Propagation of Blue Honeysuckles (*Lonicera caerulea* L.) in *In Vitro* Culture

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Abstract: The aim of this study was to develop micropropagation protocol for *Lonicera caerulea* L. Clone 44, Clone 46 and Brązowa, three important invasive woody horticultural plants. Actively growing shoots from the shrubs grown in the field were used for initiation of culture. Shoots were surface sterilized with ethanol, then with sodium hypochlorite and mercury sulfate. MS medium supplemented with cytokinin BAP at concentrations of 1.0 - 4.0 mg·dm^{-3} had no statistically significant effect on the shoot initiation of selected blue honeysuckle genotypes. Multiplication rate varied depending on the genotype and plant growth regulator concentrations. The highest number of microshoots produced per explant of Clone 44 and Clone 46 was obtained at using 2.0 mg·dm^{-3} BAP, while of cultivar Brązowa – 1.0 mg·dm^{-3} BAP. Shoots were rooted *in vitro* in the presence of IBA and IAA. Microshoots have rooted differently depending on the treatment and genotype. In the case of Clone 4458% rooting was achieved at 2.5 mg·dm^{-3} IBA and MS basal nutrient medium treatment.

Keywords: *In vitro*, *Lonicera caerulea* L., media, micropropagation, plant growth regulators.

INTRODUCTION

Lonicera belongs to the family *Caprifoliaceae* and grow extensively in Europe, Asia and North America. The finely shrubs or woody plants may be used for providing food, ornamental purposes and also as a shelter for wildlife [1]. *Lonicera* (honeysuckle) species are popular plants suitable for vegetative mass propagation *via* tissue culture [2]. The germination rate of blue honeysuckle seeds is very low. Therefore tissue culture can be a useful tool for rapid clonal propagation and distribution of good health plant material. *In vitro* culture is used to obtain more plants in a short time with less labor input and at lower production costs. An additional feature of the method is independence of the vegetative season [3, 4]. Several *Lonicera* spp. have been manipulated in *in vitro* culture. In most reports, adventitious shoots have been induced from leaf explants [5], callus cultures [6, 7] or axillary shoot production has been achieved using *L. caerulea* [2]. Unfortunately, it was found that these methods can not be broadly applicable because of the effectiveness of the micropropagation which is highly genotype-specific [8].

Hence, the aim of this research *in vitro* propagation was to provide an efficient plant production system of three genotypes of *Lonicera caerulea* L.

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MATERIAL AND METHODS

Initial explants were taken from four young shoots derived from approximately 4-year-old Polish blue honeysuckle from the experimental orchard of the Pomology Department. The leaves were removed prior to soaking the shoots for 15 minutes in water with a detergent (Ludwik washing-up liquid), then immersed in 70% (v/v) ethanol solution for 30 s. After the preliminary disinfection, the explants were disinfected with 10% (v/v) solution of sodium hypochlorite (NaOCl) for 10 minutes and with 0.2% (v/v) solution of mercury sulfate ($HgSO_4$) for 10 minutes. Under a sterile laminar flow hood, the shoot tips were rinsed three times with sterile distilled water. Shoot fragments of size 1.0 - 2.0cm, with an apex or node with lateral meristems were used as primary explants for *in vitro* culture. From 59 to 94 explants were cultured in an Erlenmayer flasks, each with 20 ml of MS medium [9] supplemented with 1.0, 2.0 and 4.0 mg·dm^{-3} 6-benzyloaminopurine (BAP). Plants placed on MS medium without the addition of growth regulators was the control at all stages of the experiment. The initiation stage lasted 6 weeks. The contamination rate and survival of the explants after sterilization were analyzed.

Explants initiated for growth were put onto multiplication media MS differed concentration of micro- and macroelements (full strength, ½ and ¼ micro- and macroelements), supplemented with BAP at concentrations of 1.0 and 2.0 mg·dm^{-3}, 25 explants per medium.

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Proliferated shoots were placed on rooting MS media (full strength and ½ micro- and macroelements supplemented with auxins: indole-3-butyric acid (IBA) at the concentrations of 2.0 and 2.5 mg·dm^{-3} and indole-3-acetic acid (IAA) at the concentration of 5.0 mg·dm^{-3}.

