Combining DOE With Neurofuzzy Logic for Healthy Mineral Nutrition of Pistachio Rootstocks *in vitro* Culture

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The aim of this study was to determine the effects of Murashige and Skoog (MS) salts on optimal growth of two pistachio rootstocks, *P. vera* cv. "Ghazvini" and "UCB1" using design of experiments (DOE) and artificial intelligence (AI) tools. MS medium with 14 macro—and micro-elements was used as base point and its concentration varied from 0 to 5 × MS concentrations. Design of experiments (DOE) software was used to generate a five-dimensional design space by categorizing MS salts into five independent factors (NH_4NO_3, KNO_3, mesos, micros and iron), reducing the experimental design space from 3,125 to just 29 treatments. Typical plant growth parameters such as shoot quality (SQ), proliferation rate (PR), shoot length (SL), and some physiological disorders including shoot-tip necrosis (STN) and callus formation at the base of explants (BC) were evaluated for each treatment. The results were successfully modeled using neurofuzzy logic software. The model delivered new insights, by different sets of "IF–THEN" rules, pinpointing the key role of some ion interactions ($SO_4^{2-} \times Cl^-$, $K^+ \times SO_4^{2-} \times EDTA^-$, and $Fe^{2+} \times Cu^{2+} \times NO_3^-$) for SQ, PR, and SL, whilst physiological disorders (STN and BC) were governed mainly by independent ions as Fe^{2+} and $EDTA^-$, respectively. In our opinion, the methodology and results obtained in this study is extremely useful to understand the effect of mineral nutrients on pistachio *in vitro* culture, through discovering new complex interactions among macro—and micro-elements which can be implemented to design new media of plant tissue culture and improve healthy plant micropropagation for any plant species.

Keywords: DOE, micropropagation, health shoots, neurofuzzy logic, pistachio rootstock, physiological disorders

INTRODUCTION

Most authors recommend the use of MS as a basal culture medium for the micropropagation for different species of *Pistacia* as *P. vera, P. khinjuk, P. lenthiskus, P. atlantica* (Barghchi and Alderson, 1983a,b, 1985; Yang and Lüdders, 1993; Benmahioul et al., 2012; Akdemir et al., 2014). Other media specifically design for woody plants, as DKW (Driver Kuniyuki Walnut medium; Driver and Kuniyuki, 1984) or WPM (Woody Plant Medium; Lloyd and McCown, 1980) for *P. vera* L. (Gonzalez and Frutos, 1990; Benmahioul et al., 2009) have also been applied. However, the use of

these standard media increases the cost of micropropagation of *Pistacia sp.* due to the appearance of physiological disorders, such as shoot tip necrosis (STN), hyperhydricity or the formation of callus (BC) at the base of the shoots (Abousalim and Mantell, 1992, 1994; Dolcet-Sanjuan and Claveria, 1995; Onay, 1996).

To avoid those problems, different alternatives have been suggested, based mainly on the combination of macro-, micro-elements and/or vitamins previously proposed for improving the effectiveness of the multiplication of shoots (Onay, 2000; Tilkat et al., 2005, 2008; Benmahioul et al., 2012), or on the incorporation of additional ingredients to the basal culture media, such as calcium gluconate, silver nitrate, ascorbic acid or vanillin, among others (Abousalim and Mantell, 1992, 1994; Parfitt and Almehdi, 1994; Dolcet-Sanjuan and Claveria, 1995; Mederos-Molina and Trujillo, 1999; Akdemir et al., 2012; Kilinç et al., 2015; Marín et al., 2017).

The establishment of an adequate design space should allow to improve the understanding of the role of mineral nutrition in response of pistachio shoots and, ultimately, to determine a culture medium suitable for mass micropropagation of *Pistacia*.

The design of experiments (DOE) provides researchers to program investigations by dramatically reducing the number of combinations to be studied and distributing them appropriately in the *n*-dimensional design space (Niedz and Evens, 2016). As a result, it is possible to obtain general conclusions, from the generated databases under statistically optimal conditions. As an example, a five-dimensional design space has been constructed on the basis of MS medium composition using a DOE software in order to optimize mineral nutrition of different species as *Citrus sinensis* (Niedz and Evens, 2007); the hybrid Gerbera (Niedz et al., 2014), *Rubus idaeus L.* (Poothong and Reed, 2014, 2015), *Corylus avellana L.* (Hand et al., 2014; Akin et al., 2017a,b), Pear sp. (Reed et al., 2016) and *Prunus armeniaca* Lam (Kovalchuk et al., 2017). As mineral nutrients of culture medium includes many components, the five-dimensional design space proposed elsewhere (Reed et al., 2016) was carried out by dividing all the mineral nutrients of MS into five factors, allowing to reduce the number of treatments from 3,125 (5^5 combinations treatments) to just 29 treatments.

If we think that mineral nutrients are just a part of the plant tissue culture medium and that other components such as vitamins or plant growth regulators need also being added and optimized, it is clear that finding adequate composition for plant tissue culture media requires to deal with high dimensional spaces, which represent a great challenge for researchers (Niedz and Evens, 2016). In this situation, artificial intelligence tools are presented as promising candidates to extract information from the big databases that can be generated during the development of the culture media using high dimensional spaces (Gallego et al., 2011). Several studies have illustrated the effectiveness of artificial neural networks (ANNs) (Gago et al., 2010a,b,c,d, 2014) and hybrid systems as neurofuzzy logic (Gallego et al., 2011; Nezami-Alanagh et al., 2014; Ayuso et al., 2017) in modeling and extracting information from experiments carried out even without a well define design space. Even more, using those artificial intelligence tools, it has been identified the negative effect of some additional ingredients that had been proposed

for improving pistachio culture, especially the silver and the gluconate ions (Nezami-Alanagh et al., 2017).

The objective of this research was to combine DOE and an artificial intelligence tool, neurofuzzy logic, first to establish a well experimental design space and second to determine the key mineral nutrients in pistachio culture. The use of two different pistachio rootstocks, "Ghazvini" and "UCB1," will make it possible to draw conclusions about the influence of genotype variations on the nutritional needs of culture.

