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Deciphering the effect of vitamins and mineral nutrients on kiwiberry micropropagation using computer-based tools

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Abstract

Mineral nutrients are essential components of basal media for optimal growth and development of plants grown in vitro. Their deficiency causes physiological disorders. Vitamins have several physiological functions, however, their role in the growth and development of micropropagated plants remains scarce and controversial. The objective of this work has been to get insight on the role of mineral nutrients and vitamins as main components of the in vitro basal medium on the quality of micropropagated kiwiberry (hardy kiwi). Two computer tools were used: design of experiments (DOE) and machine learning (ML), particularly neurofuzzy logic (NL). Two different experiments were designed using a well-sampled five-dimensional experimental design space of 33 treatments using DOE. In both experiments five independent factors were established, in the first the following mineral salts: NH_4NO_3, KNO_3, Mesos, Micros and iron, while in the second, the following five vitamins: myoinositol, thiamine, nicotinic acid, pyridoxine and vitamin E. Neurofuzzy logic models were used to identify the critical vitamins and mineral nutrients on shoot length (SL) and shoot quality (SQ). Neurofuzzy logic models showed that only 7 out of 18 mineral elements were critical factors affecting both, SL and SQ, while only 3 out of 5 vitamins were relevant. In conclusion, these results shed light on the effect of mineral nutrients as key requirements on shoot growth and quality and, for the first time, identified the relevant role of vitamin E on shoot growth of micropropagated kiwiberry.

Keywords: *Actinidia arguta*, artificial intelligence, in vitro culture, modeling, micropropagation, vitamin E, plant tissue culture

INTRODUCTION

Vitamins are organic compounds synthesized by all plants, with vital functions such as cofactors and antioxidants (Smith et al., 2007; Asensi-Fabado and Munné-Bosch, 2010; Li et al., 2021). Vitamins are often components of plant tissue culture media, playing a key role in plant growth and development but, the type and amount of vitamins required by the plants remains unclear (Abrahamian and Kantharajah, 2011; Fitzpatrick and Chapman, 2020) and their addition in plant tissue culture formulations remains controversial (Phillips and Garda, 2019). In fact, the supply of vitamins for optimal plant growth and morphogenesis to the media varied widely depending on the plant species and genotype studied and even, for some of them, no vitamins are required (Ozsan and Onus, 2017). The commonly used vitamins in plant tissue culture belong to vitamin B complex (Arab et al., 2018; Phillips and Garda, 2019; Fitzpatrick and Chapman, 2020): thiamine (B_1), nicotinic acid (niacin; B_3), pyridoxine (vitamin B_6) and myo-inositol. Less frequently riboflavin (B_2), pantothenic acid (B_5), biotin (B_7), folic acid (B_9), ascorbic acid (vitamin C) or α-tocopherol (vitamin E).

Determining critical components to improve the plant tissue culture media is challenging, due to the large number of constituents involved, such as mineral nutrients, plant growth regulators and vitamins (Phillips and Garda, 2019). Traditionally, factorial designs and

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statistical tools were used (Nezami-Alanagh et al., 2017), but today it has been shown that reduced experimental designs as D-optimal together with machine learning (ML) tools deal with the big databases derived from those experiments, being able to establish the effect of critical components using a reduced number of treatments (Kovalchuk et al., 2017; Nezami-Alanagh et al., 2018), predict the results or even optimize the growth of the plant in vitro (Gago et al., 2010b; Landin and Rowe, 2013). Machine learning combines computational statistics and algorithms to build "intelligent" mathematical models for predictions and to make decisions. Our group was pioneer in using artificial intelligence algorithms (AI) such as neurofuzzy logic for modeling, optimization and discovering the critical factor affecting the in vitro growth in the grapevines, apricot, and pistachio (Gago et al., 2010a, 2011; Nezami-Alanagh et al., 2019). Neurofuzzy logic is a powerful AI tool that combines artificial neural networks and the fuzzy logic technique. The rules provided by this technology allow the extraction of information and the generation of knowledge, being extremely useful for decision-making. Previously, DOE and ML combination has been successful in determining the interactions among mineral nutrients and predict the best combination of minerals for a healthy micropropagation of kiwiberry (Hameg et al., 2020). In this work, we will jointly analyse the effect of mineral nutrients and vitamins on the in vitro growth parameters of kiwiberry using machine learning techniques. The previously used database (Hameg et al., 2020) will be expanded with the results of a new experiment in which the effect of five vitamins at different levels will be studied. The new analysis should make it possible to deepen the knowledge of the in vitro culture of this species and elucidate the role of vitamins on the quality of micropropagated kiwiberry.

