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Original article – Thematic Issue

Neural networks models as decision-making tool for in vitro proliferation of hardy kiwi

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Summary

Plant micropropagation is a complex procedure involving the interaction of several factors such as mineral media composition, vitamins, plant growth regulator, etc. Indeed, almost each species or cultivar seems to need specific culture conditions to achieve the highest proliferation rate. The aim of this work was to establish which of the next four factors: culture media, number of subcultures, and concentration of two plant growth regulators (BAP and GA_3), cause the most significant effect on *Actinidia arguta* (Sieb. & Zucc.) Planch. ex Miq. multiplication. In this paper, neural network models were built and used as decision-making tools. Arguta kiwi apical explants were cultured in three commonly kiwifruit (*Actinidia* sp.) micropropagation media [Murashige and Skoog (MS), Standardi (St) and Gamborg's (B5)] containing different concentrations of BAP (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1}) and GA_3 (0, 0.1, 0.5 and 1.0 mg L^{-1}) during three consecutive subcultures. Two response parameters were measured in this work: shoot number and shoot length. Database was analyzed using the neurofuzzy logic technique that allowed a simple, predictive and easy to interpret model to be obtained. The model disclosed that the key factors were BAP concentration for shoot number and the combination of BAP and GA_3 for shoot length. In fact, if *A. arguta* explants were cultivated with intermediate or high BAP (>0.6 mg L^{-1}) concentration, the highest number of shoots was obtained. But, low concentration of BAP (0–0.3 mg L^{-1}) and high of GA_3 (0.5–1 mg L^{-1}) should be needed for long shoots. The model also indicated that the number of subcultures is another key factor, being necessary at least two subcultures to observe improvement on the shoot number, but only one to achieve the longest shoots. Finally, the model pointed out that all media caused a positive effect on shoot length but only St a slight positive in shoot number, suggesting that their composition need to be optimized. Our results point out the use of neurofuzzy logic as decision-making tool and its utility on obtaining knowledge of the effects of the factors on the plant micropropagation.

Keywords
Actinidia arguta, baby kiwi, computer-assisted modeling, neurofuzzy logic, plant tissue culture, prediction, optimization

Significance of this study

What is already known on this subject?

- The need of new tools for making the best decision in order to optimize media composition (mineral nutrients, vitamins, plant growth regulators and culture conditions) to achieve the best micropropagation conditions. MS promotes several physiological disorders during micropropagation of several woody plants, including kiwi (*Actinidia* sp.).

What are the new findings?

- Neurofuzzy logic models in comparison to traditional statistics can easily and accurately predict key factors for a successful *A. arguta* micropropagation, facilitating decision-making objectively.

What is the expected impact on horticulture?

- Neural networks models can be used for predicting and optimizing in vitro proliferation conditions and for designing specific medium for any plant species. Furthermore, neural networks technology can be adopted for other horticultural experiments in which large databases are used. Here we used neurofuzzy logic tool to improve the decision-making process saving time, resources and avoiding complicated statistical analysis.

Introduction

Actinidia arguta (Sieb. et Zucc.) Planch. ex Miq. kiwifruit has a hairless, smooth and edible skin. It is smaller than other kiwifruit species like *A. chinensis* Planch., and for this reason it can be eaten whole (Williams et al., 2003). Hardy kiwifruit (also known as baby kiwi) grows widely in East Asia and Siberia (Ferguson and Huang, 2007) and is commercially cultivated in North America, New Zealand, some European countries (Belgium, France, Poland) and Japan (Okamoto and Goto, 2005). However, it has not yet been commercially successful due to its limited production and postharvest conservation, compared to other kiwifruit species.

There are several studies focused on determining the optimal culture media and combination of plant growth regulators (PGRs) for *Actinida* spp. (Akbas et al., 2007; Prado et al., 2005). The most commonly used cultured medium is MS (Murashige and Skoog, 1962) containing two PGR (Akbas et al., 2007; Cañal et al., 2000; Moncaleán et al., 2002): 6-benzylaminopurine (BAP) and gibberellic acid (GA_3). However, there are no studies focused on improving *A. arguta* medium proliferation.

