vitro* procedures in the last 20 years has opened the possibility of successful micropropagation of a number of woody species. Micropropagation provides the possibility of a plant source for direct sales, and the production of vigorous, juvenile mother plants is additionally important. Successful propagation of chestnut by traditional methods such as cuttings is only possible in combination with juvenile mother plant material (Osterc, 2001).

Several reports in the literature deal with micropropagation in chestnut. All micropropagation stages have been covered in these experiments. Axillary buds and meristems have been shown to be successful explants sources. Successful induction percentages differed greatly, between 0 and 90%, depending on the maturity level of the mother plants and on the induction date (Chauvin and Salesses, 1988). The cluster propagation ability in the process of micropropagation of chestnut has also often been studied. A low total N level and low NH_4^+/NO_3^- ratio has been suggested (Piagnani and Eccher, 1988) for successful proliferation. Vieitez *et al*. (1983) compared several media to obtain the cluster propagation ability in different chestnut clones from the same hybrid group *Castanea sativa* × *Castanea crenata*. The highest number of shoots per culture formed was achieved when the explants were grown on Heller's medium, explants grown on Heller's + $(NH_4)_2SO_4$ medium (Heller, 1953) and on MS medium (Murashige and Skoog, 1962) with the addition of $NH_4NO_3^-$ were slightly worse. The shoot elongation process was highest on MS medium with a half concentration of NO_3^- and on Lepoivre medium (Quoirin and Lepoivre, 1977). The effects of different cytokinins on proliferation have also been discussed. Vieitez and Vieitez (1980) reported that BAP (6-benzylaminopurine) showed the most satisfactory effect on promoting the proliferation of axillary shoots, whereas zeatin slightly inhibited the development of axillary shoots but increased the induction rate and caused more vigorous shoots. Tetsumura and Yamashita (2004) achieved similar results with the addition of zeatin also causing the highest proliferation rate. They also compared three different media in their experiment, and concluded that standard MS media with half strength NO_3^- gave the worse shoot survival results but caused the longest shoots. Better survival results were achieved with combined (BW) medium (Tetsumura and

Yamashita, 2004) from half strength broad leaved tree (BT) medium (Chalupa, 1984) and half strength woody plant (WP) medium (Lloyd and McCown, 1981), and walnut (DKW) medium (Driver and Kuniyuki, 1984). Both media showed a worse effect on shoot growth.

In our experiment we tried to establish an efficient propagation protocol for the Slovenian chestnut clone 'Sobota' on two different basal media, supplemented with cytokinins BAP and zeatin.

2 MATERIALS AND METHODS

2.1 Plant material and regeneration of shoots

Axillary winter buds of chestnut clone 'Sobota' were used for culture initiation. The buds were removed from several year old grafted mother plants grown at the Nut Nursery Station of the Biotechnical Faculty in Maribor, Slovenia.

Surface sterilization was carried out by rinsing the buds for 30 sec in 70% ethanol. The buds were then sterilized in 20 g/l DCCA (dicloroisocyanuric acid Na_2 salt, Sigma D-2536) with the addition of a few drops of Tween 20 for 20 min and rinsed three times in sterilized water. After surface sterilization, the bud scales were removed as thoroughly as possible. The buds were cultured in 100×20 mm petri dishes (8 buds per dish) on induction medium (Ki) for 30 days. The basal Ki medium consisted of MS macro- and microelements (Murashige and Skoog, 1962), 882 mg/l $CaCl_2 \times 2H_2O$, 361 mg/l $MgSO_4$, 0.1 mg/l IBA (indole-3-butyric acid), 1.0 mg/l BAP, 35 g/l sucrose, pH 4.5 (Xing *et al.*, 1977), 8 g/l agar. After the induction period, buds were subcultured on petri dishes containing differentiation medium (WPMd). The basal WPMd medium consisted of WPM (McCown Woody Plant Medium) macro- and microelements and vitamins (Lloyd and McCown, 1981), 2 mg/l glycin, 100 mg/l inositol, 0.1 mg/l NAA (naphthaleneacetic acid), 0.1 mg/l BAP, 20 g/l sucrose, 8 g/l agar, pH 5.6.

2.2 Propagation of shoots

After the regeneration period, shoots and nodes about 0.5 cm long (15 shoots or nodes per dish, Figure 1A) were subcultured on petri dishes containing two different propagation media (MS-½NO_3 or BW) with two cytokinins (1mg/l BAP or 1 mg/l zeatin). MS-½NO_3 medium (Vieitez *et al.*, 1983) consisted of MS macro- and microelements with half strength nitrates and 100 mg/l inositol, 1 mg/l thiamine, 30 g/l sucrose, 8 g/l agar, pH 5.6. BW medium (Tetsumura and Yamashita, 2004) consisted of half strength broad leaved tree (BT) medium (Chalupa, 1984) and half strength woody plant (WP) medium (Lloyd and McCown, 1981).

