In vitro and *ex vitro* rooting of *Spiraea betulifolia* subsp. *aemiliana* (Rosaceae), an ornamental shrub

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Abstract. Two methods of rhizogenesis – *in vitro* and *ex vitro* of *Spiraea betulifolia* subsp. *aemiliana* (C.K. Schneid.) H. Hara microshoots have been compared. Pulse treatment of microshoots with aqueous solutions of 4% "Heteroauxin" or 2% succinic acid (*ex vitro* rooting) did not effective – rooting frequency ranged from 3 to 19%. It was established that the *in vitro* rooting on nutrient media supplemented with auxins was a more effective technique, providing a high percentage of rooted microshoots. The use of half- strength MS medium supplemented with 0.1 μM indolyl-3-butyric acid (rooting frequency 88%, root number 3.5 ± 0.3 per plantlet) was found to be the most efective for *in vitro* rooting. The *in vitro* rooted regenerated plantlets were successfully acclimatized with 55% of survival rate.

1 Introduction

The genus *Spiraea* L. representatives are highly ornamental plant and have many garden forms and varieties, furthermore have diverse biological activity and various other beneficial properties [1, 2]. *Spiraea betulifolia* subsp. *aemiliana* (C.K. Schneid.) H. Hara (syn. *S. aemiliana* C.K. Schneid.) is a part of the polymorphic plant complex of *Spiraea* genus (Rosaceae Juss.), *Calospira* C. Coch section. The subspecies is distributed only in the island part of Asian Russia (Sakhalin Island, Kuril Islands). Distinctive features of the subspecies are a shrub height up to 30 cm, a compact crown, a dense white corymbose inflorescences, a small rounded lamina and an absence of the inflorescence follicles pubescence [3]. Besides Russia, *S. betulifolia* subsp. *aemiliana* also grows in Japan [4]. The taxon has a narrow range and can be recommended for inclusion in the Red Data Book of the Russian Federation or the regional Red Data Book of the Sakhalin Region. *S. betulifolia* subsp. *aemiliana* is a promising plant for landscape design in the European part of Russia and has decorative qualities [5]. Currently, propagation methods this taxon have not been developed.

The development of effective micropropagation protocols of ornamental plants allows scaling the process of producing plant material, while preserving all the ornamental characteristics of mother plants, the loss of which is possible during seed propagation.

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Moreover, the success of *in vitro* propagation depends not only on the optimization of the multiplication stage conditions, but also on the selection of the most effective methods of rooting and *ex vitro* acclimatization conditions. Rooting microshoots is carried out in several ways: 1) cultivation on nutrient media supplemented with auxins; 2) by pulsed treatment for several hours (2-24 hours) of microshoot bases with auxin solutions, followed by passaging on hormone-free nutrient media or planting microplants directly in a soil substrate; 3) treatment of shoot bases with auxin-containing powders, followed by planting in a soil substrate [6, 7]. Techniques in which there is a cultivation stage on nutrient media are called *in vitro* rooting, where microplants, after treatment with auxins, are planted immediately in the soil – *ex vitro* rooting. *Ex vitro* rooting reduces the duration of cultivation due to the combination of the rooting stage and acclimatization. Moreover, acclimatization to *ex vitro* conditions is the final and often critical stage at any micropropagation protocol; in the case of death of regenerated plants at this stage, the efficiency of clonal micropropagation can significantly decrease [6, 8].

The aim of the study was to develop an effective method of rooting *Spiraea betulifolia* subsp. *aemiliana* microshoot.

2 Material and methods

Spiraea betulifolia subsp. *aemiliana* introduced at the experimental field of the Laboratory of phytochemistry of the Central Siberian Botanical Garden (Novosibirsk, Russia) was used. Intact plant material of *Spiraea betulifolia* subsp. *aemiliana* was collected at the site of its natural habitat in 2016 in the Kunashir Island (Kuril Islands, Russia).

