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Interactive Effects of Culture System and Carbon Source on Mineral Nutrition, Growth, and Shoot Proliferation in Chestnut Micropropagation

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Abstract

This study investigated the interactive effects of the culture system and carbon source on growth, shoot proliferation, and mineral nutrition dynamics in the in vitro propagation of chestnut. Explants of the 'Akyüz' cultivar were used in the Woody Plant Medium. Both plant tissues and culture media were analyzed for Fe, Cu, Mn, Zn, and Mg concentrations. Morphological parameters, nutrient accumulation, and depletion patterns were evaluated. The results demonstrated that the liquid culture system supplemented with sucrose significantly enhanced plant growth, chlorophyll content, callus development, and shoot multiplication. Sucrose treatments promoted higher accumulation of Fe, Cu, Zn, and Mg in plant tissues, whereas glucose treatments resulted in significantly higher Mn accumulation. Correlation and principal component analysis revealed strong positive relationships between growth parameters and Fe, Mg, Cu, and Zn, whereas Mn exhibited significant negative correlations. Among the machine learning models, Support Vector Regression showed the highest predictive performance for plant length (R^2 = 0.74) and SPAD (R^2 = 0.87). Nutrient depletion analysis showed substantial reductions in mineral concentrations in all treatments after four weeks. Overall, the combination of liquid culture systems with sucrose provides optimal conditions for chestnut micropropagation by promoting favorable nutrient interactions and minimizing antagonistic effects.

Keywords: *Castanea* spp.; bioreactor-based culture; in vitro propagation; woody species

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1. Introduction

Chestnut is an important nut crop that grows in the Mediterranean and temperate regions [1]. Propagating chestnuts is important because it supports the production of valuable products, such as timber, honey, and nuts [2]. Additionally, chestnut trees play a vital ecological role in preventing soil erosion and supporting biodiversity, especially in mountainous areas. However, the conventional propagation of chestnuts is limited by their low rooting ability, genotype dependency, and susceptibility to biotic stress [3–5].

Among vegetative propagation methods, in vitro propagation has emerged as a powerful alternative for rapid and uniform plant production [6]. This technique allows for the mass multiplication of plants under controlled environmental conditions, minimizing

the risk of contamination and genetic variation [7]. In recent years, studies on chestnut propagation have also focused on in vitro propagation techniques [8–11]. Traditionally, agar has been used to solidify the medium in the in vitro propagation of chestnut. However, in recent years, liquid media with temporary (TIS) or continuous immersion systems (CIS) have been used [8,12–16]. These bioreactor systems have the advantage of improving shoot proliferation, leaf development, and photosynthetic capacity in several woody and herbaceous species [17,18]. Despite these advantages, the interaction between medium composition and carbon sources remains unclear in chestnut.

Carbohydrates supplied in culture media not only serve as energy sources. They also regulate osmotic potential, metabolic pathways, and signaling processes that influence the in vitro development of the explant. Among them, sucrose is the most commonly used carbon source, and numerous studies have demonstrated its superiority over other sugars such as glucose in promoting shoot organogenesis, callus formation, and chlorophyll synthesis [19–25]. However, glucose has been reported to be a better carbon source depending on the species [26–29].

In addition to growth responses, mineral nutrition dynamics in in vitro systems represent a critical but often overlooked component of plant development. Nutrient uptake in tissue culture is a complex process influenced by the medium composition, physical state, and plant metabolic activity [30]. Micronutrients such as Fe, Zn, Cu, Mn, and Mg play key roles in enzymatic activity and chlorophyll biosynthesis [31–36]. These elements do not function independently and exhibit synergistic and antagonistic interactions. Although mineral balance is recognized as a key factor in plant development, limited information exists on how culture systems and carbon sources interact to influence nutrient accumulation and uptake dynamics in chestnut.

The present study aimed to evaluate the interactive effects of culture systems and carbon sources on shoot growth, callus development, mineral nutrient accumulation in plant tissues, nutrient depletion dynamics in culture media, and the relationships among these variables using correlation and principal component analyses. By integrating physiological and nutritional insights, this study contributes to a deeper understanding of the key factors regulating the in vitro propagation performance of chestnut.

2. Materials and Methods

2.1. Plant Material

In this study, the 'Akyüz' chestnut cultivar was used. The 'Akyüz' cultivar was obtained by hybridizing the 'King Arthur' (*C. mollissima* × *C. seguine*) and 'Lockwood' (*C. crenata* × *C. sativa* × *C. dentata*) cultivars [37]. It is known for its resistance to the Asian chestnut gall wasp [38]. Explants of the 'Akyüz' cultivar were initially established in culture through the integration of serial grafting and the PlantForm™ (PlantForm, Hjarup, Sweden) bioreactor system within the framework of a TÜBİTAK-funded project (Project No. 122O251).

