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MICROPROPAGATION

Artificial neural network and decision tree–based models for prediction and validation of *in vitro* organogenesis of two hydrophytes—*Hemianthus callitrichoides* and *Riccia fluitans*

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Abstract

The application of plant tissue culture protocols for aquatic plants has been widely adopted in recent years to produce cost-effective plants for aquarium industry. *In vitro* regeneration protocol for the two different hydrophytes *Hemianthus callitrichoides* (Cuba) and *Riccia fluitans* were optimized for appropriate basal medium, sucrose, agar, and plant growth regulator concentration. The MS No:3B and SH + MSVit basal medium yielded a maximum clump diameter of 5.53 cm for *H. callitrichoides* and 3.65 cm for *R. fluitans*. The application of 20 g/L sucrose was found appropriate for yielding larger clumps in both species. Solidification of the medium with 1 g/L agar was optimized for inducing larger clumps with rooting for both species. Provision of basal medium with any concentration of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) was found detrimental for inducing larger clumps for both species. The largest clumps of *H. callitrichoides* (5.51 cm) and *R. fluitans* (4.59 cm) were obtained on basal medium without any plant growth regulators. The attained data was also predicted and validated by employing multilayer perceptron (MLP), random forest (RF), and extreme gradient boosting (XGBoost) algorithms. The performance of the models was tested with three different performance metrics, namely, coefficient of regression (R^2), means square error (MSE), and mean absolute error (MAE). Results revealed that MLP and RF models performed better than the XGBoost model. The protocols developed in this study have shown promising outcomes and the findings can irrefutably assist to produce *H. callitrichoides* and *R. fluitans* on a large scale for the local aquarium industry.

Keywords Aquatic plants · Artificial intelligence · Basal medium · Plant growth regulators · Prediction · Validation

Introduction

Aquatic plants, also known as hydrophytes, are a group of plants that spend their whole life or part of their life cycle in water bodies (Saini *et al.* 2010). These aquatic plants survive their life and continue their growth and reproduction under suitable conditions. They can be classified as floating, submerged, and emergent hydrophytes based on their part in water bodies. The aquatic plants develop an aquatic ecosystem and are considered primary producers of the aquatic ecosystem by providing oxygen to the water bodies. The other advantages of aquatic plants include the provision of organic matter, food, or food materials like vitamins, proteins, carbohydrates, and minerals for humans and animals along with providing shelter to fish for laying eggs, protection from the prey, and providing the environment for breedings of organisms living in the water (Cirik 2001; Oyedeji and Abowei 2012). In recent years, these hydrophytes have been used for other purposes like medicinal plants, phytoremediation (Singh *et al.* 2012), and biomonitoring of water ecosystems (Zurayk *et al.* 2001).

Dwarf baby tear (*Hemianthus callitrichoides*, Griseb.) belongs to the Scrophulariaceae family and is a small rocky plant that is considered an important ornamental aquatic plant (Othman *et al.* 2015). The plant is delicate and found in clump form and is used for either cushion-forming or

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as a carpeting plant in the aquarium industry (Barpete *et al.* 2015). The plant is generally slow growing, and flowers are tiny and white which require ambient conditions (light, minerals, CO_2, and pH) to bloom in the water (Othman *et al.* 2015). Besides its use as an ornamental aquarium plant, it has also been used for the phytoremediation of water bodies (Othman *et al.* 2015). Crystalwort (*Riccia fluitans*) is a floating hydrophyte of the genus *Ricciaceae*. It is a quick-growing plant in ponds or aquariums and develops a thick mass (clump) on the surface and underwater surface from a single branch or buds (Türkoğlu and Parlak 2014). Additionally, studies on phytoremediation have also utilized this plant (Heredia *et al.* 2002).

The role of aquatic plants in aquaculture is also increasing substantially due to the high demand for maintaining nutrients. The process is known as biological filtration, and aquatic plants maintain the concentration of nitrogenous compounds in aquaculture or aquariums (Roslan *et al.* 2021). However, the selection of proper plants and their appropriate propagation technique is important. The aquarium industry or water gardens in recent years have become a popular and profitable industry all over the world and the industry comprises multi-million dollar (Mansour *et al.* 2022). These aquatic plants are native to tropical and subtropical regions and these regions are also the major producers and exporters to other parts of the world (Aasim *et al.* 2019a). In general, aquatic plants are propagated through traditional vegetative propagation methods or by using plant tissue culture techniques. In recent years, the propagation of aquatic plants through tissue culture has increased immensely all over the world to meet the local demand (Aasim *et al.* 2022a). However, the optimization of plant tissue culture techniques along with cost-effective economic plants is the major objective of commercial labs.

Machine learning (ML) is a sub-field of artificial intelligence (AI). It identifies a specific output in light of specific inputs to achieve prediction or classification objectives (Géron 2022). The AI-based ML models are capable of overcoming the said issues by analyzing and predicting complicated and multivariate datasets (Katirci 2015; Jafari and Shahsavar 2020; Kul *et al.* 2020) and learning complex interrelations (Jamshidi *et al.* 2020; Sadat-Hosseini *et al.* 2022). The adoption of ML techniques has the benefit of giving computers the ability to learn on their own and turn data into meaningful knowledge without the need for human programming (Hesami *et al.* 2022). These machine learning methods have been popular in recent years for the analysis of datasets in several fields of plant sciences. However, the application of ML models is relatively limited in plant tissue culture studies as compared to other scientific fields. *In vitro* micropropagation is the manipulation of multifactor that leads to the complicated biological process of organogenesis or embryogenesis (Jamshidi *et al.* 2019; Hesami *et al.* 2020). The role of genotypes/cultivars along with other important interacting parameters like culture mediums and conditions is necessary for optimizing the aforementioned process (Hesami *et al.* 2017; Niazian and Niedbała 2020; Hesami and Jones 2021; Viswanathan *et al.* 2022). The use of traditional statistical methods to decipher all the data that has been encrypted over the enormous datasets of biological interactions like micropropagation is quite difficult, due to complicated, noisy, and misleading datasets involving multifactorial processes (Pepe *et al.* 2021). However, using multiple ML models, successful accuracy, prediction, and optimization process of plant tissue culture procedures have recently been accomplished. These include the use of neural networks (García-Pérez *et al.* 2020; Hesami *et al.* 2020), and decision tree–based ML models (Aasim *et al.* 2022b). It is interesting to know that the use of ML models for predicting or optimizing micropropagation is very limited, and is recently reported for the ornamental aquatic plant *Alternanthera reineckii* (Aasim *et al.* 2022a). The present study was designed to optimize the cost-effective plant tissue culture protocol for two major submerged hydrophytes, followed by prediction and validation through artificial intelligence models.

Material and Methods

Plant material and surface sterilization Both *H. callitrichoides* and *R. fluitans* plants were purchased from local dealers from the aquariums as *in vitro* culture containers. Bacterial and fungal contamination can also occur even in plants purchased *in vitro*. Therefore, all plant material supplied was surface-sterilized in 1.25% commercial bleach (NaOCl) along with 1–2 drops of Tween-20 for 10 min and then rinsed 3 times with sterile water. Contamination continued when the plants were cultured on a nutrient medium after sterilization. These plants were again surface-sterilized as described above and were decontaminated after the third sterilization process.

Culture medium and conditions Twelve different nutrient media and various concentrations of sucrose, agar, and plant growth regulators (PGRs), namely 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA), were used in the experiments (Tables S1–S4). After adjusting the pH of all media to 5.6–5.8, they were autoclaved at 121°C under 1.4 kg/cm^2 pressure for 20 min. The small cluster (approximately 10 shoots) was taken from the shoot clumps with forceps, and cultured on nutrient medium in Duchefa Sterivent Containers. Four clusters of shoots (explants) were placed in each culture vessel. All cultures were incubated in a 16-h photoperiod at 24°C under cool white fluorescent light (35 μmol photons m^{-2} s^{-1}) in growth cabinets. The experiments were performed in series and an optimized input parameter was used in the next experiment.

Optimization of basal nutrient medium For the optimization of the most suitable basal medium, twelve different commercial basal media were tested at their standard rates (Table S1). These media were also provided with 30 g sucrose and 5 g/L agar. The best-optimized basal medium was later on used for the optimization of sucrose, agar, and plant growth regulator contents.

