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Envisaging the Regulation of Alkaloid Biosynthesis and Associated Growth Kinetics in Hairy Roots of *Vinca minor* Through the Function of Artificial Neural Network

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Abstract Artificial neural network based modeling is a generic approach to understand and correlate different complex parameters of biological systems for improving the desired output. In addition, some new inferences can also be predicted in a shorter time with less cost and labor. As terpenoid indole alkaloid pathway in *Vinca minor* is very less investigated or elucidated, a strategy of elicitation with hydroxylase and acetyltransferase along with incorporation of various precursors from primary shikimate and secoiridoid pools via simultaneous employment of cyclooxygenase inhibitor was performed in the hairy roots of *V. minor*. This led to the increment in biomass accumulation, total alkaloid concentration, and vincamine production in selected treatments. The resultant experimental values were correlated with algorithm approaches of artificial neural network that assisted in finding the yield of vincamine, alkaloids, and growth kinetics using number of elicits. The inputs were the hydroxylase/acetyltransferase elicitors and cyclooxygenase inhibitor along with various precursors from shikimate and secoiridoid pools and the outputs were growth index (GI), alkaloids, and vincamine. The approach incorporates two MATLAB codes; GRNN and FFBPNN. Growth kinetic studies revealed that shikimate and tryptophan supplementation triggers biomass accumulation (GI=440.2 to 540.5); while maximum alkaloid (3.7 % dry wt.) and vincamine

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production (0.017±0.001 % dry wt.) was obtained on supplementation of secologanin along with tryptophan, naproxen, hydrogen peroxide, and acetic anhydride. The study shows that experimental and predicted values strongly correlate each other. The correlation coefficient for growth index (GI), alkaloids, and vincamine was found to be 0.9997, 0.9980, 0.9511 in GRNN and 0.9725, 0.9444, 0.9422 in FFBPNN, respectively. GRNN provided greater similarity between the target and predicted dataset in comparison to FFBPNN. The findings can provide future insights to calculate growth index, alkaloids, and vincamine in combination to different elicits.

Keywords *Vinca minor* · Artificial neural network · MATLAB · Generalized regression neural network

Abbreviations

ANN	Artificial neural network
GRNN	Generalized regression neural network
FFBPNN	Feed forward back propagation neural network
MAE	Mean absolute error
NMSE	Normalized mean square error

Introduction

Human brain has the ability to learn process information, predict, and generalize features of complex data for future purpose [1]. Mathematical models used commonly for modeling of complex nonlinear data [2]. In artificial neural network, the term refers to inter-connection between the neurons in different layer of each system [3]. ANN has widely been used in many areas that include agriculture, medical, pharmaceutical, disease pattern, etc. [4]. Besides biology, it is used in many fields such as aerospace, banking, defense, electronics, entertainment, financial, industrial, oil and gas, robotics, telecommunication, speech, etc. The most widely employed algorithm for training ANNs is back propagation neural network [BPNN]. It is based on searching of error surface (error as a function of ANN weights) using gradient descent for point(s) with minimum error [5]. The predicted values are compared against the experimental value to test the network performance [6].

The biological processes vary and two of the important causes of this variation are the genetic and environmental effects which play a major role in defining a process and its behavior with respect to the time. The plant cells and tissues also goes through with these nondeterministic and nonlinear nature as the genetic and environmental factors play a key role under in vitro conditions too that results in high degree of variability leading to an array of biological developments [7]. In vitro generation of tissue is basically targeted for either manipulating the genome and growth of the plants or to produce plants at mass level in lesser time in predefined conditions. The thermodynamic restrictions, culture conditions, growth kinetics of in vitro tissue and conversions of energy to mass and vice versa are important factors and there is an urgent requirement for a modeling systems which can easily define and predict these important phenomenon in a realistic manner [8]. For a standard, realistic and objective evaluation of different biological processes occurring inside under in vitro system, the neuronal network technology (NNT) can be proven as an alternate effective technique [7]. Different complex mathematical functions and their approximations related to a process come under NNTs which deals with interpretation of various sets of data. The NNT can be considered as the network of human neurons as it involves processing of the information and abilities to take decisions. They can also help in identifying and modeling of nonlinear relations of a bioprocess with their precise and high learning capabilities. The implementation of NNTs under in vitro conditions finds ample use as recent works of different in vitro plant systems showed the beneficial effects on NNT approaches. Recently, the optimal culture condition predictions for the efficient growth of the hairy root cultures of *R. serpentina* were made with the combinatorial approaches of ANN-HMM [9]. The pattern recognitions of somatic embryos, photometric, and photosynthetic assessments of the regenerated plants under in vitro conditions can also be performed with the ANN approaches. Apart from it, biomass assessments and production of secondary metabolite can also be evaluated online through ANN-based approaches [10, 11].

