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Research Article

Optimization of sterilization process of plant explants when introduced into culture in vitro based on artificial neural networks (by the example of the family Labiatae Juss. (Lamiaceae))

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ABSTRACT

This article:

* analyzes the results of experiments at the stage of plant explants sterilization when introducing medicinal plants into the culture in vitro (for example, representatives of the family Labiatae Juss. (Lamiaceae) growing in the Belgorod Region of the Russian Federation);
* suggests a method for determining the optimal parameters of the sterilization process and evaluating its results based on the application of neural network approach;
* presents the models for estimating and predicting the outcome of sterilization which implement the proposed method with two neural paradigms: a multilayer perceptron and an artificial neural network that uses radial basis functions (RBFN).

A comparative analysis of the models showed that the lowest mean squared error in training was obtained using the RBFN. An imitation experiment using the developed model was carried out, the optimal parameters of the sterilization stage (the type of sterilizing agent, its concentration and the time of plant explants processing) were predicted, providing the highest percentage of sterile explants and the highest percentage of aseptic viable seedlings. Laboratory experiments confirmed the adequacy of the developed models.

Keywords: micropropagation, plant explants sterilization, cell and tissue culture, optimization, modeling, artificial neural networks, simulation experiment

1. INTRODUCTION

At present, due to the economic activity of man, there is a rapid decrease in the distribution areas of many plant species. The problem of conservation of plant biodiversity, especially of rare, endangered and medicinal species becomes extremely urgent [1]. One of the effective solutions to this issue is the use of micropropagation technology, which makes it possible to preserve valuable, rare, endangered and medicinal plants, creating banks and collections in ‘in vitro’ conditions [2-8]. Plants from such banks can be used to restore their natural populations or for cultivation in special areas for further use in agriculture or pharmacy. Micropropagation is a promising advanced method of vegetative propagation, based on the method of cell and tissue culture, and it allows healthier planting material to recover from viral,

bacterial and fungal infections, while saving the areas occupied by parent plants [4-8]. Another advantage here is the possibility of carrying out propagation work throughout the year, as well as obtaining planting material the reproduction of which is hampered by conventional methods. Applying this method allows preserving unique genotypes of different populations of plant objects, creating genetic banks in 'in vitro' conditions and cryobanks of meristem cultures of these species, which can be stored for a long time. These cryobanks allow (in case of loss of plant organisms in populations) growing and restoring them from cryopreserved apical meristems [9, 10].

The process of micropropagation is multi-stage and includes selecting and preparing plant explants (different organs, tissues, cells and seeds); sterilizing plant explants; introducing them into culture in vitro; producing cultivating aseptic plants in a synthetic nutrient medium; micropropagating regenerating plants; and adapting microclones to soil conditions [2-8]. It should be noted that parameters optimization at each stage is a time-consuming and costly process that requires setting and repeating a significant number of laboratory experiments. At the same time, there is a large expenditure of expensive components of all nutrient media, as well as of significant time and human resources used to provide (before each series of experiments) sterile instruments, dishes, nutrient media and indoor conditions. Identifying the optimal parameters of these stages is also complicated by the need to work with a large amount of heterogeneous, sometimes poorly structured information.

All of the above said determines the prospects of applying modern information technology tools and modeling methods, including data mining, which are now successfully used in forecasting and managing processes and objects in various fields, including biotechnology [11-18].

It should be noted that one of the basic and at the same time labor-intensive stages of micropropagation, the results of which are crucial for obtaining high-quality material, is the sterilization stage, individual for each plant species. The result of aseptic viable seedlings formation directly depends on the correct choice of the effective sterilizing agent, as well as of its concentration and processing time of plant explants.

The purpose of this research was to conduct and analyze experimental studies at the sterilization stage introducing into the culture in vitro the representatives of the family Labiatae which are rare and disappearing medicinal plants, and to optimize sterilization parameters by methods of mathematical estimation and prediction based on artificial neural networks.

