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In vitro propagation of bastard balm (*Melittis melissophyllum* L.)

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Summary

An efficient method for *in vitro* propagation of bastard balm by enhanced axillary shoot branching has been developed. The material to establish *in vitro* culture was shoot tips collected from three-year-old plants in May. The shoots obtained from initial explants were placed on MS/B5 medium containing 0.1, 0.5 or 1.0 mg/l BA with 0.01 mg/l NAA or without the auxin. The highest number of shoots per explant was obtained on the medium with 1.0 mg/l BA (3.9 shoots per explant). For the rooting of shoots ½ MS/B5 with IBA (0.25, 0.50 and 1.0 mg/l) medium was used. The medium without plant growth regulators served as a control. The best root regeneration was observed on the medium without IBA (87.1% of cuttings rooted). IBA used in the medium for shoot rooting affected the morphological traits of obtained microcuttings but not affected their weight. Irrespective of auxin concentration in this medium, obtained microcuttings acclimated in *ex vitro* conditions very well.

Key words: *in vitro, axillary shoots, MS/B5 medium, NAA, BA, IBA, ex vitro adaptation*

INTRODUCTION

Bastard balm (*Melittis melissophyllum* L., *Lamiaceae*) is a medicinal plant, wild growing in South and Central Europe. In Poland, the species is under partial legal

protection. It grows in well-lit deciduous and mixed forests, mainly in the East-South part of the country, on fresh, eutrophic soils [1, 2]. Bastard balm is a rhizomatous perennial plant, with a single stem, up to 30-50 (80) cm high. Its leaves are short-petioled, from oval to elliptic lanceolate, 7-15 cm long with notched-serrated edges. The plant blooms from May to June, producing several large, white-pink flowers in the axils of leaves [2]. The whole plant has an intensely lemon-like scent. The herb of bastard balm is a raw material rich in coumarin compounds [3, 4] but it also contains some essential oils [5] as well as flavonoids [6] and phenolic acids [7]. The antioxidant activity of this raw material was confirmed in the studies of Grujić *et al.* [8] and Kaurinovic *et al.* [9]. In folk medicine bastard balm herb has applications as a sedative, sporofic, antispasmodic, diuretic and antiulcer remedy [10]. It has been used in eye inflammations and against insomnia. Dry herb has been also utilised for the preparation of aromatic herbal teas, drunk after meals to improve digestion. Moreover, the raw material was applied to treat sore throat and cough [4]. In Poland, bastard balm herb is used mainly for the aromatization of alcohol and tobacco products [11]. The species has become endangered mainly due to overexploitation of its wild growing populations. According to Parfenov *et al.* [12] most of these populations have low levels of viability, they occupy small areas, occur at low density, have low seed yield and their age structure is incomplete. Information on bastard balm development are very scarce [2]. Preliminary studies on the generative reproduction indicate that long-term stratification of seeds is needed to induce its germination. Vegetative propagation with application of standard procedures (by stem cuttings) is also difficult and not effective enough [13, 14]. Thus, application of tissue culture techniques, used extensively for propagation and conservation of many rare species [15-17], may be a promising method of the propagation of this plant. Up to now, information on bastard balm *in vitro* cultures concern the accumulation of phenolic compounds and polysaccharides in callus [18, 19]. Little is known on the *in vitro* regeneration of this species [10]. The aim of the study was to develop an efficient protocol to obtain bastard balm microcuttings from shoot tips grown on agar-solidified medium.

MATERIAL AND METHODS

Stock culture

The material for the initiation of *in vitro* culture was shoot tips (apical bud with 3-4 mm long shoot) collected in May 2013 from three-year-old maternal plants grown in the collection of medicinal and aromatic plants of Department of Vegetable and Medicinal Plants, Warsaw University of Life Sciences – SGGW. The plants originated from Botanical Garden in Koryciny. The explants were surface-disinfected with 0.2% solution of mercuric chloride for one minute and rinsed four times with sterile distilled water. Afterwards, they were placed in test tubes on

MS/B5 medium containing 0.1 mg/l BA (6-benzyladenine). The basal medium was MS/B5 containing inorganic macro- and microelements from MS medium [20], and the organic components of B5 medium [21]. It was also supplemented with 30 g/l of sucrose and 7 g/l of agar. The cultures were kept at 25°C in the 16-hour photoperiod. Photosynthetic photon flux density (PPFD) was 50 μmol/m^2·s. The shoots developed from the initial explants were transferred on the basal medium without plant growth regulators. They were used as a stock culture.