2.2. Methods

2.2.1. Initiation of In Vitro Culture

The uniform explants consisting of nodal micro shoots (approximately 1 cm in length, containing 1 node) were selected from previously established in vitro cultures. They were initially established in Woody Plant Medium (WPM) (Duchefa Biochemie, Haarlem, The Netherlands) [39]. Zeatin-riboside (Gold Biotechnology, Inc., St. Louis, MO, USA) was used as the cytokinin source at a concentration of 2 mg L^{-1}. The medium was supplemented with 30 g L^{-1} sucrose (Merck KGaA, Darmstadt, Germany) and solidified with 7 g L^{-1} Bacto agar (BD Difco™, Becton, Dickinson and Company, Franklin Lakes, NJ,

USA). The pH of the culture medium was adjusted to 5.6–5.7 before sterilization at 121 °C and 15 psi for 20 min (Hirayama Manufacturing Corporation, Kasukabe, Saitama, Japan). The explants were subcultured every four weeks. Following the initial establishment phase, explants were subcultured twice (eight weeks). Only healthy, contamination-free explants with a similar size and visible callus formation after two subcultures were selected for the experiment.

In the experiment, two different multiplication systems (solid and liquid) and carbon sources (sucrose and glucose) were tested. The same concentration and medium (WPM) were used in the solid and liquid systems. Only in the liquid system was no agar added to the medium. For the solid system, 473 mL culture jars were used. Each jar contained 100 mL of medium. In contrast, a PlantForm™ temporary immersion bioreactor was used for the liquid system. Each bioreactor contained 500 mL of liquid medium. To better compare the two systems, 4 explants were placed in a jar and 20 in a PlantForm™ bioreactor (25 mL per explant) with three replicates (each jar and bioreactor considered an experimental unit). The bioreactor was operated under a temporary immersion system, consisting of 1 min of immersion every 6 h, followed by 2 min of aeration.

All cultures were maintained under controlled growth room conditions. The explants were cultivated at 24 ± 2 °C under a 16 h light/8 h dark photoperiod. Cool-white fluorescent lamps (approximately 40–50 µmol m^{-2} s^{-1}) were used to provide illumination. The relative humidity within the growth chamber was maintained under standard laboratory conditions. Solid culture vessels and PlantForm™ bioreactor systems were placed under identical environmental conditions to ensure treatment comparability of the treatments according to the completely randomized design. The explants were cultured for four weeks. At the end of the fourth week, the explants were collected for mineral nutrient analysis. In addition, culture media were sampled before plant introduction and after one subculture to assess the nutrient depletion and uptake dynamics.

2.2.2. Determination of Nutrient Content

Explants were washed with distilled water to remove agar. They were then oven-dried at 65 °C until a constant weight was achieved (MM Ecocell 55 MMM Medcenter Einrichtungen GmbH, Planegg, Germany). Subsequently, the plant material was dried and ground into a powder of uniform consistency by hand. To determine the mineral nutrient concentrations, 0.5 g of desiccated plant material was weighed and incinerated in a muffle furnace at 550 °C for 4–8 h. The ash obtained after combustion was dissolved in hydrochloric acid (HCl) and prepared for analysis according to standard procedures [40]. The concentrations of magnesium (Mg), iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu) were determined by atomic absorption spectrophotometry (Shimadzu AA-7000, Kyoto, Japan) [41]. The concentrations of Fe, Cu, Mn, Zn, and Mg in the culture media were determined in both liquid bioreactor media and agar-solidified media using flame photometry (BWB-1, BWB Technologies, Newbury, Berkshire, UK) and atomic absorption spectrophotometry [41]. Liquid medium samples were homogenized and analyzed directly. Agar-solidified medium samples were diluted 50-fold with distilled water to reduce matrix interference and viscosity-related analytical limitations and then homogenized before measurement. The obtained values for agar-solidified media were corrected by applying the corresponding dilution factor, and the final concentrations were calculated accordingly. Calibration curves were established for each element using a series of multi-element standard solutions prepared from high-purity stocks (Merck KGaA, Darmstadt, Germany), ensuring high linearity ($R^2 > 0.99$). All analyses were conducted in triplicate, and results were calculated and are reported as milligrams per kilogram of dry weight, expressed as parts per million (ppm).

2.2.3. Correlation Analysis and Principal Component Analysis

In principal component analysis (PCA), Pearson's correlation coefficients were calculated between plant length, number of leaves, callus width, callus height, shoot multiplication, SPAD values, and mineral elements (Fe, Cu, Mn, Zn, and Mg). The significance levels of the correlations were assessed at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Correlation matrices were visualized using heatmaps with hierarchical clustering to illustrate the patterns between variables.