At all stages of experiments the media were supplemented with 8.0 g·dm^{-3} agar (Biocorp), 30 g·dm^{-3} sucrose and 100 mg·dm^{-3} inositol and their pH was adjusted to 5.7 by adding 0.1M of NaOH or HCl. After adding growth regulators, the media were autoclaved for 20 minutes at the temperature of 121^{0}C. The cultures were maintained in a growth room at a temperature of 24 ± 1°C under 16h photoperiod from a fluorescent lamp (photosynthetic photon flux density 40 μmol·m^{-2}·s^{-1}).

The mean values of measurements at initiation and proliferation stages (shoot length, number of: axillary shoots per explant, internodes and leaves), and at the rooting stage (plant height, root length, number of roots and leaves) obtained in the experiments are presented in tables.

The results were analyzed statistically. The significance of differences was determined by means of variance analysis and Tukey's test, at a level of significance of α=0.05.

RESULT AND DISCUSSION

Sterilization of plant explants is one of the major problems in tissue culture. Chemical disinfectants such as ethanol, mercuric chloride, sodium hypochlorite and hydrogen peroxide are generally used for surface sterilization of explants for raising shoot cultures *in vitro* [10, 11]. Sedlák and Paprštein [8] used 0.15% solution of mercuric chloride ($HgCl_2$) for disinfection of *Lonicera kamtschatica* (Sevast.), Dziedzic [3] for disinfection of vegetative axillary buds of blue honeysuckle cultivars Czelabinka and Duet used 10% solution of calcium hypochlorite ($Ca(OCl)_2$), and [2,12] applied sodium hypochlorite (NaOCl) solution.

The results of sterilization procedures and development of *Lonicera* shoots from initial explants are recorded in Table **1**. In this paper, two disinfectants with different disinfection potencies were used. For Clone 44 and Clone 46 better results were obtained after the using of 0.2% $HgSO_4$ for disinfection (Table **1**). Out of the 29 explants of Clone 44 and of Clone 46, only 10 explants were contaminated with fungal infection after the first four weeks of culture. Out of the left 19 uncontaminated explants of Clone 44 and 19 of Clone 46, 16 and 18 explants developed shoots (respectively). After using 10% NaOCl solution for disinfection, 7 (23.3%) explants of Clone 44 and 10 (28.6%) explants of Clone 46 were subjected for further growth. However, for the cultivar Brązowa, after the application of 0.2% $HgSO_4$ for disinfection, 27 explants were infected and 23 explants were selected for further growth (Table **1**). Less infected plants (3 explants) were observed after using 10% NaOCl for the disinfection, whereas out of the remaining 20, only 8 explants 1were selected for further growth.

The addition of cytokinin BAP to MS medium did not significantly affect the initiation of shoots from the examined clones and the cultivar of blue honeysuckle (Figure **1**). The highest plants (5.0cm) were obtained for the cultivar Brązowa in MS medium supplemented with 2.0 mg·dm^{-3} BAP, while the lowest (1.5cm) were reported for Clone 44 in MS medium supplemented with 4.0 mg·dm^{-3} BAP (Table **2**). A high number of new shoots (2.0) were formed by Clone 46 and Brązowa on media with BAP at concentrations of 4.0 and 2.0 mg·dm^{-3} (respectively). Karhu, Sedlák and Paprštein and Dziedzic [2, 3, 8] also achieved efficient production of high quality microshoots of *L. caerulea* f. *caerulea* by

Table 1: Surface Sterilization with 70% Alcohol Followed by 10% NaOCl and 0.2% $HgSO_4$ for 10 min

Cultivar		contaminated explants		uncontaminated explants that developed shoots		uncontaminated explants that did not develop shoots	
		number	%	number	%	number	%
Clone 44	10% NaOCl	17	56.7	7	23.3	6	20
	0.2% $HgSO_4$	10	34.5	16	55.2	3	10.3
Clone 46	10% NaOCl	22	62.9	10	28.6	3	8.5
	0.2% $HgSO_4$	10	34.5	18	62.1	1	3.4
Brązowa	10% NaOCl	3	13.0	8	34.8	12	52.2
	0.2% $HgSO_4$	27	54.0	23	46.0	0	0