The lesser periwinkle (*V. minor*) belongs to the family Apocyanaceae and contains more than 50 indole alkaloid in which vincamine holds most important position as it has neuronal homeostasis properties, modulatory effects on the brain circulation, and neuroprotective as well as antihypoxic abilities [12]. Hairy roots have proven their worth by showing multifold increment of secondary metabolite production as compared to the other in vitro culture systems. *V. minor* hairy roots that were devoid of vincamine were elicited with hydroxylases and acetyltransferases with simultaneous inhibition of cyclooxygenases and incorporation of various terpenoid indole alkaloid precursors to modulate the flux towards vincamine production [13]. The present study was carried out by comparing the empirical data obtained and prediction based on GRNN and FFBPNN model to reveal the accurate predictive efficiency of ANN model for optimal growth and production kinetics of elicited hairy roots of *V. minor*. In the current study, artificial neural network was used in prediction of best possible culture in terms of elicits/inhibitors and obtained outputs in the form of growth index, alkaloids, and vincamine production. For this, two methods of ANN were applied. First was feed forward back propagation neural network [FFBPNN] that is a multilayer perceptron, trained by the standard back propagation (BP) learning algorithm and the second was generalize regression neural network [GRNN] [14] which is a kind of networks based on a standard statistical technique called kernel regression. After optimization, predicted values were compared with experimental values to find best neural network model for growth and production kinetics of hairy roots of *V. minor*.

Material and Methods

Establishment of Hairy Roots and Generation of Experimental Data

Four hairy root clones were established from leaf explants from multiple shoot cultures of *V. minor* [12]. The vincamine less hairy root clone PV8 (Fig. 1) was used for studying regulation of vincamine biosynthesis [13]. Various elicitation treatments along with inhibitors and simultaneous feeding of precursor molecules were according to Verma et al. [13]. For inhibiting serpentine type of alkaloid, cyclooxygenase inhibitors, i.e., naproxen and benzotriazole were used at the concentration of 8.4 mg/l and 0.5 μM/l, respectively, to the 20-day-old hairy roots growing in liquid medium. Hydrogen peroxide (20 μg/l) and acetic anhydride (32.4 mg/l) were added to enhance the activities of hydroxylase, peroxidase, and acetyltransferases. Five precursor molecules of terpenoid indole alkaloid pathway, i.e., shikimate (100 mg/l), tryptophan (100 mg/l), tryptamine (100 mg/l), loganin (10 mg/l), and secologanin (10 mg/l) have been tested in various combinations with cyclooxygenase inhibitors, hydroxylase, and peroxidase elicitors to screen out the best treatment favoring the vincamine biosynthesis (Table 1) [13]. The estimation of growth kinetics, total alkaloids, and HPLC-based vincamine detection was done according to Verma et al. [13]. For ANN-based comparisons, growth index, total alkaloid, and vincamine production on various treatments were taken into account and predictions were made.

