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Application of Artificial Neural Network for Modeling and Studying In Vitro Genotype-Independent Shoot Regeneration in Wheat

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Abstract: Optimizing in vitro shoot regeneration conditions in wheat is one of the important steps in successful micropropagation and gene transformation. Various factors such as genotypes, explants, and phytohormones affect in vitro regeneration of wheat, hindering the ability to tailor genotype-independent protocols. Novel computational approaches such as artificial neural networks (ANNs) can facilitate modeling and predicting outcomes of tissue culture experiments and thereby reduce large experimental treatments and combinations. In this study, generalized regression neural network (GRNN) were used to model and forecast in vitro shoot regeneration outcomes of wheat on the basis of 10 factors including genotypes, explants, and different concentrations of 6-benzylaminopurine (BAP), kinetin (Kin), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), zeatin, and $CuSO_4$. In addition, GRNN was linked to a genetic algorithm (GA) to identify an optimized solution for maximum shoot regeneration. Results indicated that GRNN could accurately predict the shoot regeneration frequency in the validation set with a coefficient determination of 0.78. Sensitivity analysis demonstrated that shoot regeneration frequency was more sensitive to variables in the order of 2,4-D > explant > genotype < zeatin < NAA. Results of this study suggest that GRNN-GA can be used as a tool, besides experimental approaches, to develop and optimize in vitro genotype-independent regeneration protocols.

Keywords: plant tissue culture; in vitro regeneration; artificial intelligence model; optimization algorithm; genotype-independent

1. Introduction

Hexaploid (or common) wheat (*Triticum aestivum* L.) is the third largest important cereal after rice and maize and occupies almost one-fifth of the world's cultivated land. The nearly 1% annual genetic gains in five major food crops including wheat has been the result of conventional breeding methodologies [1], which exploited the existing genetic variation. Tester and Langridge [2] indicated that the current genetic gains are insufficient to increase crop production by 70% by 2050 that is needed to feed the increasingly growing demand. To address challenges such as the increasing global population, and global climate change with changes in the intensity and patterns of abiotic and biotic stresses, plant breeders must leverage information from recent advancement in rapid and precise

plant breeding, facilitated by genome-editing. Biotechnological tools such as in vitro culture can be considered as a solution for this aim. Therefore, there is a need to adjust in vitro genotype-independent shoot regeneration in wheat [3].

In vitro plant regeneration is mainly dependent on exogenous and endogenous phytohormones [4]. Genotypes and the type of explant are different in their levels of endogenous phytohormones [4,5]. Indeed, in vitro shoot regeneration is controlled by the exogenous cytokinin and auxin balances, and also by concentrations of endogenous phytohormones [4,5]. The levels of endogenous phytohormones regulate the in vitro explant differentiation and are assumed to be the major variation between various genotypes and explants with different degrees of competence [4]. The interactions among the exogenous and endogenous phytohormones and their effects on in vitro organogenesis need to be extensively studied [6,7], with the goal of understanding phytohormone metabolism signaling and their roles in in vitro organogenesis.

In the context of multiple endogenous and exogenous phytohormones, in vitro organogenesis can be viewed as a multi-variable procedure impacted by different phytohormones such as auxins, cytokinin, and their interaction [8]. Also, in vitro organogenesis consists of non-linear and non-deterministic developmental processes [9]. Conventional computational techniques are inefficient to model non-linearity in complex systems that exist in plant tissue culture [10–12]. Artificial intelligence (AI) models such as artificial neural networks (ANNs) and fuzzy logic have proven to be appropriate approaches for modeling the non-linearity and ill-defined systems in in vitro culture [12]. Examples include the use of the adaptive neuro-fuzzy inference system (ANFIS) in modeling somatic embryogenesis of chrysanthemum [12] and the use of radial basis function (RBF) for modeling of in vitro shoot proliferation in pear rootstock [13].

Among ANN models, the generalized regression neural network (GRNN), proposed by Specht [13], can efficiently solve the non-linear problems due to the simplicity of network structure, the strong non-linear mapping capability, and high fault tolerance and robustness [14]. While the GRNN model has been frequently used in several fields to solve short-term load forecasting [15], pattern recognition [16], the modeling and monitoring of batch processes [17], medicinal chemistry [18], exchange rates forecasting [19], and wind speed forecasting [20], it has been used in plant tissue culture modeling very rarely.

