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Approaches to Conservation of Biodiversity of Rare and Endangered Medicinal Plants on the Basis of Microclonal Multiplication With Optimization of Parameters by Methods of Neural Network Modeling.

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ABSTRACT

This article suggests an approach that provides the possibility of obtaining the necessary amount of planting material related to rare and endangered medicinal species, which is based on the use of microclonal propagation technology of plants with optimization of parameters of its stages based on neural network modeling. One of the most important and costly stages of microclonal propagation, the stage of sterilization, is closely studied. The quality of the resulting material largely depends on this stage. Laboratory investigations and analysis of the results of obtaining aseptic viable sprouts with introduction of medicinal plants into culture on the example of representatives of the Labiatae Juss (Lamiaceae) family were carried out. A method is proposed for determining the optimal parameters of the sterilization process (the type of sterilizing agent, its concentration, and the time of treatment of plant explants with a sterilizing agent), and evaluating its results (the number of sterile explants and the number of viable seedlings) is based on the application of an artificial neural network apparatus. The results of the development of neural network models for estimating and predicting the results of sterilization, realizing the proposed method and reflecting the cause-effect relationships between the parameters of this stage are presented. The models of two types are constructed: a multilayer perceptron and a network with radial basis functions (RBF-network). A comparative analysis of the models showed that the smallest mean-square error of learning and generalization was provided by the RBF network. Simulated experiments were performed using the selected model in the form of an RBF network, where the type of sterilizing agent and the concentration and processing time of plant explants varied in steps of 0.01% (from 0 to 100%) and 1 minute (from 0 to 30 minutes) respectively, which cannot be done under laboratory conditions. Optimal parameters of the sterilization stage (the type of sterilizing agent, its concentration, and the time of processing of plant explants) are optimized, ensuring the highest percentage of sterile viable seedlings.

Keywords: biodiversity, medicinal plants, microclonal reproduction, sterilization of plant explants, optimization, artificial neural networks.

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INTRODUCTION

In modern socio-economic and environmental conditions, the problem of conserving the biodiversity of rare and endangered plants is extremely urgent because of the rapid decrease in the distribution areas of many species due to active human economic activity [1]. Thus, from 1400-1500 species of plants common in the Belgorod region, which is the region of Russia with an actively developing economy), more than 30 plant species are included in the Red Book of the country, more than 200 species require effective protection as rare and disappearing at the regional level [2, 3]. Moreover, many of them are medicinal plants, which are valuable raw materials for pharmacy, as well as perfumery, cosmetics, and other industries [2,3]. If now there is no solution to the problem of preserving the biodiversity of such plants, then environmental problems will inevitably turn into socio-economic ones.

One of the most promising ways of obtaining planting material of rare and endangered species of medicinal plants is the technology of clonal micropropagation of plants, based on the use of the method of cell and tissue culture. Today, this approach is actively developing and has a wide range of practical applications [4-12]. Isolated plant cells and callus tissues grown under in vitro conditions are capable of producing valuable biologically active substances: alkaloids, steroids, glycosides, hormones, essential oils, flavonoids, etc. [9-12]. The main advantages of cellular technologies include (a) the possibility of using secondary plant metabolites for synthesis, which are rare and disappearing from the territory of the region, and (b) obtaining environmentally friendly products all year round. The productivity of the cultured cells as a result of cell selection can significantly exceed the productivity of whole plants and yield a greater yield of synthesized a biologically active substance [10-12].

PROBLEM STATEMENT AND PURPOSE OF THE RESEARCH

The process of microclonal multiplication is multi-stage and includes such steps as (a) selection and preparation of plant explants, which can act different organs, tissues, cells, and seeds of plants; (b) the process of sterilizing plant explants; (c) introducing them into the culture in vitro; (d) production and cultivation of aseptic plants on a synthetic nutrient medium; (e) microclonal reproduction of regenerating plants; (f) adaptation of microclones to soil conditions [7-10].

