Obtaining plant raw material of Siberian iris (Iris sibirica L.) by biotechnology methods

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Abstract. The biotechnology for obtaining plant raw material I. sibirica L. has been developed. The required content of 6-BA for clonal micropropagation of I. sibirica was 2.5–5.0 μM. The introduction of cytokinins into the growth medium together with auxins, L-glutamine and adenine sulfate 100 mg/L, as well as the alternation of low and high concentrations of cytokinin enhanced the regenerative effect of 6-BA. When a year-round cultivation of regenerated plants under aeroponics conditions, the amount of biomass of plant raw materials I. sibirica in this method was approximately 31.2 kg/m² in wet weight for one year. It was established that intact plants and regenerant plants of I. sibirica, obtained on the basis of the developed biotechnology, had an identical group composition and comparable quantitative content of biologically active substances (flavonoids, tannins, coumarins and triterpene glycosides).

1. Introduction
Biotechnology methods make it possible to obtain high-quality medicinal plant raw material in a short time, in large quantities without destroying natural reserves. Using methods of suspension culture and “hairy roots”, a number of technologies have been developed that allow to receive valuable products of secondary metabolism of plants, such as glycosides, alkaloids, and some other biologically active substances. Nevertheless, according to experts, these methods have not been fully implemented in practice, since there is a problem of maintaining stable culture lines. High cost and complexity is the disadvantage of most bioreactors [1-3].

Irises are promising medicinal and ornamental perennials [4]. Blinova K.F. and her colleagues [5] isolated xanthon glycosides in Iris ensata Thunb. The content of mangiferin xanthonic glycoside (antioxidant, immunomodulator, and as part of antiviral drugs) was determined in some plants of the genus Iris, including Iris lactea Pall., Iris ensata, Iris reichenbachii Heuffel., Iris sibirica [6-11].

Biotechnological approaches such as aeroponic technology have the potential for large-scale iris cultivation and secondary metabolite production. Microclonal propagation makes it possible to obtain healthy planting material in the required quantity, regardless of the time of year. The combination of these two technological approaches will allow the development of biotechnology year-round production of medicinal plant materials of Siberian iris.
2. Materials and methods

Regenerant plants, hydroponic and intact *I. sibirica* plants were used as objects of study. Intact 6-year-old plants were harvested in the vicinity of Novoaltaysk, Altai Krai in 2015. The raw materials were dried to an air-dry state, packed in plastic bags and stored in a desiccator.

The methods used are generally accepted in biotechnology [12, 13]. *I. sibirica* flowers were taken in the budding phase, when they were tightly closed with wrapper leaves, and sterilized under the conditions of a laminar box. Parts of the perianth tube and pedicel were divided into fragments no larger than 3 × 3 mm and placed on the growth medium.

For clonal micropropagation, growth medium was prepared according to the MS formulation [14], containing 30 g/l of sucrose. At the injection stage, 3 μM NAA (α-naphthylacetic acid) was added to the culture in combination with 8 μM 6-BA (6-benzylaminopurine). At the stage of micropropagation, growth medium was prepared with the addition of 2.5-10.0 μM BAP (No. 98-2.5; No. 100 - 5.0; No. 117 - 7.5; No. 137 - 10.0). Media with auxins (A) 1 μM NAA and 0.1 μM IBA (3-indolebutyric acid) had the following hormonal composition: No. 127 - 2.5 6-BA+ A; No. 3 - 5.0 6-BA+ A; No. 129 7.5 6-BA+ A; No. 130 - 10, 0 6-BA+ A. Also, L-glutamine and adenine sulfate were introduced into the medium in an amount of 100 mg/l (L-gl. and ad.s. 100 mg / l). To root *I. sibirica*, a medium containing 3 μM NAA was used. The pH of the medium was adjusted to 5.8-5.9 and 0.6% agar was added. The medium was poured into plastic containers (30 ml each) or into culture bottles (10 ml each). Prepared growth medium was autoclaved for 20 minutes at 120°C. The explants were cultivated under a photoperiod of 16/8 hours light/dark at 24 - 26°C.