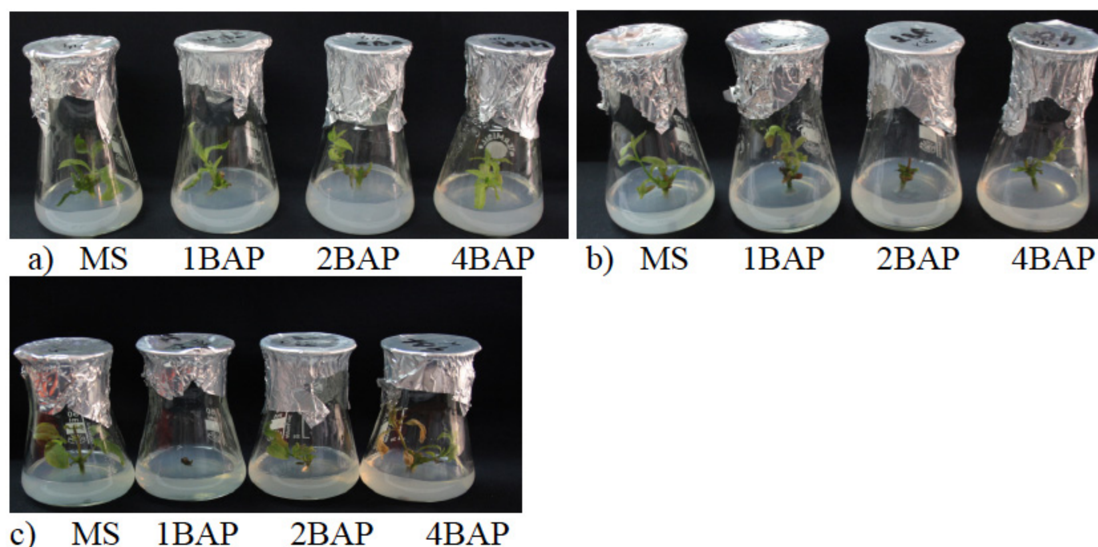

Figure 1: Comparison of shoot production of three blue honeysuckle genotypes in response to BAP concentrations: **a**) Clone 44, **b**) Clone 46, **c**) cultivar Brązowa.

Table 2: Shoot Production of *Lonicera caerulea* L. in Response to BAP Concentrations

Genotype	shoot length [cm]		new shoots per explant		number of internodes		number of leaves	
Clone 44								
MS	3.75	ab*	1.50	a	4.13	ab	10.50	a
MS + 1.0 mg·dm^{-3} BAP	4.40	a	1.75	a	5.0	a	13.75	a
MS + 2.0 mg·dm^{-3} BAP	2.62	bc	1.0	a	2.0	b	5.5	a
MS + 4.0 mg·dm^{-3} BAP	1.5	c	1.0	a	2.25	b	5.0	a
mean	3.07		1.31		3.35		16.81	
$LSD_{0.05}$	**1.56**		**0.81**		**2.42**		**9.16**	
Clone 46								
MS	4.0	a	1.0	bc	4.0	ab	8.0	b
MS + 1.0 mg·dm^{-3} BAP	4.0	a	1.5	ab	4.5	a	13.0	a
MS + 2.0 mg·dm^{-3} BAP	1.75	b	0.5	c	0.5	c	3.0	c
MS + 4.0 mg·dm^{-3} BAP	3.33	a	2.0	a	2.75	b	7.5	b
mean	3.27		1.25		2.81		7.88	
$LSD_{0.05}$	**1.53**		**0.84**		**1.57**		**3.03**	
Brązowa								
MS	3.37	a	1.5	ab	3.25	a	4.5	b
MS + 1.0 mg·dm^{-3} BAP	4.5	a	0.5	b	2.0	a	8.0	ab
MS + 2.0 mg·dm^{-3} BAP	5.0	a	2.0	a	3.0	a	15.0	ab
MS + 4.0 mg·dm^{-3} BAP	4.5	a	1.5	ab	2.5	a	17.5	a
mean	4.34		1.38		2.69		11.25	
$LSD_{0.05}$	**3.46**		**1.05**		**3.3**		**11.88**	

*Means in the same column followed by the same letter are not significantly different ($p < 0.05$; Least Significant Differences test LSD).

supplementing nutrient media with 2.0 mg·dm^{-3} BAP. According to Sedlák and Paprštein [8] increases in the concentration of BAP in the medium had a negative impact on the number of new shoots observed in the cultivar Altaj.