Petri dishes were sealed with Parafilm and the explants were exposed to a 16/8 photoperiod at 23 ± 1°C and illumination of 40 μmol $m^{-2}s^{-1}$.

One-way ANOVA followed by Duncan's multiple-range test was conducted to evaluate differences among the treatments.

3 RESULTS AND DISCUSSION

Comparison of different propagation media showed differences when different parameters were observed. The highest number of newly formed shoots was counted on standard MS medium with half concentration NO_3^- with 1 mg/l BAP as cytokinin (Figure 1) and on BW+BAP medium. Both other media showed a significant lower intensity of new shoots formation (Figure 2). The use of cytokinin BAP was most

efficacious in our experiment. This result does not correspond to some literature data, in which the cytokinin zeatin was recommended for the multiplication phase of chestnut *in vitro* propagation (Tetsumura and Yamashita, 2004). The use of BW medium was comparable with standard MS medium. It is very important regarding the MS medium that the N concentration is low. This has been already investigated (Vieitez *et al*., 1983; Piagnani and Eccher, 1988; Soylu and Ertürk, 1999).

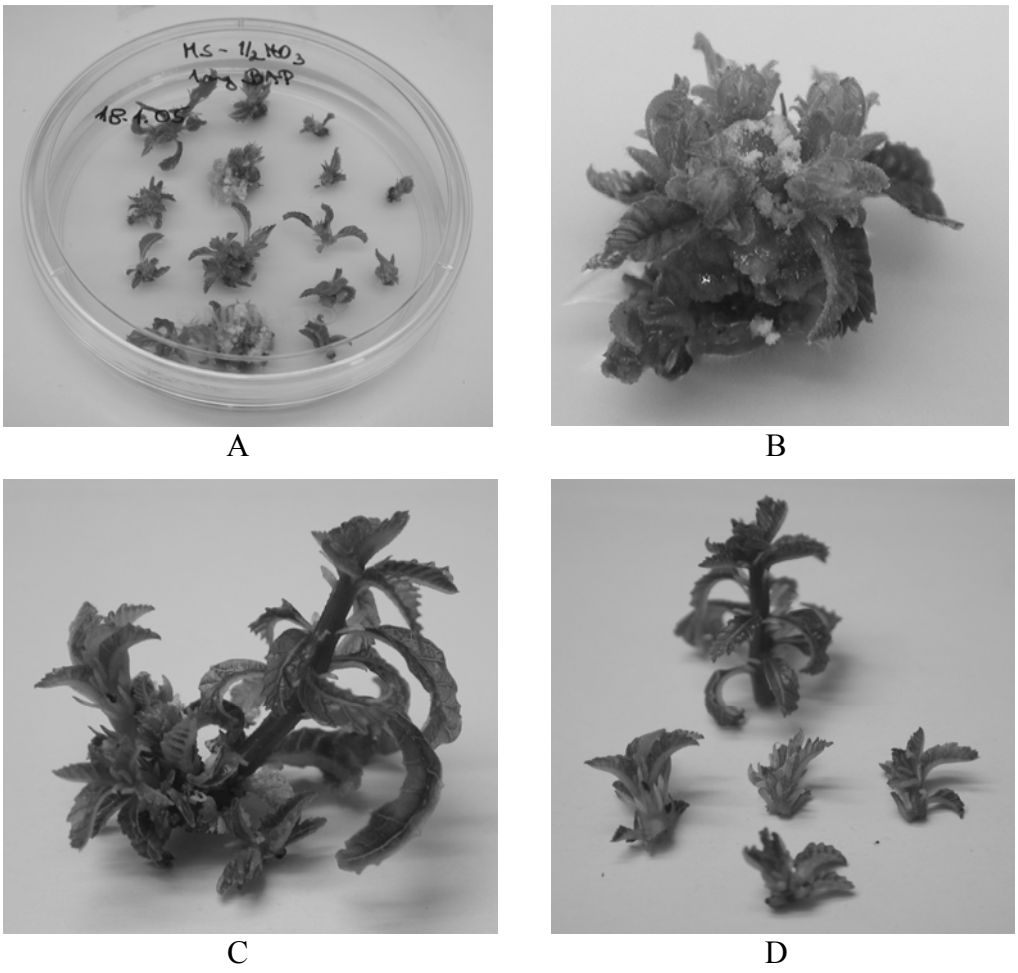

Figure 1: *In vitro* propagation of chestnut: A - shoots and nodes on propagation medium; B - multiple shoots proliferation; C - cluster of shoots; D - shoots from cluster