Shoot multiplication

The apical explants from stock culture (5-8 mm long shoot tips with one pair of leaves and apical bud) were the material used in the experiment concerning the effects of growth regulators on the formation of axillary shoots. The MS/B5 medium with 0.1, 0.5 or 1.0 mg/l BA and 0.01 mg/l NAA (1-naphtalene acetic acid) or without the auxin, was used. The medium without any plant growth regulators was the control one. The experiment was performed in three replications using 30 explants in each treatment. The number of axillary shoots obtained from the explant was determined after four weeks.

Rooting of axillary shoots

The shoots obtained on the medium with 1.0 mg/l BA were transferred on the rooting medium with a half-reduced concentration of inorganic macronutrients (½MS/B5) supplemented with 20 g/l of sucrose. For the rooting IBA (indole-3-butyric acid), in following concentrations: 0.25, 0.5 and 1.0 mg/l, was applied. The medium without IBA was the control. The experiment was established in three replications using 30 shoots in each treatment. After six weeks of culture, the percentage of rooted shoots and morphological traits of newly developed microcuttings were evaluated. They were assessed regarding their weight, length of shoots, number of leaves, number of roots and length of roots. The morphological observations were performed using six microcuttings in three replications.

Acclimatization

Rooted microcuttings obtained in the previous experiment were washed to remove the agar from the roots. The plantlets were planted in multi-pots (50 x 50 mm) filled with the peat substrate (peat, sand, perlit; in volume ratio of 3:1:1, pH=5.8) enriched with multicomponent fertilizer MIS4 (1 kg/m^3, Intermag) containing macro- and microelements. The plants were maintained in a growth chamber under 25°C temperature and 16 h photoperiod. PPFD provided by fluorescent

tubes was 70 μmol/m^2·s. During three weeks the plants were covered with perforated foil, in order to reduce excessive evaporation, and the daily airing time was gradually increased. Then the covering was removed. After four weeks, plants were transferred to greenhouse conditions. The survival rate of the plantlets was recorded two weeks later.

Statistical analysis

Statistical analysis of results was done with STATGRAPHICS Plus software, version 4.1. One-way analysis of variance was performed and the Tukey's test at the probability level $\alpha=0.05$ was used to evaluate differences between the means.

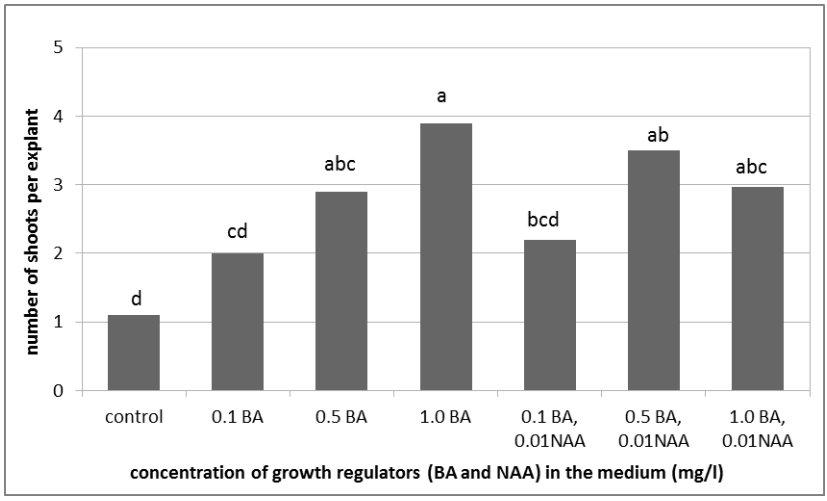
RESULTS AND DISCUSSION

Bastard balm, with a status of partly protected species, is one of the most endangered aromatic plants in Poland [1, 11]. There is little information on the biology of development of the species, e.g. on its reproduction mechanisms. Only few publications on propagation of bastard balm are available [13, 14]. The preliminary studies on traditional propagation methods of this plant were conducted in Department of Vegetable and Medicinal Plants, SGGW-WULS. They indicate that stratification is needed to induce germination of bastard balm seeds, but even after stratification the rate of germination is low and the seedling emergence are uneven (unpublished data). Little work has been undertaken also on the micropropagation of bastard balm. Some reports on *in vitro* propagation with usage of explants obtained from seedlings (with seeds as a material to initiate the culture) were published [10, 19].

