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Machine Learning Deciphers Genotype and Ammonium as Key Factors for the Micropropagation of *Bryophyllum* sp. Medicinal Plants

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Abstract: *Bryophyllum* constitutes a subgenus of succulent plants that have been widely employed worldwide in traditional medicine. Micropropagation is required to optimize their growth and reproduction for biotechnological purposes. The mineral composition of culture media is usually an underestimated factor in the design of the in vitro culture protocols of medicinal plants. Universal and highly cited media mineral formulations, such as the Murashige and Skoog (MS) medium, are generally employed in plant tissue culture studies, although they cause physiological disorders due to their imbalanced mineral composition. In this work, neurofuzzy logic is proposed as a machine-learning-based tool to decipher the key factors (genotype, number of subcultures, and macronutrients) that are involved in the establishment of the *Bryophyllum* sp. in vitro culture. The results show that genotype played a key role, as it impacts both vegetative growth and asexual reproduction in all of the species that were studied. In addition, ammonium was identified as a significant factor, as concentrations above 15 mM promote a negative effect on vegetative growth and reproduction. These findings should be considered as the starting point for optimizing the establishment of the in vitro culture of *Bryophyllum* species, with large-scale applications as biofactories of health-promoting compounds, such as polyphenols and bufadienolides.

Keywords: artificial intelligence; neurofuzzy logic; Crassulaceae; *Kalanchoe* genus; medicinal plants; plant biotechnology; in vitro mineral nutrition; macronutrients; MS basal medium

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1. Introduction

In recent decades, the use of medicinal plants has gained much attention in both the biotechnological and pharmacological industries, due to their effectiveness in the folk medicines of several regions throughout the world. In this sense, the in vitro culture of plants constitutes a widely applied and efficient methodology for obtaining valuable and "true-to-type" plant-derived products. Compared with conventional techniques for plant macropropagation, plant tissue culture (PTC) methodology confers great advantages, such as total independence of geoclimatic conditions and genetic conservation, and offers the possibility of improving the biosynthesis of secondary metabolites with health-enhancing properties [1].

Different species belonging to the subgenus *Bryophyllum* (genus *Kalanchoe*, Crassulaceae) have been used in popular medicine in various regions across Asia, Africa, and South America for the treatment of different ailments, such as infections and cardiovascular and neoplastic diseases [1,2]. Pharmacognostical and phytochemical studies have

shown that these medicinal properties are mainly developed by phenolic compounds and bufadienolides [3]. However, these studies have been carried out by following low-throughput protocols, as large amounts of raw materials of plants are required to obtain sufficient active ingredients for widespread use. Therefore, novel approaches must be applied to overcome such limitations, particularly in the face of the further exploitation of *Bryophyllum* as a reliable biotechnological system for industrial purposes.

Plant tissue culture is a challenging methodology, as it depends on multiple factors. Among them is the elucidation of proper mineral nutrition for healthy plant growth in vitro, which is a highly complex task, as minerals show innumerable interactions that play an essential role in many physiological processes of plants [4]. In this sense, predicting the optimal nutritional requirements of plants is a rational approach that should be developed in establishing in vitro plant culture protocols, as such nutritional requirements affect not only growth and multiplication rates, but also the biosynthesis of secondary metabolites and their derived associated properties, as recently reported for *Bryophyllum* [5].

As a multifactorial procedure, the identification of significant factors in establishing in vitro plant culture protocols generally requires the implementation of intricate and time-consuming experimental designs that lead to the construction of large unmanageable databases, which could be difficult to analyze by traditional statistical methods [6]. However, machine learning (ML) technology makes possible the modeling of such databases by offering a powerful artificial-intelligence-based tool to identify the key factors needed to improve a specific response [7]. This is possible because artificial-intelligence algorithms can organize huge amounts of data into useful information, learning from the data without the intervention of computer programmers.

In this study, the application of artificial neural networks (ANNs) combined with fuzzy logic (neurofuzzy logic) allowed the interpretation of prediction models by the formulation of simple "IF-THEN" rules that facilitate, in a very simple way, the identification of optimal responses [8]. Neurofuzzy logic has previously been applied very successfully to different plant tissue culture techniques, including micropropagation [6], germination [9], organogenesis [10], and the biosynthesis of bioactive compounds from plant and cell in vitro cultures [11,12]. On these bases, the combination of neurofuzzy logic and PTC has been recently proposed as a cutting-edge strategy in achieving efficient valorization of medicinal plants [13].