MATERIALS AND METHODS

Plant Materials and *in vitro* Culture Conditions

Pistacia vera cv. "Ghazvini," a high tolerant to salinity rootstock (Karimi et al., 2009) and "UCB1"(interspecific hybrid *P. atlantica* × *P. integrima*), a vigorous rootstock that has resistance to wilt by *Werticillium*, were micropropagated on MS medium (Murashige and Skoog, 1962) supplemented with 1.1 mg L^{-1} N^6-benzyladenine (BAP), 0.1 mg l^{-1} indole-3-butyric acid (IBA), 30 g L^{-1} sucrose and 5.7 g L^{-1} agar. The pH was adjusted to 5.7 prior autoclaving (121°C, 1 kg cm^{-2} s^{-1} for 20 min). The cultures were kept under 16 h photoperiod (white fluorescent tubes; irradiance of 65 μmol m^{-2} s^{-1}) and day/night temperature of 25/20 ± 2°C and subcultured into a fresh medium every 30 days. Pistachio explants, with almost the same shoot length (∼1 cm) and containing 1–2 axillary buds, were randomly selected just before placing in glass boxes (180 ml) containing 25 ml on the set of treatment combinations. After 3 subcultures (30 days interval) on the same culture media, growth parameters and physiological disorders were evaluated as follows: shoot quality (SQ; scored as 1 = necrotic, 2 = poor, 3 = moderate, 4 = good), proliferation rate (PR; number of new regenerated shoots per explant), shoot length (SL; length of new regenerated shoots per explant in cm), shoot-tip necrosis (STN; scored as 1 = none, 2 = very low, 3 = moderate, 4 = high), and basal callus (BC; callus formation at the cut edge of shoots in grams).

Each treatment consisted of two replicates glass boxes (180 ml) sealed with crystal caps, containing five explants each. The experiments were carried out in triplicate.

Design of Experiment (DOE) and Data Acquisition

For the modification of MS medium, the 14 salts of MS basal medium were subdivided into five independent factors including: (i) NH_4NO_3, (ii) KNO_3, (iii) mesos, (iv) micros, and (v) iron, over a range of concentrations expressed as × MS levels (**Table 1**).

The five-factor experimental design was a 28-model-point using IV-optimal response surface using software application Design-Expert®8 (Design-Expert, 2010) plus another point with MS salt concentration as control (**Table 2**).

Modeling Tools

The database was modeled using commercial software (FormRules® 4.03, Intelligensys Ltd., UK) that combines artificial neural networks (ANNs) and fuzzy logic tools. A detailed description of the software package has reported

TABLE 1 | The five mineral nutrient factors used to construct the experimental design space based on MS salts, and concentration range expressed as × MS levels.

Factors	MS salts	Range
Factor1	KNO_3	0.0–1.0×
Factor 2	NH_4NO_3	0.2–1.1×
Factor 3 (Mesos)	$CaCl_2.2H_2O$ KH_2PO_4 $MgSO4.7H2O$	0.25–3.0×
Factor 4 (Micros)	$MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ $CuSO_4.5H_2O$ KI $CoCl_2.6H_2O$ H_3BO_3 $Na_2MoO_4.2H_2O$	0.1–4.0×
Factor 5 (Iron)	$FeSO_4.7H_2O$ $Na_2EDTA.2H_2O$	1.0–5.0×

TABLE 2 | Five factor design including 29 treatment points based on ×MS concentrations of mineral nutrients.

Treatments	Factor 1 KNO_3	Factor 2 NH_4NO_3	Factor 3 Mesos	Factor 4 Minors	Factor 5 Iron
#1	1.00	0.20	0.25	4.00	2.33
#2	0.00	0.20	2.08	4.00	1.00
#3	1.00	1.10	2.08	0.10	1.00
#4	0.00	1.10	0.25	1.40	3.66
#5	0.00	1.10	3.00	4.00	1.00
#6	0.50	0.20	3.00	0.10	5.00
#7	0.00	0.20	0.25	0.10	1.00
#8	0.00	1.10	3.00	4.00	5.00
#9	0.33	0.50	0.25	0.10	5.00
#10	0.00	0.50	3.00	0.10	2.33
#11	1.00	1.10	0.25	4.00	1.00
#12	0.00	0.20	1.16	2.70	5.00
#13	1.00	0.65	0.25	0.10	1.00
#14	0.33	0.20	3.00	4.00	3.66
#15	1.00	0.20	1.16	0.10	3.66
#16	0.66	1.10	3.00	0.10	3.66
#17	0.00	1.10	1.62	0.10	1.00
#18	1.00	0.80	3.00	4.00	2.33
#19	1.00	0.80	0.25	2.70	5.00
#20	1.00	1.10	0.25	0.10	3.66
#21	0.50	0.65	1.62	2.05	3.00
#22	1.00	1.10	3.00	2.70	5.00
#23	1.00	0.20	3.00	2.70	5.00
#24	0.50	0.65	1.62	2.05	2.00
#25	0.66	0.20	3.00	1.40	1.00
#26	0.00	0.80	3.00	1.40	5.00
#27	0.00	0.80	0.25	4.00	2.33
#28	0.33	1.10	1.16	4.00	5.00
Control (MS)	1.00	1.00	1.00	1.00	1.00

elsewhere (Shao et al., 2006). During the training process 24 variables were included. As *inputs*, 19 independent variables or factors including genotype ("UCB1" and "Ghazvini" rootstocks) and 18 ions (NH_4^+, NO_3^-, K^+, Ca^{2+}, Mg^{2+}, PO_4^{2-}, SO_4^{2-}, Cl^-, Fe^{2+}, BO_3^-, Mn^{2+}, Zn^{2+}, Cu^{2+}, MoO_2^{2-}, Na^+, Co^{2+}, I^- and $EDTA^-$) and as *outputs*, five dependent variables or parameters (SQ, PR, SL, STN, and BC mean responses of 10 shoots per treatment for each genotype).

Modeling process was carried out as previously described by Nezami-Alanagh et al. (2017). Individual models were developed for each *output*, the predictability of which was tested using the Train Set R^2 value or coefficient of determination expressed as percentage, which is indicative of the percentage of variation of an *output* that is explained by the *inputs* in the model, it is defined by equation 1.

$$R^2 = \left(1 - \frac{\sum\limits_{i=1}^{n} \left(y_i - y_i' \right)^2}{\sum\limits_{i=1}^{n} \left(y_i - y_i'' \right)^2} \right) \times 100\% \qquad (1)$$

Where y is the experimental point in the data set, y' is the predicted value calculated by the model and y'' is the mean of the dependent variable. The larger the value of the train set R^2, the more the model captured the variation in the training data. Values of R^2 between 70 and 99.9% are considered indicative of good model predictabilities (Colbourn and Rowe, 2005, 2009).

The Analysis of Variance (ANOVA) was used to evaluate the differences between experimental outputs and predicted by the model ones. F ratios higher than the critical f for the degrees of freedom of the model indicate that there are not significant differences between predicted by the model and the experimental *outputs*, and therefore, that the models are accurate. Models were developed using the default parameters in the software as shown in **Table 3**.

To carry out the modeling, avoiding ion confounding problem pointed out by Niedz and coworkers (Niedz and Evens, 2006), the ionic composition of each treatment was calculated from its corresponding macro—and micro-elements (**Tables 4** and **S1**).