Establishment of working concentrations of tissue culture medium ingredients is a tedious task and requires execution of complex experimental designs with numerous independent factors. This research investigated whether AI can predict the outcomes of shoot regeneration based on influencing factors stably and accurately. We further elucidated whether a genetic algorithm (GA) can optimize a solution for wheat shoot regeneration. This study shows high stability and accuracy of using the GRNN in modeling in vitro shoot regeneration of wheat. However, the weakness of using ANNs such as GRNN is that it is hard to obtain an optimized solution. Jamshidi, et al. [21] used GA to optimize nutrition for pear rootstocks tissue culture media formulation. Also, Hesami, et al. [22] applied the non-dominated sorting genetic algorithm-II (NSGA-II) to optimize medium composition for shoot proliferation of chrysanthemum. However, most studies selected the optimized solution only by performing considerable bench work experiments [23–27].

In this study, data mining by using the GRNN model was implemented to determine the effect and importance of different phytohormones, genotypes, and explants in wheat shoot regeneration. We assembled data from multiple wheat in vitro shoot regeneration studies which considered the use of phytohormones, genotypes, and explants. To the best of our knowledge, this study is the first report of the application of AI and GRNN-GA modelling in the field of wheat in vitro culture.

2. Material and Methods

2.1. Data and Model Development

Data for this study was collected from previous in vitro shoot regeneration studies [3,28–34]. Different types and concentrations of phytohormones, explant types, and genotypes on percent shoot regeneration frequency are summarized in Table S1. To construct the GRNN model (Figure 1), different genotypes, various explants, and different concentrations of 6-benzylaminopurine (BAP), kinetin (Kin), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), Zeatin, and $CuSO_4$ were considered as inputs, and shoot regeneration frequency was considered as output for modeling wheat in vitro shoot regeneration. For model development and validation, the dataset was randomly divided into two subsets of 70% for training and 30% for validation.

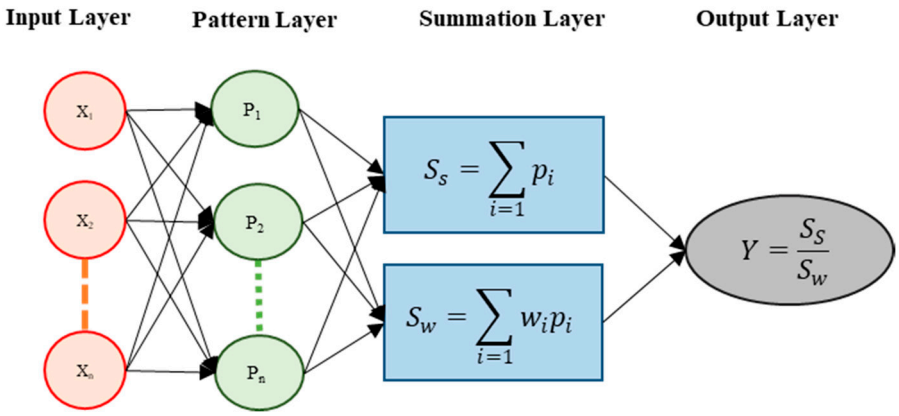

Figure 1. Schematic diagram of the proposed generalized regression neural network (GRNN) model.

GRNN with a very fast training process was established by Specht [13]. The input layer, pattern layer, summation layer, and output layer are four layers of GRNN. The input layer is completely joined to the pattern layer. Each neuron of the pattern layer is linked to S-summation and D-summation neurons from the summation layer. S-summation and D-summation neurons, respectively, measure the sum of the weighted and unweighted outputs of the pattern neurons. The connection weight between S-summation neuron and a neuron of the pattern layer is equal to the target output, while the connection weight for D-summation is unity. The output layer obtains the unknown output value corresponding to the input vector, only via dividing the output of each S-summation neuron through the output of each D-summation neuron. GRNN uses the following equations to calculate an output:

$$\hat{y} = \frac{\sum_{i=1}^{n} y_{ie}\, exp\left(-\frac{D_i^2}{2\sigma^2}\right)}{\sum_{i=1}^{n} exp\left(-\frac{D_i^2}{2\sigma^2}\right)} \quad (1)$$

$$D_i^2 = (x - x_i)^T (x - x_i) \quad (2)$$

where n, $\hat{y}$, y_i, σ, and $D_i{}^2$, and T are the number of input-output datasets, the output data (average of all the observed values), ith output data (connecting weight), width parameter, a scalar function, and the target related to the ith observation, respectively. In each iteration, a model was developed by using the training data and used to predict the outcome of the validation set. To assess the predictive ability of the GRNN model, three performance measures including R^2 (coefficient of determination), root mean square error (RMSE), and mean bias error (MBE) were used. Greater values of R^2 and smaller values of RMSE and MBE indicate the higher predictive ability and performance of the constructed model.

2.2. Process Optimization via Genetic Algorithm (GA)

The relationship of in vitro shoot regeneration of wheat with genotypes, explants, BAP, Kin, 2,4-D, IAA, IBA, NAA, Zeatin, and $CuSO_4$ was established according to the GRNN. The relationship between GRNN and GA was shown in Figure 2. The roulette wheel was implemented as the selection method to obtain the suitable fitness. The GA was run by setting the initial population size at 200, generation size at 1000, crossover probability (Pc) at 0.7, and mutation rate (Pm) at 0.04.

