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RESEARCH PAPER

In vitro Barnardia japonica (Thunb.) Schult. et Schult. fil. micropropagation by direct regeneration

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ABSTRACT

In vitro micropropagation of rare Far Eastern species *Barnardia japonica* by the direct regeneration method was studied for the first time. The effect of different concentrations of 6-benzylaminopurine and kinetin in combination with auxins on the regeneration potential of young seedlings using Murashige and Skoog media was estimated. It was found that the largest number of new plants (61 new shoots on average) is formed when explants are cultivated on the medium with a high concentration of kinetin (10 mg/L) and a minimum of α-naphthylacetic acid (0.1 mg/L). The resulting plants are rooted easily on a hormone-free medium of the same mineral composition. The least effective for the mass production of plants by direct regeneration was a medium with a combination of 2 mg/L kinetin and 1 mg/L α-naphthylacetic acid. The developed approach can be applied for the effective propagation of *Barnardia japonica* and returning the species to its natural habitat.

Keywords: *in vitro* culture, micropropagation, regeneration, *Scilla scilloides*, conservation

РЕЗЮМЕ

Пьянова (Бердасова) А.С., Салохин А.В., Лончакова Т.Е., Сабуцкий Ю.Е. Микроразмножение *Barnardia japonica* (Thunb.) Schult. et Schult. fil. методом прямой регенерации в культуре *in vitro*. Методом прямой регенерации в культуре *in vitro* впервые исследовано микроклональное размножение *Barnardia japonica* – вида, находящегося в РФ на грани исчезновения. Изучено влияние различных концентраций 6-бензиламинопурина и кинетина в комбинации с ауксинами на регенерационный потенциал молодых сеянцев с использованием в качестве базальной среды компонентного состава по прописи Murashige и Skoog. Найдено, что наибольшее число новых растений (в среднем 61 новый побег) образуется при культивировании эксплантов на среде с высоким содержанием кинетина (10 мг/л) и минимальным α-нафтилуксусной кислоты (0.1 мг/л). Полученные растения легко укореняются на безгормональной среде того же минерального состава. Наименее эффективной для массового получения растений путем прямой регенерации была среда с использованием сочетания 2 мг/л кинетина и 1 мг/л α-нафтилуксусной кислоты. Разработанную методику можно применять для эффективного размножения и возвращения вида в его естественные условия обитания.

Ключевые слова: микроразмножение, регенерация, пролеска, *Scilla scilloides*, сохранение

Barnardia japonica (*Scilla scilloides* (Lindl.) Druce) is a perennial bulbous autumn-flowering plant of the family Hyacinthaceae. It is distributed mainly in Northeastern China, Japan and the Korean Peninsula (Ohwi 1965, Kitagawa 1979, Lee 1993, Lee 1996). On the territory of the Russian Far East, there is the northern border of the species distribution. It was considered as disappeared from the flora of Russia for a long time (Barkalov 2008). Recently discovered populations in the Khasansky District and on Sakhalin Island made it possible to change the rarity category from 0 (EX) to 1 (CR) (Goncharova et al. 2013, Pshennikova et al. 2019).

The experience of cultivating *Barnardia japonica* in the Botanical Garden-Institute FEB RAS indicates its stability in culture. Late flowering and re-vegetation since August give a special decorative effect. However, even in culture, the indicators of seed reproduction (the coefficient of fruitfulness, the percentage of seed production, potential and real seed productivity) are quite low, which probably explains the rarity of detection in natural habitats (Goncharova et al. 2013).

An alternative preservation method and an effective reproduction technique is the study of cultivation and micropropagation processes *in vitro*. There are many works devoted to the tissue culture exploration of the genus *Scilla* L. representatives (McCartan & van Staden 1998, McCartan & van Staden 1999, Barykina & Churikova 2001, Chaudhuri & Sen 2002, Banciu et al. 2010). Fragments of bulbous scales and leaves are mainly used as explants for obtaining a sterile culture (Nair 1989, McCartan & van Staden 1998, Kamaleswari et al. 2016), however, due to frequent deep fungal and bacterial infections, it is possible to use floral

explants and flower stalk fragments (Chakravarty & Sen 1989, Ozdemir et al. 2016).

Earlier, Korean scientists obtained an embryogenic callus of *Barnardia japonica* from plants of various cytogenetic types and have studied the somaclonal variability of the resulting regenerated plants (Bang & Choi 1996). It was found that somaclonal variability was determined by the cytogenetic type and ploidy level of the original plants. In some cases, the percentage of the variability was very high and reached 87.7 %. It is known that micropropagation using the direct regeneration method minimizes the possibility of somaclonal variability of the resulting regenerated plants (Butenko 1999) which allows preserving their genetic uniformity and use them to restore populations of certain cytogenetic types. Also in the work of Romanian scientists (Banciu et al. 2010) was shown that *Scilla autumnalis* L. plants obtained by the direct regeneration did not have any genetic changes. Our work aimed to establish optimal conditions for effective *in vitro* micropropagation of *B. japonica* by direct regeneration.