The implementation of efficient protocols for the in vitro culture of medicinal plants involves multiple factors that generally interact in a complex and non-linear way; therefore, the optimization of these processes becomes an arduous task [14]. Consequently, the simultaneous study of several parameters requires the design of large multifactorial datasets that are difficult to analyze, and the interpretation of the results becomes challenging. Due to this complexity, neurofuzzy logic is proposed as a machine learning-based tool to decipher the critical factors that impact the establishment of efficient protocols for in vitro culture of *Bryophyllum* medicinal plants. This study represents the first step in optimizing a formulation of individualized culture media for these medicinal plants, seeking their establishment as biofactories of bioactive compounds through large-scale biotechnological strategies.

2. Materials and Methods

2.1. Plant Material and In Vitro Culture Conditions

Three different *Bryophyllum* species were selected: *Bryophyllum daigremontianum* Raym.–Hamet et Perr. (BD); *Bryophyllum tubiflorum* Harv. (BT); and *Bryophyllum* × *houghtonii* D.B. Ward (*B. daigremontianum* × *tubiflorum*, BH).

Fully-developed epiphyllous plantlets from 18-month-old *Bryophyllum* plants grown in a local greenhouse were harvested and disinfected, as previously indicated [15]. After disinfection, epiphyllous plantlets were transferred to glass culture vessels containing 25 mL of culture media. Two culture media were used for nutrition experiments: Murashige

and Skoog (MS) full-strength basal medium [16] and the same medium with half-strength macronutrient concentration, 1/2 MS (Table S1). Both media were supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar at pH 5.8 before autoclaving at 121 °C and 1.1 atm for 20 min. Later, cultures were transferred into a growth chamber under a photoperiod of 16 h light (55 μmol m^{-2} s^{-1}) and 8 h dark at 25 ± 1 °C.

2.2. Experimental Design and Data Acquisition

Cultures were maintained for four periodical subcultures of 12 weeks using newly formed epiphyllous plantlets as the starting explant for the next subculture. Specifically, three plantlets of every species were placed into culture vessels and four culture vessels were used as replicates for each treatment (n = 12). At the end of each subculture, five parameters were measured in order to monitor plant growth and plantlet reproduction: the shoot length (SL), the longest root length (RL), the newly formed plantlet number (PN), the leaf number (LN), and the fresh weight (FW). One-way analysis of variance (ANOVA) followed by a post hoc Tukey's HSD test was performed by using STATISTICA v.12 software (StatSoft Inc., Tulsa, OK, USA, 2014) to analyze statistical differences between treatments (α = 0.005).

All mineral salts supplemented in the basal media were converted to the corresponding ions, as explained previously [17]. The macronutrient-derived ions were then merged into a unique database to build the model. A total of 15 factors were included as *inputs*: genotype (BD, BH, and BT), number of subcultures (ONE, TWO, THREE, and FOUR), and ion concentrations (NO_3^-, NH_4^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}, HPO_4^{2-}, and SO_4^{2-}). In parallel, the five growth and reproductive physiological parameters (SL, RL, PN, LN, and FW) measured at the end of each subculture were included as outputs.

2.3. Modeling Tools

The commercial neurofuzzy logic software FormRules 4.03 (Intelligensys Ltd., UK), which combines artificial neural networks (ANNs) and fuzzy logic [18,19], was used to build the model. A detailed description of the software package has been reported elsewhere [20]. Modeling was performed using the following training parameters: the ridge regression factor: 1×10^{-6}; C1 = 0.80 < x < 0.946, C2 = 4.8; the number of set densities: 2; the set densities: 2, 3; the adapt nodes: TRUE; the maximum inputs per submodel: 2; the maximum nodes per input: 15. The Adaptive Spline Modeling of Data (ASMOD) algorithm was used to achieve parameter minimization, as it improves accuracy even with few data parameters and reduces model complexity [21], by dividing the model into submodels to easily interpret the results by generating a set of rules [22]. Separate models were developed for each output, and a model assessment criterion was used to prevent over-fitting of the data. Among different statistical fitness criteria, the structural risk minimization principle (SRM) was chosen, as it allows finding the best model with minimum generalization error [23], although several criteria were also tested, i.e.,: cross validation (CV), leave-one-out cross validation (LOOCV), Bayesian information criterion (BIC), and minimum description length (MDL).