It should be noted that the necessary optimization of the parameters at each stage is a long, time-consuming, and costly process that requires setting and repeating a significant number of laboratory experiments. At the same time, a large expenditure of expensive components is included in all nutrient media, as well as considerable time and human resources (for collecting materials for experiments, ensuring sterile instruments before each series of experiments, dishes, etc.). The situation of analyzing the results of experiments and identifying the optimal parameters of the stages of microclonal propagation is complicated by the need to work with a large volume of heterogeneous, sometimes poorly structured information.

The foregoing determines the prospects for using modern information technology tools and modeling methods, including methods of data mining, which are now successfully used in forecasting and managing processes and objects in various fields, including when solving various tasks in biotechnology [13-20]. Specialized models will allow to identify cause-effect relationships both between (a) the parameters of processes within individual stages of microclonal propagation and (b) between parameters that are outputs and inputs of various stages of this technology.

It should be noted that the sterilization of plant explants is one of the main and, at the same time, laborious stages of microclonal propagation, the results of which are crucial for obtaining high-quality materials [8-10].

The empirical studies have shown that the result of obtaining a sterile culture that would have a good growth directly depends on the correct choice of parameters such as the type of sterilizing agent, its concentration, and the processing time of the sterilizing agent of plant explants [8-10].

The purpose of this research was to develop an approach to the conservation of biodiversity of rare and endangered medicinal plants based on the microclonal multiplication technology with optimization of parameters by methods of neural network modeling using the example of the stage of sterilization of plant explants.

To achieve this goal, the following tasks were accomplished:

- Conducting a series of laboratory experiments at the sterilization stage when introducing *in vitro* the representatives of rare and endangered medicinal plants growing on the territory of the Belgorod region into the culture;
- Analyzing experimental results;
- Developing models for estimating and predicting the results of the sterilization step, using models of artificial neural networks, with the determination of optimal parameters that ensure the maximum percentage of viable aseptic seedlings;
- Analyzing the constructed models;
- Carrying out computer experiments to optimize the parameters of the sterilization stage, as well as evaluating the results.

MATERISLS AND METHODS

Materials

For research purposes, plants of the family of *Labiatae Juss*. and *Lamiaceae Lindl*. were chosen, which were growing both in the wild state (*Prunella grandiflora* (L.) Sholl. and *Hyssopus cretaceus* Dubjan.), as well as other species (*Salvia sclarea* L.). The species *P. grandiflora* and *H. cretaceus* are included in the regional Red Book of the Belgorod Region [2], and *H. cretaceus* is also protected at the federal level [3]. These representatives contain valuable biologically active substances, aromatic essential oils in their composition, which are widely used in cosmetology, medicine, and pharmacy [21].

Prunella grandiflora (L.) Sholl. is a perennial herbaceous plant, which has a third category in the Red Book of the Belgorod Region [2]. It is a promising source of many secondary metabolites; being a medicinal raw material, it is used as a hemostatic, antimicrobial, antipyretic, wound-healing, anti-inflammatory, tonic, expectorant, and anticomplementary agent [22].

Hyssopus cretaceus Dubj. is a relict plant, preserved from the time of the Tertiary period. *Hyssopus cretaceus* often settles on precipices, steep slopes, mainly southern exposure. Due to its influence, soil is formed due to the destruction of the parent rock [23]. In the Red Book of the Belgorod Region, the species has the category VI and the status of a particularly valuable species. *H. cretaceus* is not only an essential oil and ornamental plant, but it is also a valuable species necessary for phyto-melioration of the cretaceous slopes [23].

Salvia sclarea L. is a biennial plant, a promising source of many secondary metabolites. The chemical composition of *S. sclarea* includes such substances as: flavonoids (apigenin, rutin, quercetin, and cinaroside), phenolic compounds (dihydroquercetin, rutin, coumarin, umbelliferone, gallic, chicory and ferulic acids), phenol carboxylic acids (chlorogenic, gallic, coffee, and ferulic) [24, 25]. Many of these substances cause an increased interest of pharmacologists due to their wide spectrum of biological activity.