Among the statistical fitness criteria used by FormRules®, Structural Risk Minimization (SRM) was selected because it allowed obtaining the models with the highest predictabilities together with the simplest and more intelligible rules. FormRules® has been designed on the basis of the ASMOD algorithm (adaptive spline modeling of data) which allows the models to be divided into several submodels to easily generate "IF-THEN" rules and the interpretation of results. The result of neurofuzzy logic technology, as described by Gago et al. (2011), is a predictive model presented as "IF-THEN" rules, together with a degree of membership that varies between 0.00, "Low value," and 1.00, "High value" (Shao et al., 2006).

RESULTS

Neurofuzzy logic succeeded in simultaneously modeling the five growth parameters studied showing high Train Set R^2 between

TABLE 3 | The training parameters setting with neurofuzzy logic.

Critical factors for neurofuzzy logic model
Minimization parameters
Ridge regression factor:$1e^{-6}$
Model selection criteria
Structural risk minimization (SRM)
C1 = 0.8–0.916; 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

experimental and predicted values ($73.2 \leq R^2 \leq 94.1\%$), which are an indication of high predictability of the algorithm (**Table 5** and **Figure 1**).

Table 5 indicates the critical factors (significant *inputs*) for each *output*, together with the Train Set R^2 and ANOVA parameters of the model (calculated F ratio, degrees of freedom and f critical for $\alpha < 0.01$). The ANOVA F ratio was always greater than the corresponding f critical value, indicating good performance and quality of neurofuzzy logic models (**Table 5**).

Neurofuzzy also succeeded in determining that just 10 out of 19 *inputs* studied (genotype, NH_4^+, NO_3^-, K^+, SO_4^{2-}, Cl^-, Fe^{2+}, BO_3^-, Cu^{2+}, $EDTA^-$) significantly affected the results (**Table 5**).

Shoot quality score (1 = necrotic, 2 = poor, 3 = moderate, 4 = good) is a subjective measure that encompasses the overall appearance of shoots, including leaf and shoot health as well as multiplication. Neurofuzzy logic split the model for this parameter into four submodels: the interaction $SO_4^{2-} \times Cl^-$, highlighted as the *inputs* with stronger effect (see submodel 2) and the linear independent effects of Fe^{2+}, K^+, and NH_4^+ ions (**Table 5**).

The "IF-THEN" rules generated by the neurofuzzy models explain in words the effects of the critical *inputs* for each *output*. **Table 6** summarizes the combinations of variables to achieve low or high results with the highest degree of membership.

As it can be easily deduced from the rules, the highest shoot quality is achieved with a combination of Mid SO_4^{-2} and Low Cl^- and Low Fe^{2+} concentration (rules 3 and 7 from **Table 6**). Negative effects of both K^+ and NH_4^+ high levels are also deduced (rules 10 and 13 from **Table 6**). The meaning of Low, Mid or High for the different *inputs* can be found in **Figures S1 A1–A4** of Supplementary Materials.

It is noteworthy that the shoots of both genotypes grown with treatment #24 (**Table 4**), have a higher quality than those grown with MS (used as control), which agrees with the results of the model (**Figures 2A–D**).

The new regenerated shoots (PR) variability is explained by two submodels: the complex interaction of K^+, $EDTA^-$ and SO_4^{2-} (strongest effect) together with the interaction between Fe^{2+} and BO_3^- as submodels 1 and 2, respectively (**Table 5**).

From the corresponding rules (**Table 6**), two critical conclusions would be extracted: (i) the convenience of using

Low $EDTA^-$ concentrations (Low < 0.3 mM; **Figure S1-B1**) for obtaining High PR values, regardless the amounts of either K^+ or SO_4^{2-} (rules 14–17; **Table S2**); and (ii) the dominant role of K^+ on the PR parameter when $EDTA^-$ and SO_4^{2-} are applied at High concentration, producing a set of respective greatest and lowest PR when K^+ at Low and High amounts (**Figure S1-B1**) are used (rules 19 and 21; **Table 6**). Submodel 2 pinpoints the negative influence of BO_3^- in the presence of Fe^{2+} at either Low or Mid concentrations, giving the highest PR when low amount of BO_3^- (**Figures S1-B2,3**) is added to the culture medium (membership degree1.00, rule 24; **Table 6**).

Neurofuzzy logic explained the shoot length (SL) variability by building three submodels: the complex combination of Fe^{2+}, Cu^{2+} and NO_3^- (strongest effect), the interaction between K^+ and Cl^- ion concentrations and the genotype (submodels 1-3; **Table 5**).

The analysis of "IF-THEN" rules (**Table 6**) for this parameter reveals that, if the culture media supplemented with High concentration of NO_3^- and Mid concentration of Cu^{2+} (**Figure S1-C1**), the inclusion of Fe^{2+} ion at Low or High concentrations (**Figure S1-C2**) leads to obtaining the longest or shortest shoots, respectively (rules 33 and 42, **Table 6**).

According to these rules, the pistachio shoots grown on MS medium (Control), which incorporates Low concentrations of Fe^{2+}, medium concentration of Cu^{2+} and high concentration of NO_3^-, has the highest lengths for both genotypes with PR indexes of 2.36 ± 0.70 cm and 1.60 ± 0.14 cm for "UCB1" and "Ghazvini" respectively (**Table 4** and **Figure 2**).

The potassium and chloride ions also affect the growth of the shoots, obtaining the longest shoots when the medium contains Low K^+ content (<5 mM) together with High Cl^- content (>10 mM) (**Figures S1-C3**, **C4**) even though with membership 0.71 (rule 49; **Table S2**).

Lastly, the model also shows differences between genotypes for the shoot length parameter, although high values are obtained for both rootstocks (rules 52-53; **Table S2**).

In this study, different types of physiological abnormalities such as STN, hyperhydricity and BC during the multiplication of pistachio shoots of both genotypes were observed. Among them, the STN formation and BC were scored and successfully modeled with the neurofuzzy logic tool (**Table 5**).

Shoot-tip necrosis variability is explained by the linear effect of four *inputs*: $EDTA^-$, SO_4^{2-}, K^+ and genotype (**Table 5** and **Figures 2E–F**). While the use of $EDTA^-$ or K^+ at Low concentration (**Figures S1-D1,3**) has deteriorative effect on STN (rules 54 and 59; **Table 6**), the inclusion of SO_4^{2-} has positive effect. The STN is reduced when culture media includes Mid to High amounts of SO_4^{2-} (rules 56-58; **Table 6** and **Figure S1-D2**). Finally, results also indicate that the severity of STN in pistachio is significantly dependent on genotype, being its occurrence lower in "UCB1" than in "Ghazvini" (rules 61–62; **Table S2**).

Callus formation produced at the cut-edge of shoots was found to be dependent of Fe^{2+} and SO_4^{2-} ion concentration (**Table 5** and **Figure 2G**). The lowest BC is produced when Fe^{2+} concentration is High, over 0.3 mM (**Figure S1-E1**) (rules 63-64; **Tables 6** and **S2**). However, it must be noted that although obtaining lowest BC may seems to be best for researchers, in fact

TABLE 4 | Ion compositions of the different culture media based on the five-factor design space to optimize micropropagation of pistachio rootstocks and average results for each parameter.