Optimization of sucrose concentration The basal medium was supplemented with 0, 10, 20, 30, 40, and 50 g/L sucrose (Table S2) to optimize the optimum sucrose concentration. The media were enriched with MS-3B (Murashige and Skoog medium including half-strength concentration of NH_4NO_3 and KNO_3) for *H. callitrichoides*, and SH-MSVit (Schenk and Hildebrandt medium with MS vitamins) for *R. fluitans.* The culture mediums were gelled with 5 g/L agar for both plants.

Optimization of agar concentration For the optimization of appropriate agar concentration, the medium was solidified with 0, 1, 2, 3, 4, 5, 6, and 7 g/L agar (Table S3). The media were enriched with MS-3B for *H. callitrichoides* and SH-MSVit for *R. fluitans* along with 20 g/L sucrose for both species.

Optimization of BAP and NAA concentrations For the optimization of the appropriate cytokinin-auxin ratio, different concentrations of BAP (0.5, 1.0, and 2.0 mg/L) alone or BAP concentration with 0.5 mg/L NAA were added in the culture medium (Table S4). The media were supplemented with MS-3B for *H. callitrichoides* (Cuba) and SH+MSVit for *R. fluitans* along with 20 g/L sucrose and 1.0 g/L agar for both plants.

Acclimatization of *in vitro* plants to the aquarium *H. callitrichoides* and *R. fluitans* shoots propagated in *in vitro* conditions were removed from the culture vessels and the agar was completely washed and cleaned. Then, the shoot clumps were transferred to aquariums with a temperature of 24°C, pH of 6.5–7.2, and a water hardness of 120–140 ppm. As the light source, 4 colors of light (daylight, white, blue, and red) were used at a height of 20 cm from the aquarium. A mixture of sera aqua clay, stream gravel, volcano, and black plant granular sand was placed on the aquarium floor. Water changes were made at the rate of 1/3 twice a wk.

Statistical analysis All experiments were set up with 4 replications; each consisted of 4 explants. The measurement of clump diameter, growth, and rooting observations was carried out 4 wk after the beginning of the culture. The data obtained were subjected to variance analysis (ANOVA) using Minitab Statistical Program. The difference between the means was determined by the Tukey test, and box plots were constructed for clump diameter and rooting of both plants.

Modeling procedures In this study, *in vitro*–induced clump diameter was the output parameter in response to different input parameters like agar, sucrose, PGRs, and types of basal mediums. ANN-based multilayer perceptron (MLP) and two decision tree–based machine learning algorithms—random forest (RF) (Pavlov 2019), and extreme gradient boosting (XGBoost; Chen and Guestrin 2016)—were used as models for data analysis. For simultaneous training of many trees, RF uses bagging, also known as bootstrap aggregation, and the outcome is determined by the decision of the trained tree. Equation 1 presents the fundamental idea behind the RF model.

$$y = \sum_{i=1}^{n} \left(\alpha_i - \alpha_i^*\right) k\left(x, x_i\right) + b \tag{1}$$

y = observed value of the data point,

n = number of samples.

With the help of boosting, XGBoost creates a group of sequential tree models whose combined efforts help to improve the final result (Chen and Guestrin 2016). The objective function of XGBoost that needs to be lowered is shown in Eqs. 2 and 3, which also represent the model of XGBoost at the *j*th iteration.

$$y_i = F\left(x_i\right) = \sum_{(d=1)}^{D} f_d\left(x_i\right), f_d \in F, i = 1, \ldots, n \tag{2}$$

$$L_j = \sum_{(i=1)}^{n} l\left(y_i, \hat{y}_i^{(j-1)} + f_j\left(x_i\right)\right) + \Omega\left(f_j\right) \tag{3}$$

A type of feedforward neural network called a multilayer perceptron (MLP) is composed of multiple layers of processing nodes connected in a feedforward manner. It has three completely interconnected layers (one or more): input, output, and hidden. Backpropagation is used to train the data until lowering Eq. 4 to update the error-related weights and biases (Katırcı *et al.* 2021).

$$E = \frac{1}{K} \sum_{k=1}^{K} \left(y_k - \hat{y}_k\right)^2 \tag{4}$$

y : observed value of data point k,

k : number of samples.

The leave-one-out cross-validation (LOO-CV) technique was employed to evaluate the model's performance (Webb *et al.* 2011). Leave-one-out cross-validation is fundamentally a special form of cross-validation, where the number of folds

and instances of data are equal. The learning algorithm is applied to each instance individually while using the chosen instance as the single-item test set, and all other instances as the training set. LOO-CV is more suitable when there is a small sample size since it will use more training samples in each iteration, allowing the model to learn more efficiently from the data. To find the optimal hyperparameters and build the best model, grid search approach was used. The open-source Python programming language (Van Rossum and Drake 2009) was used for coding purposes with the aid of the sklearn library (Pedregosa *et al.* 2011). Three separate performance indicators were used to evaluate the effectiveness of each model. The coefficient of determination (R^2) demonstrates how well the model and dependent variables are related (Eq. 5). Its values range between $0 \leq R^2 \leq 1$, and values closer to 1 suggest greater model performance. The mean square error (MSE) shows how well a regression line corresponds to the observed data points (Eq. 6), and its value varies between $0 \leq MSE \leq \infty$. The average size of the differences between the observed and predicted value is measured by mean absolute error (MAE) (Eq. 7).

$$R^2 = 1 - \left(\frac{\sum_{i=1}^{n} \left(Y_i - \hat{Y}_i \right)^2}{\sum_{i=1}^{n} \left(Y_i - \overline{Y} \right)^2} \right) \tag{5}$$

$$\mathrm{MSE} = \frac{\sum_{i=1}^{n} \left(Y_i - \hat{Y}_i \right)^2}{n} \tag{6}$$

$$\mathrm{MAE} = \frac{1}{n} \sum_{i=1}^{n} \left| Y_i - \hat{Y}_i \right| \tag{7}$$

where

Y_i = observed value

$\hat{Y}_i$ = predicted value

$\overline{Y}$ = observed values mean

n = count of samples

Data scaling is a technique that is typically used in machine learning during the data preparation phase. It allows the inputs to be transformed to become dimensionless and/or have similar distributions, and ultimately helps to increase the data quality and algorithm performance. In this study, all input variables were normalized to have values between 0 and 1 by using the below formula (8).

$$X_n = \frac{X_i - X_{\min}}{X_{\max} - X_{\min}} \qquad 0 \leq X_n \leq 1 \tag{8}$$

where X_i is the observed data, X_n is the normalized data, and $X_{\max}$ and $X_{\min}$ are the maximum and minimum observed data points, respectively.

Results and Discussion

In vitro regeneration of both plants was statistically (Tables S1–S4) affected by all input parameters (sucrose, agar, and plant growth regulators). Regeneration and development of new shoots started approximately 1 wk after the culture initiation (Figs. 1*a* and 2*a*). The measurement of clump diameter, growth, and observations was carried out after 4 wk of culture. The box plot analysis of the clump diameter (Fig. 3*a–h*) of both plants in response to all input variable results was also performed. Box plot presents the graphical summarization of data in five different sets of median, minimum, maximum, and 1st and 3rd quartiles. In this way, the impact of input factors on their respective output can be analyzed more precisely. The results of the boxplot analysis revealed the variable response of all input factors on output parameters for both plants (Zulfiqar *et al.* 2021). Results further revealed no abnormal data for all output parameters of both plants.