MATERIALS AND METHODS

Plant material and culture condition

Explants of *Actinidia arguta* (Sieb. et Zucc.) Planch. ex Miq. 'Issai' of about 2 cm were cultured in 200-mL culture vessels containing 30 mL of each medium for 50 days. The cultures were maintained in a growth chamber at 25±1°C under a 16-h photoperiod at 40 µmol m^{-2} s^{-1} irradiation provided by cool white fluorescent tubes, as described elsewhere (Hameg et al., 2020). The pH was set to 5.7 prior autoclaving (121°C for 20 min at 105 kPa). Each treatment included five replicates of three explants each contained in glass vessels sealed with plastic caps. The experiments were carried out in triplicate. The shoots were harvested after 50 days.

Experimental design and data acquisition

Results from two experiments were selected to be modeled together to reveal the role of the mineral and vitamins on kiwiberry micropropagation. In the first, salts of MS medium (Murashige and Skoog, 1962) were classified into 5 independent factors: 1) NH_4NO_3; 2) KNO_3; 3) Mesos ($CaCl_2$, $MgSO_4$, KH_2PO_4); 4) Micros ($MnSO_4$, $ZnSO_4$, H_3BO_3, KI, $CuSO_4$, Na_2MoO_4, $CoCl_2$), and 5) iron – $FeSO_4$, Na_2 EDTA; following a five-dimensional experimental design developed using the software Design-Expert® 8 (Design-Expert, 2010), by selecting the D-optimal criteria. These results were published previously (Hameg et al., 2020). The second experiment was carried out to decipher the effect of vitamins. In this case, the next 5 individual vitamins were used as factors (Table 1): myo-inositol (Myo), thiamine (Thia), nicotinic acid (Nic), pyridoxine (Pyr) and vitamin E (Vit E) at several levels of concentration (Table 1). Finally, all treatments from both experiments were supplemented with 2 mg L^{-1} glycine, 30 g L^{-1} sucrose, 8 g L^{-1} agar and 1 mg L^{-1} BAP and 1 mg L^{-1} GA_3.

The following growth and quality parameter responses were determined, as described previously (Hameg et al., 2020): length of the shoots (SL) as the length from the base to the tip of the shoots (cm) and the quality of the shoots (SQ) evaluating its vigor and absence of physiological disorders, categorizing the plant on a scale of 1 to 5.

Table 1. Five-factor design including 33 model points and MS media (points 34-36) as controls[a]. The vitamin concentration on each medium is expressed as mg L^{-1}.

Media (treatments)	Factor 1 Myo-inositol	Factor 2 Thiamine	Factor 3 Nicotinic acid	Factor 4 Pyridoxine	Factor 5 Vitamin E
1	0	4.00	0.00	0.00	0.00
2	0	8.50	0.00	1.50	1.00
3	0	0.55	4.38	1.50	1.00
4	1000	10.00	5.00	0.00	0.00
5	1000	10.00	0.00	0.89	1.00
6	0	7.40	5.00	0.00	0.66
7	1000	0.00	3.00	1.50	0.00
8	50	0.00	0.00	1.50	0.26
9	595	0.00	5.00	0.00	1.00
10	0	7.40	5.00	0.00	0.66
11	1000	10.00	0.00	0.89	1.00
12	0	10.00	2.38	0.86	0.03
13	1000	7.15	0.00	0.98	0.00
14	1000	3.05	5.00	0.89	0.60
15	1000	3.05	5.00	0.89	0.60
16	875	0.55	0.00	1.50	1.00
17	380	6.95	5.00	1.50	0.00
18	1000	10.00	2.50	1.50	0.55
19	525	3.30	2.65	0.16	0.11
20	475	4.35	2.38	0.77	0.52
21	1000	10.00	2.50	1.50	0.55
22	475	4.35	2.38	0.77	0.52
23	0	10.00	2.15	0.53	1.00
24	475	10.00	0.00	0.08	0.57
25	182	1.70	0.91	1.43	0.95
26	1000	0.00	0.00	0.00	0.38
27	1000	7.00	1.93	0.00	1.00
28	0	6.05	0.00	0.96	0.38
29	0	0.00	0.28	0.41	1.00
30	0	0.00	5.00	0.54	0.00
31	515	10.00	5.00	0.84	1.00
32	596	6.55	0.00	1.50	0.62
33	475	4.35	2.38	0.77	0.52
34/35/36	100	1.00	0.50	0.50	0.00