2. MATERIALS AND METHODS

2.1 Materials

The objects of research were plants of the family Lamiaceae (Labiatae Juss., Lamiaceae Lindl.) growing in the Belgorod Region both in the wild – Prunella grandiflora (L.) Sholl.and Hyssopus cretaceusDubjan., and as introduced species – clary sage, Salvia sclarea (L). It should be noted that P. grandiflora and H. cretaceus are included in the Red Data Book of the Belgorod Region [19], and H. cretaceus is also protected at the federal level [20]. These representatives contain valuable biologically active substances and aromatic essential oils, which are widely used in cosmetology, medicine and pharmacy.

Prunella grandiflora (L.)Sholl.is a perennial herbaceous plant 10-40 cm tall with upright stems 15-60 cm tall. Leaves are long-petiolate, oblong or ovate-lanceolate. The calyx is cherry-coloured. Propagated by seeds and vegetatively.Grows on steppe meadows, dry slopes, among bushes and in light forests [21]. In the Red Data Book of the Belgorod Region (2005) the species has Category III [20]. It is a promising source of many secondary metabolites. The terrestrial part of the plant is rich in triterpenoids (ursolic and oleanolic acids), phenolcarboxylic acids (rosmarinic acid up to 3%, caffeic, chlorogenic, neochlorogenic, 4-caffeic acids), flavonoids (hyperoside, quercetin, kaempferol, rutin, quercetin 3-glucoside). Pr. grandiflora as medicinal raw material is used as hemostatic, antimicrobial, antipyretic, wound-healing, anti-inflammatory, tonic, expectorant and anti-complementary agent [22].

Hyssopus cretaceus Dubj.is a half-shrub 20 to 50 cm tall. Rounded gray-green stems, slightly tomentose on top from above, with an almost imperceptible middle vein and narrow leaves. Three to seven blue, very fragrant flowers in the axils form a loose, lengthened, one-sided, racemose inflorescence. Blooms from May to September. Fruit, in the form of a nut, ripens in late August - early September. *H. cretaceus* is a relict plant preserved since the time of the Tertiary Period. Hyssop often settles on precipices, steep slopes, mainly southern exposure. Due to its influence, soil is formed because of the destruction of the parent material [23]. In the Red Data Book of the Belgorod Region the species has Category VI and the status of a particularly valuable species. *H. cretaceus* is not only an essential oilseed and ornamental plant, but also a valuable species necessary for phyto-melioration of the cretaceous slopes [24].

Salvia sclarea L.is a herbaceous biennial plant with powerful upright or slightly curved stems in the lower nodes. Blooms from early June to late July, sometimes until August.Large, racemose inflorescences, 15 to 40 cm in length, consisting of flower-bearing axes of various orders. Fruits are nuts, ripening in August-September, ovoid or ellipsoidal, of chestnut or brown color, smooth-surfaced, when wetted, strongly mucous [25]. This species is a promising source of many secondary metabolites. The chemical composition of S. sclarea includes such substances as: flavonoids (apigenin, rutin, quercetin and cynaroside), phenolic compounds (dihydroquercetin, rutin, coumarin, umbelliferone, gallic, cichoric and ferulic acids), phenol carboxylic acids (chlorogenic, caffeic and ferulic acids) [26]. Many of these substances raise an interest of pharmacologists due to their wide spectrum of biological activity. Thus, umbelliferone has bactericidal, fungicidal and anti-inflammatory impact. Coumarins possess fungistatic activity. Ferulic acid has a powerful hepatoprotective effect and is a strong antioxidant. Caffeic acid has antioxidant and hypoglycemic effects. Cichoric acid acts as an inhibitor of HIV-type I integrase [27].

2.2 Laboratory methods of research

For introduction into culture in vitro, plant seeds of P. grandiflora, H. cretaceus, S. sclarea were used as plant explants. Seeds were harvested in the summer period in the territory of the Belgorod Region and the botanical garden of the National Research University BelSU. All work on the collection, drying and storage of plant material was carried out according to generally accepted procedures [28, 29].