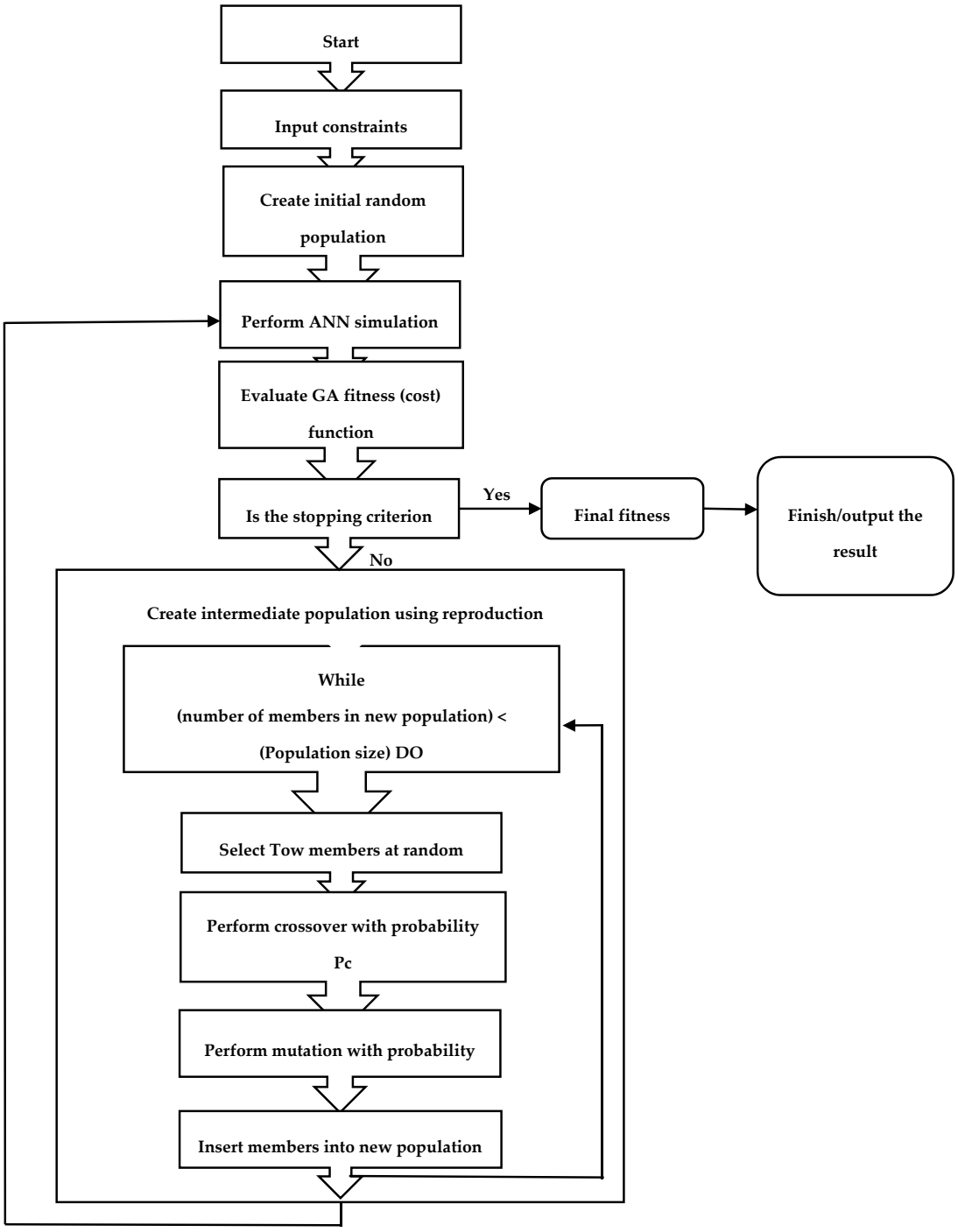

Figure 2. Schematic relationship of artificial neural network (ANN) model (GRNN) and optimization genetic algorithm (GA).

2.3. Sensitivity Analysis of Shoot Regeneration to Variations in the Input Variables

Sensitivity analysis was conducted to characterize the sensitivity of in vitro shoot regeneration to changes in genotypes, explants, and concentrations of BAP, Kin, 2,4-D, IAA, IBA, NAA, Zeatin, and $CuSO_4$. This sensitivity was measured by variable sensitivity error (VSE) value displaying the performance (RMSE) of GRNN-GA model when that input variable is removed from the model. Variable sensitivity ratio (VSR) value was determined as ratio of VSE and GRNN-NSGA-II model error (RMSE value) when all input variables are available. A higher important variable in the model was detected by higher VSR. MATLAB (Matlab, 2010) software was employed to run the model.

3. Results

3.1. Artificial Intelligence Accurately Predicted In Vitro Shoot Regeneration

In this study, GRNN was implemented for modeling in vitro shoot regeneration of wheat as an output based on ten input variables (genotypes, explants, BAP, Kin, 2,4-D, IAA, IBA, NAA, Zeatin, and $CuSO_4$).

The assessment of predicted and observed data on training and validation sets was used for describing the efficiency of the GRNN model. As can be seen in Table 1, the GRNN model was successful in predicting in vitro shoot regeneration outcomes of wheat in training ($R^2 > 0.88$, RMSE = 14.12, and MBE = −0.33) and validation ($R^2 > 0.78$, RMSE = 14.76, and MBE = −0.89) processes with correlations between observed and predicted data demonstrating a good fit (Figure 3).

Table 1. Performance criteria of the generalized regression neural network (GRNN) model for in vitro shoot regeneration of wheat in training and validation processes.

Performance Measure	Training	Validation
R^2	0.88	0.78
RMSE	14.12	14.76
MBE	−0.33	−0.89

R^2: coefficient of determination; RMSE: root mean square error; MBE: mean bias error.

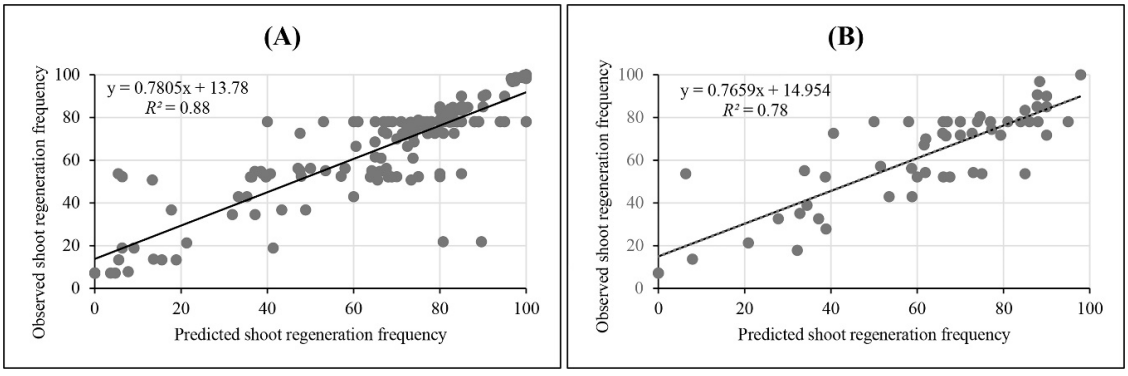

Figure 3. Experimental observed data against GRNN predicted outcomes of wheat in vitro shoot regeneration in training set (**A**) and in validation set (**B**).