Treatment	Genotype	Ions (mM)																Growth parameters			Physiological disorders	
		NH_4^+	NO_3^-	K^+	Ca^{2+}	Mg^{2+}	PO_4^{2-}	SO_4^{2-}	Cl^-	Fe^{2+}	BO_3^-	Mn^{2+}	Zn^{2+}	Cu^{2+}	Co^{2+}	I^-	$EDTA^-$	SQ	PR	SL (cm)	STN	BC (g)
#1	UCB1	4.12	22.92	19.13	0.75	0.38	0.31	1.13	1.50	0.23	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.23	1.89 ± 0.16	2.40 ± 0.40	0.74 ± 0.07	1.88 ± 0.18	0.128 ± 0.010
#2	UCB1	4.12	4.12	2.62	6.24	3.13	2.60	3.75	12.47	0.10	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.10	2.10 ± 0.13	2.85 ± 0.28	0.62 ± 0.04	1.97 ± 0.27	0.194 ± 0.017
#3	UCB1	22.68	41.47	21.40	6.24	3.13	2.60	3.24	12.47	0.10	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.10	2.73 ± 0.13	2.50 ± 0.19	0.77 ± 0.05	1.05 ± 0.05	0.225 ± 0.016
#4	UCB1	22.68	22.68	0.32	0.75	0.38	0.31	0.93	1.50	0.37	0.14	0.14	0.04	0.000014	0.0001471	0.007	0.37	1.72 ± 0.16	1.56 ± 0.29	0.81 ± 0.09	2.61 ± 0.32	0.006 ± 0.002
#5	UCB1	22.68	22.68	3.77	8.98	4.50	3.75	5.12	17.96	0.10	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.10	3.00 ± 0.07	3.20 ± 0.38	1.35 ± 0.15	1.05 ± 0.05	0.245 ± 0.029
#6	UCB1	4.12	13.52	13.14	8.98	4.50	3.75	5.02	17.96	0.50	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#7	UCB1	4.12	4.12	0.31	0.75	0.38	0.31	0.49	1.50	0.10	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.10	2.65 ± 0.18	3.00 ± 0.39	0.51 ± 0.06	1.00 ± 0.00	0.059 ± 0.014
#8	UCB1	22.68	22.68	3.77	8.98	4.50	3.75	5.53	17.96	0.50	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.50	2.00 ± 0.22	2.90 ± 0.31	0.57 ± 0.08	2.25 ± 0.35	0.074 ± 0.010
#9	UCB1	10.31	16.57	6.58	0.75	0.38	3.75	0.89	1.50	0.50	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#10	UCB1	10.31	10.31	3.75	8.98	4.50	3.75	4.75	17.96	0.23	0.40	0.40	0.12	1E-05	1.05E-05	0.0005	0.23	2.44 ± 0.29	3.89 ± 0.35	0.45 ± 0.05	1.55 ± 0.29	0.143 ± 0.016
#11	UCB1	22.68	41.47	19.13	0.75	0.38	0.31	1.00	1.50	0.10	0.40	0.27	0.08	0.000401	0.00042029	0.02	0.10	1.40 ± 0.16	2.30 ± 0.47	0.62 ± 0.11	1.60 ± 0.30	0.112 ± 0.022
#12	UCB1	4.12	4.12	1.47	3.49	1.75	1.46	2.60	6.98	0.50	0.27	0.27	0.00	0.00027	0.0002837	0.0135	0.50	2.28 ± 0.27	2.44 ± 0.24	0.68 ± 0.07	1.77 ± 0.32	0.040 ± 0.006
#13	UCB1	13.40	32.19	19.11	0.75	0.38	0.31	0.49	1.50	0.10	0.40	0.40	0.00	1E-05	1.05E-05	0.0005	0.10	1.94 ± 0.35	2.89 ± 0.35	0.46 ± 0.04	3.05 ± 0.21	0.120 ± 0.008
#14	UCB1	4.12	10.39	10.03	8.98	4.50	3.75	5.39	17.96	0.37	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.37	2.39 ± 0.13	3.11 ± 0.26	0.62 ± 0.07	1.22 ± 0.22	0.184 ± 0.019
#15	UCB1	4.12	22.92	20.25	3.49	1.75	1.46	2.13	6.98	0.37	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.37	1.63 ± 0.21	2.05 ± 0.22	0.62 ± 0.07	2.67 ± 0.32	0.079 ± 0.005
#16	UCB1	22.68	35.20	16.28	8.98	4.50	3.75	4.88	17.96	0.37	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.37	2.19 ± 0.33	2.50 ± 0.37	0.56 ± 0.08	1.75 ± 0.41	0.106 ± 0.015
#17	UCB1	22.68	22.68	2.03	4.86	2.44	2.03	2.55	9.73	0.10	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.10	2.78 ± 0.22	2.78 ± 0.32	0.50 ± 0.05	1.11 ± 0.11	0.249 ± 0.054
#18	UCB1	16.49	35.28	22.56	8.98	4.50	3.75	5.26	17.96	0.23	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.23	3.31 ± 0.36	2.50 ± 0.42	1.23 ± 0.21	1.56 ± 0.37	0.190 ± 0.023
#19	UCB1	16.49	35.28	19.12	0.75	0.38	0.31	1.23	1.50	0.50	0.27	0.27	0.08	0.00027	0.0002837	0.0135	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#20	UCB1	22.68	41.47	19.11	0.75	0.38	0.31	0.76	1.50	0.37	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.37	1.57 ± 0.20	3.00 ± 0.37	0.57 ± 0.10	2.92 ± 0.51	0.056 ± 0.007
#21	UCB1	13.40	22.80	11.44	4.86	2.44	2.03	3.01	9.73	0.30	0.21	0.20	0.06	0.000205	0.0002154	0.01025	0.30	3.05 ± 0.17	2.85 ± 0.22	0.88 ± 0.08	1.32 ± 0.16	0.184 ± 0.017
#22	UCB1	22.68	41.47	22.55	8.98	4.50	3.75	5.36	17.96	0.50	0.27	0.27	0.08	0.00027	0.0002837	0.0135	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#23	UCB1	22.68	22.92	22.55	8.98	4.50	3.75	5.36	17.96	0.50	0.27	0.27	0.08	0.00027	0.0002837	0.0135	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#24	UCB1	13.40	22.80	11.44	4.86	2.44	2.03	2.91	9.73	0.20	0.21	0.20	0.06	0.000205	0.0002154	0.01025	0.20	3.28 ± 0.21	2.78 ± 0.19	1.15 ± 0.15	1.05 ± 0.05	0.229 ± 0.016
#25	UCB1	4.12	16.65	16.28	8.98	4.50	3.75	4.79	17.96	0.10	0.14	0.14	0.04	0.000014	0.0001471	0.007	0.10	2.50 ± 0.13	3.36 ± 0.36	0.71 ± 0.07	1.13 ± 0.13	0.211 ± 0.025
#26	UCB1	16.49	16.49	3.75	8.98	4.50	3.75	5.19	17.96	0.50	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.50	2.20 ± 0.21	3.30 ± 0.33	0.45 ± 0.06	1.35 ± 0.23	0.059 ± 0.004
#27	UCB1	16.49	16.49	0.33	0.75	0.38	0.31	1.13	1.50	0.23	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.23	2.17 ± 0.21	2.33 ± 0.40	0.61 ± 0.05	2.11 ± 0.45	0.056 ± 0.007
#28	UCB1	22.68	28.94	7.74	3.49	1.75	1.46	2.77	6.98	0.50	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.50	2.63 ± 0.20	2.75 ± 0.30	0.70 ± 0.08	1.05 ± 0.80	0.115 ± 0.011
MS (Control)	UCB1	20.61	39.41	20.05	2.99	1.50	1.25	1.73	5.99	0.10	0.10	0.10	0.03	0.0001	0.00010507	0.005	0.10	3.00 ± 0.35	2.60 ± 0.50	2.36 ± 0.70	2.10 ± 0.55	0.225 ± 0.030