Optimization of basal medium Tissue culture basal media are comprised of different macro- and micronutrients along with certain vitamins at variable concentrations. The variable components of these basal media help to propagate plants under *in vitro* conditions. To date, the number of basal mediums has been optimized and used successfully for different plant species even for recalcitrant plants. However, their requirement is dependent on the number of variables like genotype, explant, and objective of the study (Malik *et al.* 2004). In aquatic plants, optimization of the most efficient and suitable basal medium is limited (Doğan 2022), and studies are confined mainly to the concentration rather than the type of basal mediums (Aasim *et al.* 2019b). The most commonly used basal mediums in aquatic plants are MS (Zote *et al.* 2018), B5 (Koul *et al.* 2014), and SH medium (Ozcan *et al.* 2021). In the present study, twelve different basal mediums were tested for two different aquatic plants, and the results revealed the need for a specific basal medium for efficient *in vitro* regeneration. Five different basal mediums were proved to be efficient for *H. callitrichoides* and statistically similar to each other (Table S1). The shoot-clump diameter in response to these five different basal mediums was 5.57 cm for MS followed by 5.53 cm for MS No:3B, 5.43 cm for Anderson's Rhododendron Medium, 5.38 cm for SH +

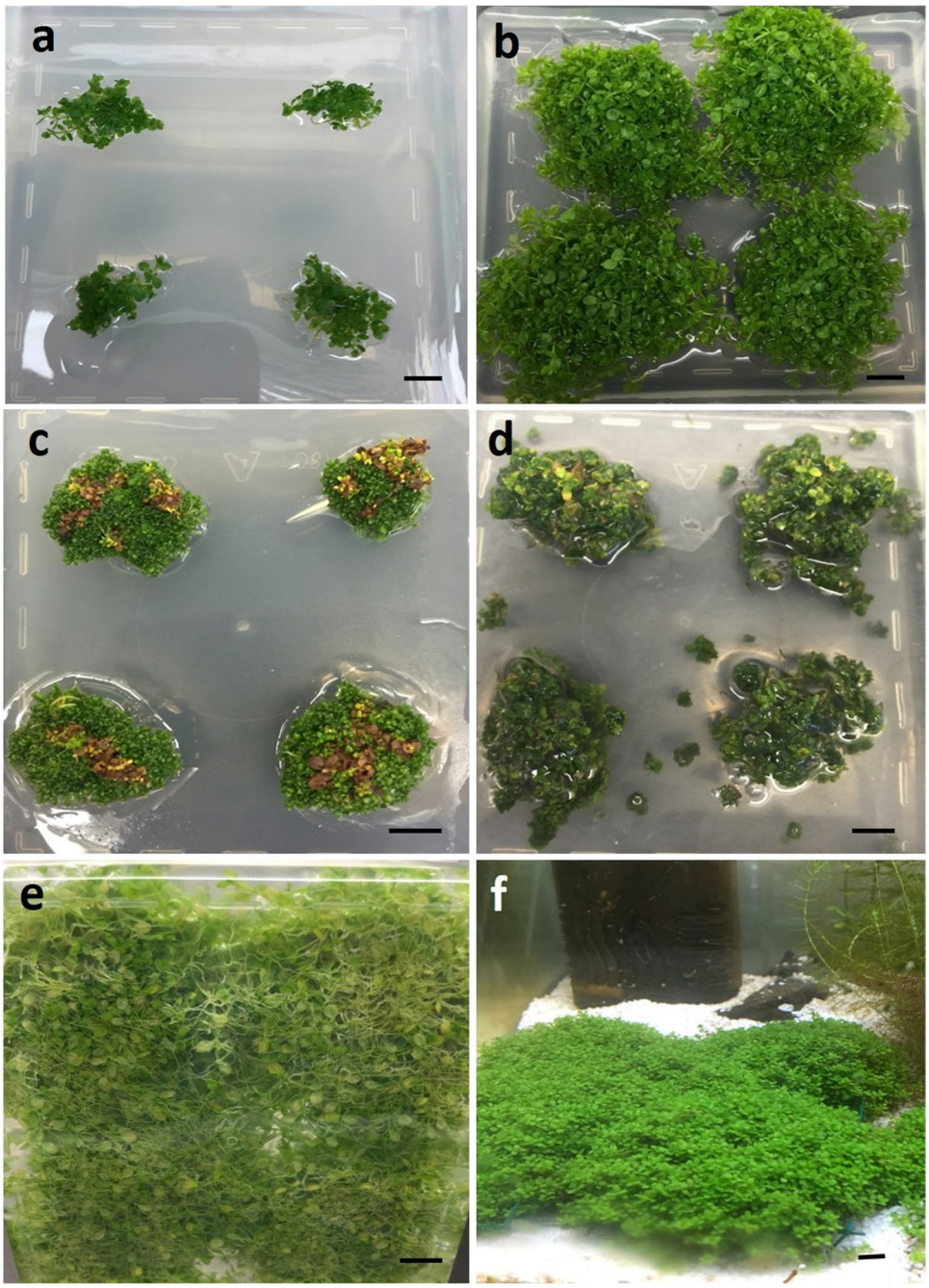


Figure. 1 *In vitro* micropropagation of *H. callitrichoides* on different tissue culture media and acclimatization to the aquarium. (***a***) Micropropagation on MS No:3B medium supplemented with 20 g/L sucrose, 1 g/L agar, and without PGR after 1 wk of culture, and (***b***) after 4 wk of culture. (***c***) Micropropagation on MS No:3B medium supplemented 60 g/L sucrose, 1 g/L agar, and without PGR. (***d***) Micropropagation on MS No:3B medium supplemented 30 g/L sucrose, 1 g/L agar, 1 mg/L BAP, and 0.5 mg/L NAA. (***e***) Root formation on regenerated shoots in MS No:3B medium supplemented 20 g/L sucrose, 1 g/L agar, and without PGR. (***f***) Acclimatization of *in vitro* plants to the aquarium. *Bar* = 1 cm.

MSVit, and 5.04 cm for SH medium. To select the best basal medium among these five, the best shoot and root development in the culture medium was attributed to MS No:3B (including ½ conc. NH_4NO_3) medium compared to other basal mediums (Fig. 1*b*, *e*; Table S1). Therefore, this medium was selected for the optimization of other parameters for *H. callitrichoides*.

The response of *R. fluitans* to different basal mediums was different and the diameter of *in vitro* regenerated shoot clumps ranged from 1.82 to 3.65 cm (Table S1). The maximum diameter was attained from medium supplemented with SH+MSVit (Fig. 2*b*) followed by Orchimax medium. However, SH+MSVit medium proved to be superior to Orchimax medium for *in vitro* root growth and development (Table 1). Supplementation of Lindemann Orchid medium was the least responsive for both plants with the lowest clump diameter without any root growth (Fig. 1*e*; Table S1). The results obtained highlighted the need for a specific basal medium for each plant. However, there is a need of optimizing the concentration of these basal mediums to produce more economic plants.