Employment of ANN Model

Two ANN methods namely FFBPNN and GRNN were applied in order to study three outputs of *V. minor* hairy roots, i.e., growth index, total alkaloids, and vincamine production. Feed forward neural network method is most widely used in ANN-based studies [3]. It uses back propagation [BPNN] algorithm. There are two parts in BPNN propagation. A feed forward phase in which the data information at the input nodes is propagated forward to compute the output information signal at the output unit, and a backward part in which modifications to the connections strengths are made based on the differences between the computed and observed information signals at output units [2]. The performance of FFBPNN depends upon its topology. FFBPNN consists of three layers; input layer, a hidden layer, and an output layer as shown in Fig. 2a [2, 6, 15]. Here, input layer has nine neurons and output layer has three neurons. The hidden layer resides between input and output layers and can have variable number of neurons depending upon the application of the network. The optimum output of the experiment is governed by customizing the hidden layer neurons. Selection of neurons in hidden layer is usually performed by trial and error method by changing the number of neurons for its good effect [15]. Weights and biases play a major role in FFBPNN. The GRNN method is based on a standard statistical technique known as kernel regression [2, 16]. It consists of four layers namely input layer, pattern layer, summation layer, and output layer. Architecture of GRNN is provided in Fig.2b. In GRNN, the number of neurons in input layer depends on the experimental parameters. Pattern layer comes after input layer and it contains neuron that represents the training pattern and its output. Summation layer resides between pattern and output layers. The summation and output layer performs together to normalize the output set [16].

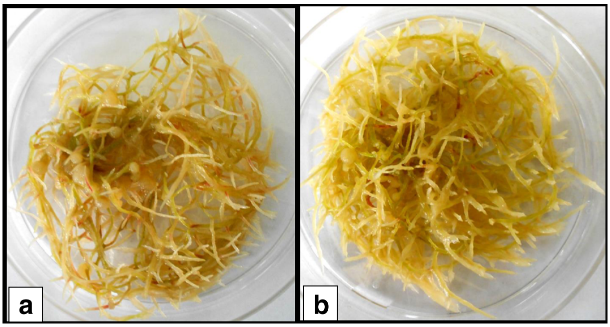

Fig. 1 Hairy root clone PV8 of *Vinca minor*. (**a**) control roots (**b**) roots treated with shikimate (100 mg/l)

Table 1 Comparison of experimental data and GRNN and FFBPNN neural network predicted values for growth index, total alkaloids (% dry wt.), and vincamine production (% dry wt.) in hairy roots of *V. minor*

S.No	Treatment	GI (target)	GI (predicted)		Total alkaloids (target)	Total alkaloids (predicted)		Vincamine (target)	Vincamine (predicted)	
			GRNN	FFBPNN		GRNN	FFBPNN		GRNN	FFBPNN
1	Control	315.0	315.0	315.0	2.6	2.6	2.4	0	0	−0.001
2	NAP (naproxen)	320.6	320.6	320.6	2.5	2.5	2.4	0	0	−0.001
3	BENZ (benzotriazole)	285.0	285.0	285.0	2.3	2.3	2.3	0	0	0.006
4	AA (acetic anhydride)	240.5	240.5	280.0	2.9	2.9	3.0	0	0	0.001
5	H_2O_2 (hydrogen peroxide)	322.4	322.4	322.3	2.9	2.9	2.9	0	0	0.002
6	SHK (shikimate)	540.5	540.5	540.4	2.7	2.7	2.1	0	0	0.002
7	TRYP (tryptophan)	440.2	440.2	440.3	2.5	2.5	2.4	0	0	−0.001
8	TRY (tryptamine)	345.6	345.6	314.0	2.5	2.5	2.5	0	0	0.001
9	LOG (loganin)	205.0	205.0	187.2	2.1	2.1	1.6	0	0	0.001
10	SECO (secologanin)	300.0	300.0	265.4	2.6	2.6	2.6	0	0	0.006
11	NAP+ H_2O_2	330.4	330.4	350.2	3.1	3.1	2.8	0	0	−0.003
12	NAP+ H_2O_2 + BENZ	346.5	346.5	346.5	2.5	2.5	2.4	0	0	0.002
13	NAP+ H_2O_2 + AA	246.7	246.7	301.7	3.0	3.0	3.0	0	0	−0.004
14	NAP+ H_2O_2 + AA + SHK	343.4	343.4	343.4	3.2	3.2	2.9	0	0	−0.001
15	NAP+ H_2O_2 + AA + TRYP	350.5	350.5	350.4	3.1	3.1	3.1	0	0	−0.004
16	NAP+ H_2O_2 + AA + TRY	285.5	293.1	300.7	3	3.1	3.2	0	0.0055	−0.005
17	NAP+ H_2O_2 + AA + LOG	160.4	160.4	160.4	2.3	2.3	2.1	0	0	0.005
18	NAP+ H_2O_2 + AA + SECO	290.8	290.8	290.8	3.5	3.5	3.5	0.015	0.015	0.002
19	NAP+ H_2O_2 + AA + SHK + LOG	175.8	175.8	165.7	2.2	2.2	2.1	0	0	0.005
20	NAP+ H_2O_2+AA+SHK+SECO	280.9	280.9	280.8	3.2	3.2	3.2	0.011	0.011	0.001
21	NAP+ H_2O_2 + AA + TRYP + LOG	200.0	200.0	200.0	2.1	2.1	2.1	0	0	0.007
22	NAP+ H_2O_2 + AA + TRYP + SECO	340.5	340.5	304.8	3.7	3.7	3.6	0.017	0.017	0.002
23	NAP+ H_2O_2 + AA + TRY + LOG	140.6	140.6	162.8	2.0	2.0	2.0	0	0	0.003
24	NAP+ H_2O_2 + AA + TRY + SECO	300.8	293.1	300.7	3.2	3.1	3.2	0.011	0.0055	−0.005