MATERIAL AND METHODS

Mature seeds of *B. japonica* were collected in 2016 near settlement Zarubino (Khasansky District of Primorye Territory) and used as a starting material for obtaining a sterile culture *in vitro*. To study the micropropagation process we used two-month-old young seedlings containing 2 leaves and a formed bulb with removed roots. The number of explants in each experiment is 10, the number of repetitions is 3.

For the preparation of nutrient media, we used extra pure grade (LenReaktiv, Russia) macro- and microelements according to Murashige-Skoog composition (Murashige & Skoog 1962), sucrose (Helicon, Russia), phytohormones (Sigma-Aldrich, USA), vitamins and agar-agar (Sigma-Aldrich, USA). Silver nitrate (LenReaktiv, Russia) was used for the seeds sterilization.

Preparation and sterilization of the media were performed according to standard procedures (Butenko 1999). The solutions were adjusted to pH 5.7–5.8 using 0.1 N KOH before autoclaving. Sucrose concentration in all cases was 3 %, agar 0.6 %. Plant hormones and vitamins added after autoclaving. The media were poured into sterile test tubes (10 mL of the media) or 250 mL Erlenmeyer flask (50 mL of the media).

The seeds were pre-soaked for 24 hours before sowing and then washed for 20 minutes in a 0.1 % Tween 80 solution. Surface sterilization was carried out using a 1 % silver nitrate solution (exposure time 15 min) followed by a single wash with 1 % sterile sodium chloride solution and twice with sterile distilled water (2 minutes each). The seeds were placed on an agarized hormone-free Murashige and Skoog (MS) medium (Murashige & Skoog 1962) and germinated for 4 weeks. One month after germination the plants were transplanted onto a new medium. At the stage of micropropagation, various combinations of cytokinins (6-benzylaminopurine (BAP), 6-furfurylaminopurine or kinetin (Kn)) and auxins (2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthylacetic acid (NAA), indole-3-butyric acid (IBA)) were added to the nutrient medium at concentrations of 0.1–10.0 mg/L to induce shoot formation. The medium without growth regulators used as a control. Subsequent subculturing of explants on the fresh medium of the same composition was carried out every 3–4 weeks. The rooting of the resulting regenerated plants took place in flasks using a hormone-free MS medium. The experiments were carried out in 3 repetitions. Conditions for seed germination and explants cultivation: temperature 24 ± 2ºC, 16-hour photoperiod, illumination of 2000–3000 lux with cool-white fluorescent light (Philips, Poland).

Statistical processing and analysis of the data were performed using Statistica 6.1 software.

RESULTS

At the stage of sterile culture obtaining we showed 100 % efficiency of the *B. japonica* seed sterilization technique with 1 % silver nitrate solution. Mass germination of seeds on hormone-free MS medium was observed on the 13th day of cultivation. The average percentage of seed germination after 30 days was 85.7 %.

In our experiments, we used young seedlings as explants at the stage of two true leaves with formed bulbs and removed roots. To study the type of seedlings morphogenetic response, the resulting plants transferred to MS medium containing various combinations of cytokinins and auxins: 2 mg/L BAP + 1 mg/L NAA, 4 mg/L BAP + 1 mg/L NAA, 2 mg/L BAP + 0.5 mg/L IBA, 2 mg/L NAA + 0.5 mg/L BAP, 1 mg/L 2,4-D + 0.5 mg/L BAP, 2 mg/L Kn + 1 mg/L NAA and 10 mg/L Kn + 0.1 mg/L NAA.

When we used media with a higher concentration of auxin than the concentration of cytokinin, the process of direct regeneration was observed only in the case of combination 2 mg/L NAA and 0.5 mg/L BAP. After 2 months of cultivation, adventitious shoots were formed on the surface and at the base of the explants. This process was accompanied by an increase in the size of the explants and the formation of dense morphogenic callus tissue (Fig. 1A). After the resulting callus was transferred to a hormone-free media, active regeneration of shoots was observed. Replacement of NAA by 2,4-D at a concentration of 1 mg/L led only to a pronounced callusogenesis of the explants with the formation of a dense white callus (Fig. 1B). Cultivation of the