The MS medium with BAP as cytokinin also caused the strongest growth of newly formed chestnut shoots, with more than 1.2 cm on average. The shoots grew to about 1.0 cm on average on other media. Differences were not significant (Figure 3). The positive effect of the cytokinin zeatin on more vigorous shoots was not as strongly present as has been reported (Vieitez and Vieitez, 1980). In our experiment, the cytokinin BAP showed very comparable or better results, especially when newly formed shoots are included in the comparison. The multiplying factor of vital shoots (Figure 1) on the media with BAP was significantly better (average 2.1 shoots per cluster) than shoots developed on media with zeatin (average 1.2 shoots per cluster). Cytokinin zeatin negatively affected the propagation of shoots (Figure 2). Genetic differences between the material, the type of explant and the subculture technique used

for experiment (Sanchez *et al*., 1997) are certainly also very important. The Slovene clone 'Sobota' was used in our experiment for which no results of *in vitro* propagation have been published.

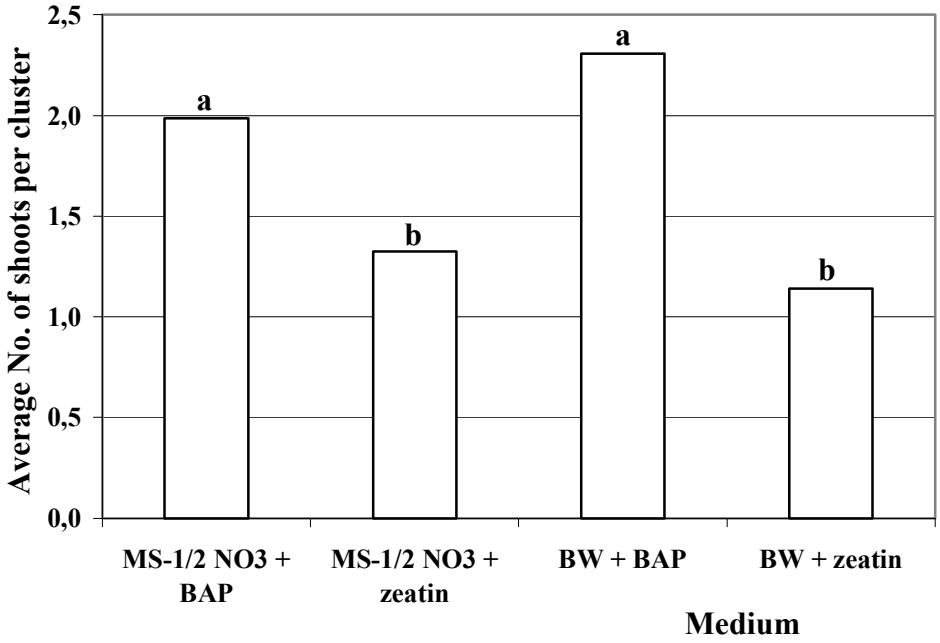

Figure 2: The proliferation intensity of chestnut clone 'Sobota' *in vitro* clusters in relation to different propagation media. Average number followed by identical letters are not significantly different according to Duncan's multiple-range test (P<0.05).

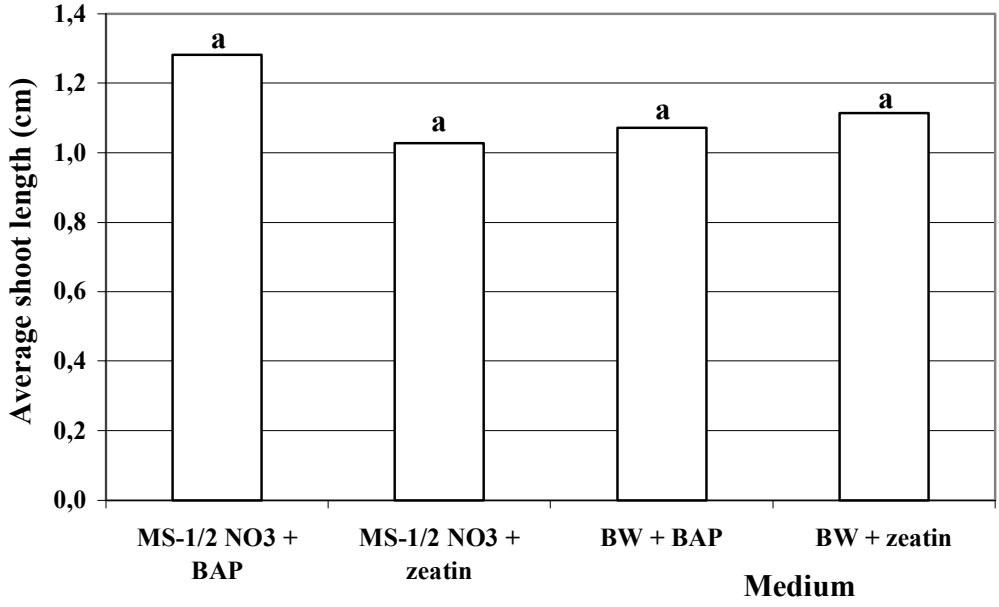

Figure 3: Average length of chestnut shoots of clone 'Sobota' in terms of different multiplication media. Average number followed by identical letters are not significantly different according to Duncan's multiple-range test (P<0.05).