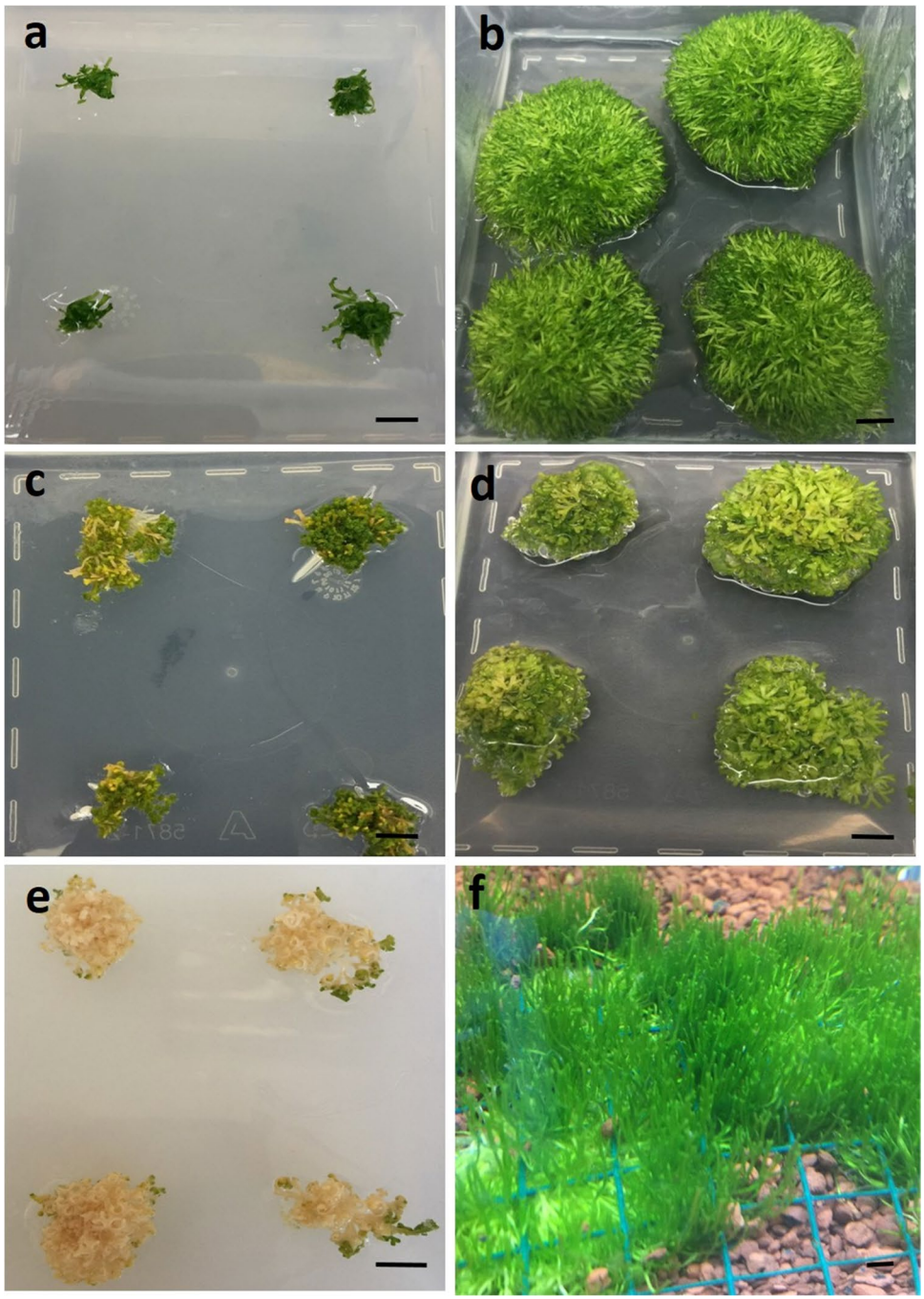


Figure. 2 *In vitro* micropropagation of *R. fluitans* on different tissue culture media and acclimatization to the aquarium. (***a***) Micropropagation on SH+MSVit medium supplemented with 20 g/L sucrose, 1 g/L agar, and without PGR after 1 wk of culture, and (***b***) after 4 wk of culture. (***c***) Micropropagation on SH+MSVit medium supplemented 60 g/L sucrose, 1 g/L agar, and without PGR. (***d***) Micropropagation on SH+MSVit medium supplemented 30 g/L sucrose, 1 g/L agar, 1 mg/L BAP, and 0.5 mg/L NAA. (***e***) Micropropagation on Lindemann Orchid medium supplemented 20 g/L sucrose, 1 g/L agar, and without PGR. (***f***) Acclimatization of *in vitro* plants to the aquarium. *Bar* = 1 cm.

Optimization of sucrose concentration The provision of sucrose in the culture medium is the source of reducing or non-reducing carbon for the *in vitro* regenerated plantlets and controls the *in vitro* morphogenesis process (Yaseen *et al.* 2013; Aasim *et al.* 2019b). The most used concentration of sucrose in the culture medium is 30 g/L with exceptions of low or high sucrose concentration used based on species and final target of the experiment. The incorporation of different sucrose concentrations exerted a significant and variable impact on *in vitro* plant regeneration of both plants (Table S2). The application of any sucrose concentration exerted a positive impact on the clump diameter of *H. callitrichoides* and resulted in a maximum of 4-fold more diameter than control explants. Clump diameter ranged from 1.64 to 5.43 cm on media supplemented with variable sucrose concentration as compared to control which resulted in minimum clump diameter (1.36). The highest clump diameter was attained from the medium provided with 20 g/L sucrose (Fig. 1*b*; Table S2). Clump diameters of 10, 20, and 30 g/L sucrose were statistically similar to each other and contrary

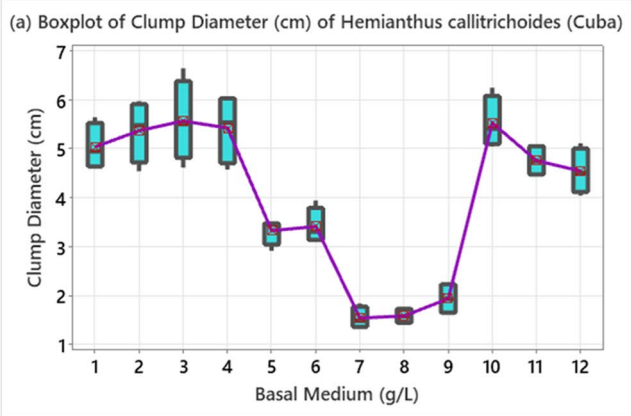
to the previous findings (Barpete *et al.* 2015). They achieved the highest clump diameter of *H. callitrichoides* at medium enriched with 30 g/L sucrose. They also achieved the highest clump diameter on a medium supplemented with 45 g/L sucrose. The further increase of sucrose was detrimental in their study, supporting the current results of this study. It was also notable that a medium without sucrose induced browning and necrosis on the explants (Barpete *et al.* 2015), whereas the application of 60 g/L sucrose induced stress on the clumps (Fig. 1*c*). On the other hand, direct rooting was also maximum on medium supplemented with 20 g/L sucrose (Fig. 1*e*) followed by 10 and 30 g/L respectively. There was no rooting on the medium enriched with 50 and 60 g/L sucrose (Table 1).

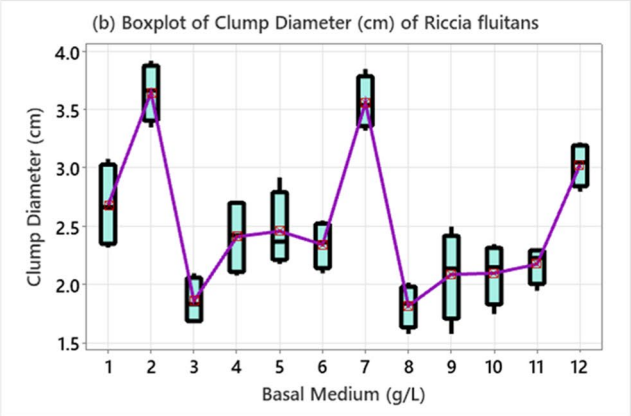
The results of sucrose concentration showed similar effects on *in vitro* regeneration of *R. fluitans* (Table 2). Supplementation of 20 g/L sucrose resulted in the highest clump diameter (Fig. 2*b*), whereas a further increase of sucrose in the culture medium reduced clump diameter significantly (Fig. 2*c*). The diameter ranged from 1.58 to 5.21 cm with maximum and minimum attributed to medium enriched with 20 g/L and 60 g/L sucrose, respectively. The rooting was only observed on medium supplemented with 20 and 30 g/L sucrose (Table 1). However, the rooting was comparatively less than *H. callitrichoides*. From these results, it is concluded that 20 g/L sucrose was found optimum for *in vitro* regeneration of both *H. callitrichoides* and *R. fluitans.* The results on both plants suggest the need for sucrose at variable concentrations for maximum regeneration. A similar type of results has been reported for other crops like *Bacopa monnieri* at the rate of 30 g/L (Ranjan and Kumar 2018) or 20 g/L (Ranjan *et al.* 2018).

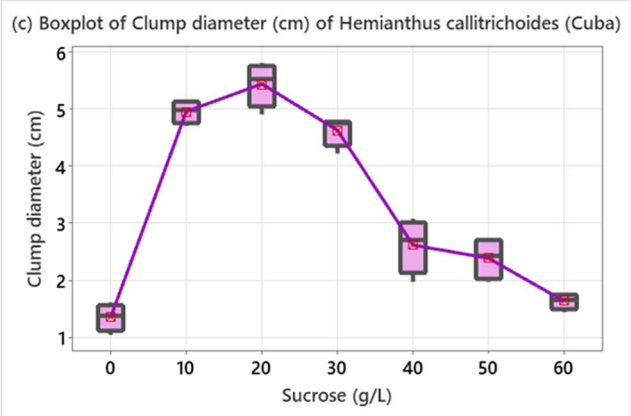
Optimization of agar concentration Gelling agents and their concentration in the culture medium play a significant role in the *in vitro* regeneration of aquatic plants (Dogan 2022). Several studies revealed the use of variable gelling concentration from solidified to semi-solidified or liquid medium (Karataş *et al.* 2014; Dogan *et al.* 2015). Gelling agents and their concentration not only regularize the *in vitro* regeneration but also control the size and the number of plants propagated (Aasim *et al.* 2019b). Supplementation of different agar concentrations exerted a significant impact on *H. callitrichoides* and insignificant for *R. Fluitans* (Table S3). Agar concentration up to 1–4 g/L induced more clump diameter (5.13–5.52 cm) compared to medium without agar (4.29 cm) for *H. callitrichoides*. The maximum clump diameter (5.52 cm) was attained from the medium supplemented with 1 g/L agar (Fig. 1*b*) followed by 3 g/L (5.30 cm), and 2 g/L agar (5.20 cm). It was also noted that regenerated clump was healthier and more greenish in appearance but with relatively small and rounded shape leaves on the control medium, solidified with 1 and 2 g/L agar. Supplementation of 5 g/L and above up to 7 g/L agar induced comparatively less clump diameter than the control. On the other hand, 1 g/L agar also induced more rooting than all other concentrations (Fig. 1*e*; Table 1).