Transfer of regenerated plants to ex vitro conditions. Growing conditions and characteristics of the aeroponic installation. A three-tier universal aeroponic installation developed by the Federal State Budget Scientific Institution of the All-Russian Research Institute of Agricultural Biotechnology (Iu. Martirosian) was used in the work. The installation is built on the principle of modularity and can be used for research on potato breeding, as well as for propagating and growing other agricultural and medicinal plants.

The installation module consists of three tiers, from each tier you can simultaneously plant and grow from 72 (for relative large-sized plants) to 144 plants (a total of 426 iris plants), the technological area is 0.98 m², the illumination is 15 kLk, and the power consumption is 1 kW.

Plants were grown on an aeroponic installation in a cultivation room equipped with a climate control unit. Water supply and mineral nutrition of plants were carried out by periodically injecting a nutrient solution (under a pressure of 3 atm), irrigating the root system of plants. The aeration of the roots took place in the pauses between the supply of the solution.

The system is provided with automatic control of the technological process of feeding the nutrient solution, the aeration regimes of the root system, the duration and cyclicity of the light period, maintaining the necessary temperature and humidity in the cultivation room.

A necessary condition for the operation of the aeroponic installation was constant air exchange: the minimum value is 5000 cubic meters/hour. The optimum growth temperature of iris is 24-26°C. Relative humidity should be 65-80 ± 5%.

Plant raw material extraction. The phytochemical study was based on the scheme described by V.M. Cosman and colleagues [15]. Extractives were extracted from plant materials by sequential samples handling with various solvents: hexane, ethanol of a downward concentration (96% and 40%), water and 1% sodium hydroxide solution. Extraction with hexane and alcohol solutions was carried out in a Soxhlet apparatus, samples handling in raw material - extractant ratio - 1:50. The treatment with water and a 1% alkali solution was carried out by keeping the samples in solution at a temperature of 50–70°C, with raw material – extractant ratio - 1:50.

Determination of the content of certain groups of biologically active substances. To quantify the content of flavonoids in the extracts, a technique based on their ability to form a colored complex with an AlCl₃ solution was used. As extractant, 90% ethyl alcohol containing 10% sulfuric acid solution was used. The optical density of the resulting solution was measured on a UV-Vis Cary 60 spectrophotometer at a wavelength of 430 nm in a cell with a layer thickness of 10 mm. As a
one of the main problems is necessary to alternate the propagation. The number of flavonoids was carried out in terms of quercetin. The contents of coumarins and triterpene glycosides were also determined spectrophotometrically at a wavelength of 272 nm and 360 nm, respectively. Quantitative determination of tannins was carried out by oxidation with potassium permanganate in the presence of indigo carmine [16-18].

Quantification of essential oil absolute. The solvent extraction process took place in several successive stages. Petroleum ether (40–70° C) was used as a solvent. The result is a valuable aromatherapy and perfume product called absolute [19].

All measurements were performed no less than three times. All calculations of the content of various substances are given on an absolutely dry mass.

3. Results and discussions
Biotecnology for obtaining raw material. In case of microclonal propagation of iris, using vegetative shoots as explants is not economically feasible [20].

The maintenance of a long-passaged culture is one of the main problems with microclonal propagation. For iris culture at the stage of micropropagation proper, it is necessary to alternate medium with a high and low content of cytokinin. At the same time, L-gl and ad.s. 100 mg/l should be added into growth medium with a low content of phytohormone. On rooting medium, shoots should be planted only after passing on medium with a low content of cytokinin supplemented with non-hormonal growth stimulants, and some shoots must be transferred to medium with a high content of cytokinin for further propagation. Thanks to this cultivation scheme, a sterile culture of varieties based on *Iris sibirica* can be maintained for a long time (table 1, figure 1).