Explants of Clone 44, Clone 46 and Brązowa honeysuckle remained alive in medium without PGRs (Plant Growth Regulators), but the proliferation rate was low (Table **3**). A greater number of microshoots were produced on MS medium containing 1.0 mg·dm^{-3} BAP for Brązowa and 2.0 mg·dm^{-3} BAP for both

Table 3: Proliferation of Shoot Tips of *Lonicera caerulea* L. Cultured on Various Media

Cultivar	shoot length[cm]		number of new shoots per explant		number of leaves	
Clone 44						
MS	2.06	b	2.5	b	3.0	b
MS + 1.0 $mg \cdot dm^{-3}$ BAP	3.2	ab	2.5	b	8.8	ab
MS + 2.0 $mg \cdot dm^{-3}$ BAP	5.0	a	5.5	a	7.0	ab
½MS + 1.0 $mg \cdot dm^{-3}$ BAP	2.75	b	2.5	b	10.0	a
½MS + 2.0 $mg \cdot dm^{-3}$ BAP	1.7	b	2.0	b	7.5	ab
¼MS + 1.0 $mg \cdot dm^{-3}$ BAP	1.5	b	1.25	b	5.5	ab
¼MS + 2.0 $mg \cdot dm^{-3}$ BAP	1.9	b	1.75	b	5.5	ab
mean	2.59		2.57		6.76	
$LSD_{0.05}$	**2.05**		**1.57**		**6.2**	
Clone 46						
MS	4.0	a	4.0	a	8.0	a
MS + 1.0 $mg \cdot dm^{-3}$ BAP	2.7	abc	2.6	abc	7.2	a
MS + 2.0 $mg \cdot dm^{-3}$ BAP	3.4	ab	3.60	ab	10.60	a
½MS + 1.0 $mg \cdot dm^{-3}$ BAP	2.2	bc	2.4	abc	6.0	a
½MS + 2.0 $mg \cdot dm^{-3}$ BAP	2.0	bc	1.8	c	7.5	a
¼MS + 1.0 $mg \cdot dm^{-3}$ BAP	1.2	c	1.0	c	6.0	a
¼MS + 2.0 $mg \cdot dm^{-3}$ BAP	2.1	bc	1.60	bc	8.0	a
mean	2.51		2.43		7.61	
$LSD_{0.05}$	**1.64**		**2.05**		**5.35**	
Brązowa						
MS	2.87	b	3.17	ab	4.5	bc
MS + 1.0 $mg \cdot dm^{-3}$ BAP	3.38	ab	3.67	a	8.67	a
MS + 2.0 $mg \cdot dm^{-3}$ BAP	3.25	ab	3.0	ab	7.33	abc
½MS + 1.0 $mg \cdot dm^{-3}$ BAP	4.38	a	2.17	bc	8.0	ab
½MS + 2.0 $mg \cdot dm^{-3}$ BAP	3.25	ab	2.83	ab	11.0	a
¼MS + 1.0 $mg \cdot dm^{-3}$ BAP	1.0	c	1.0	c	3.5	c
¼MS + 2.0 $mg \cdot dm^{-3}$ BAP	1.0	c	1.0	c	4.0	c
mean	2.73		2.40		6.71	
$LSD_{0.05}$	**1.26**		**1.25**		**3.87**	

*Means in the same column followed by the same letter are not significantly different ($p < 0.05$; Least Significant Differences test LSD).

Clones. According to Karhu [13] concentrations of BAP strongly affected the proliferation and growth of the form of L. *caerulea* shoots. Suzuki *et al*. and Osburn *et al*. [12, 14] showed that the shoots obtained by primary culture were proliferated effectively on normal strength MS medium supplemented with 5 µM BAP.