In the present study, we have used the hardy kiwi variety 'Issai' because it is monoecious, in contrast to other kiwifruit species, which are generally dioecious. The presence of male and female flowers on the same plant allows auto fertility, and pollinating male plant can be avoided as a result, saving land (SERIDA, 2014).

Several methodologies such as specific experimental designs (Niedz and Evens, 2007) and their corresponding statistical analysis (Ibañez et al., 2003) including response surface methodology and Chi-Squared Automatic Interaction Detection (CHAID) algorithm (Akin et al., 2017) have been used to optimize plant tissue culture media. In the last years, we have reported that some artificial intelligence (AI) tools, such as artificial neural networks and fuzzy logic, have clear advantages over statistics in deciphering key factors, especially in large datasets such as those used in micropropagation studies (Gago et al., 2010; Gallego et al., 2011; Nezami-Alanagh et al., 2014, 2017).

The aim of this work is to use those techniques as decision-making tools to improve the in vitro proliferation conditions for *A. arguta*, as they allow the understanding of the critical factors through the models generated with neurofuzzy logic.

Table 1. Macro and micronutrients concentrations of each medium. All media were supplemented with MS vitamins concentrations.

Salts	MS (mg L^{-1})	B5 (mg L^{-1})	St (mg L^{-1})
KNO_3	1 900	2 500	1 800
NH_4NO_3	1 650	0	400
$(NH_4)_2SO_4$	0	134	0
$Ca(NO_3)_2 \cdot 4H_2O$	0	0	1 200
$CaCl_2 \cdot 2H_2O$	440	150	0
$MgSO_4 \cdot 7H_2O$	370	250	360
KH_2PO_4	170	0	270
$NaH_2PO_4 \cdot H_2O$	0	150	0
$MnSO_4 \cdot 4H_2O$	22.3	13.2	1.0
$ZnSO_4 \cdot 7H_2O$	8.6	2.0	8.6
H_3BO_3	6.2	3.0	6.2
KI	0.83	0.75	0.08
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025
$Na_2 \cdot EDTA$	37.25	37.25	37.25
$FeSO_4 \cdot 7H_2O$	27.85	27.85	27.85

Table 2. PGR combinations assayed (20 combinations) with each medium (MS, B5 and St) and subculture (21, 42 and 63 days). Only those combinations with less or equal concentration of GA_3 than BAP were employed (for BAP 0, 0.5 and 1.0).

BAP (mg L^{-1})	GA_3 (mg L^{-1})
0.0	0.0
0.5	0.0
0.5	0.1
0.5	0.5
1.0	0.0
1.0	0.1
1.0	0.5
1.0	1.0
1.5	0.0
1.5	0.1
1.5	0.5
1.5	1.0
2.0	0.0
2.0	0.1
2.0	0.5
2.0	1.0
2.5	0.0
2.5	0.1
2.5	0.5
2.5	1.0

Materials and methods

The experiment was carried out with apical shoot explants of 'hardy kiwifruit' (*A. arguta*) proliferated previously in Cheng medium (Cheng, 1975) supplemented with 1 mg L^{-1} BAP and 1 mg L^{-1} GA_3. The cultured explants were maintained in a growth chamber under 16/8 (light/dark) photoperiod (white cold light of 40 µmol m^{-2} s^{-1}) and a temperature of 25 ± 1°C, and subcultured into a fresh medium every 21 d. In this study, we employed explants grown in Cheng medium during 40 d to obtain enough number of shoots > 1.5 cm, homogeneous and healthy.

Experimental design and data acquisition

In this work, the effect of three factors on hardy kiwi shoot growth was assayed: media mineral composition, plant growth regulators and subculture number.

Three media containing the original concentration of macro- and micro-nutrients described for MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) and St (Standardi, 1981), commonly used in *Actinidia* (Akbas et al., 2007; Cañal et al., 2000; Moncaleán et al., 2002) in vitro culture (Table 1) but the same vitamins than MS, to simplify the analysis.