#1	Ghazvini	4.12	22.92	19.13	0.75	0.38	0.31	1.13	1.50	0.23	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.23	2.28 ± 0.14	2.89 ± 0.38	0.75 ± 0.12	1.88 ± 0.18	0.084 ± 0.009
#2	Ghazvini	4.12	4.12	2.62	6.24	3.13	2.60	3.75	12.47	0.10	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.10	2.62 ± 0.14	3.45 ± 0.24	0.60 ± 0.02	1.00 ± 0.00	0.191 ± 0.012
#3	Ghazvini	22.68	41.47	21.40	6.24	3.13	2.60	3.24	12.47	0.10	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.10	1.96 ± 0.25	3.18 ± 0.32	0.83 ± 0.07	1.28 ± 0.14	0.187 ± 0.018
#4	Ghazvini	22.68	22.68	0.32	0.75	0.38	0.31	0.93	1.50	0.37	0.14	0.14	0.04	0.000014	0.0001471	0.007	0.37	1.25 ± 0.08	2.20 ± 0.24	0.70 ± 0.09	3.65 ± 0.15	0.001 ± 0.001
#5	Ghazvini	22.68	22.68	3.77	8.98	4.50	3.75	5.12	17.96	0.10	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.10	3.60 ± 0.14	3.40 ± 0.42	1.36 ± 0.14	1.75 ± 0.28	0.287 ± 0.047
#6	Ghazvini	4.12	13.52	13.14	8.98	4.50	3.75	5.02	17.96	0.50	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#7	Ghazvini	4.12	4.12	0.31	0.75	0.38	0.31	0.49	1.50	0.10	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.10	1.95 ± 0.24	4.10 ± 0.43	0.51 ± 0.04	2.38 ± 0.46	0.033 ± 0.007
#8	Ghazvini	22.68	22.68	3.77	8.98	4.50	3.75	5.53	17.96	0.50	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.50	1.89 ± 0.20	3.44 ± 0.05	0.73 ± 0.04	1.88 ± 0.45	0.060 ± 0.017
#9	Ghazvini	10.31	16.57	6.58	0.75	0.38	3.75	0.89	1.50	0.50	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#10	Ghazvini	10.31	10.31	3.75	8.98	4.50	3.75	4.75	17.96	0.23	0.40	0.40	0.12	1E-05	1.05E-05	0.0005	0.23	2.50 ± 0.17	5.45 ± 0.45	0.52 ± 0.05	1.18 ± 0.18	0.146 ± 0.019
#11	Ghazvini	22.68	41.47	19.13	0.75	0.38	0.31	1.00	1.50	0.10	0.40	0.27	0.08	0.000401	0.00042029	0.02	0.10	1.70 ± 0.18	3.00 ± 0.25	0.79 ± 0.08	2.95 ± 0.31	0.102 ± 0.006
#12	Ghazvini	4.12	4.12	1.47	3.49	1.75	1.46	2.60	6.98	0.50	0.27	0.27	0.00	0.00027	0.0002837	0.0135	0.50	1.11 ± 0.07	3.33 ± 0.47	0.64 ± 0.03	3.87 ± 0.08	0.026 ± 0.004
#13	Ghazvini	13.40	32.19	19.11	0.75	0.38	0.31	0.49	1.50	0.10	0.40	0.40	0.00	1E-05	1.05E-05	0.0005	0.10	1.90 ± 0.20	3.40 ± 0.54	0.81 ± 0.11	2.61 ± 0.37	0.086 ± 0.011
#14	Ghazvini	4.12	10.39	10.03	8.98	4.50	3.75	5.39	17.96	0.37	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.37	2.19 ± 0.04	2.63 ± 0.26	0.94 ± 0.08	1.28 ± 0.28	0.076 ± 0.018
#15	Ghazvini	4.12	22.92	20.25	3.49	1.75	1.46	2.13	6.98	0.37	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.37	1.58 ± 0.16	2.65 ± 0.31	0.67 ± 0.05	2.76 ± 0.32	0.052 ± 0.007
#16	Ghazvini	22.68	35.20	16.28	8.98	4.50	3.75	4.88	17.96	0.37	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.37	1.56 ± 0.29	1.88 ± 0.22	0.82 ± 0.18	2.91 ± 0.41	0.037 ± 0.005
#17	Ghazvini	22.68	22.68	2.03	4.86	2.44	2.03	2.55	9.73	0.10	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.10	2.50 ± 0.20	3.00 ± 0.48	0.58 ± 0.06	1.00 ± 0.00	0.227 ± 0.022
#18	Ghazvini	16.49	35.28	22.56	8.98	4.50	3.75	5.26	17.96	0.23	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.23	2.80 ± 0.24	3.00 ± 0.29	0.99 ± 0.23	1.35 ± 0.19	0.228 ± 0.048
#19	Ghazvini	16.49	35.28	19.12	0.75	0.38	0.31	1.23	1.50	0.50	0.27	0.27	0.08	0.00027	0.0002837	0.0135	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#20	Ghazvini	22.68	41.47	19.11	0.75	0.38	0.31	0.76	1.50	0.37	0.01	0.01	0.00	1E-05	1.05E-05	0.0005	0.37	1.22 ± 0.12	2.00 ± 0.33	0.76 ± 0.10	3.92 ± 0.07	0.023 ± 0.006
#21	Ghazvini	13.40	22.80	11.44	4.86	2.44	2.03	3.01	9.73	0.30	0.21	0.20	0.06	0.000205	0.0002154	0.01025	0.30	2.63 ± 0.24	2.80 ± 0.31	1.02 ± 0.09	1.81 ± 0.25	0.088 ± 0.010
#22	Ghazvini	22.68	41.47	22.55	8.98	4.50	3.75	5.36	17.96	0.50	0.27	0.27	0.08	0.00027	0.0002837	0.0135	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#23	Ghazvini	22.68	22.92	22.55	8.98	4.50	3.75	5.36	17.96	0.50	0.27	0.27	0.08	0.00027	0.0002837	0.0135	0.50	1.00 ± 0.00	0	0	4.00 ± 0.00	0
#24	Ghazvini	13.40	22.80	11.44	4.86	2.44	2.03	2.91	9.73	0.20	0.21	0.20	0.06	0.000205	0.0002154	0.01025	0.20	3.80 ± 0.06	2.55 ± 0.23	1.35 ± 0.11	1.12 ± 0.08	0.182 ± 0.014
#25	Ghazvini	4.12	16.65	16.28	8.98	4.50	3.75	4.79	17.96	0.10	0.14	0.14	0.04	0.000014	0.0001471	0.007	0.10	3.14 ± 0.21	2.86 ± 0.63	0.86 ± 0.10	1.14 ± 0.14	0.183 ± 0.007
#26	Ghazvini	16.49	16.49	3.75	8.98	4.50	3.75	5.19	17.96	0.50	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.50	1.50 ± 0.21	3.30 ± 0.53	0.59 ± 0.05	2.37 ± 0.53	0.039 ± 0.005
#27	Ghazvini	16.49	16.49	0.33	0.75	0.38	0.31	1.13	1.50	0.23	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.23	1.86 ± 0.09	2.55 ± 0.34	0.85 ± 0.12	1.61 ± 0.33	0.039 ± 0.012
#28	Ghazvini	22.68	28.94	7.74	3.49	1.75	1.46	2.77	6.98	0.50	0.40	0.40	0.12	0.000401	0.00042029	0.02	0.50	1.30 ± 0.13	2.00 ± 0.25	0.96 ± 0.10	4.00 ± 0.00	0.046 ± 0.008
MS (Control)	Ghazvini	20.61	39.41	20.05	2.99	1.50	1.25	1.73	5.99	0.10	0.10	0.10	0.03	0.0001	0.00010507	0.005	0.10	3.17 ± 0.27	3.33 ± 0.42	1.60 ± 0.14	2.83 ± 0.38	0.255 ± 0.021