Laboratory experiments

The scientific research was carried out according to the schedule of the technical task of the state task No. 40.5084.2017/БЧ “Investigation of methods and modeling of processes in biotechnology and plant systematics”.

For introduction into the culture *in vitro* as plant explants, the seeds of the following plants were used: *P. grandiflora, H. cretaceus, S. sclarea*. Seed collection takes place after the flowering phase from May 2017, taking into account the fruiting time of each species. Collection of plant material is carried out in dry sunny weather during the full ripening of seeds. The tops of the stems are cut off with fruiting inflorescences, placed in bags, and labeled. Cameral processing includes: drying the collected material in the laboratory, separating the seeds from the remains of the dry parts of the inflorescences (cups, coronets, trichomes), placing them in separate paper bags, and indicating the date and place of collection on the label.

Manipulations on introduction into culture in vitro were carried out in the Laboratory of Innovative Methods of Research of Plant Objects at the Department of Biotechnology and Microbiology of the Belgorod

National Research University, with observance of the standard rules of aseptic conditions in laminar boxes of microbiological safety (Lamsystems/II Class/A2 Type).

At the stage of sterilization, plant explants were treated with five different sterilizing agents: Lysoformin 3000, Biocide, Whiteness (5-15%), Chloramine B, Silver nitrate. For each type, the concentration of the sterilizer (%) and the processing time (min) were changed.

Sterilization of nutrient media, materials, instruments, and equipment was carried out according to the methods adopted in the work on cell and tissue culture [26, 27].

To assess the effect of aseptic solutions, plant explants were placed on the Murasige-Skoga nutrient medium without hormones [28]. Next, the seeds were cultivated in a thermostat at a temperature of 22-24°C. For each mode, 10 seeds of each species were used, taking into account the specific time and concentration of the sterilizing agent. The experiment was carried out in 3-fold replicates. The effect of the sterilization regime was evaluated by the number of sterile and viable explants.

Modeling methods

To develop an adequate model for estimating and predicting the sterilization process, reflecting the cause-effect relationship between the parameters and the result of the sterilization stage, the artificial neural network (ANN) device was chosen. Previously, this method was repeatedly used by the authors to construct models for estimating and predicting the state of natural and natural-technical objects in cases when it is impossible to analytically describe the chemical-physical and biological-chemical processes underlying their functioning (see, for example, [29- 31]).

When building neural network models, it is necessary to solve the following tasks: (a) collecting data for training; (b) preparing the data; (c) choosing a network topology; (d) experimentally selecting network characteristics; (e) network training; (f) checking the adequacy of training.

It should be noted that the areas of ANN application with different topologies may overlap, and their different paradigms can be used to solve the same problem. Which of the topologies works best can only be determined by an experiment.

In our case, the construction of the model is reduced to the solution of the approximation problem, i.e. to obtain a continuous function with respect to a finite number of discrete values. To solve this problem, the ANN of the following topologies is most often used: the multilayer perceptron and networks with radial-basis function (RBF-network). To select the best model, their estimate being based on the mean square error obtained at the training stage was used.

The processes of constructing and researching models, as well as doing simulation experiments, were carried out with the help of a package of applied programs and functions of the Neural Network Toolbox of the computer system MATLAB.

RESULTS AND DISCUSSION

1. Results of laboratory studies on introduction into the culture in vitro at the stage of sterilization of plant explants

As a result of laboratory experiments, preliminary data on the effect of the most effective sterilizing agent, its concentration, and time were obtained.

Figure 1 shows the result of a comparative analysis of the effect of sterilizing agents on the number of sterile explants and viable seedlings for an example of *H. cretaceus* species (treatment time is 20 minutes, the concentration values are shown in the diagram).

