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Micropropagation of sea buckthorn (*Hippophae rhamnoides* L.)

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A protocol for micropropagation of sea buckthorn was developed starting with shoot tips or meristems from plants up to 18 years old. Among the different media used, the best medium for both initiation and multiplication was the woody plant medium (WPM). 6-Benzylaminopurine (BAP) was the most suitable growth regulator with an optimal concentration of 0.10–0.25 mg/l for initiation and 0.4–1.0 mg/l for multiplication. On WPM medium with BAP, the average rate of multiplication in Erlenmeyer flasks was 3.3–4.0 shoots per explant per month and in test tubes 2.0–3.0 shoots. Moreover, most explants produced several to tens of adventitious buds which grew into shoots. Explants rooted spontaneously in the multiplication medium at a frequency of about 33%. With this method, explants of different origins have been successfully propagated *in vitro*; and rooted young plants which had developed root nodules were produced both in the greenhouse and in the field.

Key words: tissue culture, growth regulators, nodules, dioecious

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Introduction

Sea buckthorn, *Hippophae rhamnoides,* is a dioecious shrub or small tree species which is widely distributed on the Eurasian continent. Like other members of the family *Elaeagnaceae*, sea buckthorn can fix nitrogen in its root nodules, which contain an endophyte *Frankia*. The plant has long been of interest for its tasty and attractive fruit, which according to recent studies (Wang et al. 1982, Lachman et al. 1985, Chen et al. 1988, Lu 1988, Zhang 1988, Wahlberg and Jeppsson 1990, 1992, Yao et al. 1992, Xu 1992) has high nutrient and medicinal value. The species has the potential for multiple use as a fruit as well as a medicinal, ornamental, soil-improving and land reclamation plant. Breeding programs have been and are being conducted in many countries, among them Russia, Finland, Sweden, China, Germany, Hungary and the former Czechoslovakia (Papp 1982, Plekhanova 1988, Hiirsalmi 1988, Li 1988, Smatana et al. 1988, Wahlberg and Jeppsson 1990, 1992, Xu 1992).

In breeding programs of open-pollinated dioecious species like sea buckthorn, once a genotype is selected, it must be propagated vegeta-

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tively to maintain the characteristics. Sea buckthorn plants and cultivars are cloned by cuttings and/or suckers (Papp 1982, Osipov and Morozova 1983, Garanovich 1984, Kuznetsov 1985, Shlyapnikova 1985, Xu et al. 1988, Li et al. 1991, Xu 1992). In recent years, however, efforts have been made towards the more effective method of *in vitro* propagation. When Burdasov and Sviridenko (1988) induced shoot elongation and root formation from 0.5–0.7 cm long apical meristems (shoot tips proper) cultured *in vitro* from one-year-old seedlings, only a single shoot developed from each explant (each jar) and no plants were established in the soil (or pots). Montpetit and Lalonde (1988) succeeded in propagating seed germinates *in vitro* from plants grown in North America but not germinates from a European seed source. Liu (1989) reported having managed to micropropagate seed germinates and to induce roots in the medium, but she apparently failed to obtain rooted plants. A note in the Chinese Hippophae journal claimed that tissue culture of sea buckthorn has succeeded at the Qiang-Yang Teacher's College (Jiang et al. 1991), but gives no further information. It has been known that micropropagated plants have been produced at the Finnish Healthy Plant Centre and the Kemira Oy Espoo Research Centre (COST 87 1991). However, there are no reports on the methods they used.

The purpose of this study was to investigate the effects of the different media, growth regulators and their concentrations on shoot tip and meristem culture of *H. rhamnoides*, using both old and young plants of Chinese, Finnish and Danish origins.

Material and methods

This study was carried out in a stepwise approach. First, a primary experiment was done in rather small scale, and then the main experiment was designed on that basis.