TABLE 5 | Critical factors from the neurofuzzy logic models, coefficient of determination (train set R^2 in percentage) and ANOVA parameters for training (F ratio, degree of freedom (df1: model and df2: total), and f critical value for $\alpha = 0.01$) for each *output*.

Outputs	Submodel	Significant *inputs*	Train Set R^2 (%)	F ratio	df1, df2	f critical
SQ	1	Fe^{2+}	75.7	14.06	10, 47	2.72
	2	$\mathbf{SO_4^{2-} \times Cl^-}$				
	3	K^+				
	4	NH_4^+				
PR	**1**	$\mathbf{K^+ \times EDTA^- \times SO_4^{2-}}$	84.5	18.42	13, 44	2.56
	2	$Fe^{2+} \times BO_3^-$				
SL (cm)	**1**	$\mathbf{Fe^{2+} \times NO_3^- \times Cu^{2+}}$	94.1	21.97	24, 33	2.40
	2	$K^+ \times Cl^-$				
	3	Genotype				
STN	**1**	$\mathbf{EDTA^-}$	73.2	14.94	8, 49	2.89
	2	SO_4^{2-}				
	3	K^+				
	4	Genotype				
BC (g)	**1**	$\mathbf{Fe^{2+}}$	82.7	57.90	4, 53	3.69
	2	SO_4^{2-}				

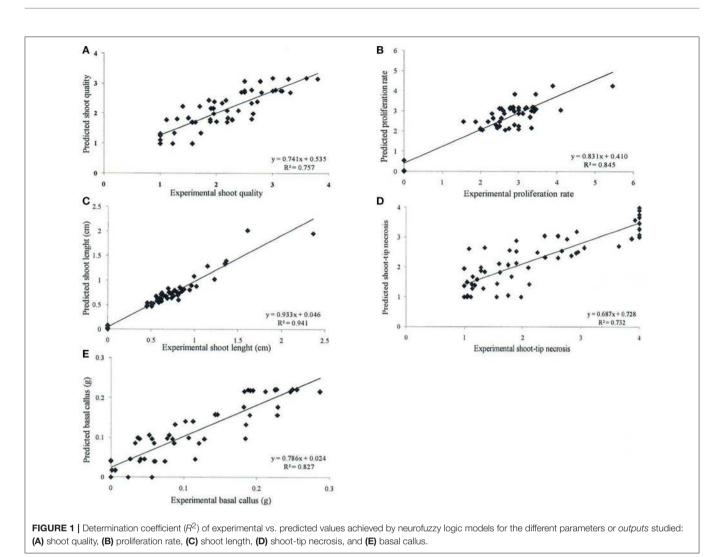*The most significant submodels have been bold by software.*

the use of Fe^{2+} at excessive concentrations suppress dramatically shoots growth and development (Data not shown). Regarding the role of SO_4^{2-} on BC, it is necessary to consider a Low concentration of this ion for preventing BC formation (rules 65–67; **Tables 6** and **S2**, **Figure S1-E2**).

DISCUSSION

The ingredients of culture medium, including mineral nutrients, significantly affect pistachio shoots growth (Akdemir et al., 2012; Kilinç et al., 2015; Marín et al., 2017). Recently, a new medium for pistachio was developed using artificial intelligence tools, even with a poorly sampled experimental design (Nezami-Alanagh et al., 2017). Despite of its good results, the study also pointed out the necessity of improving the adjustment in the concentrations of macro- and micro-elements of standard culture media. In this regard, for example, the decrease of KNO_3 to 558 mg L^{-1} (almost one-fourth) and the increase of $MgSO_4 .7H_2O$ up to 468 mg L^{-1} (1.2-fold), $CuSO_4.5H_2O$ to 0.11 mg L^{-1} (up to 4.5-fold), $FeSO_4.7H_2O$ up to 31.2 mg L^{-1} (1.11-fold) compared to MS salt levels has led to positive effects on the parameters studied (Nezami-Alanagh et al., 2017).

In the present study, we have used a IV-optimal design space using DOE software through dividing macro- and micro-elements of MS medium into five independent factors and assigning various levels for each (**Tables 1, 2**) with two purposes: (i) to establish a well sampled design space and (ii) to reduce the number of treatments to be assayed from 3125 to just 29 combinations based on MS levels. The use of neurofuzzy logic as an advanced modeling tool (Gago et al., 2010a,b,c,d, 2014; Gallego et al., 2011; Nezami-Alanagh et al., 2014; Ayuso et al., 2017) has enabled determining the key factors affecting the parameters studied. In fact, the efficiency of neurofuzzy logic can be summarized in: (i) constructing statistical significant

mathematical models characterized by high coefficients of determination (> 70%) indicating high predictability of the algorithm, since higher the R^2 value obtained, the better the predictability of the trained model (Shao et al., 2006), and (ii) development of submodels defined by a set of vague linguistic tags, expressed as "IF-THEN" rules, that allow the understanding of the complex nonlinear relationships between *inputs* and *outputs* in an easy way.