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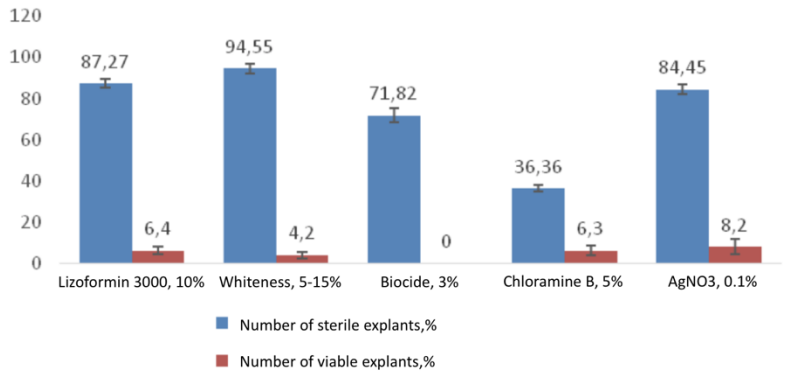

Fig 1: Influence of sterilizing agents on the number of sterile explants and viable seedlings of the species H. cretaceous

In the laboratory experiment, it was found that the optimal sterilization regime for the *H. cretaceus* species was the use of 0.1% silver nitrate solution for 20 minutes, since this method of sterilization yielded the most optimal ratio of the number of sterile plant explants (84.45%) to the number of viable seeds (8.2%). It is also possible to use 10% solution of "Lizoformin 3000" as a sterilizing agent. The use of a 3% solution of Biocide and a 5% solution of Chloramine B is not advisable, since a low percentage of viable seeds and a small percentage of sterile explants were achieved.

The results of the action of sterilizing agents on the number of sterile explants and viable sprouts of *P. grandiflora* are shown in Figure 2.

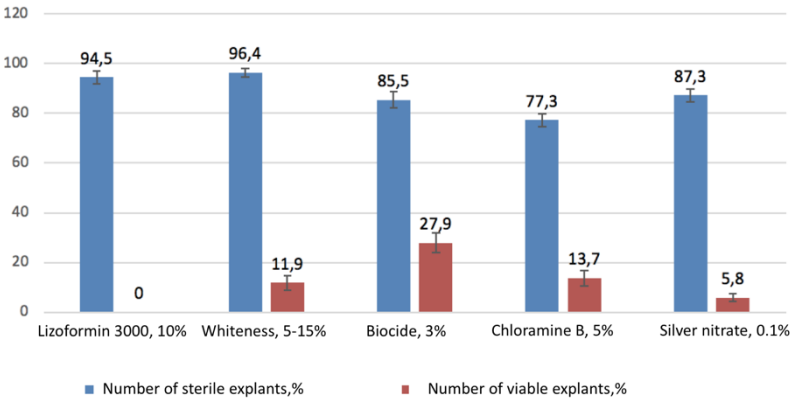

Fig 2. Influence of sterilizing agents on the number of sterile explants and viable sprouts of the P. grandiflora species

Figure 2 shows that the optimal sterilizing agent for the *P. grandiflora* species is a 5-15% Whiteness solution with a sterilization time of 20 minutes, since an optimal ratio of sterile seed percentage (96.36%) to the percentage of viable (11.83%) was achieved with this method of sterilization. The application of "Biocide" and 5% solution of "Chloramine B" is also possible. The use of a 10% solution of "Lysoformin 3000" and 0.1% solution of silver nitrate for sterilization is not rational, so the percentage of viable seeds is close to zero.

For the *S. sclarea* species, the data presented in Figure 3 were obtained.

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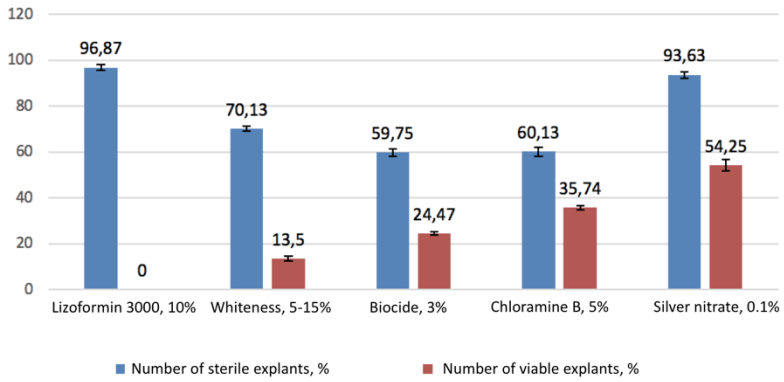