Donor plants and explants

The donor plants for both experiments were 2- to 3-year-old sea buckthorn plants of Finnish, Chinese and Danish origins growing in the nursery of Helsinki University, and 18-year-old female and male plants from a sea buckthorn plantation of Danish origin growing at Helsinki harbor. In the primary experiment, after 2–3 cm long shoot tips had been rinsed in 70% ethanol for 1 minute, sterilized in NaClO solution with 4–5% active Cl for 10 minutes and rinsed in water 3 times, they were cultured on the initial media (described below). Three weeks later the culture was passed to the multiplication media (described below). In the main experiment, the same donor plants and sterilization procedure were used; however, the explants were sterilized in 2–3% active Cl solution for 8 minutes, and meristems and 0.5 cm long shoot tips were used for initiating the culture.

Media and growth regulators

The media used in the primary experiment included MS medium (Murashige and Skoog 1962) with 0.22/1.00 mg/l 6-benzylaminopurine (BAP) for initiation/multiplication, WPM medium (woody plant medium; Lloyd and McCown 1980) with 0.22/1.00 mg/l BAP, A-medium with 80/80 mg adenine-sulphate + 2/15 mg 2iP + 0.5/4.0 mg IAA/l and Z-medium with 0.5 mg adenine-sulphate + 4 mg 2iP + 3.8 mg IAA/l. A- and Z-media were modified WPM medium in which the iron concentration has been doubled (A medium) and 1000 mg/l KNO_3 (Z medium) was added. The two media were used in our laboratory for *Chaenomeles* and roses (Lehtonen et al. 1992).

The media used in the main experiment were WPM medium and 1/10 MS medium (MS medium at 1/10 strength of all macro- and micro-elements except for Fe which was in double amount of MS) with different concentrations of BAP (0.1–1.0 mg/l) and zeatin (1.0–2.5 mg/l). The 1/10 MS medium (Uosukainen and Niskanen 1985)

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has been used successfully in our laboratory for *Rhododendron*, *Azalea* and other species. In addition, MS, WPM and modified WPM media at full, 1/2 and 1/4 strength as well as 1/10 MS medium with BAP (0.1–1.5 mg/l), zeatin (0.2–1.0 mg/l) and thidiazuron (TDZ, 0.02–0.04 mg/l) were also tested for initiation and multiplication. For rooting the plantlets, WPM and MS media at full, 1/2 and 1/4 strength as well as 1/10 MS medium with 0.1–0.3 mg/l BAP or IAA or without growth regulators and with normal or half amount of sucrose were tested.

Growth conditions

Test tubes (20x150 mm), each containing 10 ml of medium, were used throughout the experiments while Erlenmeyer flasks (100 ml), each containing 40 ml of medium, were also used at the multiplication stage in the main experiment. One explant was placed in each tube and three in each flask. The explants were incubated at 22°C with 14 h warm white fluorescent light under 100 $\mu Em^{-2}s^{-1}$ density. The culture cycle was 21 days for initiation and 35 days for multiplication in the primary experiment and 30 days in the main experiment.

The plantlets were transferred into a plant propagator with a matrix of horticultural peat (water soluble N, P, K=100, 80, 160 g/m^3, pH = 5.5). Crushed root nodules collected from sea buckthorn plants were diluted with tap water and mixed with peat before the plantlets were transferred to secure fast and even root nodulation.

Evaluation

In the primary experiment, the survived, multiplied, contaminated and browned cultures were counted for each medium. In the main experiment, survived cultures were further classified according to their growth and development (see the results).

To eliminate the effects of genotypes, the study employed a balanced experiment design in which each treatment combination had the same number of explants from each donor plants. SAS programs GLM and FREQ were used for statistical analysis.

Results

Primary Experiment

At the initiation stage, the survival rate was only 16.9% on MS, 20% on A and Z, and 21.5% on WPM. There was 15–25% cultures dead of fungous and bacterial contamination and the rest 46.7–64.6% dead of browning. Statistically, there were no significant differences among the four media. At the multiplication stage, the differences among media were highly significant ($P < 0.001$). WPM medium gave not only the highest survival rate (92.9%) but also the highest rate of multiplied cultures among the survived explants, while on each of the remaining three media only 15.4–27.3% of explants survived (Table 1).

It was observed that *in vitro* cultured explants of sea buckthorn usually formed adventitious buds directly at their basal area. In this process the basal area swelled and differentiated at the same time, and buds then became visible. During this process, the basal area remained green.