The presented study is the first attempt to *in vitro* propagation of bastard balm on the basis of vegetative initial explants obtained from plants growing in the field. In our investigations, shoot tips of maternal plants collected in May were the material to establish the *in vitro* culture.

Axillary shoot formation was observed in explants cultured on all the tested media. However, the number of regenerated shoots depended on the concentration of BA in the medium. The number of shoots per explant (multiplication factor) ranged from 1.1 for explants placed on a medium without growth regulators (control) to 3.9 for those exposed to 1.0 mg/l BA (fig. 1, 3). In the study of Skrzypczak and Skrzypczak [10], where the initial explants originated from seedlings, the most effective medium for bastard balm shoot formation was MS medium with zeatin (0.5 mg/l), in comparison to media enriched with BAP and IAA. The influence of BA on enhanced axillary branching among plants from the *Lamiaceae* family had been studied previously. Although the multiplication factor obtained for those plants was not very high, beneficial effects of BA on axillary shoot formation was proved. In case of *Salvia santolinifolia*, 3.0 mg/l BA gave the best shoot

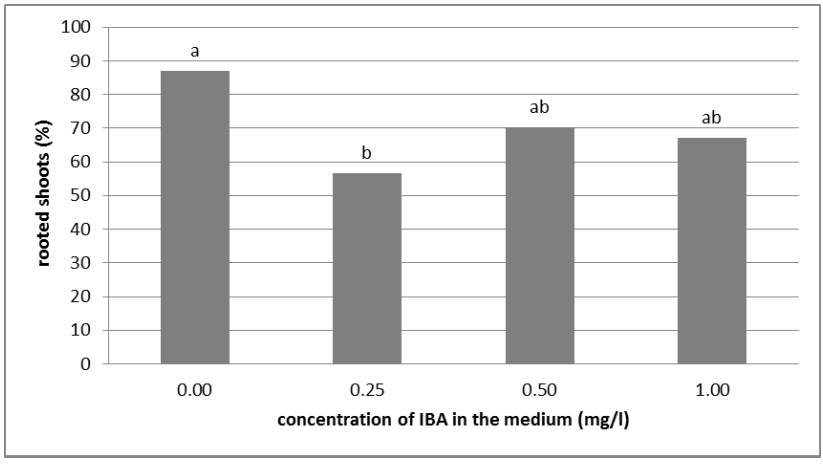
regeneration with 1.8 shoots per explant [22], whereas for *Salvia nemorosa* the best medium was the one amended with 8.9 μM (2.0 mg/l) BA with 5.7 shoots per explant obtained [23]. In above mentioned experiments, additional application of NAA did not increase the number of regenerated shoots [22]. This data are in good agreement with the results of our study.



Values followed by the same letters do not differ significantly at α=0.05

Figure 1.

Effect of plant growth regulators on axillary shoot formation; n=30



Values followed by the same letters do not differ significantly at α=0.05

Figure 2.

Effect of IBA concentration on the shoot rooting (%); n=30

The shoots regenerated in the first part of our experiment were rooted on ½MS/B5 with IBA of different concentration (0.25, 0.50, 1.00 mg/l). The highest rooting was observed at the medium without the auxin (87%). IBA limited the process significantly (fig. 2, 4, 5). This results are in contradiction with those obtained by Skrzypczak and Skrzypczak [10] where bastrad balm rooting was stimulated by both IBA and IAA. Beneficial effects of IBA on root regeneration among other species from the *Lamiaceae* family, namely *Ocimum americanum, Mentha piperita* or *Salvia recemosa*, were observed too [23-25].

Figure 3.

Axillary shoots formed on explants after four weeks of culture on MS/B5 medium with 1.0 mg/l BA

Figure 4.

Rooted microcuttings after eight weeks of culture on ½MS/B5 medium without growth regulators

Figure 5.