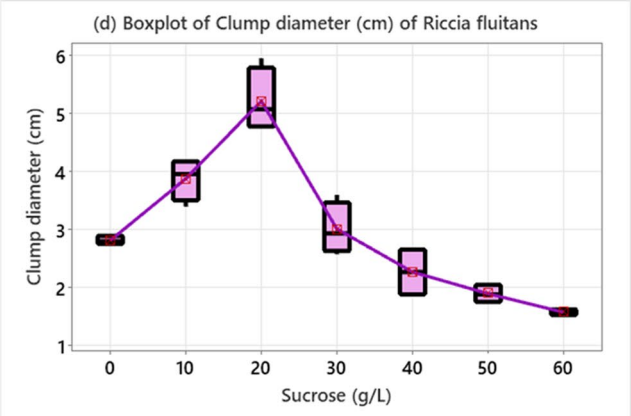
Statistically insignificant clump diameter was observed from different agar concentrations for *R. fluitans* and ranged from 3.27 to 4.04 cm (Table 3). The highest diameter (4.04 cm) was attained from medium gelled with 1 g/L (Fig. 2*b*). The lowest diameter of *R. fluitans* was recorded from medium solidified with 4 g/L agar. On the other hand, the highest rooting on clumps was obtained on the liquid medium (0 g/L agar) followed by medium gelled with 1 g/L (Table S3). It was also noted that regenerated plants grew upward with elevated agar concentration. On the other hand, an interesting outcome was the reduced rooting with elevated agar concentration (Table 1). Results revealed that medium solidified with 1 g/L agar is more suitable followed by medium without agar (liquid medium). Based on the results, medium solidified with 1 g/L was found to be more suitable for both aquatic species. Previous studies on aquatic plants revealed the use of variable gelling agents and their concentration (Aasim *et al.* 2019b). Different studies on *B. monnieri* (the most widely *in vitro* propagated aquatic plant) revealed the use of agar at higher concentrations as a gelling agent (Ranjan *et al.* 2018). However, there are studies where researchers used liquid medium for *in vitro* propagation of different aquatic plants like *Hygrophila polysperma* (Karataş *et al.* 2014), and *Ceratophyllum demersum* (Dogan *et al.* 2015). These studies highlighted the superiority of liquid medium over solid or semi-solidified culture medium. The use of a liquid basal medium with variable MS concentrations and full-strength MS medium was found more suitable for *H. callitrichoides* (Ng *et al.* 2016). On the other hand, some studies enlighten the negative impact of the liquid medium on *in vitro* regeneration of aquatic plants (Karatas *et al.* 2014). All explants of *Rotala macrandra* cultured on a liquid medium supplemented with BAP and NAA died. Similarly, both agar-solidified and liquid medium for *Veronica anagallis-aquatica* were tested and the agar-solidified medium was more responsive than the liquid medium (Shahzad *et al.* 2011).

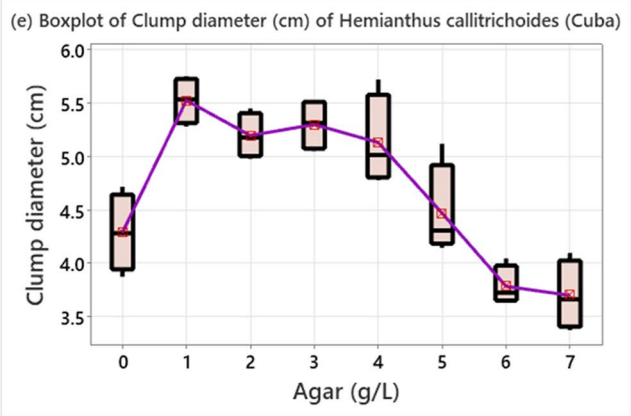
Optimization of BAP and NAA concentration Supplementation of PGRs in the culture medium plays a major role in inducing *in vitro* organogenesis of aquatic plants. Application of PGRs in the culture medium exerted a negative impact on *in vitro* regeneration of both species (Fig. 1*d*; Fig. 2*d*). The diameter of *H. callitrichoides* ranged from 2.16 to 3.20 cm (Table S4) compared to the control that was recorded highest (5.51 cm). In previous studies, *in vitro* plant development also decreased with elevated BAP concentration (Barpete *et al.* 2015) irrespective of the presence or absence of NAA in the culture medium (Ing *et al.* 2019). The negative

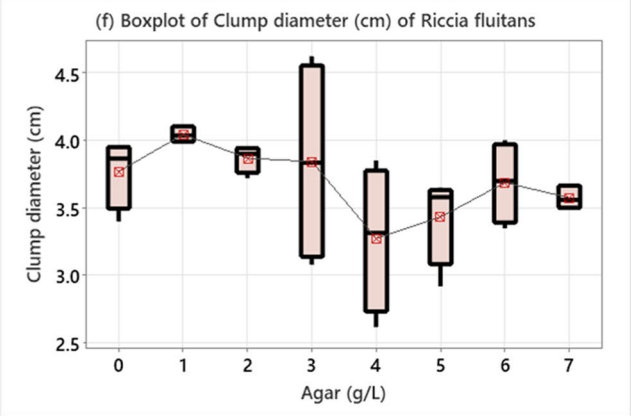

(a) Boxplot of Clump Diameter (cm) of Hemianthus callitrichoides (Cuba)
Clump Diameter (cm)
Basal Medium (g/L)

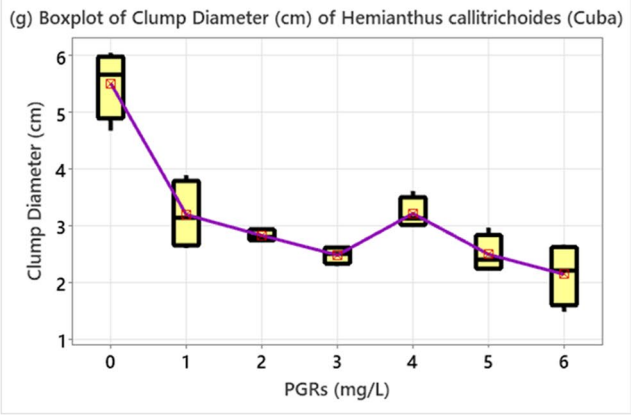

(b) Boxplot of Clump Diameter (cm) of Riccia fluitans
Clump Diameter (cm)
Basal Medium (g/L)

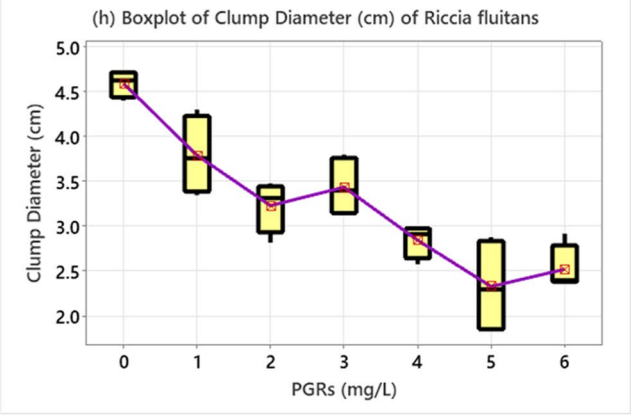

(c) Boxplot of Clump diameter (cm) of Hemianthus callitrichoides (Cuba)
Clump diameter (cm)
Sucrose (g/L)


(d) Boxplot of Clump diameter (cm) of Riccia fluitans
Clump diameter (cm)
Sucrose (g/L)


(e) Boxplot of Clump diameter (cm) of Hemianthus callitrichoides (Cuba)
Clump diameter (cm)
Agar (g/L)


(f) Boxplot of Clump diameter (cm) of Riccia fluitans
Clump diameter (cm)
Agar (g/L)


(g) Boxplot of Clump Diameter (cm) of Hemianthus callitrichoides (Cuba)
Clump Diameter (cm)
PGRs (mg/L)


(h) Boxplot of Clump Diameter (cm) of Riccia fluitans
Clump Diameter (cm)
PGRs (mg/L)

◂**Figure. 3** Box plot for the clump diameter of *H. callitrichoides* and *R. fluitans*. (***a***, ***b***) Clump diameter in response to basal medium, (***c***, ***d***) sucrose concentration, (***e***, ***f***) agar, and (***g***, ***h***) PGRs.