Main Experiment

The high rate of mortality at the initiation stage in the primary experiment was probably due to the high concentration of NaClO. Therefore in the main experiment, 2–3% active Cl solution was used and the sterilization time was reduced to 8 minutes. To reduce contamination, meristems and 0.5 cm long shoot tips were used, since meristems and vigorous shoot tips are normally less contaminated than longer ones. Based on the results of primary experiment, WPM medium

Table 1. Effect of tissue culture media on multiplication of shoot tips of sea buckthorn during the multiplication stage (results after 35 days).

Medium[1)]	N[2)]	Number of multiplied shoot tips	Number of unmultiplied shoot tips	Number of dead shoot tips
MS	11	2	1	8
A	13	0	2	11
WPM	14	10	3	1
Z	13	0	2	11

[1)] See material and methods for medium descriptions.
[2)] N = Number of shoot tips used for multiplication, i.e. all survials at initiation stage.

was again used and for further comparison, 1/10 MS medium added.

Initiation

Shoot tip culture. The results show that the media (WPM and 1/10 MS), cytokinins (BAP and zeatin) and their concentrations significantly affect the culture of shoot tips (Fig. 1). Whatever the cytokinins (BAP or zeatin) were used, WPM medium always showed better results than 1/10 MS medium ($P < 0.01$). The percentage of cultures falling into classes 3 and 2 totaled 54.5% on WPM medium and 33.6% on 1/10 MS medium. The rate of mortality was 13.2% and 19.6% on WPM and 1/10 MS media, respectively. Of the mortality, 4.1% on WPM and 8.4% on 1/10 MS median was caused by fungous and bacterial contamination, and the remaining about 10% was caused by browning.

Whatever the media (1/10 MS or WPM medium), BAP which was used in concentrations of 0.125–0.75 mg/l always gave better results than zeatin in concentrations of 1.0–2.5 mg/l ($P < 0.05$). The percentage of cultures in classes 3 and 2 on the media with BAP was 52.4% while on media with zeatin 35.5% (Fig. 1). The rate of mortality on the medium with BAP was 13% and on the media with zeatin 20%. The results showed that the optimal combination was WPM medium with BAP at the concentration about 0.125–0.250 mg/l. The poorest combination was 1/10 MS with zeatin, on which no cultures in class 3 were found.

Meristem culture. The effects of media (WPM and 1/10 MS), growth regulators (BAP and zeatin) and their concentrations on meristem culture showed the same trends as those on shoot tip culture, but were more pronounced (Fig. 2). On WPM medium 15.5% of the cultures were in class 3, while on 1/10 MS medium no cultures reached that class. On WPM medium, cultures in classes 3 and 2 accounted for 55.4% of the total cultures, but on 1/10 MS medium these classes made up only 10.7% of the total. The rate of mortality was 21.4% and 30.4% on WPM and 1/10 MS media, respectively. On both media there was virtually no contamination.

On the media with BAP the percentages of cultures in classes 3 and 2 totalled 40.5%, while on media with zeatin the percentage was 25.6%. Like the shoot tip culture, the optimal combination was WPM medium with BAP at the concentration about 0.125–0.250 mg/l and the poorest was 1/10 MS medium with zeatin.

In both shoot tip and meristem cultures, two plant types were observed: a normal type and a hyperhydrous type. The hyperhydous type was characterized by light green leaves and shoots, which was thick and brittle (easy to break) due to the high content of water. In shoot tip culture, the rate of this hyperhydrous type was 0.4% on WPM and 2.4% on 1/10 MS media, while in meristem culture, the rate was 1.8% and 10.7%, respectively. Thus, it seemed that the hyperhy-