Fig 3: Influence of sterilizing agents on the number of sterile explants and viable sprouts of the S. sclarea **species.**

The optimal sterilization mode of the S. sclarea species is the use of 0.1% silver nitrate for 20 minutes. The best ratio of sterile plant explants (93.63%) to the number of viable seeds (54.25%) was obtained in this case. The use of sterilization regimes using other sterilizing agents is not advisable because of their low effectiveness.

As can be seen from the diagrams, the sterilization parameters differently determine the results of this stage: the quantitative characteristics of seed sterility are not sufficient to ensure a high percentage of aseptic viable germinated explants.

2. Constructing optimization models and evaluating the sterilization stage based on the apparatus of artificial neural networks

In order to obtain sufficient initial data for the construction and training of models in the form of ANN, an additional series of laboratory experiments on the sterilization of plant seeds was carried out. As a result, 135 experiments were performed for each plant species: 45 with each of the sterilizing agents at different concentrations of the agent and the time of its effect on the seeds. The results were divided into a training (100 experiments) and a test (35 experiments) of the sample.

To construct a model that provides the ability to evaluate and predict the results of the sterilization steps of plant explants with the choice of optimal parameters, we used two ANN paradigms, namely the multilayer perceptron and the RBF-network.

A model was constructed with the topology of the multilayer perceptron with one hidden layer. To determine the optimal number of neurons of the hidden layer and their activation functions, a series of computer experiments was performed: the number of neurons in the hidden layer with a linear and sigmoidal activation function changed. The root-mean-square error in learning is п = 0.12÷0.2. The best result is obtained for ANN with 12 neurons in the hidden layer and a sigmoid activation function. The structure of this network, being performed in the MATLAB computer system, is shown in Figure 4.

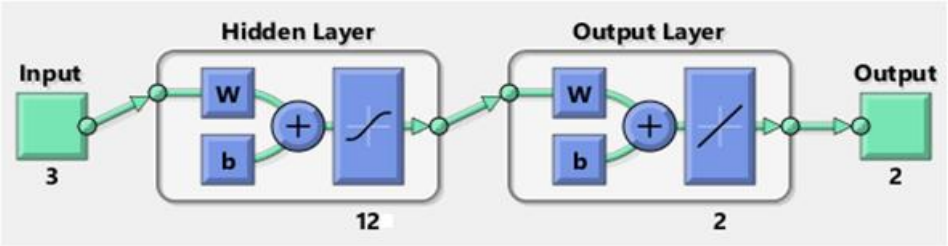

Fig 4: The structure of the obtained multilayer perceptron being performed in the MATLAB system.

Also, an RBF network with an allowable mean square error RBF = 0.1 was performed. The structure of the network being constructed in the MATLAB system is shown in Figure 5.

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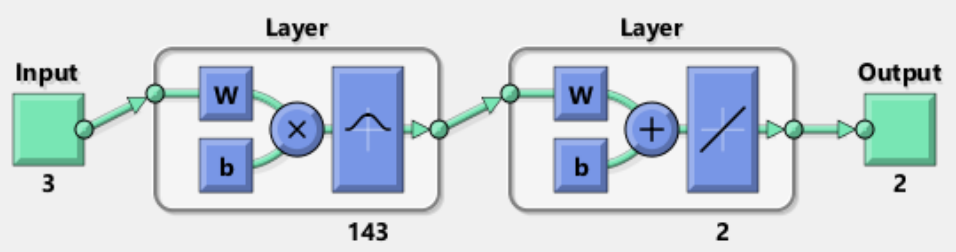

Fig 5: Structure of the RBF-network for simulating sterilization of plant explants being implemented in the MATLAB system.

A comparative analysis of mean square errors shows that ?n<?RBF, i.e. a model based on the RBF-network gives a better level of approximation of the original data.