Manipulations on introduction into culture in vitro were carried out in the laboratory of 'Innovative methods of research of plant objects' of the department of biotechnology and microbiology of the NRU BelSU with observance of the standard aseptic conditions rules in laminar boxes of microbiological safety Lamsystems class II type A2.

At the stage of sterilization, plant explants were treated with five different sterilizing agents: lysoformin 3000, biocide, liquid bleach (5-15%), chloramine B, and silver nitrate. The following values of the parameters varied: the concentration of the sterilizer (c,%) and the treatment time (t, min).

Sterilization of nutrient media, materials, instruments and equipment was carried out according to the techniques adopted in the work on cell and tissue culture [30, 31]. After sterilizing, plant explants were washed with sterile distilled water three times for 15 minutes.

To evaluate the effect of aseptic solutions, plant explants were placed in the Murashige-Skoog nutrient medium without hormones [32]. 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 time and concentration of the sterilizing agent. The experiment was carried out three times. The effect of the sterilization mode was evaluated by the number of sterile and viable explants.

2.3 Modeling methods

To develop an adequate model of optimization and evaluation of 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 that underlie their functioning (see, for example, [33- 35]).

When building neural network models based on ANN, the following tasks should be solved: data collection for training; data preparation; choice of network topology; experimental selection of network characteristics; network training; checking the training adequacy.

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 experiment. In the present case, the model construction is reduced to solving the approximation problem, that is, to obtaining 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: multilayer perceptron and networks with radial-basis function (RBFN). The processes of constructing and researching models, as well as simulation experiments, were carried out using a package of applied programs and functions of the Neural Network Toolbox of the MATLAB computer system.

3. RESULTS AND DISCUSSION

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

As a result of laboratory experiments, preliminary data on the most effective sterilizing agent, its concentration and time were obtained. The average results of the sterilization mode impact on the preparation of sterile explants of P. grandiflora, H. cretaceus, S. sclarea with an assessment of their viability are presented in Table 1.

Table 1 - Modes of sterilization and evaluation of their effect by the number of sterile and viable plant explants of P. grandiflora, H. cretaceus, S. sclarea.

			Number of obtained sterile explants, % / number of viable explant species (%)		
Sterilizing agent	Time, min	Concentration, %	*H. cretaceus*	*P. grandiflora*	*S. sclarea*
Lysoformin 3000	10	5	64,1±1,2/ 13±1,5	77±1,8/ 12,6±2,1	79,3±2,1/ 9,92±1,4
		10	72,2±1,3/ 11,1±0,8	81,8±3,4/ 7,9 ±1,2	82,8±1,2/ 7,2±0,7
		15	80,0±2,1/ 10,1±1,7	86,9±2,4/ 5,7±1,1	87,9±1,6/ 5,1±2,0
	20	5	82,6±2,1/ 7,3±1,2	91,9±2,1/ 0 ±0	92,3±2,6/ 2,1±0,9
		10	87,6±2,6/ 6,4±2,7	94,5±2,5/ 0 ±0	96,8±1,3/ 0 ±0
		15	89,9±2,4/ 3,7±1,1	98,3±2,1/ 0 ±0	97,9±2,1/ 0 ±0
	30	5	91,9±2,1/ 0 ±0	97,2±2,5/ 0 ±0	98,0±1,9/ 0 ±0
		10	95,5±3,0/ 0 ±0	99,1±0,8/ 0 ±0	98,5±1,2/ 0 ±0
		15	98,0±1,7/ 0 ±0	98,9±0,5/ 0 ±0	98,8±1,1/ 0 ±0
Biocide	10	1	6,4±3,8/ 0 ±0	12,4±3,1/ 8,8±3,9	1,1±1,3/ 1±0,8
		3	11,4±3,8/ 0 ±0	15,4±3,1/ 9,8±3,9	2,1±1,3/ 2,7±0,8
		5	12±3,8/ 0 ±0	17,4±3,1/ 10,8±3,9	3,1±1,3/ 3,7±0,8
	20	1	56,4±3,8/ 0 ±0	85,4±3,1/ 11,8±3,9	55,1±1,3/ 33,7±0,8
		3	76,4±3,8/ 0 ±0	85,4±3,1/ 11,8±3,9	61,1±1,3/ 35,7±0,8
		5	78,4±3,8/ 0 ±0	86,4±3,1/ 12,8±3,9	65,1±1,3/ 36,7±0,8
	30	1	79,4±3,8/ 0 ±0	85,4±3,1/ 11,8±3,9	45,1±1,3/ 26,7±0,8