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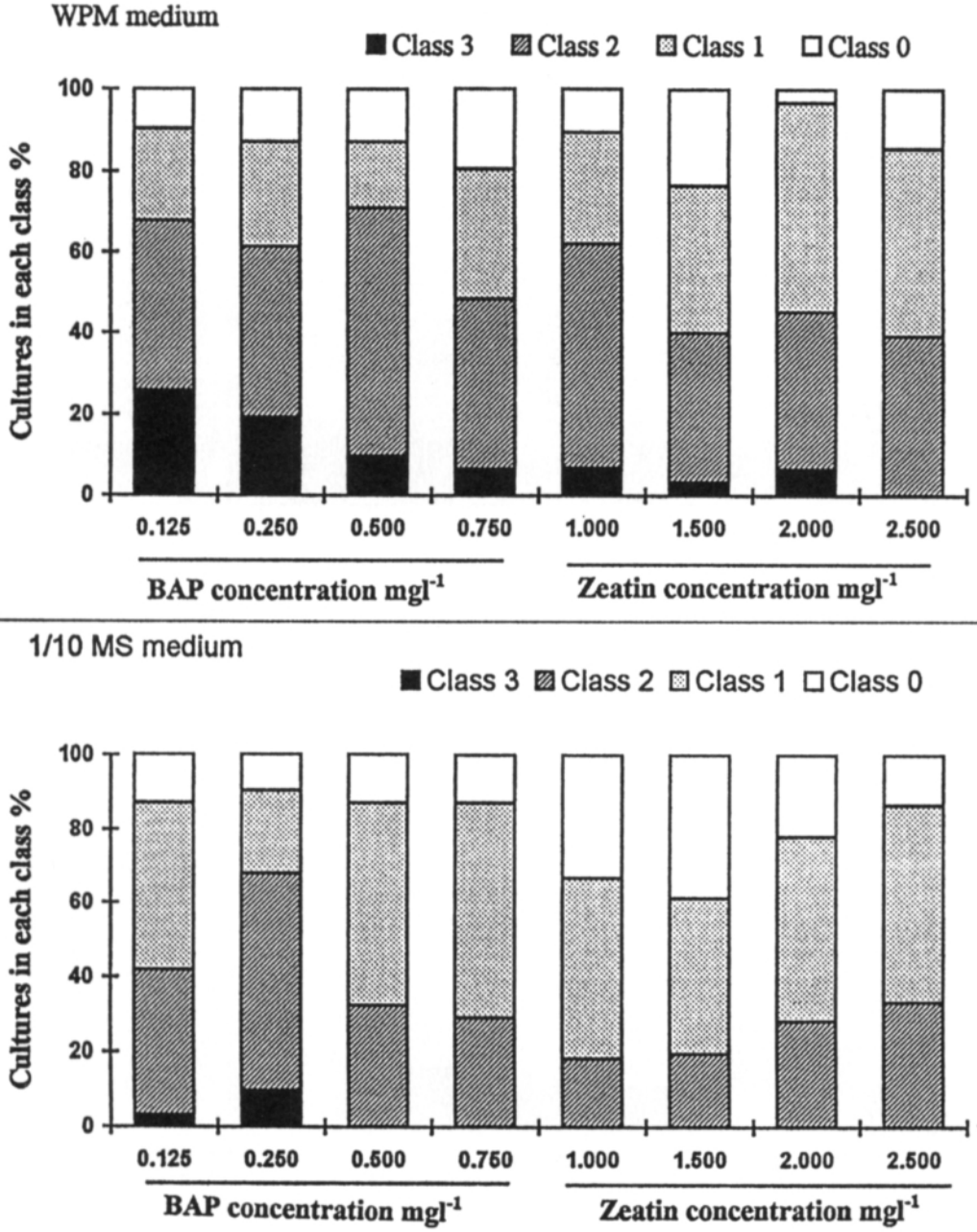

Fig. 1. Effects of media WPM (woody plant medium) and 1/10 MS (MS medium at 1/10 strength, see Material and methods) and concentration of growth regulators BAP (6-benzylaminopurine) and zeatin on initiation of shoot tips (N = 31 for each treatment combination, results after 30 days of culture).
Class 3 = vigorous growth and/or more than 4 adventitious buds.
Class 2 = good growth and/or 1 – 4 adventitious buds.
Class 1 = poor growth and no adventitious buds.
Class 0 = dead.