Independent predictive models were provided for every output, whose quality was assessed by the coefficient of determination of the training set (train set R^2) expressed as a percentage, according to Equation (1), where y_i is the experimental value in the dataset; y_i' is the predicted value obtained by the model; and y_i'' is the mean value of the dependent variable. Train set R^2 values between 70 and 99.9% were considered acceptable predictive values, whereas values higher than 99.9%, indicated that the model may have been overfitted and required readjustment [18]. Finally, in order to assess model accuracy, the differences between predicted and experimental data were checked by ANOVA.

$$R^2 = \left(1 - \frac{\Sigma_{i=1}^{n}(y_i - y_i')^2}{\Sigma_{i=1}^{n}(y_i - y_i'')^2}\right) \times 100 \quad (1)$$

Once submodels were established, the application of fuzzy logic was applied to improve the interpretation of results by ranging as low, medium, or high the model results and providing a membership degree, with values between 0 and 1 [24], to indicate the influence of each input and their interaction(s) on the different outputs.

3. Results and Discussion

Table 1 shows the dataset subjected to both statistical analysis and neurofuzzy modeling, including the inputs (e.g., genotype, subculture number, and macronutrient ion concentrations) and the outputs used for monitoring *Bryophyllum* growth- and reproduction-related physiological responses (e.g., shoot length, SL; root length, RL; plantlet number, PN; leaf number, LN; and fresh weight, FW).

ANOVA revealed that genotype BH exhibited the highest values ($p < 0.005$) for SL, PN, or FW, depending on the MS media strength and the number of subcultures (Table 1). In contrast, BT caused the highest LN ($p < 0.005$) after a low number of subcultures (1 and 2), particularly if full-strength MS was used (Table 1). However, the obtained information did not provide deep insight into the key factor(s) responsible for the observed results.

As a solution, neurofuzzy logic modeling was employed for deciphering cause–effect relationships among the inputs and outputs in previous reports on the plant biotechnology field, regardless of the different natures of experimental data and the sizes of datasets [10,12,13]. The neurofuzzy software employed used the ASMOD algorithm as an intelligent data processing tool to build empirical non-linear multivariate models. It was able to successfully model four out of five response parameters (train set $R^2 > 70\%$) [18]: SL, PN, LN, and FW (Table 2). The scatter plots containing the experimental and predicted values for R^2 fitting are shown in Figure S1. Moreover, for each of these parameters, *F* ratio values were higher than those of critical *f* (Table 2), indicating that no statistical differences between experimental and predicted data were found ($\alpha = 0.05$). This means that the model was statistically accurate and showed high predictability. In contrast, RL was the only output that did not reach 70% of predictability, suggesting that this parameter was not influenced by the factors involved in this study.

SL, PN, LN, and FW were found to be influenced by only three factors out of the 15 tested: genotype, subculture number, and NH_4^+ concentration (Table 2). In the case of SL, the model identified two critical factors: genotype was predicted to cause the strongest effect, whereas the number of subcultures was found to have a secondary influence (Table 2). PN was explained by the interaction between genotype and NH_4^+ concentration, whereas LN was mainly affected by the genotype, as well as by the NH_4^+ concentration as a second independent factor. Finally, genotype was the only critical factor regarding FW changes. It is worth noting that SL, LN, and FW were preferentially explained exclusively by genotype. These three parameters may be related to the typical characteristics of the succulent nature of *Bryophyllum*: the different foliar water storage capacities and the leaf morphology and size of each species could determine the differences detected by the model [25,26].

In order to interpret how the inputs influenced the different outputs, the set of "IF-THEN" rules generated by the model, together with their membership degree (MD), are shown in Table 3.

Table 1. Inputs (genotype: genot.; subculture number; subc.; and ion concentration in mM) and outputs (SL, RL, PN, LN, and FW) used for ANN modeling. Outputs are expressed as the mean ± standard deviation. Bold letters indicate maximum values for each treatment (treat.). Different letters in the same column indicate significant differences ($p < 0.005$). Different super-script letters in the same column indicate significant differences ($p < 0.005$).