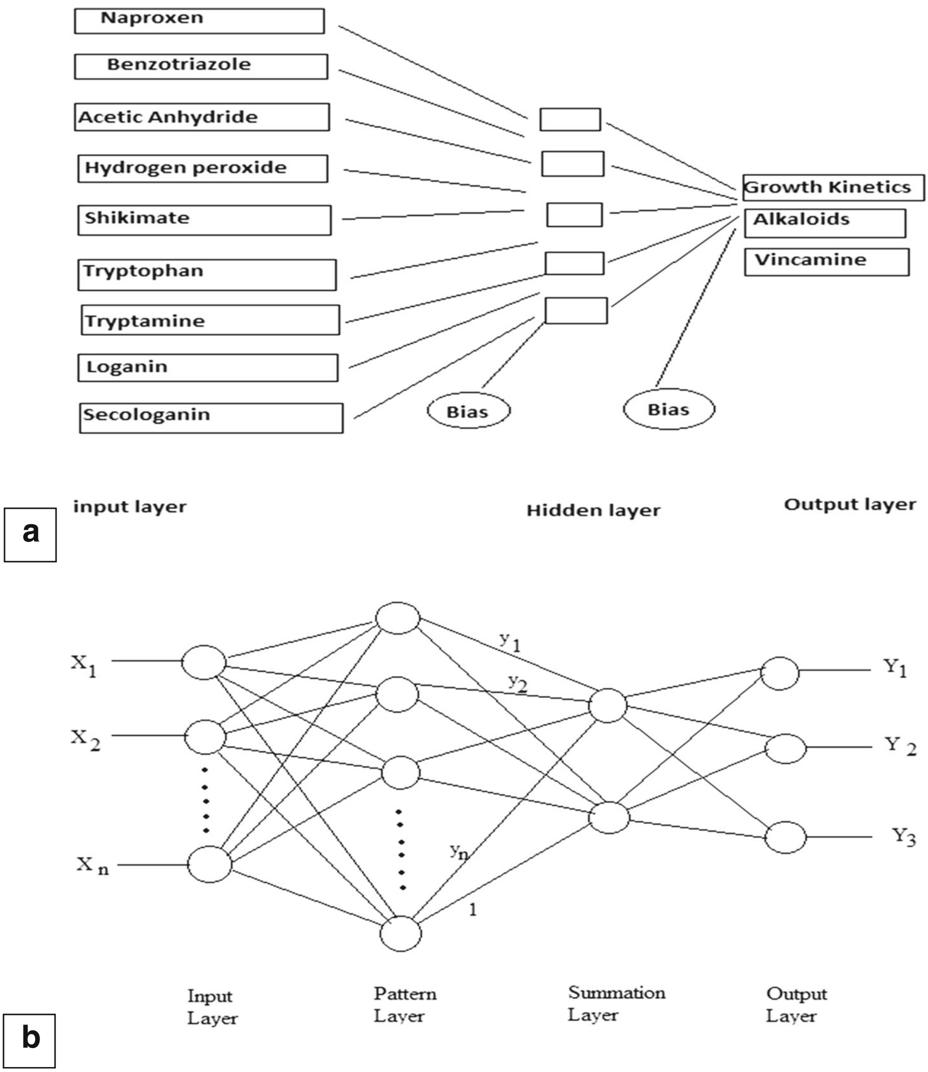

Fig. 2 **a** Architecture of Feed Forward Back Propagation Neural Network with hidden layer. **b** Architecture of generalize regression neural network

Training Set Data

In FFBPNN method, the whole data was divided into three parts for training to obtain the optimal output [17, 18]. It was performed by taking 14 records to train the network, five entries were used to validate the prediction quality of the network and remaining five data were used to evaluate the performance of trained network. A number of different networks having 1 to 50 neurons in hidden layer were created. It was followed by training of the network with Levenberg-Marquardt [6, 19] optimization that updates weight and bias state. For GRNN, spread value 0.001 to 1 was taken to find the accurate results. It indicates that there were 1000 different spread values. The best network model on 0.001 spread value that showed the maximum correlation coefficient was selected. For training the neural network, data obtained as per the above calculations were used. The target values of growth index, total alkaloids, and vincamine thus obtained from the training data were compared with predicted respective values.

Statistical Analysis

The performance of ANN was also demonstrated by calculation of errors between experimental and predicted outputs. The mean absolute error (MAE) and normalize square error (NMSE) for both ANN methods were determined along with correlation coefficient. Standard deviation of GI, alkaloid, and vincamine production was also calculated for experimental as well as GRNN and FFBPNN data.

Results and Discussion

Two MATLAB codes for ANN method viz; GRNN and FFBPNN were used for obtaining maximum correlation between experimental data and predicted data. Total of 24 treatments were taken into account that provided outputs for growth index, total alkaloids, and vincamine production in *V. minor* hairy roots. According to experimental conditions and by incorporating FFBPNN, input layer contains nine neurons, five neurons as hidden layer and three neurons as output layer (Fig. 2a). The experimental data and predicted growth index, alkaloids, and vincamine for FFBPNN and GRNN (Table 