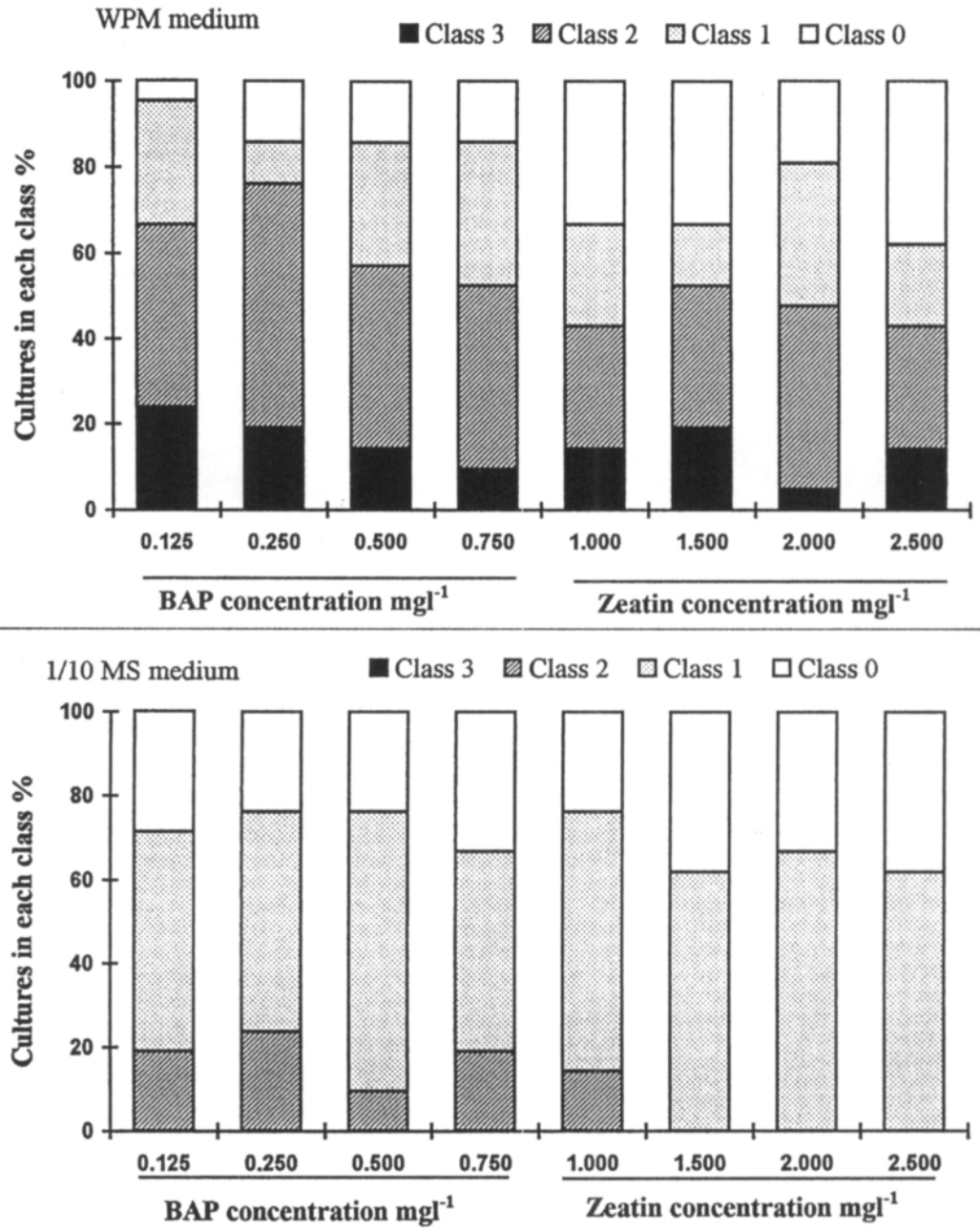

Fig. 2. Effects of media WPM (woody plant medium) and 1/10 MS (MS medium at 1/10 strength, see Material and methods) and concentration of growth regulators BAP (6-benzylaminopurine) and zeatin on initiation of meristems (N = 21 for each treatment combination, results after 30 days of culture).
Class 3 = vigorous growth and/or more than 4 adventitious buds.
Class 2 = good growth and/or 1 – 4 adventitious buds.
Class 1 = poor growth and no adventitious buds.
Class 0 = dead.

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drocity was correlated with 1/10 MS medium ($P<0.01$).