Two plant regulators: GA_3 and BAP, at different concentrations were tested in each culture medium (Table 2) and finally, all explants were transferred into a fresh medium every 21 days during 3 subcultures. All media were supplemented with 30 g L^{-1} sucrose, and 8.0 g L^{-1} agar and their pH adjusted to 5.8 before autoclaving.

In total 180 combinations (3 media × 20 PGR combinations × 3 subcultures) were assayed. As response parameter, the shoot number (SN) and shoot length (SL; mm) of explants were recorded at the end of each subculture. A unique database was made (Table S1) including all inputs, outputs and results obtained, expressed as mean and standard deviation. Each treatment consisted of 4 replicates glass jars (180 mL) sealed with caps, containing 3 explants each. The experiments were carried out in triplicate (n = 2,112).

Neurofuzzy logic modeling

The software FormRules v4.03 (Intelligensys Ltd., 2008) was used due to a user-friendly interface and easy interpretation of the results (Gallego et al., 2011). Neurofuzzy logic combines fuzzy logic with artificial neural networks to generate models able to disclose information and knowledge, facilitating the decision making process. Here, it was used

Table S1. Plant Growth Regulator (PGR) concentrations (expressed as mg L^{-1}), culture media (MS, B5 and St), subculture time (21, 42 and 63 days) and mean response values of the parameters shoot number (SN) and length (SL) expressed as average and standard deviation used to characterize plant growth. This dataset was used for neurofuzzy logic model.