impact of elevated BAP concentration in the liquid medium was also reported in *H. polysperma* (Karataş *et al.* 2014). On the other hand, increased shoot regeneration frequency and shoot counts of *C. demersum* were also reported with elevated PGRs (Karatas *et al.* 2014). Enrichment of medium with BAP and NAA also showed a negative impact on rooting in the current study. Earlier rooted clumps of *H. callitrichoides* (Barpete *et al.* 2015) cultured on medium without any PGRs were also documented. A similar type of toxic impact of BAP and NAA was also attributed to *R. fluitans* on diameter and rooting compared to the control group (Table 1). The diameter of *in vitro* regenerated *R. fluitans* ranged from 2.52 to 3.79 cm (Table S4) on a medium supplemented with BAP and NAA as compared to the control. Supplementation of NAA with BAP was more toxic and detrimental than medium enriched with BAP alone. The provision of BAP and NAA was more detrimental to rooting and recorded zero. Overall, rooting was also low on clumps of *in vitro* regenerated *R. fluitans.* These results suggested that culture medium without any PGRs is more suitable for inducing larger clumps for both species. Results on both species revealed that supplementation of PGRs in the culture medium was more detrimental than medium devoid of any PGRs. Therefore, both species can be propagated without supplementing any PGRs and it will also enable to produce economic plants *in vitro*.

Acclimatization of *in vitro* plants to the aquarium After shoot clumps of *H. callitrichoides* and *R. fluitans* species formed, they were removed from the culture containers, and the agar contained in their roots was completely washed in tap water. Then, the shoots were transferred to aquariums where temperature, light, and pH were controlled. All transplanted plants have adapted to aquarium conditions. After 10 d of transferring to the aquarium, the plants started to grow and 3 wk later there was a significant increase in the growth and new shoots started to appear from the bottom parts of the plants (Fig. 1*f*; Fig. 2*f*). The successful use of *in vitro* regenerated plants in the aquariums has been documented for different aquatic plants by employing different substrates and aquarium conditions (Aasim *et al.* 2019b). Acclimatization of aquatic plants in aquariums or water bodies is the critical step and documented for different aquatic species (Karimi Alavijeh *et al.* 2022; Parzymies *et al.* 2022). However, studies revealed the provision of different culture conditions ranging from substrate to gases (CO_2, O_2), and light from different sources.

Machine learning modeling analysis The majority of *in vitro* culture procedures are challenging to comprehend due to their complex behavior (Qureshi *et al.* 2021). Although the use of different traditional statistical approaches to interpreting the dataset generated from *in vitro* regeneration experiments is extensive, these statistical approaches are not efficient to interpret the results when compared with AI-based models (Niazian and Niedbała 2020; Hesami and Jones 2021; Jafari *et al.* 2022). However, it is not a simple methodology, and certain conditions like data (size, collection, quality) and model fit may lead to uncertainty (Jamshidi *et al.* 2020; Pepe *et al.* 2021; Hesami *et al.* 2022). Therefore, the selection of a proper model, normalization of data, optimization of hyperparameters, validation of models, etc. are highly significant. In plant tissue culture studies, the use of AI/ML-based models is increased substantially in recent years, with a successful application of regression (Aasim *et al.* 2023a) classification (Aasim *et al.* 2022b; Mirza *et al.* 2022), or hybrid (Hesami *et al.* 2019; Salehi *et al.* 2021; Aasim *et al.* 2023b) models for optimization have been employed for specific targets. In this study, ANN-based MLP and decision tree–based RF and XGBoost models were employed for data prediction and validation of the clump diameter and *in vitro* rooting of two different aquatic plants. A recent study on the aquatic plant also demonstrated the successful use of these three models to check the impact of LEDs lights (Aasim *et al.* 2022a).

The impact of different input parameters like nutrient medium, sucrose concentration, agar concentrations, and PGRs exhibited relatively high R^2 scores for the clump diameter of *H. callitrichoides.* It is evident from the results that XGBoost has minimum R^2 scores for all input variables compared to the RF and MLP models. However, maximum R^2 scores were recorded from either the RF or MLP model, depending on the input variable. Maximum R^2 scores of clump diameter in response to input variables were recorded for RF for nutrient medium (0.890) and agar concentration (0.775), whereas the MLP model exhibited maximum R^2 scores of 0.957 for sucrose and 0.845 for PGRs. In comparison to R^2, the maximum MSE and MAE (0.462) values were recorded from the XGBoost model for all input parameters studied (Table 2). The impact of different input parameters like nutrient medium, sucrose, agar concentrations, and PGRs demonstrated relatively low R^2, MSE, and MAE score values. The maximum R^2 score of 0.915 was recorded for the RF model from sucrose followed by 0.794 from the MLP model. It was also evident that the maximum R^2 score for all models was higher from sucrose concentration compared to other mediums. The maximum MSE score was recorded from XGBoost models for all input parameters and recorded as 0.155 for agar concentration, 0.148 for sucrose, 0.130 for PGRS, and 0.091 for the nutrient medium variable. In the case of MAE, the maximum score value of

Table 1. Effects of different input parameters on *in vitro* rooting frequency (%) of *H. callitrichoides* and *R. fluitans*

	H. callitrichoides	*R. fluitans*
Nutrient medium		
SH (Schenk and Hildebrandt medium)	43.2±8.22 c	81.5±2.82 b
SH + MSVit (Murashige and Skoog vitamin mixture)	44.0±3.94 c	94.5±6.50 a
MS (Murashige and Skoog medium)	30.2±7.72 cde	40.9±6.77 e
Anderson's Rhododendron medium	6.5±2.90 fg	15.2±3.99 f
CHU(N6) medium	22.7±5.38 de	55.2±8.12cd
McCown woody plant medium	18.1±6.59 ef	43.1±5.44de
Orchimax medium	80.2±7.14 ab	64.1±7.92 c
Lindemann Orchid medium	0.0±0.00 g	0.0±0.00 g
Knudson C Orchid medium	0.0±0.00 g	0.0±0.00 g
MS Mod.No:3B (½ concentration NH_4NO_3 and KNO_3)	95.1±12.29 a	43.2±2.44de
MS Mod.No:1B (½ conc. macroelements)	94.4±6.56 a	38.4±3.20 e
SH+B_5 vitamins	37.5±4.40 cd	61.5±3.45 c
BAP +NAA (mg 1^{-1})		
0+0	81.1±4.74 a	54.1±3.02
0.5+0	0.0±0.00	0.0±0.00
1.0+0	7.2±1.29 c	0.0±0.00
2.0+0	0.0±0.0 d	0.0±0.00
0.5+0.5	17.1±2.90 b	0.0±0.00
1.0+0.5	0.0±0.00 d	0.0±0.00
2.0+0.5	0.0±0.00 d	0.0±0.00
Sucrose (g L^{-1}) (*p* 0.05)		
0	0.0±0.00 e	0.0±0.0 d
10	60.7±3.85 b	8.2±2.88 c
20	79.2±5.71 a	50.6±5.43 a
30	44.9±5.15 c	24.8±5.12 b
40	21.4±3.12 d	6.3±3.93 cd
50	0.0±0.00 e	0.0±0.00 d
60	0.0±0.00 e	0.0±0.00 d
Agar (g L^{-1}) (*p* 0.05)		
0	24.3±4.40 de	98.1±2.31 a
1	97.5±3.65 a	59.5±6.43 b
2	41.1±2.77 b	39.6±2.37 c
3	40.6±6.38b	45.7±3.69 c
4	36.4±5.36 bc	22.5±4.67 d
5	31.1±5.92 bcd	17.4±5.34 d
6	27.8±5.15 cde	8.1±2.58 e
7	18.9±5.20 e	0.0±0.00 e

All *letters* presented in the table are statistically significant at *p* 0.05 + SD

agar concentration was from the RF model while maximum MAE scores for all input parameters were recorded from the XGBoost model of *R. fluitans* (Table 2). The graphical presentation of the actual and predicted values of clump diameter of different models is presented in Fig. 4*a–d* (*H. callitrichoides*) and Fig. 4*e–h* (*R. fluitans*).