3.2. Determining an Optimized Solution for Regeneration by Using Generalized Regression Neural Network (GRNN)-GA

We further the investigation and optimized the GRNN by using GA in order to present and avail a precise condition for wheat in vitro shoot regeneration based on the concentrations of phytohormones, types of explants, and genotypes. We would caution that the GRNN gives appropriate accuracy for interpolation but not for extrapolation. Thus, the upper and lower bounds of input data (Table S1) were set as constraints. According to GRNN-GA (Table 2), our model predicted that the highest shoot

regeneration frequency (97.63%) can be obtained from immature embryo explant on medium containing 0.15 mg/L BAP, 0.73 mg/L Kin, 0.17 mg/L 2,4-D, 0.37 mg/L IAA, 0.04 mg/L IBA, 0.01 mg/L NAA, 4.51 mg/L Zeatin, and 13.08 mg/L $CuSO_4$. It is worth to say that shoot regeneration frequencies > 90% were obtained when we replaced the best genotype with any other genotype in the model (Table S2).

Table 2. The optimized solution for shoot regeneration achieved by using GRNN-GA in wheat.

Type of Explant	BAP (mg/L)	Kin (mg/L)	2,4-D (mg/L)	IAA (mg/L)	IBA (mg/L)	NAA (mg/L)	Zeatin (mg/L)	$CuSO_4$ (mg/L)	Shoot Regeneration Frequency (%)
Immature embryo	0.15	0.73	0.17	0.37	0.04	0.01	4.51	13.08	97.63

BAP: 6-benzylaminopurine; Kin: kinetin; 2,4-D: 2,4-dichlorophenoxyacetic acid; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; NAA: 1-naphthaleneacetic acid.

3.3. The Importance of Input Variables in Shoot Regeneration

The importance of each input in the developed model was assessed via the whole database to evaluate the general VSR. The VSR was obtained for the shoot regeneration frequency with respect to genotypes, explants, BAP, Kin, 2,4-D, IAA, IBA, NAA, Zeatin, and $CuSO_4$ (Table 3). Sensitivity analysis demonstrated that shoot regeneration frequency was more sensitive to 2,4-D, followed by explant, genotype, zeatin, BAP, IAA, Kin, $CuSO_4$, IBA and NAA (Table 3).

Table 3. The results of sensitivity analysis on the developed GRNN model to rank the importance of factors involved in in vitro shoot regeneration of wheat.

Item	Genotype	Explant	BAP	Kin	2,4-D	IAA	IBA	NAA	Zeatin	$CuSO_4$
Variable sensitivity ratio (VSR)	1.35	1.83	1.22	1.08	1.97	1.17	0.93	0.54	1.29	1.02
Rank	3	2	5	7	1	6	9	10	4	8

4. Discussion

The in vitro shoot regeneration in wheat has been widely studied. Establishing wheat in vitro shoot regeneration systems were associated with obstacles such as chimeric callogenesis consisting of both non-embryogenic and embryogenic calli, low efficiency of shoot regeneration, and genotype-dependency [3,28–34]. Computational approaches may help in reducing trial and errors in the process of optimizing regeneration systems. Artificial intelligence (AI) models can be considered as one method to develop and optimize in vitro shoot regeneration protocols. Although there are no reports to use AI models in in vitro culture of wheat, several studies have previously proved the reliability and accuracy of AI methodology to predict and optimize different in vitro culture processes such as in vitro sterilization [22,35], callogenesis [36–38], cell growth and protoplast culture [39,40], somatic embryogenesis [37,41,42], shoot regeneration [43–46], androgenesis [9], hairy root culture [47,48], and rhizogenesis [49] in other plants.