1) are provided. In FFBPNN method, the results revealed that amongst all hidden layers, the best correlation coefficient (regression) was obtained on five neurons in hidden layer. But when correlation coefficient was calculated for each output separately, it was found that alkaloids and vincamine had poor correlation (between experimental and predicted values) that needs to be improved. It is assumed that it happened due to the number of neurons in hidden layer, weights, and biases which needs to be adjusted for optimal output. Weights were assigned randomly in FFBPNN process and are applicable only for growth index. Weights play a very important role in optimization [20, 21]. FFBPNN algorithm has some drawbacks. Initialization of weight is a random process. It affects the performances each time when the same network architecture was simulated. This is due to the different initial random weight assignment in the beginning of each training simulation [20]. Therefore, for best optimization that can be done is to train the network separately for all outputs. In current study, three network models were generated. Each network model has nine neurons in input layer and output layer has only one neuron. In case of hidden layer, there were five neurons in growth index network, five in total alkaloids network, and four neurons in vincamine network. The architecture of these three networks was 9-5-1, 9-5-1, and 9-4-1 for growth index, alkaloids, and vincamine, respectively. In GRNN, there was no need of iteration. In order to obtain the optimal output, spread value 0.001 to 1 was taken. Input and output data for 1000 different spread values (result of all spread values is not provided) were optimized and best network model on 0.001 spread value which showed the maximum correlation coefficient was selected, provided in Table 2. The best Pearson correlation coefficient for 0.001 spread value was obtained. Pearson correlation coefficient value showed that how output and experimental values correlate to each other. The correlation value 1 indicates that both the groups (output and experimental value) are close to each other and while correlation value 0 shows random correlation [4, 6, 8]. The detailed comparison