		Shoot number									Shoot length (mm)								
Media		MS			B5			St			MS			B5			St		
Subcultures		21 d	42 d	63 d	21 d	42 d	63 d	21 d	42 d	63 d	21 d	42 d	63 d	21 d	42 d	63 d	21 d	42 d	63 d
BAP	GA_3																		
0	0	1.5±0.52	1.0±0.00	1.1±0.28	1.0±0.00	1.0±0.00	1.1±0.33	1.1±0.29	1.0±0.00	1.2±0.40	0.8±0.20	0.5±0.00	0.5±0.04	0.9±0.31	0.5±0.04	0.5±0.05	0.8±0.16	0.5±0.03	0.5±0.03
0.5	0	1.9±0.90	3.0±1.06	2.6±0.88	1.7±0.89	3.0±1.03	3.0±0.86	1.3±0.65	2.9±0.70	3.6±0.88	1.0±0.42	0.7±0.21	0.7±0.11	1.1±0.37	0.7±0.17	0.8±0.22	1.4±0.41	0.9±0.13	0.8±0.24
1	0	1.7±0.89	2.6±0.77	3.0±0.95	2.0±1.21	3.1±0.64	3.3±0.56	1.8±0.75	3.4±0.90	3.9±0.90	1.3±0.67	0.8±0.17	0.7±0.17	1.3±0.50	0.6±0.06	0.7±0.12	1.5±0.50	0.7±0.16	0.7±0.11
1.5	0	2.0±0.95	2.8±1.11	2.5±0.83	1.4±0.67	2.9±0.73	2.9±0.78	2.2±1.11	3.6±0.98	3.9±0.72	1.3±1.64	0.8±0.33	0.6±0.26	1.1±0.41	0.6±0.12	0.6±0.13	1.0±0.34	0.7±0.12	0.6±0.11
2	0	1.5±0.52	3.3±1.25	3.4±0.70	1.3±0.67	2.7±0.48	2.9±0.69	1.5±1.00	3.9±0.83	4.0±0.95	0.9±0.33	0.6±0.10	0.6±0.09	1.0±0.33	0.6±0.11	0.6±0.05	1.4±0.42	0.6±0.09	0.6±0.11
2.5	0	2.0±0.85	2.6±0.92	3.3±0.99	2.0±1.21	3.2±1.08	3.5±0.61	1.9±1.08	4.0±1.00	3.8±1.07	1.1±0.51	0.6±0.11	0.6±0.11	1.0±0.47	0.6±0.07	0.6±0.08	1.1±0.30	0.6±0.14	0.6±0.14
0.5	0.1	2.0±0.85	2.5±0.90	3.1±1.18	1.3±0.45	2.9±0.88	2.8±0.70	2.0±0.95	2.8±0.79	2.8±1.05	1.2±0.43	0.8±0.20	0.7±0.28	1.2±0.59	0.7±0.15	0.7±0.10	1.3±0.37	1.0±0.21	0.9±0.21
1	0.1	2.8±1.48	2.7±0.80	3.3±1.09	1.7±1.00	3.2±0.97	3.7±0.99	2.0±1.21	3.4±1.05	3.8±0.87	1.1±0.41	0.7±0.11	0.6±0.11	1.2±0.38	0.7±0.07	0.7±0.11	1.1±0.38	0.8±0.24	0.8±0.17
1.5	0.1	1.4±0.67	3.4±0.63	3.5±1.28	1.8±0.83	3.5±0.76	4.0±0.69	1.9±1.00	2.7±0.79	3.5±0.87	1.2±0.44	0.6±0.12	0.6±0.05	1.1±0.52	0.7±0.15	0.7±0.12	1.2±0.59	0.8±0.44	0.7±0.18
2	0.1	1.9±0.90	3.3±0.96	3.3±1.61	1.9±0.90	3.3±0.95	3.4±0.86	1.5±0.80	3.3±1.14	3.2±0.58	0.9±0.36	0.6±0.13	0.6±0.07	0.8±0.21	0.6±0.08	0.6±0.05	1.3±0.74	0.7±0.15	0.6±0.07
2.5	0.1	1.9±1.00	2.5±0.98	3.3±1.36	1.8±0.94	2.7±0.63	3.5±0.67	1.2±0.39	3.6±0.90	3.1±1.12	1.1±0.48	0.7±0.18	0.7±0.09	0.9±0.41	0.6±0.10	0.6±0.08	1.3±0.53	0.6±0.16	0.7±0.18
0.5	0.5	1.8±0.87	2.9±0.74	2.5±1.15	1.5±0.67	2.5±0.78	2.9±1.04	1.7±0.89	3.1±0.70	2.9±0.65	1.2±0.35	0.7±0.16	0.8±0.20	1.4±0.43	0.9±0.19	0.8±0.17	1.4±0.48	0.9±0.11	1.0±0.35
1	0.5	1.9±0.79	3.2±0.83	2.6±0.73	2.2±0.94	3.5±0.55	3.7±0.65	2.5±1.09	3.3±0.91	3.5±0.67	1.2±0.50	0.7±0.19	0.7±0.15	0.8±0.19	0.6±0.03	0.7±0.07	2.0±0.44	0.8±0.18	0.7±0.15
1.5	0.5	2.3±0.87	3.4±0.85	2.8±1.13	1.8±0.87	3.2±1.33	3.2±0.83	1.7±0.49	3.5±0.90	3.1±1.00	1.2±0.58	0.6±0.11	0.7±0.10	0.9±0.32	0.7±0.27	0.6±0.11	1.1±0.45	0.7±0.13	0.8±0.19
2	0.5	1.5±0.67	2.8±1.14	2.4±0.86	1.5±0.67	3.3±0.96	3.2±0.97	1.4±0.67	3.9±0.85	3.4±1.37	1.1±0.33	0.7±0.17	0.7±0.17	0.9±0.36	0.6±0.05	0.6±0.07	1.2±0.42	0.6±0.05	0.7±0.12
2.5	0.5	2.1±1.00	2.9±0.35	3.1±1.10	1.8±0.83	2.9±1.16	4.1±1.22	1.5±0.67	3.7±0.72	3.7±0.99	1.0±0.39	0.6±0.10	0.6±0.07	0.8±0.31	0.6±0.14	0.7±0.07	1.4±0.45	0.6±0.09	0.6±0.09
1	1	1.7±0.78	2.8±0.75	2.7±0.91	2.1±1.17	*	*	1.8±0.75	3.5±0.62	3.6±1.32	1.0±0.30	0.7±0.14	0.7±0.11	1.0±0.35	*	*	1.4±0.43	0.7±0.10	0.8±0.19
1.5	1	2.0±1.21	2.6±0.74	2.5±1.55	1.5±0.80	2.7±0.98	4.4±0.97	1.8±1.19	3.8±0.95	3.5±0.68	1.1±0.41	0.6±0.07	0.8±0.25	1.1±0.43	0.7±0.19	0.7±0.12	1.2±0.33	0.7±0.13	0.8±0.12
2	1	1.7±0.65	2.7±0.79	2.4±1.13	2.4±1.24	3.4±1.34	3.6±1.14	2.3±1.14	3.1±0.86	4.1±0.89	1.2±0.55	0.6±0.17	0.7±0.14	1.0±0.40	0.6±0.10	0.7±0.11	0.9±0.47	0.6±0.08	0.6±0.07
2.5	1	1.5±0.52	2.4±0.85	2.5±0.74	1.8±1.03	*	*	1.5±0.67	3.6±0.69	3.6±0.86	0.9±0.36	0.6±0.18	0.7±0.17	0.9±0.31	*	*	1.2±0.55	0.6±0.07	0.7±0.18