			Inputs								Outputs				
Treat.	**Genot.**	**Subc.**	**NO_3^-**	**NH_4^+**	**K^+**	**Cl^-**	**Ca^{2+}**	**Mg^{2+}**	**HPO_4^{2-}**	**SO_4^{2-}**	**SL (cm)**	**RL (cm)**	**PN**	**LN**	**FW (g)**
1	BH	ONE	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	7.1 ± 0.4 ^{ab}	5.4 ± 0.2 ^{bcde}	28.8 ± 4.9 ^{abc}	12.7 ± 0.5 ^{cde}	2.3 ± 0.2 ^{abc}
2	**BH**	**TWO**	**39.4**	**20.6**	**20.0**	**5.99**	**2.99**	**1.50**	**1.25**	**1.76**	7.1 ± 0.3 ^{ab}	5.2 ± 0.2 ^{bcde}	27.5 ± 2.1 ^{bc}	13.3 ± 0.3 ^{cde}	**2.7 ± 0.1 ^{a}**
3	BH	THREE	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	4.6 ± 0.2 ^{defgh}	3.3 ± 0.3 ^{de}	15.3 ± 1.7 ^{cd}	10.3 ± 0.2 ^{efgh}	1.8 ± 0.1 ^{bcdef}
4	BH	FOUR	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	5.4 ± 0.4 ^{cdefg}	4.2 ± 0.4 ^{cde}	13.5 ± 2.3 ^{de}	10.5 ± 0.4 ^{efg}	2.0 ± 0.2 ^{abcd}
5	BH	ONE	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	6.9 ± 0.4 ^{abc}	4.6 ± 0.3 ^{cde}	38.4 ± 3.8 ^{ab}	12.7 ± 0.3 ^{cde}	2.1 ± 0.2 ^{abcde}
6	**BH**	**TWO**	**19.7**	**10.3**	**10.0**	**2.99**	**1.50**	**0.75**	**0.62**	**1.01**	**8.0 ± 0.4 ^{a}**	5.4 ± 0.5 ^{bcde}	**42.4 ± 3.8 ^{a}**	13.6 ± 0.6 ^{cde}	2.5 ± 0.2 ^{ab}
7	BH	THREE	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	5.8 ± 0.2 ^{bcde}	5.3 ± 0.3 ^{bcde}	33.2 ± 1.7 ^{ab}	12.0 ± 0.3 ^{cdef}	2.1 ±0.1 ^{abcde}
8	BH	FOUR	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	5.9 ± 0.3 ^{bcde}	5.9 ± 0.3 ^{bcde}	32.1 ± 3.5 ^{ab}	11.8 ± 0.2 ^{def}	2.4 ± 0.2 ^{abc}
9	BD	ONE	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	3.4 ± 0.2 ^{h}	4.2 ± 0.3 ^{cde}	8.7 ± 1.6 ^{de}	7.3 ± 0.3 ^{gh}	0.9 ± 0.1 ^{h}
10	BD	TWO	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	3.6 ± 0.2 ^{h}	5.0 ± 0.4 ^{cde}	10.1 ± 1.0 ^{de}	7.7 ± 0.2 ^{gh}	1.0 ± 0.1 ^{gh}
11	BD	THREE	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	3.3 ± 0.2 ^{h}	6.4 ± 0.5 ^{bc}	7.2 ± 1.2 ^{de}	7.2 ± 0.3 ^{gh}	1.0 ± 0.1 ^{gh}
12	BD	FOUR	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	3.2 ± 0.1 ^{h}	5.9 ± 0.5 ^{bcd}	7.1 ± 1.0 ^{de}	7.3 ± 0.4 ^{gh}	1.0 ± 0.1 ^{gh}
13	BD	ONE	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	3.9 ± 0.2 ^{fgh}	9.2 ± 0.7 ^{a}	32.5 ± 3.3 ^{ab}	7.3 ± 0.3 ^{gh}	1.3 ± 0.1 ^{efgh}
14	BD	TWO	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	4.0 ± 0.2 ^{fgh}	6.1 ± 0.8 ^{bc}	25.7 ± 3.1 ^{bcd}	6.8 ± 0.3 ^{h}	1.1 ± 0.1 ^{gh}
15	BD	THREE	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	3.1 ± 0.3 ^{h}	8.2 ± 1.0 ^{ab}	33.3 ± 4.5 ^{ab}	7.3 ± 0.4 ^{gh}	1.1 ± 0.1 ^{fgh}
16	**BD**	**FOUR**	**19.7**	**10.3**	**10.0**	**2.99**	**1.50**	**0.75**	**0.62**	**1.01**	3.6 ± 0.2 ^{gh}	**9.6 ± 0.4 ^{a}**	30.6 ± 2.1 ^{ab}	8.0 ± 0.0 ^{fgh}	1.3 ± 0.1 ^{defgh}
17	BT	ONE	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	6.8 ± 0.6 ^{abc}	5.4 ± 0.7 ^{bcde}	1.8 ± 1.1 ^{de}	19.5 ± 1.6 ^{ab}	1.6 ± 0.2 ^{cdefgh}
18	BT	TWO	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	6.6 ± 0.4 ^{abc}	5.9 ± 0.7 ^{bcd}	2.1 ± 0.6 ^{de}	21.0 ± 1.2 ^{a}	1.7 ± 0.2 ^{cdefg}
19	BT	THREE	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	3.8 ± 0.1 ^{gh}	3.0 ± 0.3 ^{e}	0.4 ± 0.3 ^{e}	14.8 ± 0.5 ^{cd}	0.9 ± 0.1 ^{h}
20	BT	FOUR	39.4	20.6	20.0	5.99	2.99	1.50	1.25	1.76	4.2 ± 0.2 ^{efgh}	4.7 ± 0.3 ^{cde}	0.9 ± 0.5 ^{de}	16.0 ± 0.5 ^{bc}	1.0 ± 0.1 ^{gh}
21	BT	ONE	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	5.3 ± 0.2 ^{cdefg}	5.5 ± 0.3 ^{bcde}	6.7 ± 1.6 ^{de}	20.8 ± 0.7 ^{a}	1.4 ± 0.1 ^{defgh}
22	**BT**	**TWO**	**19.7**	**10.3**	**10.0**	**2.99**	**1.50**	**0.75**	**0.62**	**1.01**	5.6 ± 0.2 ^{bcde}	5.0 ± 0.3 ^{bcde}	9.8 ± 1.9 ^{de}	**22.3 ± 0.7 ^{a}**	1.3 ± 0.1 ^{defgh}
23	BT	THREE	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	5.5 ± 0.2 ^{bcdef}	6.2 ± 0.4 ^{bc}	9.7 ± 1.8 ^{de}	20.7 ± 0.7 ^{a}	1.2 ± 0.1 ^{fgh}
24	BT	FOUR	19.7	10.3	10.0	2.99	1.50	0.75	0.62	1.01	6.2 ± 0.3 ^{bcd}	6.2 ± 1.2 ^{bc}	14.4 ± 1.5 ^{de}	21.5 ± 0.9 ^{a}	1.4 ± 0.1 ^{defgh}