At the beginning of culturing, shoot tips grew more quickly than meristems and later the shoot tip plantlets looked more mature than those grown from meristems. If cultures were left to grow for a longer time (30–40 days), new shoots or buds developed from the basal area and the axilla of the lower leaves; and the shoots that had developed from shoot tips began to die. In general, meristems produced more buds and shoots from the basal area, but shoot tips produced more axillary buds and shoots from axilla.

Table 2. Effects of BAP (6-benzylaminopurine) concentration on multiplication of sea buckthorn shoot tip cultures in WPM (woody plant medium) medium during the multiplication stage (N=21, results after 30 days of culture).

BAP mg/l	Mean number of shoots/explant[1)]
0.1	2.0 a
0.2	2.1 a b
0.4	2.6 a b c
0.6	3.0 c
1.0	2.9 b c

[1)] Means with the same letter are not significantly different at the 0.05 level (Tukey test).

Multiplication

After initiation, plantlets derived from meristem and shoot tip cultures were subcultured on WPM medium with 0.1–1.0 mg BAP/l for multiplication. The results showed that the optimal concentration was between 0.4–1.0 mg/l (Table 2). The multiplication rate at this concentration was 2.6–3.0 shoots per explant per month.

A further comparison was made with different vessels on WPM medium with 0.5 and 1.0 mg BAP/l (Table 3). The results indicated that the two types of vessels had significant effects on the number of shoots and adventitious buds ($P = 0.017$). On the other hand, there were no significant differences between the two BAP concentrations, in agreement with the results in Table 2. At both concentrations of BAP, the explants produced more shoots in flasks (3.96 and 3.33 shoots/explant) than in tubes (2.1 and 2.75 shoots/explant). In both types of vessels shoots were shorter on the medium with 1.0 mg BAP/l than with 0.5 mg BAP/l. Spontaneous rooting was observed in both tubes and flasks, and in all cases the frequencies were about 33%.

Most of the explants produced a cluster of several to tens of adventitious buds. If there were enough space and nutrients, the clusters continued to grow. Actually, when there was no space to extend outward, the cluster grew down into the medium. In general, the explants from meristems grew more rapidly and produced more adventitious buds than those from shoot tips. If the culture was left in the same medium for 15–20 days or more, tens of shoots grew up from these buds; however, the shoots that had formed early became too old or even died. By taking into account the newly formed adventitious buds, the propagation rate was about 6 shoots per explant per month.

The number of shoots and adventitious buds generated from explants depended on the donor plants and their origins. By visual observation (no statistical data collected), plants of Chinese origin, in general, responded better than those of Finnish and Danish origins, perhaps due to their more vigorous vegetative growth (shoot elongation) seen in the field trials. Although there were different responses among individuals of the same origin, no clear differences were observed between the groups of old and young, male and female donor plants of same origin.

In the additional experiments, MS, WPM and modified WPM media at full, 1/2 and 1/4 strength as well as 1/10 MS medium with BAP (0.1–1.5 mg/l), zeatin (0.2–1.0 mg/l) and thidiazuron (TDZ 0.02–0.04 mg/l) did not appear any better than correspondent WPM medium with BAP for initiation and multiplication. It was observed that young leaves started to differentiate into buds when they touched the growth medium. Four leaves were then cultured and one of them grew into a plantlet.

Table 3. Effects of culture vessels (test tubes 20 x 150 mm and Erlenmeyer flasks 100 ml) and BAP (6-benzylaminopurine) concentrations on shoot and adventitious bud multiplication and rooting frequency in WPM (woody plant medium) medium (results after 30 days of culture).

	1.0 mg/l BAP		0.5 mg/l BAP	
	Test tube	Erlenmeyer flask	Test tube	Erlenmeyer flask
Number of explants cultured	10	27	8	27
shoots/explant (mean ± se)	2.1 ± 0.4	4.0 ± 0.4	2.8 ± 0.4	3.3 ± 0.4
Adventitious buds/explant	> 4	> 4	1–4	> 4
Root frequency %	30	33	37	33

Rooting

In multiplication medium, plantlets of sea buckthorn rooted spontaneously at about 33% (Table 3). MS and WPM media at 1/2 and 1/4 strength as well as 1/10 MS medium did not perform any better than the multiplication medium. Medium with IAA alone showed very poor results. Medium without growth regulators and with less sucrose depressed multiplication and promoted shoot elongation but did not increase rooting frequency. To attain a certain height for transfer into soil, it is better to use a lower concentration of BAP before the transfer.