Shoot quality is a complex parameter that includes the evaluation of several macroscopic observations such as the appearance of shoots health together with absence of physiological disorders (Hand et al., 2014; Niedz et al., 2014). Neurofuzzy logic models have managed to discover the interaction or independent effects of SO_4^{2-}, Cl^-, Fe^{2+}, K^+ and NH_4^+ on SQ in pistachio rootstocks with dominant role of $SO_4^{2-} \times Cl^-$ (rules 1–13, **Tables 6** and **S2**). In agreement with our findings, the significant effects of different salts on SQ of different plant species as $ZnSO_4.7H_2O$ and Fe/EDTA in *Citrus sinensis* cv. "Valencia" (Niedz and Evens, 2007); *Gerbera hybrida* cv. "Pasadena" (Niedz et al., 2014); $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and KH_2PO_4 in *Pyrus sp.* (Wada et al., 2013) and *Rubus idaeus* L. (Poothong and Reed, 2014, 2015); KH_2PO_4, K_2SO_4 and NH_4NO_3 in *Corylus avellana* L. (Akin et al., 2017b); KH_2PO_4 and $MgSO_4.7H_2O$ in *Prunus armeniaca* Lam (Kovalchuk et al., 2017) *in vitro* culture have been reported. The use of salts as factors, in all of these studies, implies a problem of ion confounding, being difficult to identify exactly corresponding ion(s) impacting the parameter (Niedz and Evens, 2006). In agreement with our results, Akin et al. (2017a) using CHAID algorithm, found that hazelnut quality was affected by K^+, NO_3^-, NH_4^+ and genotype, showing that the best SQ for "Barcelona," "Jefferson," and "Wepster" was obtained when the culture media was supplemented with $K^+ \leq 46$ mM, $NO_3^- \leq 88$ mM, and $NH_4^+ \leq 20$ mM.

FIGURE 1 | Determination coefficient (R^2) of experimental vs. predicted values achieved by neurofuzzy logic models for the different parameters or *outputs* studied: **(A)** shoot quality, **(B)** proliferation rate, **(C)** shoot length, **(D)** shoot-tip necrosis, and **(E)** basal callus.

Although usually the interaction of $NH_4^+ \times NO_3^- \times K^+$ has been considered as the central attentions over *in vitro* studies because of their dominant ions in most tissue culture media formulations (Niedz et al., 2014), in present study the implementation of neurofuzzy logic strongly exploited the existence of another kind of relationship between ions ($K^+ \times EDTA^- \times SO_4^{2-}$), with critical influence of K^+ on PR; so that the integration of K^+ at Low and High concentrations should lead to achieve a set of greatest and lowest PR, respectively. The beneficial effect of K^+ at Low amount is in agreement accordance with our earlier study during micropropagation of *Prunus sp.* where K^+ was integrated in culture media up to 3.19 mM (Nezami-Alanagh et al., 2014), whereas in MS is at 20.05 mM (**Table 5**). Neurofuzzy logic models also pinpointed that explants cultured on media with Fe^{2+} / $EDTA^-$ at Low to Mid concentration, exhibited high PR. Niedz et al. (2014) reported 0.1 mM Fe^{2+} / $EDTA^-$ as optimal concentration for micropropagation of *Gerbera hybrida*. Whilst, the use of Fe^{2+} / $EDTA^-$ only at 0.23 mM was recommended by Garrison et al. (2013) during micropropagation of hybrid hazelnut. In agreement with those results, here the explants treated with

either low or too high amounts of these ions were failure to yield shoots suitable for subculture. This suggests that Fe^{2+} and $EDTA^-$ of MS (0.1 mM) can also be used in pistachio micropropagation.

The positive role of NO_3^- on SL increment, solely or in combination with other ions, has also been reported previously. Wada et al. (2015) using response surface modeling found that to attain longest shoots in diverse pear species it is necessary to integrate High NO_3^- [20–60 mM] in addition to a proper range of NH_4^+: K^+ ratios to culture medium. In the previous study using neurofuzzy logic we found that NO_3^- at 17.5–20 m M led to produce longest shoots in *P. vera* cv. 'Ghazvini' (Nezami-Alanagh et al., 2017). Interestingly, the experimental design used in the current study allowed to have global viewpoint related to role of this ion on the parameter studied. So that, based on neurofuzzy logic models it was revealed that combination of high NO_3^- with low Fe^{2+} and Mid Cu^{2+} being necessary to achieve long shoots (Rule 33; **Table 6**). Niedz et al. (2014) determined the optimal amount of some chemicals including Fe^{2+} / $EDTA^-$ and $CuSO_4.5H_2O$ in a single-factor design, followed by illustrating 0.1 and 0-0.1 mM as the optimum

TABLE 6 | Rules selection, with membership degree 1.00, generated by neurofuzzy logic showing the best combination of *inputs* to obtain the highest or lowest results for each *output*.

Rules	NH_4^+	NO_3^-	K^+	SO_4^{2-}	Fe^{2+}	$EDTA^-$	Cu^{2+}	Cl^-	BO_3^-	SQ	PR	SL (cm)	STN	BC (g)	Membership degree
3	IF			**Mid**				**Low**	THEN	**High**					**1.00**
5				**High**				**Low**		**Low**					**1.00**
6			High				High			High					1.00
7					Low					High					1.00
8					High					Low					1.00
10			High							Low					1.00
11		Low								Low					1.00
13		High								Low					1.00
18	IF		Low	Low		High			THEN		Low				1.00
19			**Low**	**High**		**High**					**High**				**1.00**
20			High	Low		High					Low				1.00
21			**High**	**High**		**High**					**Low**				**1.00**
24					Mid			Low			High				1.00
28	IF	Low			Low		Low		THEN			Low			1.00
29		Mid			Low		Low					Low			1.00
31		Low			Low		Mid					Low			1.00
32		Mid			Low		Mid					High			1.00
33		**High**			**Low**		**Mid**					**High**			**1.00**
34		Low			Low		High					Low			1.00
37		Low			High		Low					Low			1.00
40		Low			High		Mid					Low			1.00
41		Mid			High		Mid					Low			1.00
42		**High**			**High**		**Mid**					**Low**			**1.00**
44		Mid			High		High					Low			1.00
45		High			High		High					High			1.00
47			Mid				Low					Low			1.00
54	IF					**Low**			THEN				**Low**		**1.00**
55						**High**							**High**		**1.00**
56				Low									High		1.00
57				Mid									Low		1.00
58				High									Low		1.00
59			Low										Low		1.00
64	IF					**High**			THEN					**Low**	**1.00**
65				Low										Low	1.00

The inputs with stronger effects on each output have been highlighted by software.

concentration for obtaining longest shoots in *Gerbera hybrid* together with the effects of deterioration of these metals on the parameters at higher concentration which agrees with our findings. Moreover, the interaction $K^+ \times Cl^-$ was also determined as the next significant factors on SL parameter, promoting longest shoots IF Low K^+ plus High Cl^- is included in the culture media. It is interesting to note that neurofuzzy logic generated useful information about the importance of NO_3^-, Cu^{2+} and Fe^{2+}, suggesting that the first should be used at lower concentration (20 mM) compared with that use in MS (39 mM).