Table 2. Neurofuzzy logic model quality parameters and inputs with a significant effect on each output. Inputs with the strongest effect on each parameter are shown in bold.

Outputs	Submodel	Train Set R^2 (%)	*F* Ratio	df1, df2	*f* Critical ($p < 0.05$)	Significant Inputs
SL	**1**	82.77	13.61	6, 23	2.53	**Genotype**
	2					Subculture
RL	-	65.81	5.45	6, 23	2.53	-
PN	**1**	92.27	33.81	6, 23	2.53	**Genotype × NH_4^+**
LN	**1**	92.52	58.71	4, 23	2.80	**Genotype**
	2					NH_4^+
FW	**1**	83.39	33.46	3, 23	3.03	**Genotype**

Concerning SL, the model rules for genotype (Table 3) showed that BH and BT presented high values for this output, with this effect being stronger for BH according to its MD, whereas BD showed low values. These differences in vegetative growth could be explained by the different requirements for each genotype and, in the case of BD, it was observed that its growth was not optimal under these experimental conditions. Additionally, the number of subcultures was the other critical factor for this output (Table 2). The rules showed that SL was high during the first two subcultures, but became low from the third subculture to the fourth subculture (Table 3). This finding indicated that several subcultures should be performed before achieving a stable *Bryophyllum* plant in vitro culture multiplication, as plant material should adapt to these new axenic conditions [27].

The neurofuzzy model identified PN as the only output that could be explained by the interaction of two factors: genotype and NH_4^+ concentration (Table 2). In this case, the rules obtained for PN were even more meaningful. Thus, PN was high at low NH_4^+ concentrations for BD and BH, the latter showing a stronger influence, whereas it was always low for BT (Table 3). To define the levels of NH_4^+ concentration, FormRules also showed the corresponding quantitative values, according to the experimental space used: low concentrations were considered under 15 mM, while high concentrations were considered above the same value (Figure S2). Such a sensitivity towards ammonium has been previously determined for these *Bryophyllum* species, as low NH_4^+ concentrations were predicted to enhance the accumulation of phenolic compounds, either at plant or cell culture level [11,28], as well as the antioxidant activity of *Bryophyllum* leaf extracts [11,29].