[a]For mineral´s five-factor design please see Table 2 in Hameg et al. (2020).

A database was built using 23 inputs (18 ions and 5 vitamins) and 2 outputs (SL and SQ). The use of individual ions and vitamins makes easier the understanding of the specific effects of each ion avoiding the ion confounding, as described in detail elsewhere (Niedz and Evens, 2006, 2007; Nezami-Alanagh et al., 2018).

Computer-based tools

Neurofuzzy logic is a hybrid technology that combines the strength of artificial neural networks with the ability of fuzzy logic to generate meaningful rules (Gallego et al., 2011). The software FormRules® v4.03 (Intelligensys Ltd., UK) was used in this study. FormRules uses a technology based on the adaptive spline modeling of data (ASMOD) algorithm to minimize the number of relevant inputs involved in the model built for each parameter, reducing its complexity and improving the accuracy with fewer parameters (Shao et al., 2006).

Additionally, ASMOD split each model into submodels in function of the relevant inputs, to clearly infer the obtained results by generating a set of rules. Finally, FormRules®, uses various statistical fitness criteria, including structural risk minimization (SRM), cross-validation (CV), Bayesian information criterion (BIC), among others. Here, SRM provided the best results, ensuring maximum predictability with the simplest rules. To assess model quality, both model predictability – by the Train Set R2 parameter (>70% are indicative of high predictability) – and model accuracy – through the corresponding ANOVA – were tested as described in detail previously (Shao et al., 2006; García-Pérez et al., 2020; Hameg et al., 2020). All data were used for training the model (García-Pérez et al., 2020).

The neurofuzzy logic software generates for each model a set of 'IF… THEN' rules with a specific range level (from low to high) and a membership degree, a confidence level, which ranges from 0 to 1, meaning 1 that the rule is always fulfilled. More detailed information about the interpretation of these concepts ('IF…THEN' rules, range levels, and membership degrees) can be found in previous reports (Gallego et al., 2011; Nezami-Alanagh et al., 2017; García-Pérez et al., 2020).

RESULTS AND DISCUSSION

Neurofuzzy logic succeeded in modeling the effect of mineral nutrients and vitamins on the growth and healthy proliferation of kiwiberry and selecting the critical factors. The selected critical variables distributed in several submodels by the ASMOD algorithm, together with the parameters to indicate models predictability and accuracy are presented in Table 2.

Table 2. Neurofuzzy logic model train set R^2, ANOVA parameters for training (*f*-ratio, degrees of freedom (df1: model and df2: total), f-critical value for α=0.01) and critical factors (significant inputs) computed for each output. The inputs with stronger effect on each output have been highlighted in bold.

Outputs	Submodel	Critical factors	Train set R^2 (%)	*f-ratio*	df1	df2	*f*-critical
SL	1	Fe^{2+}	97.18	27.81	37	67	2.38
	2	**Mg^{2+}**					
	3	NO_3^-					
	4	Pyr × Vit E					
	5	PO_4^{3-}					
	6	BO_3^-					
	7	Myo × Vit E					
SQ	1	Fe^{2+}	93.30	63.86	12	67	2.46
	2	**$K^+ \times SO_4^{2-}$**					
	3	BO_3^-					
	4	NO_3^-					