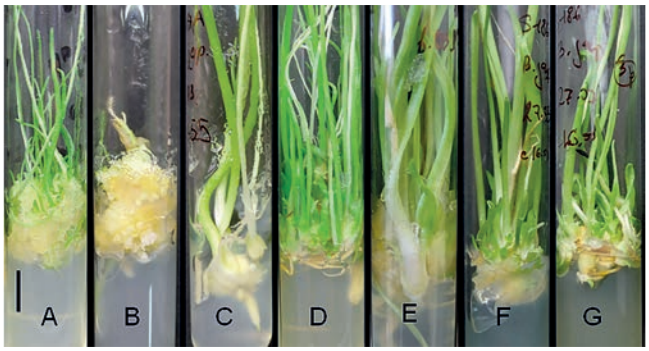

Figure 1 Types of *Barnardia japonica* seedlings morphogenetic response on MS media with different composition of cytokinins and auxins after 3 months of cultivation: A – media with 2 mg/L NAA and 0.5 mg/L BAP; B – media with 1 mg/L 2,4-D and 0.5 mg/L BAP; C – media with 2 mg/L Kn and 1 mg/L NAA; D – media with 10 mg/L Kn and 0.1 mg/L NAA; E – media with 2 mg/L BAP and 1 mg/L NAA; F – media with 4 mg/L BAP and 1 mg/L NAA; G – media with 2 mg/L BAP and 0.5 mg/L IBA. Bar: 1 cm

callus on a hormone-free medium led to active rhizogenesis without the formation of shoots.

When we cultivated young *B. japonica* seedlings on media containing a higher concentration of cytokinin (BAP or Kn) than auxin (NAA or IBA), only direct regeneration of vegetative shoots on the surface of the outer scales was observed. In the case of the medium with 2 mg/L Kn and 1 mg/L NAA, the shoot formation was weak and the development of roots and a noticeable increase in the size of the bulb occurred (Fig. 1C). Further explants cultivation on this medium was accompanied by a strong thickening of the roots without the formation of new shoots. Increasing the kinetin concentration to 10 mg/L and reducing NAA to 0.1 mg/L significantly stimulated the regeneration process and led to the appearance of numerous adventitious shoots on explants bulb surface (Fig. 1D).

Using of 6-benzylaminopurine even at a concentration of 2 mg/L in combination with 1 mg/L NAA led to the intensive formation of young shoots on the bulb surface after 1.5 months of cultivation. Increasing the concentration of BAP to 4 mg/L, or the replacement of NAA by IBA at a concentration of 0.5 mg/L also led to the active adventitious shoots formation (Fig. 1E–G).

To identify the most effective phytohormones combination for *B. japonica* micropropagation and counting of regenerated shoots, it was necessary to obtain plants with formed bulbs. According to Bang & Choi (1996), regenerated from callus tissue plants of *B. japonica* were rooted easily without additional application of auxins and quickly acclimatized to *ex vitro* conditions. In our experiment, the obtained plants also were transferred to a hormone-free MS medium after 3 months of cultivation on propagation media (Fig. 2A,B). Shoots and roots active growth and the bulbs formation were observed after 2 months of the rooting stage without auxins utilization (Fig. 2C). In all cases the resulting plants were heterogeneous and bulbs varied in size from 2 to 8 mm. The obtained results during the micropropagation experiment are shown in Table 1

DISCUSSION

Studies of some *Scilla* L. species morphogenesis in *in vitro* culture show that morphogenetic processes in different species are similar and do not depend on the type of explants. Subsequent emergence of meristematic foci and the formation of adventive structures occurs subepidermally from the abaxial side (McCartan & van Staden 1998, Barykina & Churikova 2001). Some authors note that leaf explants give a larger number of shoots compared to bulbous explants (McCartan & van Staden 1998, Kamaleswari et al. 2016).

In accordance with previously published works, kinetin and its combinations with auxins are more effective for *in vitro* micropropagation of the genus *Scilla* L. representatives. Application of MS medium with 5 mg/L Kn and 1 mg/L NAA gave 12–14 shoots for *S. hyacinthiana* leaf explants (Nair 1989). Using a mixture of 1–2 mg/L of kinetin with 1–2 mg/L indoleacetic acid (IAA) showed the best results for leaf (14.2 shoots per explant) and bulb explants (8.9 shoots per explant) for *S. natalensis* (McCartan & van Staden 1998). Similar plant hormones combination also was more effective for *S. kraussii* leaf explants (McCartan & van Staden 2002) and gave 6 shoots per explant. The combination of BAP and NAA was more effective for micropropagation of *S. pratensis* and *S. autaminalis* (Banciu et al. 2010, Balabova et al. 2013). Bulb explants of *S. pratensis* produced 5.2 shoots on medium with 2.25 mg/L BAP and 0.93 mg/L NAA. Young seedlings of *S. autaminalis* gave 14.2 shoots when used combination of 5 mg/L BAP and 1 mg/L NAA.