Table 2 Error values in terms of MAE, MSNE, and correlation coefficient (CC) for ANN prediction

Method of ANN	Growth index			Alkaloids			Vincamine		
	MAE	MSNE	CC	MAE	MSNE	CC	MAE	MSNE	CC
FFBPNN	11.75	0.01	0.9725	0.118	0.04	0.9444	6.7e-04	0.48	0.9422
GRNN	0.63	0	0.9997	0.008	0	0.9980	4.5e-04	0	0.9511

plots for growth index (Fig. 3a), total alkaloid production (Fig. 3b), and vincamine production (Fig. 3c) along with their experimental values are provided. These comparison plots clearly demonstrate that experimental values and ANNs values were approximately same to good effect. Figure 4a–c shows scatter plots between predicted values and values obtained experimentally. It can be clearly seen that there is a strong linear relation between predicted and experimental values, especially in case of GRNN model. The performance of ANN was also demonstrated by calculation of errors between experimental and predicted outputs [6]. The mean absolute error (MAE) and normalize square error (NMSE) for both ANN methods were determined (Table 2). It is evident from the data provided that error values in terms of MAE, NMSE, and correlation coefficient (CC) for growth index, alkaloid content, and vincamine were found better in GRNN method as compared to the FFBPNN method. The values of growth index for MAE, NMSE, and CC were found to be 11.75, 0.01 and 0.9725 in FFBPNN method while for GRNN method, the MAE, MNSE, and CC values were 0.63, 0.00, and 0.9997, respectively. In case of alkaloid, the FFBPNN and GRNN methods gained the values of 0.118 and 0.008 (MAE), 0.04 and 0.0 (NMSE) but again the correlation coefficient (CC) values were better in case of GRNN method (0.9980) when compared to FFBPNN method (0.9444). Again, in case of vincamine, the correlation coefficient (CC) values were found to be better for GRNN method (0.9511) than FFBPNN method (0.9422) (Table 2). The standard deviation (SD) of growth index (GI), alkaloids, and vincamine for experimental, FFBPNN, and GRNN data were also calculated. It showed that SDs of experimental data of growth index, alkaloid content, and vincamine (GI 87.51228, alkaloid 0.4614, and vincamine 0.0053) were having nearer values to GRNN method based values (GI 87.4837, alkaloid 0.4604, and vincamine 0.0050) in comparison to FFBPNN method based values (GI 85.7609, alkaloid 0.4614, vincamine 0.0049) (Table 3). The topology of ANN plays a vital role in process of a network model. Command interpreter in ANN topology is resolute by the number of layers, the number of neurons in each layer, and the transfer functions [22]. The use of ANN is increasingly becoming most preferred methodology to model the complex biological responses. ANNs can also play central role as highly potential predictive modeling tool under in vitro tissue culture studies. Neural computing offers reliable and realistic approach for describing in vitro culture of plant species even with minimal available information. All results were showing that GRNN provided better result than FFBPNN. This confirms the earlier finding which states that GRNN method is better than FFBPNN method [2, 14, 21, 23, 24]. The success obtained after applying neural network technology has been phenomenal with a relatively modest experimental effort while consuming minimum amount of time. Maximum inference has been derived from relatively simplistic experimental procedures. Assessment and interpretation of biological interactions are not easy as it consists of complex and vague data. Exploration of such data sets requires multidimensional network systems that can model, process, and show first-rate potential in decision-making [25]. The practice directed to the