Again, genotype was predicted as the most critical factor regarding LN (Table 2). According to the rules, this output was high in the case of BT, whereas it was low for BH and BD, the latter presenting the strongest effect (Table 3). As shown in Figure 1, the plant morphology for the three *Bryophyllum* species cultured in vitro is clearly differential: tubular BT leaves are longer and thinner than those from BD and BH, which present boat-shaped, folded leaves with a higher surface [30,31]. In addition, the second submodel obtained for this output was NH_4^+ concentration (Table 2); it showed that LN was high at low NH_4^+ concentrations (Table 3). The quantitative values for NH_4^+ concentrations were the same as that obtained for PN (Figure S2).

It should be noted that PN and LN showed a close relationship, according to the model. This correlation could be explained based on the asexual reproduction strategy observed in the *Bryophyllum* species. Reproduction in BD, BH, and BT is mediated by the constitutive plantlet formation in the leaf margins of grown plants, which drives the invasiveness that is attributed to these species [32,33] (Figure 1). However, little is known about this adaptative reproduction mechanism, which involves complex genetic, hormonal, and embryogenic processes [34,35]. Due to genotypic reasons, BT showed the lowest PN, as plantlet formation is restricted to the distal leaf end in this genotype, whereas they are formed along the whole leaf margin in BH and BD [36,37] (Figure 1).

Table 3. Rules selection obtained by neurofuzzy logic. Bold letters indicate input/s with the strongest effect on low and high values for each output. MD, membership degree.

Rules		Subculture	Genotype	NH_4^+		SL (cm)	PN	LN	FW (g)	MD
1			**BH**			**High**				**0.98**
2			**BD**			**Low**				**1.00**
3			BT			High				0.63
4	IF	ONE			THEN	High				0.54
5		TWO				High				0.64
6		THREE				Low				0.95
7		FOUR				Low				0.79
8			**BH**	**Low**			**High**			**0.86**
9			BH	High			Low			0.50
10	IF		BD	Low	THEN		High			0.72
11			BD	High			Low			0.81
12			BT	Low			Low			0.78
13			**BT**	**High**			**Low**			**0.98**
14			BH					Low		0.80
15			**BD**					**Low**		**1.00**
16	IF		**BT**		THEN			**High**		**1.00**
17				Low				High		0.57
18				High				Low		0.61
19			**BH**						**High**	**0.76**
20	IF		**BD**		THEN				**Low**	**0.89**
21			BT						Low	0.76

Murashige and Skoog (MS) medium [15] is considered the most-used basal universal media formulation for plant tissue culture, as it has been successfully employed to culture a great number of plant species without presenting apparent physiological disorders. However, mineral requirements vary between the plant genotypes and plant tissue culture techniques, and some authors have claimed the supraoptimal composition of MS formulation [8,38]. In this sense, preferential MS modifications have been addressed to decrease macronutrient concentrations and the ratio among different nitrogen sources, mainly ammonium and nitrate [39,40]. Furthermore, as members of the Crassulaceae family, the *Bryophyllum* species perform the crassulacean acid metabolism (CAM), due to their adaptation to arid climates, to which they are native [41]. Such climates are characterized by poor mineral accessibility that is driven by scarce water availability, so the uptake of mineral nutrients is limited [42] and, consequently, CAM species are adapted to poor mineral soil conditions. In this way, the decrease in the mineral composition of MS medium to half (1/2 MS) constitutes a rational approach for the establishment of *Bryophyllum* in vitro culture.

The importance of the nitrogen source on CAM activity has been previously reported on *Bryophyllum* growth through open-field cultivation approaches. As described by Pereira et al. [43], the concentration and balance between soil nitrate and NH_4^+ influenced the metabolism of several species belonging to the *Kalanchoe* genus. In fact, toxicity effects were found in BT at ammonium concentrations of 5 mM, and different authors have highlighted the soil nitrate preference upon soil ammonium in several species, including *Bryophyllum* [41,44]. Different hypotheses have been established regarding soil ammonium toxicity in this genus: (i) the lowest nocturnal rates of organic acid transport inside the vacuole in the presence of NH_4^+; and (ii) the energetic cost coupled to the ammonium release from plants, which constitutes an effective strategy for their invasiveness in arid ecosystems [43,45,46]. In this work, a next-generation approach was employed to elucidate whether nitrate or ammonium is the key factor in the parameters that were studied.