Microcuttings rooted differently depending upon the treatment and species [12]. Sedlák and Paprštein [8] rooted blue honeysuckle shoots on MS medium supplemented with 2.5 $mg \cdot dm^{-3}$ IBA achieving 100% rooting and roots of good quality. In our study, the majority of shoots (58%) which developed roots were observed for Clone 44, and the least (12.5%) for the cultivar Brązowa (data not shown). Morphologically, Clone 44 roots were very variable. Roots produced in media with ½MS supplemented with 2.0 $mg \cdot dm^{-3}$ IBA and 5.0 $mg \cdot dm^{-3}$ IAA were thicker and shorter (2.0cm) than roots formed in ½MS media with 2.5 $mg \cdot dm^{-3}$ IBA (4.83cm) (Table **4**). Clone 46 developed roots only in MS and ½MS medium supplemented with 2.0 $mg \cdot dm^{-3}$ IBA and 5.0 $mg \cdot dm^{-3}$ IAA. In contrast, the cultivar Brązowa exhibited the weakest rooting *in vitro*. Dziedzic [3] suggested that the best rooting of the cultivars Duet and Czelabinka was achieved on WPM media supplemented with 2.0 $mg \cdot dm^{-3}$ IBA and 5.0 $mg \cdot dm^{-3}$ IAA. On the other hand, Karhu [15] suggested that for establishing plants *ex vitro* focus should be

Table 4: Rooting on Various MS Medium Supplemented with Different Concentrations of IBA and IAA

Cultivar	shoot length [cm]		root length [cm]		number of roots per one shoot		number of leaves	
Clone 44								
MS	0.83	a	0	b	0	b	10.8	a
½MS + 2.5 mg·dm^{-3} IBA	2.5	a	4.83	a	3.67	ab	10.67	a
MS + 2.5 mg·dm^{-3} IBA	1.0	a	4.67	a	7.0	a	3.33	a
½MS + 2.0 mg·dm^{-3} IBA+ 5.0 mg·dm^{-3} IAA	0.33	a	2.0	b	3.0	ab	6.0	a
MS + 2.0 mg·dm^{-3} IBA+ 5.0 mg·dm^{-3} IAA	1.17	a	0	b	0	b	6.0	a
mean	1.17		2.3		2.73		7.36	
$LSD_{0.05}$	**2.37**		**2.0**		**4.29**		**10.32**	
Clone 46								
MS	1.07	bc	0	a	0	a	7.14	ab
½MS + 2.5 mg·dm^{-3} IBA	0.5	c	0	a	0	a	6.0	bc
MS + 2.5 mg·dm^{-3} IBA	1.0	c	0	a	0	a	4.0	c
½MS + 2.0 mg·dm^{-3} IBA+ 5.0 mg·dm^{-3} IAA	1.78	ab	1.17	a	0.33	a	9.28	a
MS + 2.0 mg·dm^{-3} IBA+ 5.0 mg·dm^{-3} IAA	2.0	a	0.25	a	0.5	a	5.0	bc
mean	1.27		0.28		0.17		6.28	
$LSD_{0.05}$	**0.74**		**1.89**		**0.75**		**3.03**	
Brązowa								
MS	2.13	a	0	a	0	b	9.33	a
½MS + 2.5 mg·dm^{-3} IBA	0.63	b	0	a	0	b	2.67	b
MS + 2.5 mg·dm^{-3} IBA	0.63	b	0	a	0	b	4.67	b
½MS + 2.0 mg·dm^{-3} IBA+ 5.0 mg·dm^{-3} IAA	0.5	b	0	a	0	b	4.67	b
MS + 2.0 mg·dm^{-3} IBA+ 5.0 mg·dm^{-3} IAA	1.0	ab	0.67	a	1.0	a	4.67	b
mean	0.98		0.13		0.2		5.20	
$LSD_{0.05}$	**1.19**		**0.72**		**0.93**		**2.4**	

*Means in the same column followed by the same letter are not significantly different ($p < 0.05$; Least Significant Differences test LSD).

placed on the quality of rooted plants rather than on the root number.

CONCLUSION

1. Among the solution chosen for the disinfection of shoots from Clone 44 and Clone 46, the best results were obtained after the application of 0.2% $HgSO_4$ solution, while for the cultivar Brązowa it was 10% NaOCl solution.

2. MS medium supplemented with cytokinin BAP at concentrations from 1.0 to 4.0 mg·dm^{-3} had no statistically significant effect on the shoot initiation of selected clones and the cultivar Brązowa.

3. The multiplication rate varied depending on the genotype and the concentrations of BAP. The highest multiplication rate was obtained on MS medium supplemented with 2.0 mg·dm^{-3} BAP for Clone 44 and Clone 46 and for the cultivar Brązowa – MS with 1.0 mg·dm^{-3} BAP.

4. The best microshoot rooting rates (58%) were achieved for Clone 44 when MS basal nutrient medium with 2.5 mg·dm^{-3} IBA was used.

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