After a period of 45 days growing in propagators, the plants were transplanted to pots growing in a greenhouse. At this time the plants had developed root nodules, a sign of Frankia activity. After about 40 days, some plants were more than 30 cm high. The plants have been planted in the field and grow normally.

Discussion

MS medium was the only medium used by Montpetit and Lalonde (1988) and by Liu (1989) to culture seed germinates. However, this study showed that WPM medium, the woody plant medium, is definitely better than MS (including 1/10 MS medium) and the other media tested, both for initiation and for multiplication of sea buckthorn.

According to these results, BAP is more effective than other growth regulators for *in vitro* culture of sea buckthorn plants, a finding which agrees with previous studies. The suitable concentration of BAP is 0.125–0.25 mg/l for initiation and 0.4–1.0 mg/l for multiplication. In the main experiment, zeatin was used at higher concentration (1.0–2.5 mg/l) than BAP (0.1–1.0 mg/l) though the former is more active than the latter. However, in the additional experiments, lower concentration of zeatin (0.2–1.0 mg/l) was used, BAP still gave better results. Thus the conclusion of BAP is better than zeatin valid for all concentration.

With WPM medium combined with BAP, the average rates of multiplication were 3.3–4.0 shoots per month per explant in flasks and 2.0–3.0 shoots per explant in test tubes. In addition, most explants produced many adventitious buds, which in turn produced several to tens of shoots. This result is obviously better than that of a previous study (Montpetit and Lalonde 1988) in which the best result was 3–5 shoots per explant.

Field plants are exposed to all kinds of microbes. Contamination could be a problem when 2–3 cm long shoot tips were used in the *in vitro* culture. Using short shoot tips, or meristems can effectively reduce contamination. During initiation, meristems had a higher rate of mortality than shoot tips did because the former are more sensitive to environmental conditions. None the

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less, in general, the surviving meristems developed better than the shoot tips did.

With the method used in the present study, sea buckthorn plants of different origins have been successfully propagated *in vitro*. However, studies are still needed to improve the rate of multiplication, in particular, to induce adventitious buds that will grow into shoots quickly. Methods of increasing rooting frequency, both in media and in soil, should also be improved.

It appears, however, that adequate micropropagation methods can be worked out for practical use in conjunction with breeding programs and plant production of sea buckthorn. In future studies, more research is needed on the effects of root nodulation and host plant/*Frankia*-interaction on the successful micropropagation and growth of sea buckthorn.

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SELOSTUS

Tyrnin mikrolisäys

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Tyrnin mikrolisäystä tutkittiin aloittamalla viljelmät sekä versonkärjistä että kasvupistesolukoista.

Vanhimmat emotaimet olivat 18-vuotiaita. Tutkimuksessa verrattiin useita eri ravintoalustoja. Parhaimmaksi aloitus- ja lisääntymisvaiheen alustaksi osoittautui puuvartisille kasveille kehitetty WPM-alusta. Sopivin kasvunsääde oli 6-benzyyliaminopuriini (BAP). Aloitusvaiheessa optimaalisin BAP-pitoisuus oli 0,10 – 0,25 mg/l ja lisääntymisvaiheessa 0,4 – 1,0 mg/l. BAP-kasvunsäädettä käyttämällä WPM-ravintoalustalla keskimääräinen lisääntymiskerroin Erlenmeyerpulloissa oli 3,3 – 4,0 versoa/viljelmä/kk ja koeputkissa 2,0 – 3,0 versoa/viljelmä/kk. Edellä mainittujen versojen lisäksi useimmat viljelmät tuottivat lukuisia, jopa kymmeniä jälkisilmuja, jotka kehittyivät versoiksi. Viljelmistä keskimäärin 33% juurtui lisäysalustalla ilman erityistä juurrutuskäsittelyä. Kehitetyllä menetelmällä eri alkuperää olevia tyrniviljelmiä onnistuttiin lisäämään *in vitro*. Juurtuneita nuoria kasveja, joihin muodostui juurinystyröitä, tuotettiin sekä kasvihuoneessa että avomaalla.