PCA was performed using the covariance matrix of the standardized variables. Eigenvalues and percentages of explained variance were calculated for each principal component. Variables with higher loadings were considered to be more influential in defining the principal components. The relationships among variables were visualized using a correlation circle (variable factor map), where the direction and length of the vectors indicate the strength and contribution of each variable to the principal components.

2.2.4. Modeling Procedure

Machine learning (ML) approaches were applied to investigate the predictive relationship between mineral composition and in vitro growth parameters. Plant length and SPAD were defined as output variables, while mineral elements (Fe, Cu, Mn, Zn, and Mg) were used as predictor variables. As datasets are small-sized in in vitro studies compared to field studies, a leave-one-out cross-validation (LOOCV) strategy was used to ensure robust and unbiased performance estimation. In this approach, each observation was iteratively used as a test sample while the remaining data were used for model training. This strategy reduces the risk of overfitting in ML. Multiple linear regression (MLR), Random Forest (RF), and Support Vector Regression (SVR) ML models were used to predict the output variables. These models were selected because they can work better with small datasets. MLR was employed to assess linear relationships between mineral nutrients and growth parameters. In addition, RF regression models were constructed using 500 trees to capture potential non-linear interactions among variables. The variable importance in RF models was quantified using the percentage increase in mean squared error (%IncMSE). SVR with a radial basis function (RBF) kernel and epsilon-regression formulation was applied to model complex non-linear relationships. Model performance was evaluated using the coefficient of determination (R^2) (Equation (1)), root mean square error (RMSE) (Equation (2)), and mean absolute error (MAE) (Equation (3)). Additionally, scatter plots of observed versus predicted values were generated to visually assess model accuracy, with a 1:1 reference line indicating perfect agreement.

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

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2} \quad (2)$$

$$MAE = \frac{1}{n}\sum_{i=1}^{n}\left|Y_i - \hat{Y}_i\right| \quad (3)$$

2.2.5. Statistical Analysis

The experiment was conducted using a completely randomized design in a 2 × 2 factorial arrangement, with culture system (solid and liquid) and carbon source (sucrose and glucose) as the main factors. Before analysis, the assumptions of normality and homogeneity of variances were checked using the Shapiro–Wilk and Levene's tests, respectively. The

results confirmed that datasets followed a normal distribution and all variables exhibited homogenous variances ($p > 0.05$). Data were subjected to two-way analysis of variance (ANOVA) to evaluate the effects of the factors and their interactions. When significant differences were detected, the means were separated using Duncan's multiple range test at a significance level of $p < 0.05$. All data in the tables are presented as mean values ± standard error (SE). Statistical analyses were performed with the R software (version 4.5.1) using the "Agricolae" package.

3. Results

3.1. Effects of Culture System and Carbon Source on Shoot Proliferation and Morphological Traits of Explants

The interaction between the culture system and carbon source significantly affected plant length, leaf number, and SPAD values (Table 1).

Table 1. Effects of culture system and carbon source on shoot growth and chlorophyll content of explants.

Culture System	Carbon Source	Plant Length (cm)	Number of Leaves (*n*)	SPAD
Solid	Sucrose	5.46 ± 0.18 c	14.20 ± 1.48 a	34.02 ± 1.09 b
	Glucose	4.96 ± 0.11 d	9.80 ± 0.83 b	26.98 ± 1.03 d
Liquid	Sucrose	7.20 ± 0.15 a	15.40 ± 1.14 a	40.38 ± 0.90 a
	Glucose	6.10 ± 0.10 b	10.20 ± 1.30 b	29.68 ± 1.20 c
Factor Means				
Culture System	Solid	5.21 ± 0.29 b	12.0 ± 2.58	30.50 ± 3.84 b
	Liquid	6.65 ± 0.59 a	12.8 ± 2.97	35.03 ± 4.72 a
Carbon Source	Sucrose	6.33 ± 0.93 a	14.8 ± 1.39 a	37.20 ± 3.48 a
	Glucose	5.53 ± 0.60 b	10.0 ± 1.05 b	28.33 ± 1.77 b
p				
Culture System		***	N.S.	*
Carbon Source		*	***	***
Culture System × Carbon Source		***	***	***

Means followed by different lowercase letters within the same column indicate statistically significant differences according to Duncan's multiple range test ($p < 0.05$). Asterisks in the *p*-value section denote the level of statistical significance: * $p < 0.05$; *** $p < 0.001$; N.S.: Not significant. Standard error (±SE) is provided for each mean.