* Contaminated (discarded) treatments.

Table 3. Training parameters setting with neurofuzzy logic.

Property
Minimization parameters (ASMOD)
Ridge regression factor: $1e^{-6}$
Model selection criteria
Structural risk minimization (SRM): C1=1.0; C2= 4.8
Number of set densities: 2
Set densities: 2, 3
Adapt nodes: TRUE
Max. inputs per SubModel: 4
Max. nodes per Input: 15

for describing the influence of the four factors studied (culture medium, number of subcultures and two PGR concentrations) on the plant growth responses (shoot number and length).

Culture media (St, B5 and MS), subculture number (1, 2 and 3) and plant growth regulator concentrations (BAP and GA_3) were considered as factors or inputs on growth. As outputs, the two parameters evaluated: the total new shoots produced and the lengths of the shoots obtained were selected. All treatments were included for training the model.

The training process was conducted according to previous work (Nezami-Alanagh et al., 2014). Although several statistical fitness criteria were probed during the modeling, the Structural Risk Minimization (SRM) gave the best results (the highest R^2 values, together with the simplest and more intelligible set of IF-THEN rules for each output). Minimization parameters are summarized in Table 3.

Once trained, the accuracy of the neurofuzzy logic model was assessed using the train set R^2 parameter and ANOVA parameters. While the train set R^2 parameter describes how much of the variability of the outputs (dependent variable) is explained by the inputs included for the model, the ANOVA parameters allow finding statistical differences between experimental and predicted results.

Train set R^2 parameter was calculated by the following equation:

$$R^2 = \left(1 - \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{\sum_{i=1}^{n} (y_i - \bar{y}_i)^2} \right) x100$$

where $\bar{y}$ is the mean of the dependent variable, and $\hat{y}$ is the predicted value from the model.

The model predictability was tested using R^2 parameter: the larger the value the better the model captured the variation in the training data. Train set R^2 values between 70 and 99% are indicative of good to excellent model predictabilities (Colbourn and Rowe, 2005).

Finally, the results obtained are presented through simple 'IF-THEN' rules with a certain membership degree. The membership degree represents a degree of truth, which varies from 0 to 1, meaning 1 that the output expected value is always a full member of the fuzzy set "Low" or "High". More information about neurofuzzy logic theory and applications can be found elsewhere (Gallego et al., 2011; Nezami-Alanagh et al., 2014, 2017).

Results and discussion

Optimization of plant tissue culture conditions is difficult due to the complex interactions among the multiple factors involved in the process, and kiwifruit micropropagation is not an exception. Although most species of *Actinidia* respond to in vitro multiplication without substantial problems, some combinations of mineral nutrients cause physiological disorders (Standardi and Catalano, 1984). In this paper, we compare two well-known culture media such as MS and B5 (a simple medium with low NH_4 content) previously used in *Actinidia* sp. (Tanaka et al., 1997; Cañal et al., 2000; Akbas et al., 2007) with the specific media designed for *Actinidia* sp. (Standardi, 1981). In order to elucidate which mineral nutrient plays a key role in kiwifruit multiplication, all media were provided with MS vitamins. Also, *Actinidia* micropropagation is greatly stimulated by PGRs, mainly cytokinins for cell divisions and shoot formation, and gibberellins for shoot elongation (Standardi and Catalano, 1984). Finally, the effects of PGR can be observed during several subcultures, with a decrease in shoot number and/or length depending on the PGRs used and the number of subcultures (Mustafa and Taha, 2012).