Rooted microcuttings after eight weeks of culture on ½MS/ B5 with 1.0 mg/l IBA

In our study, IBA used in the rooting medium influenced some morphological traits of obtained microcuttings. The plants cultured on the medium with higher concentration of IBA (1.00 and 0.50 mg/l) were characterized with shorter shoots and roots and lower number of this organs per plant in comparison to those cultured on the medium without IBA or with very low concentration of this regulator (0.25 mg/l). According to De Klerk [26] IBA, as a relatively stable auxin, remain present in plant tissues and medium for a longer time and may inhibit not only the outgrowth of root primordia but also may stimulate massive ethylene accumulation in the tissue culture container. In such a case, high concentrations of ethylene inhibit the elongated growth of plant organs, what was seen in our experiment. However, the weight of obtained microcuttings was similar and was not affected by IBA concentration. After six weeks of *ex vitro* acclimatization, microcuttings adapted to the greenhouse conditions very well regardless the content of IBA in the rooting medium (tab. 1, fig. 6).

Table 1.

Morphological traits of microcuttings (observations directly before *ex vitro* planting) and percentage of plants acclimated in *ex vitro* (observation six weeks after planting)

Rooting media with IBA (mg/l)	Mass of microcutting [g/plant]	Shoot length [cm]	Number of leaves per shoot	Number of roots per shoot	Root length [cm]	Plants acclimated in *ex vitro* (%)
½ MS/B5 0.00 IBA	0.37 a	8.8 a	5.3 a	5.0 a	3.8 a	90.7 a
½ MS/B5 0.25 IBA	0.46 a	5.2 b	4.0 ab	4.6 a	3.5 a	84.5 a
½ MS/B5 0.50 IBA	0.32 a	4.1 b	3.5 ab	3.2 ab	3.0 b	90.1 a
½ MS/B5 1.00 IBA	0.38 a	3.2 c	3.3 b	2.3 b	2.7 b	88.2 a

Values followed by the same letters in columns do not differ significantly at $\alpha=0.05$

Figure 6.

Plants acclimated in *ex vitro* conditions – six weeks after planting

CONCLUSIONS

1. BA (concentrations 0.1–1.0 mg/l) used in the medium for bastard balm axillary shoots formation enhanced the process. Additional application of NAA (0.01 mg/l) did not affect shoot branching.
2. IBA used in a medium for shoot rooting suppress root formation. The highest percentage of rooted shoot was obtained on the medium without IBA.
3. IBA in the medium for shoot rooting influenced analyzed morphological traits of obtained microcuttings but did not affected their weight.
4. The regenerated, rooted shoots successfully acclimated in *ex vitro* conditions regardless the growth regulators concentration (IBA) applied in the medium for the rooting.

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ROZMNAŻANIE MIODOWNIKA MELISOWATEGO (*MELITTIS MELISSOPHYLLUM* L.) W KULTURACH *IN VITRO*

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S t r e s z c z e n i e

Opracowano metodę szybkiego rozmnażania miodownika melisowatego w kulturach *in vitro* na drodze stymulacji tworzenia pędów bocznych. Materiałem do inicjacji kultury *in vitro* były wierzchołki pędów pobierane z roślin 3-letnich w maju i odkażane 0,2% roztworem sublimatu. Otrzymane z eksplantatów inicjalnych pędy wykładano na pożywkę MS/B5 zawierającą 0,1; 0,5 lub 1,0 mg/l BA z dodatkiem 0,01 mg/l NAA lub bez dodatku auksyny. Najwięcej pędów bocznych otrzymano na pożywce zawierającej 1,0 mg/l BA (3,9 pędów na eksplantat). Do ukorzeniania pędów zastosowano pożywkę ½ MS/B5 z dodatkiem IBA (0,25; 0,50 i 1,0 mg/l). Kontrolę stanowiła pożywka bez regulatorów wzrostu. Najwięcej ukorzenionych mikrosadzonek obserwowano na pożywce bez dodatku IBA (87,1%). IBA zastosowane w pożywce do ukorzeniania pędów wpłynęło na cechy morfologiczne uzyskanych mikrosadzonek, ale nie wpłynęło na ich masę. Niezależnie od stężenia IBA w tej pożywce mikrosadzonki dobrze adaptowały się do warunków *ex vitro*.

Słowa kluczowe: *in vitro, pędy boczne, pożywka MS/B5, NAA, BA, IBA, adaptacja ex vitro*