**Table 1.** The composition of the growth media and the cultivation scheme of *I. sibirica* of King of King variety.

| N of experiment | Option of media (6-BA, μM) | Stage of micropropagation | Stage of rooting |
|-----------------|----------------------------|---------------------------|------------------|
|                 | N<sub>e</sub> of shoots     | number of shoots           | height of the plant | number of roots | length of roots |
|                 |                            |                           | mm               |               | mm            |
| 1               | control                    | 93(1.0)                   | 1.5±0.1          | 67.0±8.6      | 86.6±8.6      | 3.2±0.3       | 17.1±1.6     |
| 2/1             |                            | 98(2.5)                   | 1.8±0.2          | 69.5±3.7      | 101.6±9.4     | 6.8±0.3*      | 14.8±1.1     |
| 2/2             | 127(2.5+A)                 | 1.2±0.1                   | 65.0±7.1         | 101.3±2.9     | 7.0±1.1*      | 13.3±0.8*     |              |
| 2/3             | 98→78                      | 1.5±0.1                   | 58.1±4.3         | 78.0±2.4      | 12.3±0.8*     | 14.3±0.7      |              |
| 2/4             | 127→78                     | 2.0±0.3*                  | 78.0±6.3         | 93.6±3.4      | 6.8±0.9*      | 18.4±0.6      |              |
| 3/1             | 100(5,0)                   | 2.0±0.7*                  | 53.3±3.5         | 113.6±4.8*    | 5.3±1.0       | 9.2±1.0*      |              |
| 3/2             | 3(5.0+A)                   | 1.2±0.1                   | 65.9±5.1         | 63.5±1.1*     | 7.0±1.1*      | 9.5±0.7*      |              |
| 3/3             | 100→78                     | 1.8±0.3                   | 63.6±6.04        | 103.5±10.8    | 9.0±0.6*      | 12.2±0.7*     |              |
| 3/4             | 3→78                       | 1.7±0.04                  | 58.0±2.7         | 77.6±4.1      | 7.2±0.5*      | 19.4±0.6      |              |
| 4/1             | 117(7,5)                   | 1.2±0.1                   | 51.9±3.2         | 60.0±4.6*     | 6.6±0.2*      | 11.7±0.8*     |              |
| 4/2             | 129(7,5+A)                 | 1.2±0.2                   | 52.2±3.0         | 84.0±7.2      | 4.0±0.2*      | 10.1±0.8*     |              |
| 4/3             | 117→78                     | 2.0±0.3*                  | 61.6±4.1         | 107.5±1.6*    | 6.3±0.3*      | 19.8±0.7     |
| 4/4             | 129→78                     | 1.7±0.2                   | 62.6±6.2         | 87.9±7.2      | 6.7±0.8*      | 15.2±0.9     |
| 5/1             | 137(10,0)                  | 1.0±0.2                   | 49.2±4.3         | 94.6±5.9      | 4.2±0.5       | 12.0±0.7*     |              |
| 5/2             | 130(10.0+A)                | 1.1±0.3                   | 67.5±5.1         | 76.0±5.2      | 3.3±0.9       | 7.8±0.5*     |
| 5/3             | 137→78                     | 1.5±0.1                   | 60.5±3.3         | 81.8±3.4      | 6.4±1.0*      | 13.4±0.9     |
| 5/4             | 130→78                     | 1.7±0.4                   | 84.0±6.9         | 83.3±1.4      | 7.3±0.8*      | 11.4±0.6*    |

Note. + A - 1.0 μM NAA + 0.1 μM IBA; Growth medium 78 - MS +1 μM 6-BA+ L-gl and ad.s. 100 mg/l; * - differences with control are reliable at P ≤ 0.05.
Obtaining plant material *I. sibirica* in aeroponics. The average mass of the plant-regenerant planted in an aeroponic installation was 0.4 g. On an area of 0.98 m$^2$, 432 plants were grown, 3 plants per planting place. After three months of cultivation, the average weight of one plant was approximately 18.0 ± 1.5 g, respectively, the growth of 432 plants was 7776 g. In year-round cultivation, the amount of biomass of plant material *I. sibirica* was approximately 31.2 kg/m$^2$ in terms of fresh weight per year (figure 1).

**Figure 1.** A) Regenerant plants of *I. sibirica* at the stage of micropropagation, B), C) Obtaining plant material *I. sibirica* in aeroponics.