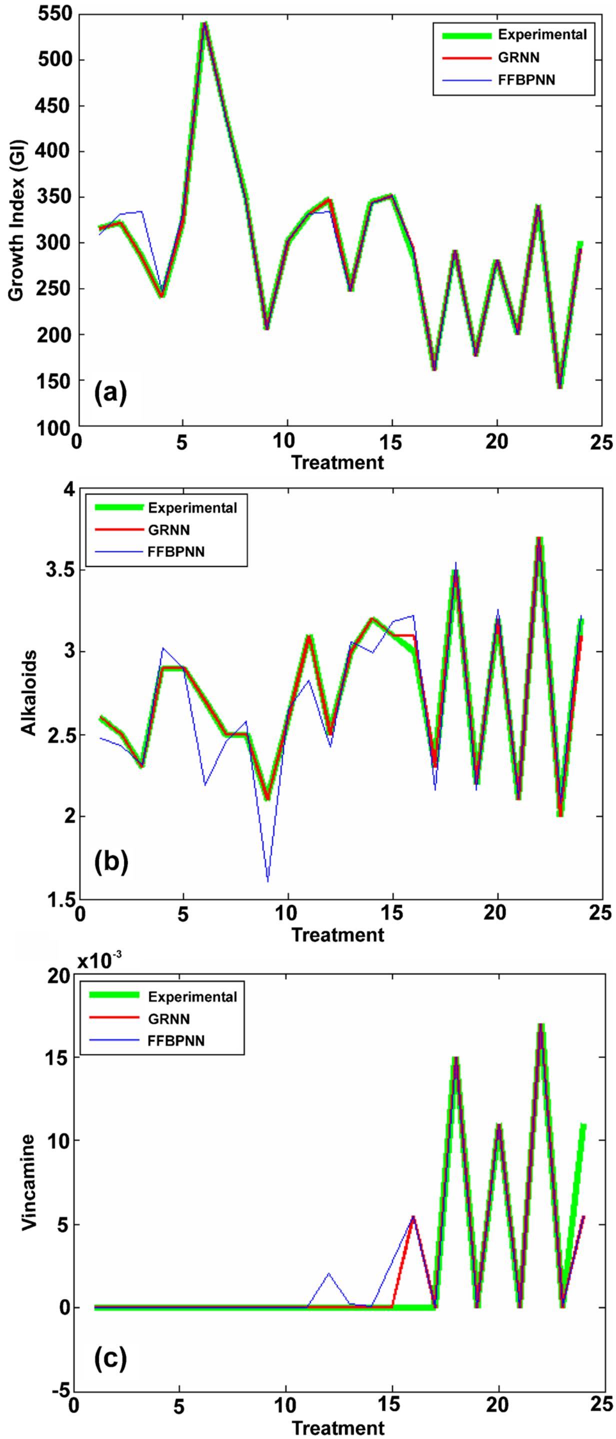

Fig. 3 Comparison between experimental and predicted values based on GRNN and FFBPNN. **a** Growth index, **b** total alkaloids (% dry wt.), and **c** vincamine production (% dry wt.). *Green line*: experimental data, *blue line*: prediction values based on FFBPNN, *red line*: prediction values based on GRNN