The effects of various input factors (nutrient medium, sucrose concentration, agar concentrations, and PGRs) on the rooting rate of *H. callitrichoides* revealed a similar pattern for the MLP, RF, and XGBoost model for the rooting rate of *H. callitrichoides*. The RF model generated relatively more R^2 scores for nutrient medium (0.943), sucrose (0.983), and PGRs (0.984). On the contrary, the maximum R^2 score of agar was recorded from the MLP medium. The minimum MSE and MAE scores were recorded from models with maximum R^2 scores for all input parameters (Table 3). The rooting rate of *R. fluitans* also exhibited a similar pattern to the rooting rate of *H. callitrichoides*. The maximum R^2

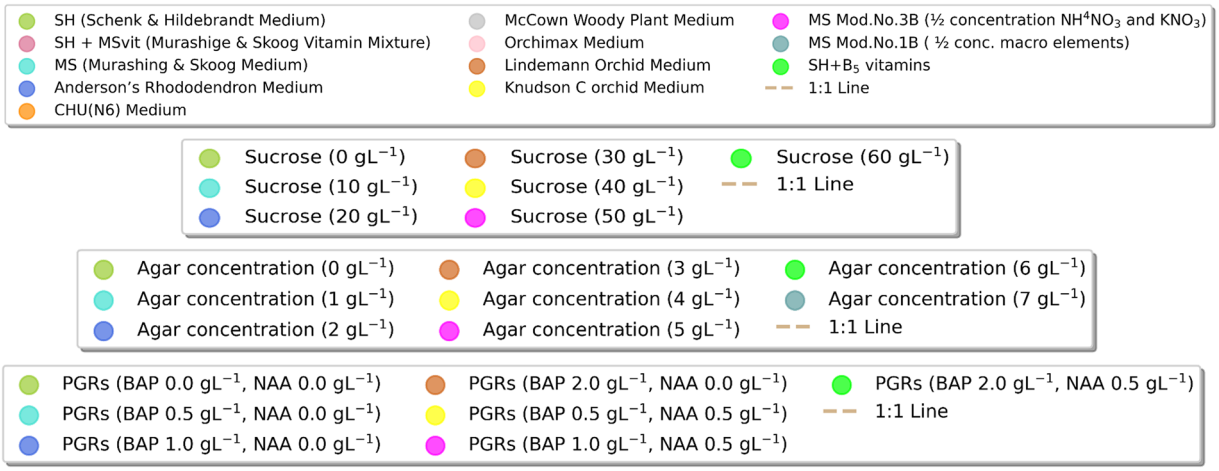
Table 2. Performance metrics of ANN and decision tree–based ML models for clump diameter

Performance metrics	*H. callitrichoides* (clump diameter)					
	Nutrient medium			Sucrose		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.885	0.890	0.859	0.957	0.951	0.938
MSE	0.285	0.271	0.348	0.106	0.121	0.154
MAE	0.423	0.409	0.462	0.253	0.274	0.309
	Agar concentration			PGRs		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.774	0.775	0.749	0.845	0.843	0.806
MSE	0.116	0.115	0.128	0.187	0.189	0.234
MAE	0.296	0.285	0.298	0.336	0.335	0.381
Performance metrics	*R. fluitans* (clump diameter)					
	Nutrient medium			Sucrose		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.794	0.773	0.770	0.907	0.915	0.897
MSE	0.081	0.089	0.091	0.133	0.123	0.148
MAE	0.228	0.247	0.259	0.287	0.261	0.293
	Agar concentration			PGRs		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.282	0.091	0.055	0.794	0.789	0.788
MSE	0.118	0.149	0.155	0.126	0.129	0.130
MAE	0.277	0.284	0.280	0.283	0.295	0.297

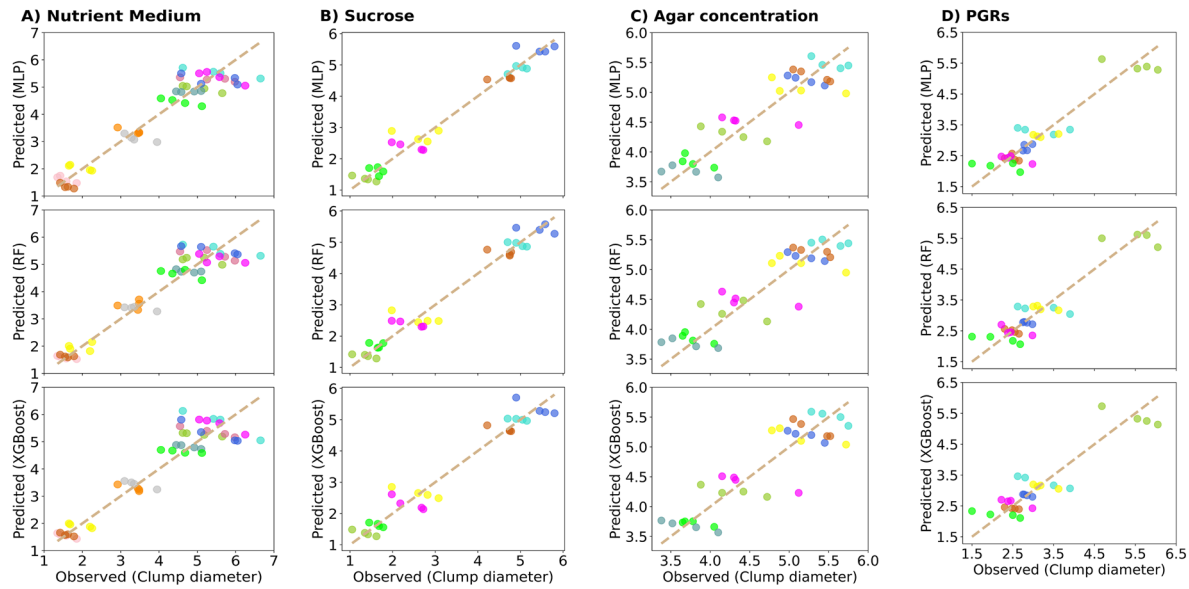
Table 3. Performance metrics of ANN and decision tree–based ML models for rooting rate

Performance metrics	*H. callitrichoides* (rooting rate)					
	Nutrient medium			Sucrose		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.938	0.943	0.844	0.970	0.983	0.918
MSE	58.611	54.227	147.138	27.968	15.478	75.197
MAE	5.757	5.663	9.183	3.787	2.653	5.431
	Agar concentration			PGRs		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.959	0.945	0.946	0.979	0.984	0.903
MSE	22.673	30.251	29.464	16.423	11.986	74.412
MAE	4.212	4.792	4.725	3.057	1.786	4.572
Performance metrics	*R. fluitans* (rooting rate)					
	Nutrient medium			Sucrose		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.927	0.959	0.841	0.929	0.961	0.879
MSE	59.645	33.145	128.820	22.116	12.296	37.609
MAE	5.749	4.170	8.650	2.962	2.322	3.534
	Agar concentration			PGRs		
	MLP	RF	XGBoost	MLP	RF	XGBoost
R^2	0.980	0.980	0.898	0.987	0.996	0.995
MSE	17.689	18.473	91.615	4.736	1.402	1.850
MAE	3.051	3.134	6.417	0.843	0.415	0.481

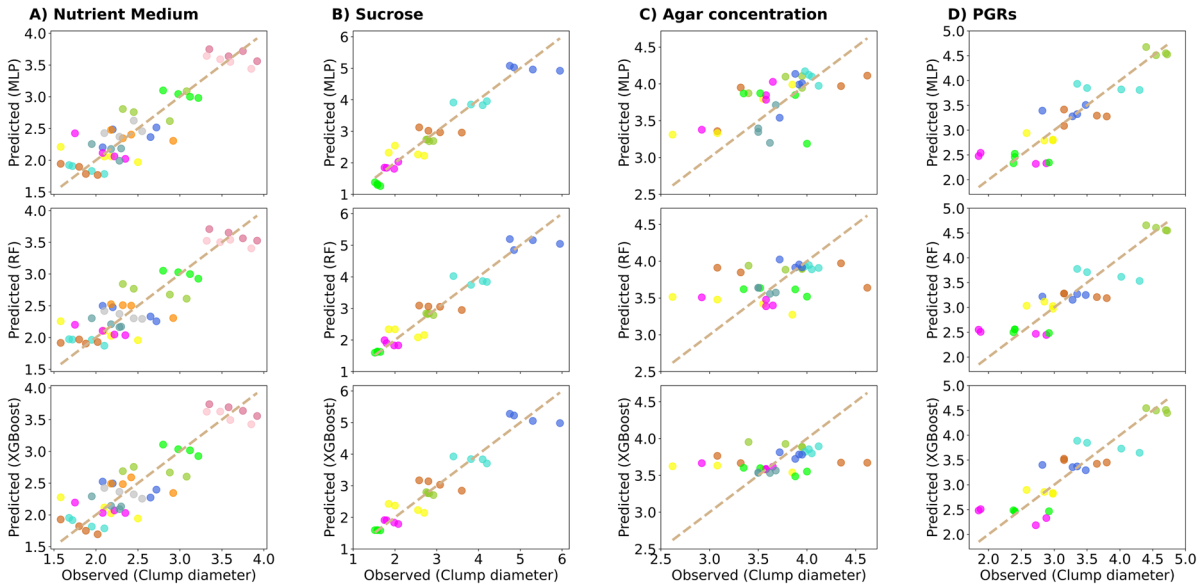
scores with low MSE and MAE scores for nutrients, sucrose, and PGRs were documented from the RF model. On the contrary, the maximum R^2 scores with minimum MSE and MAE scores were documented from the MLP model (Table 3).



a) Hemianthus callitrichoides (Clump)



b) Riccia fluitans (Clump)



◂**Figure. 4** Observed and predicted clump diameter values for the tested models aimed at different input parameters for *in vitro* regeneration of *H. callitrichoides* and *R. fluitans*.