		3	80,4±3,8/ 0 ±0	85,4±3,1/ 11,8±3,9	75,1±1,3/ 38,7±0,8
		5	81,4±3,8/ 0 ±0	86,4±3,1/ 12,8±3,9	77,1±1,3/ 39,7±0,8
Liquid bleach (5-15%)	10	50	54,7±1,8/ 6,8±1,2	65,4±1,3/ 30,8±2,7	70,2±1,2 3,5±1,0
		100	74,7±1,9/ 6,2±1,5	75,4±1,5/ 29,9±2,7	50,2±1,6 6,5±0,2
	20	50	80,7±1,2/ 5,2±1,9	80,4±1,5/ 28,8±2,1	68,2±1,3 12,5±0,8
		100	94,7±1,8/ 4,2±1,9	85,4±1,9/ 27,8±2,6	70,2±1,1 13,5±0,9
	30	50	96,7±1,9/ 0±0	90,4±1,6/ 17,8±2,9	72,2±1,1 12,1±0,9
		100	98,7±1,9/ 0±0	95,4±1,8/ 7,8±2,3	75,2±1,1 10,5±0,9
Chloramine B	10	1	3,4±1,6/ 0 ±0	10,3±2,3/ 1,7±12	9,7±1,5/ 0 ±0
		5	6,4±1,6/ 0 ±0	13,3±2,3/ 3,7±3,2	12,7±1,5/ 4,5±0,7
		10	16,4±1,6/ 0 ±0	20,3±2,3/ 5,7±3,2	13,7±1,5/ 4,6±0,7
	20	1	26,4±1,6/ 5,3±1,3	70,3±2,3/ 14,7±3,2	55,7±1,5/ 20,5±0,7
		5	36,4±1,6/ 6,3±1,3	77,3±2,3/ 13,7±3,2	59,7±1,5/ 24,5±0,7
		10	46,4±1,6/ 3,3±1,3	80±2,3/ 10,7±3,2	60,7±1,5/ 0 ±0
	30	1	50,4±1,6/ 0 ±0	57±2,3/ 9,7±3,2	51,7±1,5/ 22,5±0,7
		5	56,4±1,6/ 0 ±0	87,3±2,3/ 7,7±3,2	59,7±1,5/ 0 ±0
		10	61,4±1,6/ 0 ±0	89,3±2,3/ 5,7±3,2	62,7±1,5/ 0 ±0
Silver nitrate	10	0,05	5,5±2,3/ 0 ±0	3,3±2,4/ 0 ±0	3,6±1,3/ 0 ±0
		0,1	15,5±2,3/ 0 ±0	7,3±2,4/ 0 ±0	13,6±1,3/ 4,2±1,1
	20	0,05	55,5±2,3/ 3,2±2,2	57,3±2,4/ 1,8±1,5	73,6±1,3/ 56,2±1,1
		0,1	84,45±2,3/ 8,2±2,2	87,3±2,4/ 5,8±1,5	93,6±1,3/ 54,2±1,1
	30	0,05	65,5±2,3/ 5,2±2,2	72,3±2,4/ 1,5±1,5	80,6±1,3/ 50,2±1,1
		0,1	84,3±2,3/ 7,2±2,2	89,3±2,4/ 1,8±1,5	95,6±1,3/ 44,2±1,1

As can be seen from the table, the sterilization parameters differently determine the results of this stage: the sterility of the seeds is not sufficient to ensure a high percentage of aseptic viable germinated explants.