For the SL, the model showed high predictability (Train Set R^2=97%) and accuracy (*f*-ratio = 27.81). The model is divided into seven submodels, revealing than 5 ions, independently, and three vitamins explained 97% of the variability of the length of the shoots (Table 2). Moreover, the independent effect of Mg^{2+} was pinpointed as the main factor (strongest effect) affecting this parameter, as described previously (Hameg et al., 2020). The other ions (Fe_2^+, NO_3^-, PO_4^{3-}, BO_3^-) found from neurofuzzy also contributed to explain the variability found on this parameter, but of lesser magnitude than Mg^{2+} (Hameg et al., 2020). Additionally, neurofuzzy logic generated two submodels involving the interaction of three vitamins: Pyr with Vit. E and other interaction between Vit. E and Myo (Table 2).

For the SQ, neurofuzzy logic generated four different submodels, being the interaction between K^+ and SO_4^{2-} was selected as the main factor with a stronger effect on this parameter, as described on previous studies (Hameg et al., 2020). The model also showed other mineral elements that also contributed to explain the variability of the SQ (Fe_2^+, BO_3^-, and NO_3^-). Neurofuzzy logic did not detect any effect of the vitamins within the limits of the study (Table

2).

The neurofuzzy logic FormRules® software generated a total of 58 rules, including all factors described in Table 2, but only the rules related with highest membership degree are shown in Table 3.

Table 3. Rules for both parameters SL and SQ related with growth and quality responses, respectively and their membership degree (MD). The inputs with the strongest effect indicated by the model have been highlighted in bold.

Rule		$[Mg^{2+}]$	$[K^{+}]$	$[SO_4^{2-}]$	[Myo]	[Pyr]	[Vit E]		SL	SQ	MD
3		**Low**							**Low**		**1.00**
4		**Mid**							**High**		**1.00**
5		High							Low		1.00
14						Low	High		High		1.00
17	IF					Mid	High	THEN	High		0.68
20						High	High		High		1.00
33					Low_1(4)		Mid		High		1.00
36					Mid_2(4)		Mid		High		1.00
39					Mid_3(4)		Mid		High		1.00
42					High_4(4)		Mid		High		0.86
46			Low	Low						Low	0.68
47			**Low**	**Mid**						**Low**	**1.00**
48	IF		Low	High				THEN		Low	0.53
49			High	Low						Low	1.00
50			**High**	**Mid**						**High**	**1.00**
51			High	High						High	0.95

SL was mainly predicted by the independent effect of the magnesium ion. In this way, neurofuzzy suggests that IF the media is supplemented with Mg^{2+} at Mid concentration (1.41-3.47 mM; Hameg et al., 2020) THEN the SL is always High (Table 3; rule 4; membership 1.00). The MS culture media supplies the ion Mg^{2+} at 1.5 mM as the salt $MgSO_4 \cdot 7H_2O$ (440 mg L^{-1}). Other authors (Poothong and Reed, 2014, 2015) in raspberries suggested that "high mesos" (including $MgSO_4$ up to 555 mg L^{-1}) also promoted the highest shoot length. However, they were not able to establish if the effect was due to the ion Mg^{2+} or the ion SO_4^{2-} independently, or due to the interaction between them. Our results fully agree with those findings and demonstrated that $MgSO_4 \cdot 7H_2O$ supplied at mid concentrations always promoted long shoots (Hameg et al., 2020). Interestingly, neurofuzzy logic was able to distinguish the role of Mg^{2+} and SO_4^{2-}, due to the previously mentioned ion confounding effect (Niedz and Evens, 2006; Niedz and Evens, 2007). Further details about the role of other mineral elements on SL of micropropagated kiwiberry can be found in our previous work (Hameg et al., 2020). SQ was mainly predicted by the effect of the interaction between K^+ and SO_4^{2-}. The rules stated that in order to get healthier plants, high levels of K^+ (>12.37 mM) were always needed in combination with mid to high SO_4^{2-} (1.67 mM < x < 5.20 mM) (Table 3; rules 50-51). Regarding this, it is widely known that increasing the Mesos salts ($CaCl_2$, $MgSO_4$, KH_2PO_4; up to 1.5×), salts which contain these two ions, generally promotes a positive effect on growth parameters such as shoot length, shoot number (Poothong and Reed, 2015) or chlorophyll content (De Carvalho et al., 2018), and thus contributing to a better overall shoot quality. Additionally, Kovalchuk et al. (2017) indicated that the best shoot quality of *Prunus americana* were achieved with up to 2.4× WPM culture medium levels of KH_2PO_4 and up to 0.8× $MgSO_4$ (12.61 mM K^+ and 7.41 mM SO_4^{2-} mM), fully agreeing with our results, but way above of our testing levels for SO_4^{2-}.