Data analysis and interpretation of big datasets requires the use of specific statistical tools depending on the type of data obtained (Mize et al., 1999; Ibañez et al., 2003) and the results obtained could be less specific and difficult to interpret than other based on computer generated optimal design and analysis (Akin et al., 2017). One advantage of neural networks, as computer-assisted tool, is that all parameters can be analyzed simultaneously independently of the experimental design and the type of data used (Gallego et al., 2011; Nezami-Alanagh et al., 2017), which could help plant propagators to make the right decisions about media design.

In this work, a large data set (Table S1) including the four inputs (number of subcultures, culture medium, BAP and GA_3 concentrations) and the two outputs (SN and SL) was modeled using neurofuzzy logic, an artificial neural networks tool. The neurofuzzy logic (ASMOD algorithms) succeeded in modelling both, SN and SL parameters (Table 4). As the train set R^2 values were higher than 78% and the ANOVA results indicate that there are not statistical significant differences between experimental and predicted results (*f* ratio was always greater than the *f* critical values for all parameters), the models are accurate and have high predictability (Colbourn and Rowe, 2005). Interestingly, the PGR concentrations have been highlighted as the factors with stronger effect for both parameters.

SN is explained by the single effect of three factors (number of subcultures, culture medium, and BAP concentration), being BAP the variable with highest incidence on the number of shoots. However, the SL variability is explained by the four inputs, particularly the concentration of BAP and GA_3, whose interaction is a critical aspect (Table 4).

Table 5 presents the whole set of "IF…THEN" rules generated by the model. It can be easily deduced that IF BAP concentration is Low THEN the SN value always will be Low (rule 1; MD = 1.00). When no BAP was added to the medium, the lowest SN was obtained (Table S1). However, medium or high BAP concentrations give high values of SN (rules 2–3; MD = 0.90–0.80).

Number of subcultures has also an effect on SN. So, IF the subculture number is Low THEN the SN is always Low (rule 4, MD = 1.00). The number of subcultures should be high to obtain high SN values (rule 6; MD = 0.81).

Finally, St medium gives the best results regarding SN

Table 4. Inputs (critical factors) selected by neurofuzzy logic to explain the variability of the outputs. Train set R^2 values and ANOVA parameters for each output: *f* ratio, degrees of freedom (df1: model and df2: total), and *f* critical value for $\alpha = 0.01$. Inputs with stronger effect are highlighted.

Output	Critical factors	Train set R^2 (%)	*F ratio*	df1	df2	*f* critical ($\alpha < 0.01$)
SN	**BAP** Number of subcultures Culture medium	78.47	87.50	7	175	3.67
SL	**BAP*GA_3** Number of subcultures Culture medium	85.13	94.50	10	175	3.14

Table 5. IF-THEN rules generated for each submodel by neurofuzzy logic software for SN and SL parameters with their corresponding membership degree (MD). The stronger effect on SN and SL indicated by the model are highlighted.

Rules		BAP	GA_3	Media	Subculture		SN	SL	MD
1		**Low**					**Low**		**1.00**
2		**Mid**					**High**		**0.90**
3		High					High		0.80
4					Low		Low		1.00
5	**IF**				Mid	**THEN**	Low		0.67
6					High		High		0.81
7				MS			Low		0.80
8				B5			Low		0.63
9				St			High		0.50
10		**Low**	**Low**					**Low**	**1.00**
11		**Low**	**High**					**High**	**1.00**
12		Mid	Low					Low	1.00
13		Mid	High					Low	1.00
14		High	Low					Low	1.00
15		High	High					Low	1.00
16	**IF**				Low	**THEN**		High	1.00
17					Mid			Low	0.76
18					High			Low	0.75
19				MS				High	0.66
20				B5				High	0.55
21				St				High	0.86

values (rule 9, MD = 0.50). However, taking into account the membership values obtained for all media, it can be deduced that none of them are efficient in obtaining a high number of new shoots and suggests that a new optimized media for *A. arguta* should be designed.