In this study some physiological disorders such as STN and BC are described. STN has been considered as one of

the main drawbacks in micropropagation of different species including *Pistacia* (Onay, 2003; Bairu et al., 2009a; Bariu et al., 2009b; Chiruvella et al., 2011; Akdemir et al., 2014) and has been attributed to certain mineral nutrients deficiencies such as calcium and/or boron (Barghchi and Alderson, 1996; Akdemir et al., 2012). Neurofuzzy logic has pointed out the significant influence of other factors on this type of abnormality. $EDTA^-$ has been highlighted by the model as the factor with the strongest effect on STN, since at High concentration promotes the highest STN (rule 55; **Table 6**). Probable reasons explaining this effect could be the toxicity effects of $EDTA^-$ when using at excessive amounts or chelating with other metals causing mineral deficiency in shoots (George et al., 2008).

FIGURE 2 | Response of pistachio rootstocks to mineral nutrients "Ghazvini" rootstock on **(A)** MS (control), **(B)** Treatment #24; "UCB1" rootstock on **(C)** MS (control), and **(D)** Treatment #24. The occurrence of some physiological disorders including **(E)** shoot-tip necrosis, leaf necrosis and hooked leaves in "Ghazvini," **(F)** shoot-tip necrosis in "UCB1"and **(G)** basal callus in "UCB1".

Furthermore, production of formaldehyde as a result of the $EDTA^-$ oxidization mediated by the fluorescence lamps within the growth chamber, which can also became accumulated until inhibitory levels (Hangarter and Stasinopoulos, 1991). Therefore, Low $EDTA^-$ (0.1 mM) should be used for healthy pistachio micropropagation.

Reed et al. (2013) using RSM methodology reported that low levels of some MS ingredients such as KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, NH_4NO_3 and KNO_3 contributed to promote necrosis in some species of *Pyurus* including *P. ussuriensis* 'Hang Pa Li'. Again, the problem of ion confounding prevents the establishment of causality between the specific ions and the physiological abnormality. Our results clearly determined that low concentrations of K^+ and Mid-High level of SO_4^{2-} inhibit the STN symptoms (rules 56-60; **Tables 6** and **S2**). Finally, neurofuzzy logic pointed out additionally, the differences between genotypes with regard STN parameter, being the occurrence of the symptoms lower in "UCB1" than in "Ghazvini" (rules 61–62; **Table S2**).

Callus formation at the base of shoots is also considered as one of common *in vitro* physiological disorders in diverse

woody plants species, which is affected mainly by chemical composition of culture medium. For instance, the integration of some mineral ingredients at a certain concentrations such as KH_2PO_4, $MgSO_4$ and $CaCl_2$ in some *Prunus* cultivars (Reed et al., 2013), $MgSO_4 \cdot 7H_2O$ in *Prunus armeniaca* Lam., (Kovalchuk et al., 2017), or NO_3^- ion in Robus germplasms (Poothong and Reed, 2016) caused basal callus formation of *in vitro* shoots. In a recent study using CHAID analysis has been reported the significant effects of NH_4^+, followed by genotype and SO_4^{2-} on callus formation in hazelnut shoots (Akin et al., 2017a). Neurofuzzy logic has pinpointed the independent significant effect of Fe^{2+} and SO_4^{2-} as the two most important factors on this parameter, predicting the lowest BC in pistachio rootstocks when these components are used at High and Low concentrations, respectively (rules 63–67; **Tables 6** and **S2**). These results suggest that SO_4^{2-} at lower concentration than in MS (1.173 mM) is better to reduce STN and BC in pistachio.

CONCLUSIONS

From this study two main conclusions can be drawn. First, pistachio growth parameters SQ, PR, SL, STN and BC can be improved by using medium which include the next ions concentration: K^+ (<10 mM); Fe^{2+} and $EDTA^-$ (0.1 mM); BO_3^- (<0.2 mM); Cu^{2+} (≈0.0002 mM); NO_3^- (<20 mM) and SO_4^{2-} (≈4 mM). This combination, which is in accordance with our previous studies (Nezami-Alanagh et al., 2017), allows to increase the healthy micropropagation of pistachio.

Second, the acquisition of knowledge about the performance of complex systems, such as *in vitro* culture, can be greatly increased by combining the use of DOE and computer-based mathematical models generated by artificial intelligence. While DOE lets to reduce the number of treatments in complex factorial designs, but ensuring a good sampled design space, neurofuzzy logic models facilitate the analysis of large databases and the establishment of the critical factors for all parameters studied, simultaneously.

AUTHOR CONTRIBUTIONS

EN-A performed the experiments. G-AG contributed with reagents materials. EN-A, G-AG, and PG conceived and designed the experiments. ML and PG contributed DOE/modeling/analysis tools. All authors contributed to writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01474/full#supplementary-material

Figure S1 | Graphical representation of the fuzzyfication process developed by neurofuzzy logic per each parameter **(A1–A4)** shoot quality, **(B1–B3)** Proliferation rate, **(C1–C4)** shoot length (in cm), **(D1–D3)** shoot-tip necrosis and **(E1,2)** basal callus (in gram) and their domains (Low, Mid, High, ….). Neurofuzzy divided the input range (x axis) of a variable between several domains to which a symbolic name is attached. In this sense, two (Low and High) and three (Low, Mid and High) were established for the significant submodels developed by neurofuzzy logic for each parameter (see **Table 5**). The y axis represents the membership function ranging from 0 to 1: the closer the membership function is to 1, the closer the value is to truly Low or truly High (if two domains were established, as for Cl^- in **A2**). This process facilitate the interaction between the membership function (0 and 1) and the linguistic terms (Low, Mid, High) making the terms meaningful to a computer. After the modeling the knowledge extracted is presented by "IF-THEN" rules. As example, if we refer in Rule 3 Mid SO_4^{2-} concentration having a look to panel **A1**, this means around 3 mM with a membership degree of 1.00, whereas 4 mM can be either considered Mid (membership 0.6) or Low (membership 0.3). In other words, SO_4^{2-} at 3 mM is pondered by the model as Mid concentration rather than low and never High (membership 0).

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