Neurofuzzy logic also highlighted the positive effect of the interaction of three vitamins on shoot elongation. If high vitamin E was supplied to the basal medium in combination with

Pyr, at any level, the SL was higher. Additionally, when mid vitamin E was combined with myoinositol, at any the concentration, the SL was also higher (Table 3; rules 33-42, membership 1.00). These results suggest that the role of this non-commonly supplied vitamin to plant tissue culture media should be reassessed.

Vitamin E (α-tocopherol) has been related to saline, cold and UV-related stress due to its essential role on maintaining membrane integrity (Munné-Bosch and Alegre, 2002). Only few authors have supplemented this vitamin into the basal culture when working with plant cell suspensions. For example, Larkin (1981) supplied vitamin E at 0.01 mg L^{-1} for culturing sugarcane protoplast, while Oswald et al. (1977) supplemented cells suspensions media of white clover (*Trifolium repens*) with 1 mg L^{-1}, and finally, Sroga (1983) tested vitamin E up to 2.5 mg L^{-1} for culturing callus and cell suspensions of *Lupinus angustifolius*. However, the studies regarding the effect of vitamin E on micropropagation is very scarce (George et al., 2008). Here, the use of neurofuzzy logic model pointed support the use of mid-high levels of vitamin E to achieve larger shoots, independently of the Pyr nor Myo concentrations.

Pyr (Vitamin B_6) has been reported to act as co-enzyme in enzymatic reactions to metabolize carbohydrates, fats, and proteins, and is also involved in photosynthesis and respiration (Hendawy and El-Din, 2010). Kazemiani et al. (2018) did not find a significant effect on the number or the length of shoots of potato (*Solanum tuberosum*) when media were supplemented with a high Pyr concentration (50 mg L^{-1}) in combination with Thia (10 mg L^{-1}). However, a positive effect of the Pyr on shoot elongation of other tree fruit plants such as *Pistacia vera* has been recently described (Nezami-Alanagh et al., 2017). Our results showed that the addition of Pyr combined with vitamin E, has a positive effect on the shoot number of kiwiberry.

Myo is also commonly added in small quantities to the culture media in order to stimulate cell growth. The metabolic route of this vitamin leads to its breakdown to ascorbic acid and pectin (Saad and Elshahed, 2012). For this reason, Myo is considered, together with Thia, an essential organic compound for plant tissue cultures (George et al., 2008). Our results indicated an interaction between Myo and Vit E not previously reported.

Taken together, our results pointed out an interesting role of vitamin E in the length and quality of kiwiberry shoots and suggest that this vitamin should be supplied to the basal medium at mid-high concentration (0.75 mg $L^{-1} < x < 1$ mg L^{-1}).

Finally, it should be noted that the structure of the study has not allowed to establish the potential interactions between minerals and vitamins, as it is the result of two independent experimental studies. However, our results reveal the critical role of three vitamins that could otherwise be masked by the stronger effect that mineral nutrients have compared to vitamins on the multiplication and growth of shoots, during the micropropagation of plants, in this case the kiwiberry, as has been demonstrated here.

CONCLUSIONS

In conclusion, the suitability of computer tools such as DOE and ML to predict and select those minerals and vitamins that are potentially critical for micropropagation of kiwiberry has been demonstrated. The results shed light for very first time, on the possible effect of some vitamins such as pyridoxine, myoinositol and particularly vitamin E on the kiwiberry micropropagation. Further studies are needed to validate these results and fully understand the effect of the interaction between mineral nutrients and vitamins and/or other media essential components, such as plant growth regulators.

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