Phytochemical analysis of the obtained raw material. Air-dried samples of biotechnological raw material were analyzed for ash, moisture and high molecular weight components content in comparison with leaves and rhizomes with roots of intact plants. The ash content in Sterkh regenerated plants was 7.7%, which corresponds to the content of intact plants in leaves. In the aeroponic leaves of Sterkh, ash content was 2 times higher than that of intact plants.

The maximum yield of extractives was determined in the plant biomass of Cambridge and Sterkh regenerated plants - 21.6% and 19.6%, respectively, and in Sterkh aeroponic plants - 32.0%. Thus, the total content of extractives in the hydroponic leaves of *I. sibirica* of the Sterkh variety is 1.77 times higher than in the traditional one.

The essential oil absolute quantitative content was determined for Cambridge and Sterkh regenerated plants, as well as for different periods of collection of intact raw material (spring, autumn). The maximum content of essential oil was noted in the leaves of Cambridge spring vegetation - 1.63% and Cambridge regenerated plants - 1.12%. The content of the essential oil in the plants of releasing and in the aeroponic leaves of Sterkh does not differ much from the amount of essential oil accumulated in the rhizomes with the roots of intact plants, but it is 26% higher in comparison with the leaves of intact plants.

The quantitative content of biologically active substances (BAS) is one of the main indicators of the medicinal raw material quality [21]. We have not found a decrease in the content of secondary metabolites (flavonoids, tannins, coumarins and triterpene glycosides) in the raw material of Siberian iris when grown under controlled conditions. In this regard, biotechnological raw material *I. sibirica* can be a natural source of these substances for humans (tables 2, 3).

**Table 2.** The quantitative content of the groups of biologically active substances *I. sibirica* of Cambridge variety, % by a.w.

| BAS | Biotechnological raw materials regenerated plants | Traditional raw materials | leaves | rhizome with roots |
|-----|-------------------------------------------------|---------------------------|--------|-------------------|
|     | spring                                          | autumn                    | spring | autumn            |

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We have enhanced the regenerative effect of adenine sulfate 100 mg/L, as well as the alternation of low and high concentrations of cytokinin μM. The introduction of cytokinins into the micropropagation proper, the necessary for the formation of the largest number of adventitious shoots of optimal length at the stage of rooting.

4. Conclusion
For the formation of the largest number of adventitious shoots of optimal length at the stage of micropropagation proper, the necessary 6-BA content in the culture media for *I. sibirica* was 2.5-5.0 μM. The introduction of cytokinins into the growth medium together with auxins, L-glutamine and adenine sulfate 100 mg/L, as well as the alternation of low and high concentrations of cytokinin enhanced the regenerative effect of 6-BA.

In year-round cultivation of regenerated plants under aeroponics conditions, the amount of biomass of plant raw material *I. sibirica* was approximately 31.2 kg/m² in wet weight for one year.

It has been established that intact plants and regenerant plants of *I. sibirica*, obtained on the basis of the developed biotechnology, had the identical group composition of biologically active substances. We have not found a decrease in the content of secondary metabolites (flavonoids, tannins, coumarins and triterpene glycosides) in the raw material of Siberian iris when grown under controlled conditions.

| Table 3. The quantitative content of groups of biologically active substances *I. sibirica* of Sterkh variety, % by a.w. |
|---------------------------------------------------------------|
| **BAS** | **Biotechnological raw materials** | **Traditional raw materials** |
|        | regenerant plants | hydroponic leaves | leaves | rhizome with roots |
| Flavonoids | 5.75±0.08 | 4.80±0.09 | 4.87±0.05 | 2.32±0.07 |
| Tannins | 1.80±0.05 | 2.41±0.08 | 2.69±0.07 | 3.62±0.07 |
| Coumarins | 0.96±0.07 | 0.10±0.05 | 0.82±0.06 | 0.40±0.04 |
| Triterpene glycosides | 0.83±0.05 | 2.34±0.06 | 1.79±0.07 | 3.04±0.06 |

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