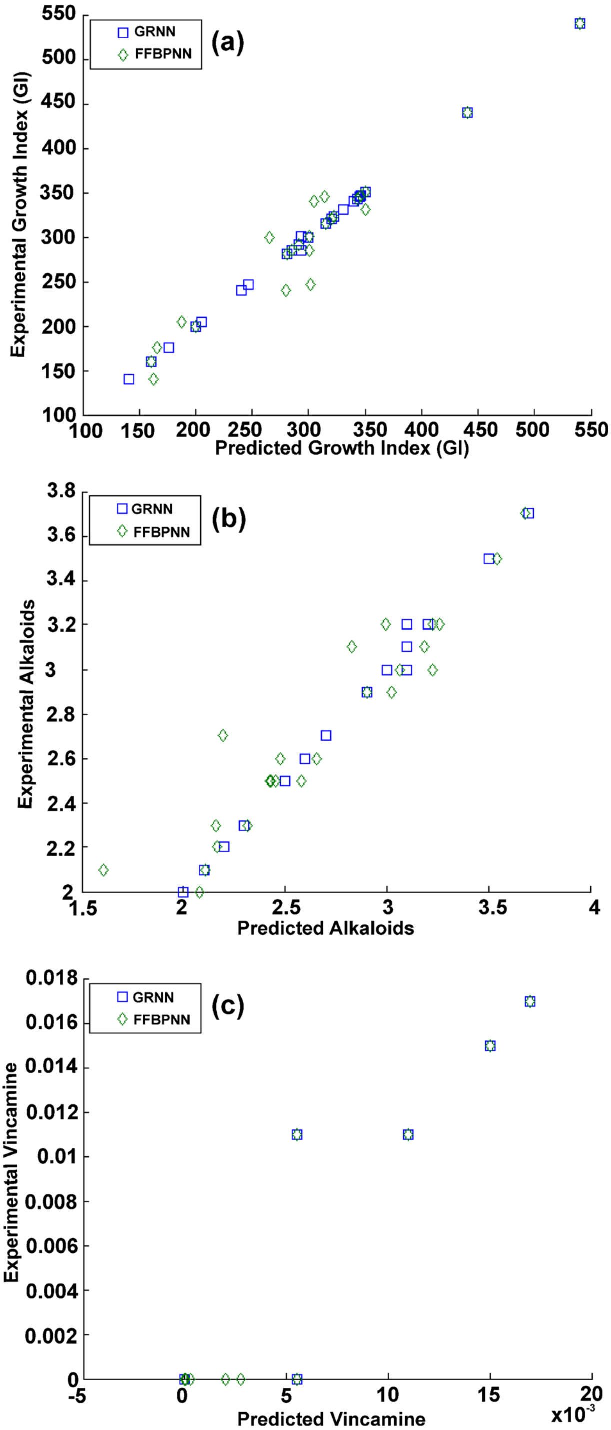

Fig. 4 Scatter plots showing relation between predicted and experimental values for **a** Growth index. **b** Alkaloids. **c** Vincamine

Table 3 Standard deviation of growth kinetics, alkaloids, and vincamine for experimental, FFBPNN, and GRNN data

	Growth index	Alkaloids	Vincamine
Experimental	87.51228	0.4614	0.0053
FFBPNN	85.7609	0.4614	0.0049
GRNN	87.4837	0.4604	0.0050

establishment and utilization of hairy root culture systems for biomass and metabolite production is subjected to the optimization of chemical parameters that influence their growth and productivity. Such optimizations were done through offline experimental analysis, resulting in an outflow of time, money, and labor. Therefore, it is required to develop such modeling systems which can authentically analyze the culture conditions up to an optimal level that can accumulate with complex developmental patterns of this commercially important biological system and ultimately lead to high yields [9]. The ability of the ANN to accurately simulate even under altered conditions could be highly encouraging in designing of cultivation systems on large scale. Various image processing methods have been developed successfully for assessing culture types, biomass production etc. but in order to bring them to a usable form, neural network solutions offer attractive incentives. ANN can be modulated to simulate the metabolism of the in vitro plants under a given set of conditions. It could be useful in estimating the amount of secondary metabolites that could accumulate at a specified time period and also the time at which one can derive maximum yield.

Conclusion

The strategy of regulating the vincamine biosynthesis in *V. minor* hairy root cultures succeeded in delivering the desired metabolic product. This was achieved by blocking the serpentine/type of alkaloids (through the addition of naproxen), promoting the transformation of tabersonine towards vincamine through various elicitor and precursor feeding treatments. In the current study, two methods of ANN (GRNN and FFBPNN) were compared for the yield of vincamine, growth kinetics, and total alkaloids. The experimental data was provided via hairy root clone of *V. minor* plant applying different elicit in vitro. Both methods gave acceptable results in case of growth kinetics, while alkaloid and vincamine production was not supported by FFBPNN. GRNN method was found to be useful in predicting all the three studied parameters. FFBPNN methods are sensitive due to randomly assigned weights which are not present in GRNN simulation. The GRNN results were more acceptable. The study provided acceptable correlation between experimental values and predicted values and to obtain best working neural network model.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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