The actual and predicted values of clump diameter and rooting frequency were also presented through a graphical presentation, inclined on 1:1 line. The graphical presentation of the actual and predicted values of clump diameter of different models is presented for *H. callitrichoides*) (Fig. 4*a–d*) and *R. fluitans* (Fig. 4*e–h*). Figure 5*a–d* and Fig. 5*e–h* present the distribution of the actual and predicted values of rooting frequency respectively for *H. callitrichoides* and *R. fluitans*. The predicted values close to actual data are placed on or near the line. The distribution of data is also dependent

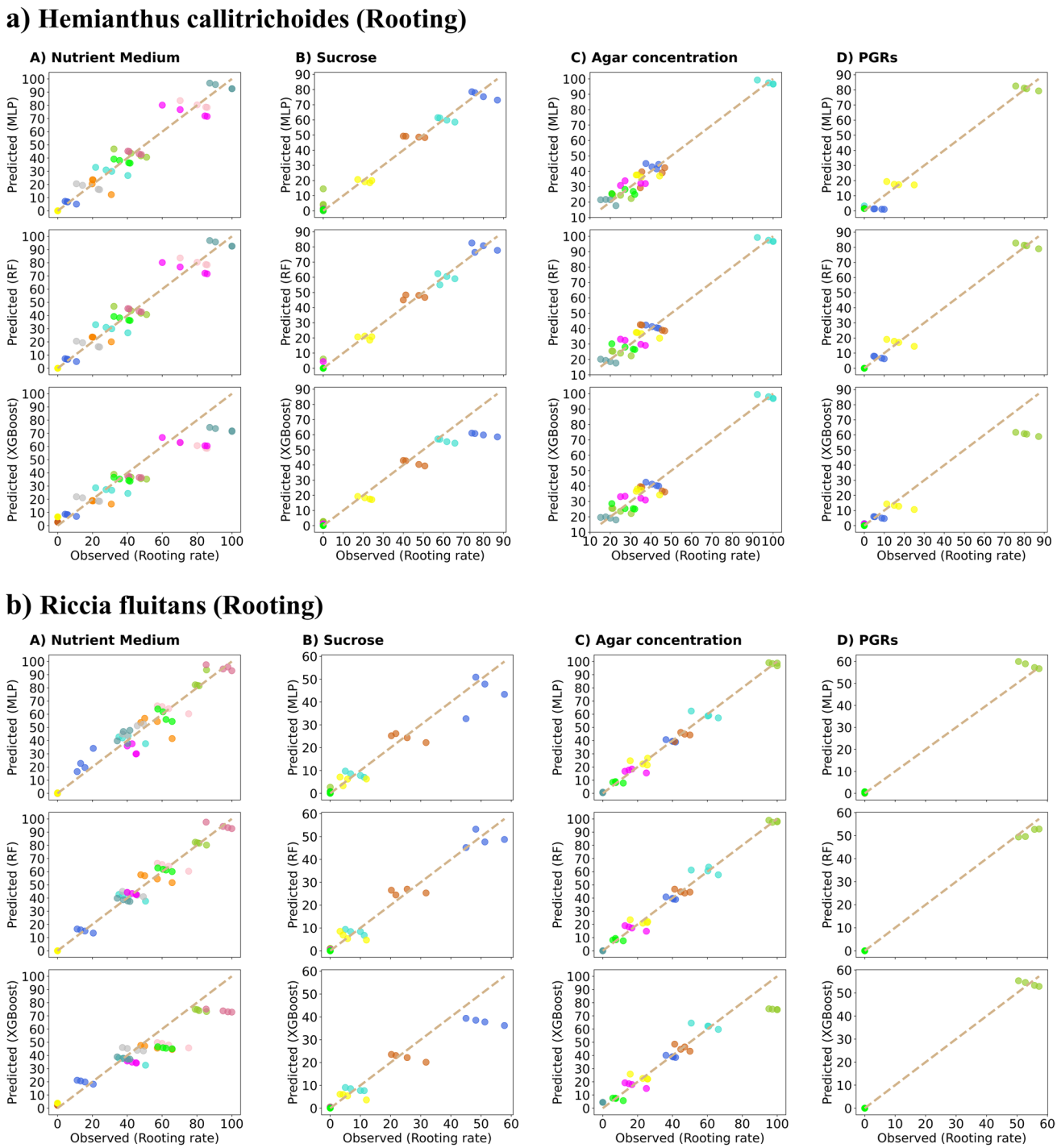


Figure. 5 Observed and predicted rooting rate values for the tested models aimed at different input parameters for *in vitro* regeneration of *H. callitrichoides* and *R. fluitans*.

on the values of R^2, and high R^2 values generally result in data inclined on the line (Aasim *et al.* 2022a).

All performance scores were found to be relatively high and close to each other. It was also evident from the results that a similar pattern was documented for all three used performance metrics. The high R^2 scores were supported by low MSE and MAE scores irrespective of the type of model, output parameters, and plant type. Based on these observations, the performance of the models was also evaluated. The performance of the models by three different performance scores revealed the better performance of MLP and RF models. A comparison of models depicted that the performance of the XGBoost model was relatively less when compared to the other models while considering all three performance scores. The performance of both MLP and RF models revealed that the best performance was input dependent for both clump diameter and *in vitro* rooting. An investigation of previous studies depicted the use of AI/ML models for *in vitro* regeneration (Arab *et al.* 2016; Niazian and Niedbała 2020; Niazian and Shariatpanahi 2020), and *in vitro* rooting (Arab *et al.* 2018). The results attained in this study showed the supremacy of AI/ML models for analyzing and optimizing culture conditions in plant tissue culture studies (Jamshidi *et al.* 2019; Farhadi *et al.* 2020; Salehi *et al.* 2021).

Conclusion

This study was designed to optimize the culture conditions for commercial propagation of two aquatic plants with relatively low cost by optimizing the use of expensive inputs like sucrose, agar, and PGRs, at relatively low concentrations. It was determined that the best basic nutrient medium for *H. callitrichoides* was MS No:3B (including ½ conc. NH_4NO_3) and for *R. fluitans* SH medium containing MS vitamins. The use of 20 g/L sucrose as a sugar source gave the highest *in vitro* micropropagation results in both plants, and it was determined that the supplementation of 20 g/L sucrose is sufficient compared to the standard use of 30 g/L sucrose. Results revealed that the addition of 1 g/L agar generated larger clumps for both plants. The provision of BAP and NAA caused a negative effect on the *in vitro* propagation of *H. callitrichoides* and *R. fluitans*, and results showed that these plants can be propagated without any PGRs. The validation of data through ANN and the ML-based models confirmed the attained results by exhibiting high R^2 scores with low MSE and MAE scores for all input parameters used in this study. In conclusion, with this study, a low-cost, fast, and efficient *in vitro* production method was developed for the important aquarium and phytoremediation plants *H. callitrichoides* and *R. fluitans*.

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Author contributions EO did all the laboratory works, statistical analysis, and manuscript writing. HHA designed the experiments, provided guidance for the study, and did a critical reading of the manuscript. MA performed statistical analysis, article writing, and editing. SAA performed artificial intelligence analysis and article editing.

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Data availability The data derived during artificial intelligence studies will be provided after evaluating the request.

Declarations

Ethical approval There is no need for ethical approval for the research.

Competing interests The authors declare no competing interests.

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