3. Carrying out simulation experiments

With the help of the constructed models, simulation experiments were carried out to identify the optimal conditions for the sterilization of plant explants: the type of sterilizing agent, its concentration, and the time of sterilization.

In the framework of the experiments, all possible combinations of these parameters were submitted to the input of the models with a change in the concentration of the sterilizing agent from 0 to 100% with steps of 0.01 and the sterilization time from 0 to 30 minutes with steps of 1. On the basis of the data obtained, the optimal sterilization parameters were selected, which corresponded to the highest percentages of sterile seeds and viable seedlings. The results obtained are shown in Table 1.

Table 1: Optimal parameters of sterilization being obtained on the basis of computer experiment.

Plant	Sterilizing agent	Time, min	Concentration, %	Number of viable germinated explants, %
H. cretaceus	Lysoformin 3000	9	7,12	9,1
P. grandiflora	Whiteness (5-15%)	16	77,1	15,1
S. sclarea	Silver nitrate	18	0,12	58,3

The verification laboratory experiments confirmed the good quality of training and acceptable predictive capabilities of the constructed model.

CONCLUSION

Based on the results of the studies, the following conclusions can be made.

In the course of laboratory experiments on the introduction of rare and endangered medicinal plant species into the culture in vitro in the process of their microclonal multiplication (on the example of the representatives of the family Lamiaceae: *H. Cretaceous, P. Grandiflora, S. Sclarea*; the sterilization stage), it was established that the number of viable and the number of sterile shoots were different depending on the effect of the type of sterilizer, its concentration, and time.

To ensure effective simulation experiments to evaluate and predict the results of the sterilization step with the determination of the optimal concentration values of the sterilizing agent and the time of its effect on the explants of the studied plants, the process of constructing and studying models in the form of ANN was carried out. A series of additional laboratory experiments was carried out, and two samples were generated: training (100 experiments) and testing (35 experiments). Two types of ANN are constructed and investigated: (a) the multilayer perceptron with one hidden layer and (b) the RBF-network with an allowable mean square

error σRBF= 0.1. ANN inputs: type, concentration of sterilizing agent, treatment time. ANN outputs: the percentage of the sterile seedlings received and the percentage of viable seedlings.

A comparative analysis of the constructed models shows that the RBF-network gives a smaller learning error due to the presence of a hidden layer of neurons with radial-basis activation functions that can track the slightest changes in the levels of the original data.

With the help of this model, simulation experiments were performed, on the basis of which the optimal sterilization parameters corresponding to the highest percentages of sterile and viable seeds for the plant species tested were selected: *H. Cretaceous* – a sterilizing agent Lizoformin 3000 with a concentration of 7.12% and a sterilization time of 9 minutes; *P. Grandiflora* – Whiteness (5-15%) with a concentration of 77.1% and a sterilization time of 16 minutes; *S. sclarea* – Silver nitrate with a concentration of 0.12% and a sterilization time of 18 minutes. The number of viable sterile shoots was 15.9%, 32.1%, and 55.3%, respectively.

The results of testing laboratory experiments confirm the obtained data of a computer experiment on choosing the type of sterilizer, its concentration, and the time of exposure.

Thus, the developed model can be successfully applied for carrying out simulation experiments on the optimal selection of sterilization parameters with the provision of the output of the maximum percentage of viable explants.

The proposed method can be extended to any plant species, and the developed model can be used in the evaluation and prediction of the results of the sterilization process carried out under microclonal propagation of various plants belonging to the family under consideration.

The application of the approach described in the paper will allow to reduce the time and financial expenses for the purchase of nutrient medium components when selecting an effective sterilizing agent in the process of microclonal propagation of plants at the stage of introduction into the culture *in vitro*, to reduce the human resources in the preparation and setting of the experiment, and to reduce the risks associated with human factors.

The use of the proposed approach will contribute to the conservation of biodiversity of rare and endangered species of medicinal plants by obtaining the necessary quantity of the quality planting material.

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