The conglomerates of shoots obtained during clonal micropropagation were separated individually and used to optimize the stages of rooting and acclimatization to *ex vitro* conditions. Rhizogenesis induction was carried out in two ways. In the case of *in vitro* rooting, root formation was induced on Murashige-Skoog (MS) media [9] complete or with a half-strength content of macro- and microsalts, supplemented with indolyl-3-butyric acid (IBA) at a concentration of 0.1 and 1.0 μM. The passage duration at the *in vitro* rooting stage lasted 50-60 days. For *ex vitro* rooting, the shoot bases were pulse-treated with aqueous solutions of 4% "Heteroauxin" (indolyl-3-acetic acid at concentrations 50 g/kg; Orton, Russia) for 3.5 h or 2% succinic acid (STK, Russia) for 16 h, and then transferred immediately to a soil substrate for the rhizogenesis induction, thus combining the stages of rooting and acclimatization. Microshoots planted in a substrate without preliminary soaking in root formation stimulating solutions were a control. To acclimatize microshoots to *ex vitro* conditions, the soil mixture (Fasko +, Russia) supplemented with sand (3:1) was used as a substrate. Before planting, the substrate was preliminarily spilled with an aqueous solution of $KMnO_4$. Microplants were planted in containers filled with the soil substrate and covered with a film to prevent desiccation. For 2 weeks after the beginning of acclimatization, a high humidity was maintained in the containers, gradually reducing it, then the film was completely removed. After 2.5 months plants were transplanted into individual pots (0.4 l) and transferred to greenhouse conditions.

All cultures at the *in vitro* rooting stage were incubated at 23 ± 2 °C, under a light intensity of 54 μmol m-2 s-1 under a 16-h photoperiod. Acclimatization experiments were carried out at 23 ± 2 °C under a 16-h photoperiod (room condition).

Data are presented as mean ± standard error (M ± SE). The signifcance level accepted was $P \leq 0.05$. The statistical analysis package Microsoft Excel was used to process the results.

3 Results and discussion

The rooting of *in vitro* raised microshoots is carried out on media supplemented with auxins: IBA, indolyl-3-acetic acid (IAA), or 1-naphthylacetic acid. Using IBA as the main inducer of rhizogenesis in the study is explained by numerous evidences of the effectiveness of this growth regulator for *in vitro* rooting various Rosaceae woody plants [10-12].

High rhizogenesis frequency (not less than 60%) of shoots was obtained, the maximum frequency of *in vitro* rooting was 88% on ½ MS + IBA 0.1 μM medium. Cultivation on media supplemented with auxin stimulated the root formation, without callus formation on all nutrient media studied. It was established experimentally that the use of half-strength media led to the activation of root formation (Fig. 1), while an increase the IBA concentration to 1.0 μM reduced the rhizogenesis frequency. The maximum root number and high rooting frequency was obtained on a ½ MS supplemented with 0.1 μM IBA, that allowed to consider the medium to be the best for *in vitro* rooting induction.

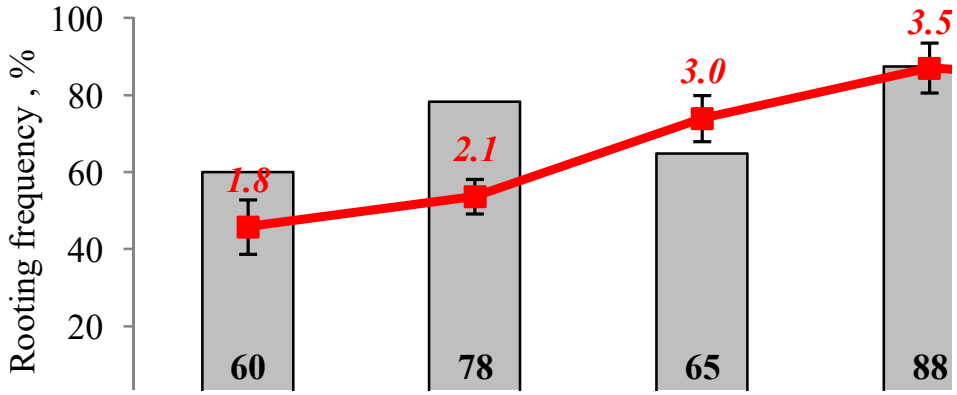

Fig. 1. *In vitro* rhizogenesis of *Spiraea betulifolia* subsp. *aemiliana* at the end of passage, MS and ½ MS nutrient media, supplemented with 0.1and 1.0 μM IBA