In terms of shoot quality, both MS media showed better results than the BW medium. On the MS media, on average 84% of the shoots survived or were suitable for propagation, 16% brown and intensive hyperhydration shoots were decayed or eliminated. On the BW media, only 71.25% of shoots survived with the remaining 28.75% being decayed (Figure 4, Table 1). The shoots multiplicated on MS medium, regardless of the cytokinin used, showed lower strong hyperhydration and browning ratios and more of them could remain in the culture. The hyperhydration and browning ratios or necrosis symptoms of shoots cultivated on both BW media reached values over 28% (Figure 4, Table 1).

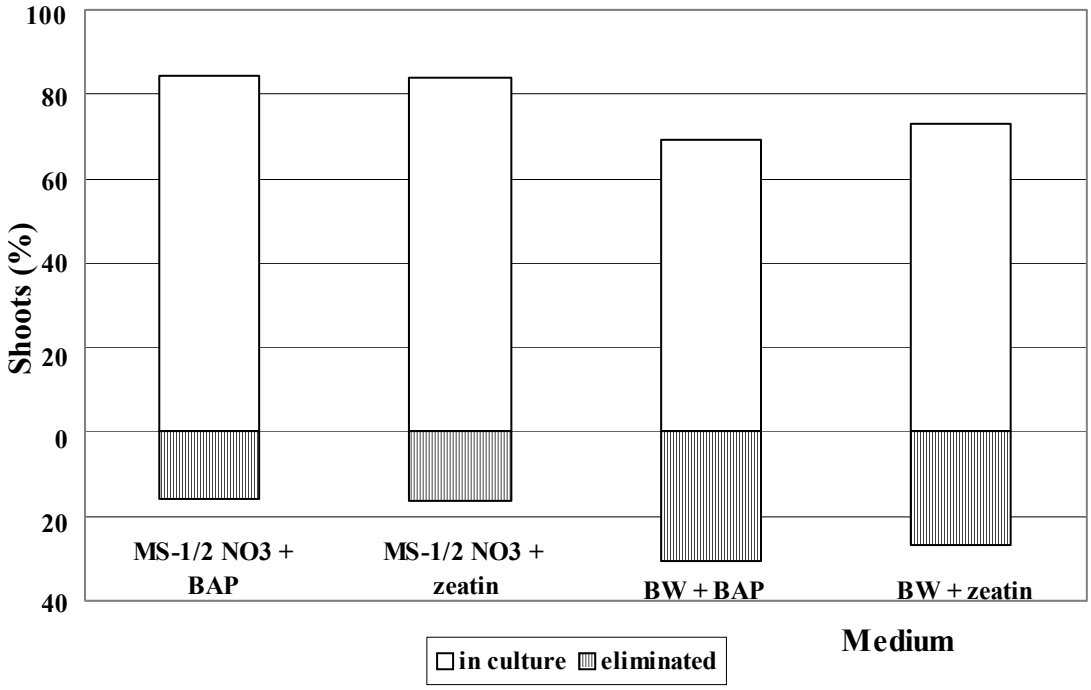

Figure 4: The ratio (%) of well formed *in vitro* shoots remaining in the culture and the ratio (%) of eliminated *in vitro* shoots with the chestnut clone 'Sobota'.

Table 1: The quality of newly formed shoots of chestnut clone 'Sobota' in terms of different propagation media. Percentages followed by identical letters are not significantly different according to Duncan's multiple-range test ($P<0.05$).

Medium	Shoots (%)		Shoots remained in culture (%)	Shoots (%)		Elimina-ted shoots (%)
	vigorous	little hyperhy-dration		intensive hyperhy-dration	browning, necrosis symptoms	
MS-½NO_3 + BAP	76.8	7.5	84.3 a	4.8	10.9	15.7 a
MS-½NO_3 + zeatin	74.4	9.3	83.7 a	12.8	3.5	16.3 a
BW + BAP	53.2	16.2	69.4 b	27.7	2.9	30.6 b
BW + zeatin	69.7	3.4	73.1 b	20.2	6.7	26.9 b

The shoot quality was especially critical, because the intensive hyperhydration rate was extremely high (Table 1). These results showed that the BW medium was not optimal in our experiment with the clone 'Sobota' (*Castanea sativa*) because the hyperhydration and browning problems or necrosis symptoms can be very problematic during *in vitro* culture. These results are in contrast to those of Tetsumura and Yamashita (2004) who worked with Japanese chestnut (*Castanea crenata*). The differences may be related to the different plant materials used.

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