The overall results demonstrated the negative effect of high NH_4^+ concentrations on PN and LN for all of the tested species. Although our experimental design could seem limited (only two concentrations of MS were used), neurofuzzy logic was able to determine that ammonium may play a toxic effect on *Bryophyllum* growth, suggesting the suitability of other media formulations with ammonium concentrations under 15 mM. Alternatively, other commonly used formulations, such as Gamborg B5 medium or woody plant medium (WPM), contain lower ammonium concentrations (2.03 mM and 5 mM, respectively) to overcome such toxicity in a wide range of plants [47]. The same experimental design allowed neurofuzzy logic to unravel the critical factors affecting the production of phenolic compounds on *Bryophyllum* sp. cultured in vitro, thereby predicting that NH_4^+ concentrations below 15 mM caused an accumulation of such metabolites [11]. In addition, neurofuzzy logic successfully predicted the organogenetic process on *Bryophyllum*, emphasizing the differences between genotypes [10].

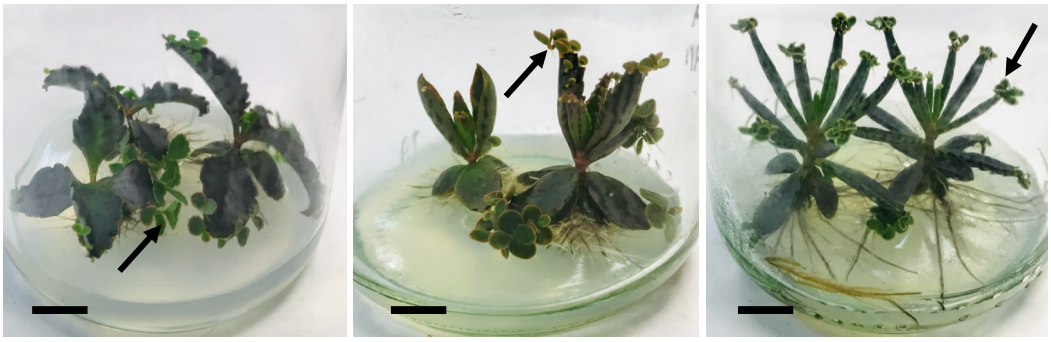

Figure 1. In vitro-cultured *Bryophyllum* plants. From left to right: *B. daigremontianum* (BD), *B.* × *houghtonii* (BH), and *B. tubiflorum* (BT). Arrows point at plantlets formed at the leaf margins. Bars = 1 cm. Figure adapted from [1].

Finally, all these results suggest, that regardless of culture medium composition: (i) BH plants presented a high shoot length with a low number of leaves but, due to their morphology, the overall plant fresh weight was high; (ii) BD plants presented a low shoot length, with a low number of leaves and low fresh weight, revealing that culture conditions are not optimal for this species; and (iii) BT plants presented a high shoot length with a high number of leaves, but their thin, tubular morphology caused a low fresh weight.

4. Conclusions

The machine-learning tool, neurofuzzy logic, was able to decipher the critical factors that impact the establishment of the in vitro culture of *Bryophyllum* medicinal plants, demonstrating that genotype plays a key role, as it impacts both vegetative growth and asexual reproduction in all of the studied species. In addition, ammonium was identified as a significant factor, as concentrations above 15 mM promote a negative effect on vegetative growth and reproduction. On these bases, our results indicated the need to optimize genotype-based formulations following the establishment of PTC, as even closely related species may show differential requirements in terms of mineral nutrition. In response to such a paradigm, the combination of machine learning and PTC has been evaluated as a fast and robust approach in achieving such a goal, thereby facilitating the biotechnological exploitation of unknown medicinal plants, as demonstrated here with *Bryophyllum* sp.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8110987/s1, Figure S1: Determination coefficients (R^2) for experimental vs. predicted values for all outputs, generated by the neurofuzzy logic model. A. SL (cm). B. PN. C. LN. D. FW (g).; Figure S2: Graphical interpretation of the *fuzzification* process performed by ANN tool for PN (left) and LN (right); Table S1: Salt composition of culture media used in this study.

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