In the current study, GRNN was used, for the first time in wheat, to develop a suitable model for in vitro shoot regeneration. According to our results, GRNN was a promising and powerful tool for modeling and predicting the system. Although there is no report regarding the application of the GRNN model in plant tissue culture studies, in line with our results, studies in other fields revealed the good performance of the GRNN model [50,51]. One of the weaknesses of using AI models is that it is hard to obtain an optimized solution [52–57]. To tackle this problem, several studies [21,22,44,52,57,58] used GA and NSGA-II to optimize in vitro culture conditions. In the current study, GA was linked to the GRNN model for the optimization process. Based on our results, a hybrid GRNN and GA can be considered as an efficient computational methodology for predicting and optimizing in vitro shoot regeneration of wheat.

Sensitivity analysis demonstrated that shoot regeneration frequency was more sensitive to 2,4-D, explant, genotype, and zeatin, and less sensitive to NAA. Previous studies [3,28–34] have demonstrated

that shoot regeneration of wheat is changed by the type and concentration of phytohormones and also by the type of explants and genotype. Yadav, Malik, Kumar and Jaiwal [3] showed that 2,4-D was the best auxin, among other auxins, for in vitro shoot regeneration of wheat. Also, Kumar, Mamrutha, Kaur, Venkatesh, Grewal, Kumar and Tiwari [34] reported that better results were achieved by using 2,4-D rather than NAA or IBA. 2,4-D is one of the most powerful synthetic auxins which has a main function in many in vitro processes such as callogenesis, embryogenesis, organogenesis and shoot regeneration [5,59]. In addition, the positive impact of the appropriate concentration of 2,4-D has been shown on the biological and molecular process of in vitro shoot regeneration by adjusting and regulating the endogenous phytohormones metabolism of different explants and genotypes [59,60]. Also, our results showed that the highest shoot regeneration frequency can be achieved by immature embryo as an explant. The potential of immature embryo has been previously confirmed by several studies [3,32]. Indeed, each explant or genotype has different levels of endogenous phytohormones that have resulted in different responses to exogenous phytohormones [4,59]. This leads us to consider in vitro shoot regeneration as a genotype-dependent process. Therefore, it is necessary to adjust the type and concentrations of exogenous phytohormones based on the levels of endogenous hormones to achieve genotype-independent protocols. Our results showed that 2,4-D and zeatin can be considered as the best auxin and cytokinin, respectively, for in vitro shoot regeneration in wheat. In line with our results, Yadav, Malik, Kumar and Jaiwal [3] reported that the highest frequency of shoot regeneration in wheat was achieved from the combination of 2,4-D and zeatin.

5. Conclusions

Various factors such as genotypes, explants, and phytohormones affect in vitro culture of wheat, hindering the ability to tailor genotype-independent protocols. Optimizing regeneration conditions such as genotype, type of explant, as well as type and concentration of phytohormones can be considered as one of the critical steps to establish a genotype-independent and high-frequency regeneration protocol. Recently, different artificial neural networks have been widely applied for modeling and predicting the outcomes of in vitro culture systems. In this study, GRNN was implemented, for the first time, to model and forecast in vitro shoot regeneration of wheat. Our results showed that the GRNN model can accurately model and predict in vitro shoot regeneration of wheat for obtaining in vitro genotype-independent protocol. In addition, we have shown than GA was able to accurately optimize the system. The results of the current study demonstrate that the combination of GRNN and GA can lead to modeling and understanding in vitro organogenesis and can pave the way for further in vitro culture studies in wheat such as somatic embryogenesis. Further experimental work is needed to validate the results of this computationally optimized culture media.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/15/5370/s1, Table S1. Database obtained from studies on in vitro shoot regeneration of wheat. Table S2. The shoot regeneration response of different genotypes to the optimized solution using GRNN-GA. The optimized solution was immature embryo as explant and a medium containing 0.15 mg/L BAP, 0.73 mg/L Kin, 0.17 mg/L 2,4-D, 0.37 mg/L IAA, 0.04 mg/L IBA, 0.01 mg/L NAA, 4.51 mg/L Zeatin, and 13.08 mg/L $CuSO_4$.

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Conflicts of Interest: The authors declare no conflict of interest.

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