3.2 Building optimization models and evaluation of the sterilization stage, based on ANN

In order to obtain a sufficient number of initial data and to make a selection for the construction and training of models in the form of ANN, an additional series of laboratory experiments on plant seeds sterilization was carried out. As a result, 135 experiments were performed for each plant species, 45 with each of the sterilizing agents. The results were divided into the training (100 experiments) and the test (35 experiments) part of the samples.

To construct a model that provides the possibility of estimating and predicting the plant explants sterilization results with the choice of optimal parameters, ANNs of two paradigms were realized: multilayer perceptron and RBF-network.

A model was constructed with the topology of a multilayer perceptron with one hidden layer. To determine the optimal number of hidden layer neurons and their activation functions, experiments were performed; the top 10 results for the training error ($\square_п$) received are shown in Table 2.

Table 2 - Experiments results for the selection of a multilayer perceptron characteristics

Number of neurons in the hidden layer	Activation function of hidden layer neurons	Mean squared error, $\square_п$
9	Linear	0,18
11	Linear	0,14
15	Linear	0,15
17	Linear	0,19
19	Linear	0,2
8	Sigmoid	0,16
12	Sigmoid	0,12
13	Sigmoid	0,14
15	Sigmoid	0,17
21	Sigmoid	0,18

From the results obtained, it can be seen that the best mean squared error corresponds to a network with 12 neurons in the hidden layer and sigmoid activation functions. The structure of this network, constructed in the MATLAB system, is shown in Figure 4.

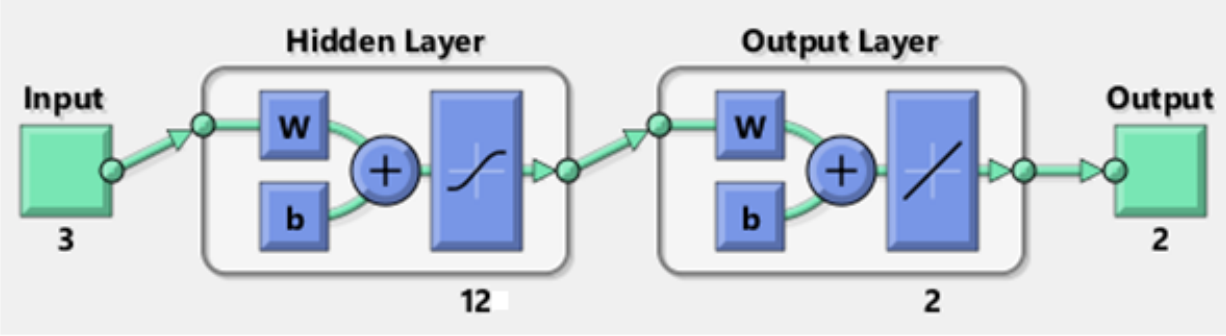

Fig. 4. The structure of the obtained multilayer perceptron, constructed in the MATLAB system.

Also, an RBFN with an allowable mean squared error $\square_{RBF}$ = 0,1was implemented. The structure of the RBFN, constructed in the MATLAB system is shown in Figure 5.

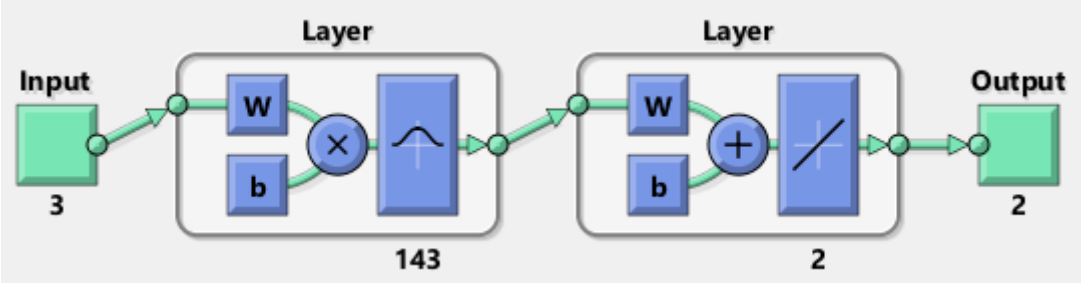

Fig. 5. Structure of the RBFN for simulating plant explants sterilization, constructed in the MATLAB system.