In our study it was found that the best phytohormones combinations which promote the most efficient micropropagation of *Barnardia japonica* were the following: 10 mg/L Kn + 0.1 mg/L NAA (multiplication factor – 61.0), 2 mg/L BAP + 0.5 mg/L IBA (multiplication factor – 47.0) and 4 mg/L BAP + 1 mg/L NAA (multiplication factor – 46.6). The least effective combination for micropropagation by the direct regeneration method was the combination of 2 mg/L Kn and 1 mg/L NAA (multiplication factor

Table 1. The average number of shoots for *Barnardia japonica* micropropagation stage on MS medium with different phytohormones

Phytohormones combination	Average shoot number
1 mg/L 2,4-D + 0.5 mg/L BAP	–
2 mg/L NAA + 0.5 mg/L BAP	35.2±1.7
2 mg/L BAP + 1 mg/L NAA	40.6±2.3
4 mg/L BAP + 1 mg/L NAA	46.6±2.1
2 mg/L BAP + 0.5 mg/L IBA	47.0±3.4
2 mg/L Kn + 1 mg/L NAA	3.6±1.6
10 mg/L Kn + 0.1 mg/L NAA	61.0±4.2

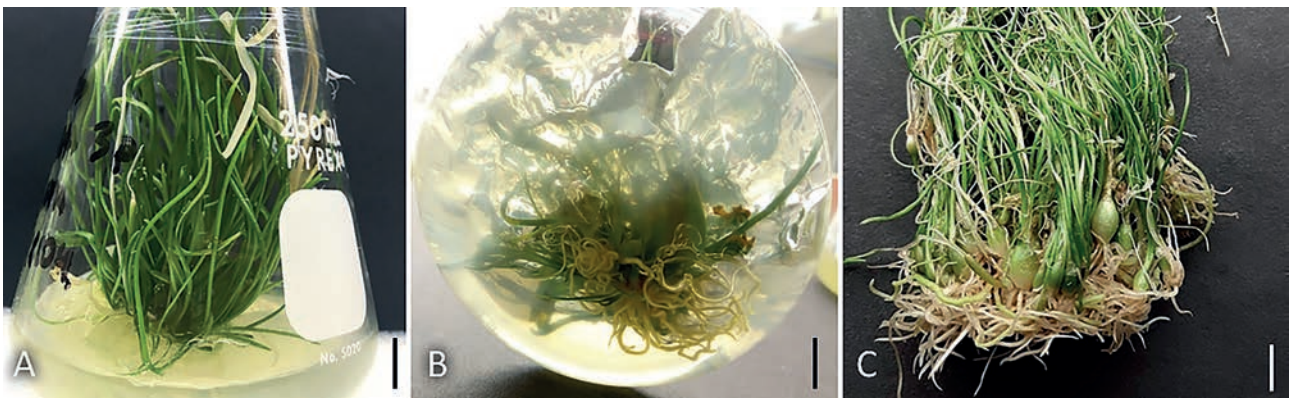

Figure 2 Development of *Barnardia japonica* plants at rooting stage (A, B) and after rooting stage (C). Bar: 1 cm.

– 3.6). The moderate multiplication rate showed media supplemented with 2 mg/L BAP + 1 mg/L NAA (multiplication factor – 40.6) and 2 mg/L NAA + 0.5 mg/L BAP (multiplication factor – 35.2). However, in the second case, the process of direct regeneration is associated with the formation of shoots from the resulting callus tissue. Rather high multiplication factor for *B. japonica* micropropagation in comparison with previously studied species can be explained by the use of young seedlings bulbs, which have significant regenerative potential in comparison with explants from adult plants.

CONCLUSION

Thus, it was shown that using MS media with a high concentration of cytokinins and low amount of auxins gives perfect results for threatened species *Barnardia japonica* reproduction by direct regeneration method in *in vitro* culture. The most effective combination is 10 mg/L Kn + 0.1 mg/L NAA (multiplication factor – 61.0). Good results also were obtained with 2 mg/L BAP + 0.5 mg/L IBA (multiplication factor – 47.0) and 4 mg/L BAP + 1 mg/L NAA (multiplication factor – 46.6). The collection of *B. japonica* plants *in vitro* is maintained in the active growth phase. Application of this micropropagation technique leads to the production of a large number of genetically homogeneous plants with a well-developed root system, suitable for *ex vitro* planting.

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