The results obtained agree with previous studies showing that the addition of BAP is crucial for shoot production (Cañal et al., 2000; Akbas et al., 2007), in fact, the control treatment (0 mg L^{-1} BAP and 0 mg L^{-1} GA_3) showed no shoot proliferation. Moreover, Akbas et al. (2007) also showed that the SN decreased as the BAP concentration increased up to 4 mg L^{-1}, with the treatments supplemented with 0.5 mg L^{-1} showing the highest SN on *A. deliciosa.* Working on the same species, Beretta and Eccher (1987) achieved the highest proliferation rate at 1 mg L^{-1} BAP. Here, neurofuzzy logic analysis also supports that mid (0.6–0.7 mg L^{-1}) concentrations of BAP are the clue to obtain large number of shoots in *A. arguta*.

Mustafa and Taha (2012), working with *Ficus carica* L., demonstrated that a minimum number of subcultures in a new medium were essential before detecting differences on explant responses with respect to those obtained in the old medium. Their study showed that only after the third subculture, the proliferation rate was higher in the new medium compared to the old one. Prado et al. (2005), working on *A. deliciosa*, described no new shoot formation on a first subculture in a new medium, due to the need for a certain adaptation time. Our studies on *A. arguta* support those results.

More recent studies (Wojtania et al., 2015) have suggested that PGR signaling can be affected by the medium (macro and micronutrients) composition on *Magnolia* sp. Low levels of nitrogen can inhibit leaf formation, regardless of cytokinin levels. Sokolov et al. (2015), working on the same genus, noted that increasing KNO_3 concentration enhanced the proliferation rate, but it could be toxic at high concentrations.

Neurofuzzy model generated for SL indicates that all factors help to explain the variability of this parameter, being the interaction between BAP and GA_3 crucial (Table 5). IF the GA_3 and BAP concentrations are Low THEN the SL is always

Low (rule 10; MD = 1.00), but IF the BAP concentration is Low but the GA_3 concentration is High THEN the SL is always High (rule 11; MD = 1.00). Those rules support the idea that GA_3 promotes the growth in length, whereas BAP, as cytokinin promotes division and generation of new shoots (Kiba et al., 2010). It has been reported that GA_3 promotes SL (Chawla, 2009). However, in our study this growth regulator only shows a positive effect on SL just in combination with low concentrations of BAP (0–0.3 mg L^{-1}). Working with *A. deliciosa*, Moncaleán et al. (2002) showed that treatments not supplemented with cytokinins had a high GA_3 absorption, suggesting that the presence of cytokinins could negatively affect explant GA_3 uptake. Our results agree with those studies.

The number of subcultures also affects the SL parameter (Table 5). IF the subculture number is Low THEN the SL is High (rule 16; MD = 1.00), and IF it is Mid or High THEN the SL is Low (rules 17–18, MD = 0.76–0.75).

The submodel 3 indicates that all media give acceptable results for SL (rules 19–21; Table 5) in *A. arguta.*

Conclusions

In conclusion, the results described in this study allow us to suggest that the presence of BAP at mid or high concentrations (here > 0.6 mg L^{-1}) is needed for a successful *A. arguta* micropropagation. Moreover, high GA_3 (here around 1.0 mg L^{-1}) concentration in combination with low BAP (here 0–0.3 mg L^{-1}) concentration was necessary for high shoot elongation. Also, both response parameters (SN and SL) were influenced by the number of subcultures, proving that it is necessary at least 3 subcultures, to obtain the highest SN although only 1 for highest shoot length. St medium, designed for *Actinidia*, showed the overall best results, although could be modified to improve SL results. Therefore, it is clear that a new optimized medium for *A. arguta* should be designed by adjusting those factors as suggested by the model. Finally, neurofuzzy logic has proved to be a useful modeling tool to obtain knowledge of the effects of the studied variables on the *A. arguta* in vitro proliferation (through simple rules) and to make efficient decisions for the design of a new culture medium (critical factors) that allows the improvement of proliferation in vitro of this species.

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