Using "Heteroauxin" and succinic acid are well established itself in the rooting of green cuttings of ornamental shrubs, including spirea [13], while "Heteroauxin" is the trade name for IAA, which is widely used in *in vitro* rooting microshoots [14]. The effectiveness of using pulsed treatment with auxin solutions followed by subsequent transfer to soil substrates for various woody plants has been proven many times: for *Acer* L., *Betula* L., *Malus* P. Mill. [15], *Balanites* Delile, *Citrus* L., *Syzygium* P. Browne ex Gaertn. [16], *Rhododendron* L. [7]. The advantages of *ex vitro* rooting are the reduction of root damage during the transfer of microplants from the nutrient medium to the soil substrate, the normal anatomy of the roots with a high proportion of conductive tissue, and the higher resistance of microplants to stress, including water deficiency. In addition, the duration of cultivation is reduced due to the combination of the rooting and acclimatization stages [15].

Rhizogenesis induction of *S. betulifolia* subsp. *aemiliana* microshoots by pulsed treatment followed by planting in the substrate (*ex vitro* rooting) was found to be ineffective. A very low percentage of rooted plants were obtained: in the control group and at treatment with succinic acid, the death of microshoot was 97%, using "Heteroauxin" was more effective – 19% of the rooted microshoot was obtained (Fig. 2).

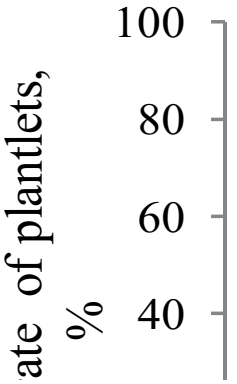

Fig. 2. Survival rate of *Spiraea betulifolia* subsp. *aemiliana* plantlets, rooted by *in vitro* and *ex vitro* methods

Such a low survival rate of plants indicated a weak development of the root system, which was formed as a result of pulse treatment. Previously M. T. McClelland et al. established experimentally that higher morphological parameters of root system growth were obtained by *in vitro* rooting, this tendency remains up to 20 weeks after transferring microplants to *ex vitro* conditions for *Acer rubrum* L. "Red Sunset" and *Malus* × *domestica* Borkh "McIntosh" [15]. In the present study, the acclimatization of *in vitro* rooted regenerated plants was almost threefold more effective and reached 55% of survival rate that indicates a better development of the root system in comparison with *ex vitro* rooting. Moreover, as noted by M. T. McClelland et al., in the early stages of development, roots formed *in vitro* or *ex vitro* vary significantly in anatomy; this is due to the availability of nutrients, water and oxygen in an *in vitro* culture, which leads to lack of need for the development of a secondary phloem and xylem. However, long-term growing in the soil substrate eliminates these differences and the anatomy of regenerated plant roots corresponds to that of intact plants [15]. In our study, the dynamics of growth and development of *S. betulifolia* subsp. *aemiliana* regenerated plants was leveled under the greenhouse conditions, all plants developed a voluminous root system and an above-ground part, which confirms the results obtained by M. T. McClelland et al.

Thus, an effective method providing a high percentage of rooting and acclimatization of *S. betulifolia* subsp. *aemiliana* regenerated plants was the *in vitro* induction of rhizogenesis on ½ MS nutrient medium supplemented with 0.1 μM IBA. The use of pulsed treatment of microshoots with solutions of 4% "Heteroauxin" (exposure 3.5 h) or 2% succinic acid (exposure 16 h) at *ex vitro* conditions was found to be ineffective for induction of root formation of the studied *Spiraea* subspecies. It is necessary to continue the study of the peculiarities of the rooting methods of *S. betulifolia* subsp. *aemiliana* microshoots to identify potential of root formation.

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