Comparative analysis of mean squared errors has shown that $\square_п$<$\square_{RBF}$, the model based on the RBF network, gives the best level of approximation of the original data.

The presented ANN models can be used to evaluate and predict the optimal conditions for the explants sterilization of the studied plant representatives introduced into the culture ‘in vitro’.

Thus, with the help of the constructed models, simulation experiments were performed to identify the optimal conditions for plant explants sterilization: the type of sterilizing agent, its concentration and the time of sterilization. In the framework of the experiments, all the possible combinations of these parameters were submitted to the models input with a change in the concentration of the sterilizing

agent from 0 to 100% (by 0.01) and the sterilization time from 0 to 30 minutes (by 1), which cannot be achieved in the laboratory conditions.

On the basis of the data obtained, optimal sterilization parameters were chosen that correspond to the highest percentages of sterile seeds and viable seedlings. The results obtained are shown in Table 3.

Table 3 - Optimal sterilization parameters obtained by the computer experiment

Plant type	Sterilizing agent	Time, min	Concentration %	Number of sterile explants, %	Number of viable explants, %
H. cretaceus	Lysoformin 3000	9	7,12	74,2	9,1
P. grandiflora	Liquid bleach (5-15%)	16	77,1	79,3	15,1
S. sclarea	Silver nitrate	18	0,12	94,3	58,3

The test laboratory experiments confirmed the good quality of training and acceptable predictive capabilities of the constructed model. The average error of approximation for the plant species studied was 6.7% (H. cretaceus), 5.1% (P. grandiflora) and 4.8% (S. sclarea).

4. CONCLUSION

Laboratory experiments on the introduction of plants in vitro in the process of their micropropagation (sterilization stage) were carried out. On the example of representatives of the family Lamiaceae – H. cretaceus P. grandiflora and S. sclarea – it was revealed that the number of viable and sterile shoots is in different dependence on the type of sterilizer, its concentration and exposure time

To ensure the implementation of simulation experiments meant to evaluate and predict the results of the sterilization step and to determine the optimal concentration values of the sterilizing agent and the time of its effect on the studied explants, a process was performed to construct and study ANN models (using representatives of the family Lamiaceae). A series of additional laboratory experiments was carried out, and two samples were generated – the training (100 experiments) and the test (35 experiments) sample. Two types of ANN were constructed and investigated: a multilayer perceptron with one hidden layer and an RBFN with an allowable mean squared error ε_{RBF}= 0,1.

Inputs: type and concentration of sterilizing agent, and treatment time; outputs - the percentage of sterile and viable seedlings obtained.

A comparative analysis of the constructed models has shown that the RBFN gives a smaller training 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 carried out, on the basis of which the optimal sterilization parameters corresponding to the highest percentages of sterile and viable seeds for the plant species under study were selected. At the same time, the results of laboratory experiments confirm the obtained data of the computer experiment on the choice of sterilizer, its concentration and the time of exposure. The average approximation error for the plant species studied was 6.7% (H. cretaceus), 5.1% (P. grandiflora) and 4.8% (S. sclarea).

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

The authors suggest that the proposed method can be extended to any plant species, and the developed model can be used in estimating and predicting the results of the sterilization process carried out in micropropagation of various plants belonging to the family under consideration.

The application of the approach described in the paper will allow reducing:

- time and financial expenses for the purchase of nutrient medium components when selecting an effective sterilizing agent in the process of micropropagation of plants at the stage of introduction into culture ‘in vitro’;

- human resources in the preparation and setting of the experiment;
- the risks associated with human factor.

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