Among the applications, the liquid culture system supplemented with sucrose resulted in the highest plant length (7.20 ± 0.15 cm). This was followed by the liquid culture system supplemented with glucose (6.10 ± 0.10 cm). In contrast, the lowest plant length was observed in the solid medium supplemented with glucose (4.96 ± 0.11 cm). Similarly, the highest leaf numbers were obtained from liquid and solid culture systems supplemented with sucrose (15.40 ± 1.14 and 14.20 ± 1.48, respectively). In terms of SPAD values, the highest value was measured in the liquid culture system supplemented with sucrose (40.38 ± 0.90). However, the lowest SPAD value was obtained from the solid culture system supplemented with glucose. In general, the liquid medium promoted shoot growth more than the solid medium, and sucrose promoted shoot growth more than glucose. While sucrose applications consistently increased leaf number in both solid and liquid media, glucose applications led to a sharp decline in leaf number in both culture systems.

The effects of the culture system and carbon source on callus formation and shoot multiplication were statistically significant, particularly according to the carbon source and its interaction with the culture system (Table 2).

Table 2. Effects of culture system and carbon source on callus formation and shoot multiplication of explants.

Culture System	Carbon Source	Callus Width (cm)	Callus Height (cm)	Shoot Multiplication (*n*)
Solid	Sucrose	1.73 ± 0.04 b *	0.87 ± 0.02 b	4.60 ± 0.89 a
	Glucose	1.47 ± 0.04 d	0.80 ± 0.01 c	2.80 ± 0.44 b
Liquid	Sucrose	1.86 ± 0.03 a	0.91 ± 0.01 a	5.40 ± 0.54 a
	Glucose	1.53 ± 0.03 c	0.82 ± 0.02 c	2.20 ± 0.44 b
Factor Means				
Culture System	Solid	1.59 ± 0.14	0.84 ± 0.04	3.70 ± 1.15
	Liquid	1.70 ± 0.17	0.86 ± 0.04	3.80 ± 1.75
Carbon Source	Sucrose	1.80 ± 0.08 a	0.89 ± 0.02 a	5.00 ± 0.81 a
	Glucose	1.50 ± 0.04 b	0.81 ± 0.01 b	2.50 ± 0.52 b
p				
Culture System		N.S.	N.S.	N.S.
Carbon Source		***	***	***
Culture System × Carbon Source		***	***	***

Means followed by different lowercase letters within the same column indicate statistically significant differences according to Duncan's multiple range test ($p < 0.05$). Asterisks in the *p*-value section denote the level of statistical significance: * $p < 0.05$; *** $p < 0.001$; N.S.: Not significant. Standard error (±SE) is provided for each mean.

The highest callus width (1.86 ± 0.03 cm) and callus height (0.91 ± 0.01 cm) were recorded in the liquid culture system supplemented with sucrose. In comparison, the lowest values were observed in the solid cultivation system supplemented with glucose (1.47 ± 0.04 cm and 0.80 ± 0.01 cm, respectively). However the main effect of the culture system alone was not statistically significant. The interaction between the culture system and carbon source revealed that liquid conditions enhanced the positive effect of sucrose on callus development. The highest shoot multiplication rate was recorded in the liquid medium with sucrose (5.40 ± 0.54 cm). This was followed by the solid medium with sucrose. In both culture systems, the use of sucrose approximately doubled the number of shoots compared to glucose. Growth rates in glucose-containing media remained significantly lower.

3.2. Effects of Culture System and Carbon Source on Mineral Nutrient Composition of Plant Tissues

The concentrations of mineral nutrients in chestnut plant tissues were significantly influenced by the carbon source and its interaction with the culture system. In contrast, the primary effect of the culture system alone was generally limited (Table 3). The use of sucrose significantly dominated mineral uptake across culture systems. The highest values of Cu (0.33 ± 0.04 ppm), Zn (1.50 ± 0.13 ppm), Fe (5.88 ± 1.05 ppm), and Mg (3.37 ± 0.41 ppm) were recorded in the sucrose-containing medium. In contrast, Mn showed an opposite pattern, with significantly higher accumulation in glucose treatments. The highest Mn concentration was detected in the liquid system–glucose combination at 4.91 ± 0.26 ppm, whereas in the sucrose-containing medium, it was 2.25 ± 0.28 ppm. Regarding Mg uptake, the difference among carbon sources was most pronounced in the liquid system. The switch from sucrose to glucose resulted in a 68% decrease in Mg accumulation (from 3.37 ± 0.41 to 1.06 ± 0.19 ppm).

Table 3. Effects of culture system and carbon source on mineral nutrient concentrations of chestnut plant tissues.

Culture System	Carbon Source	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)	Mg (ppm)
Solid	Sucrose	5.74 ± 1.02 a *	0.27 ± 0.03 b	2.39 ± 0.32 c	1.32 ± 0.44 b	1.26 ± 0.05 c
	Glucose	3.47 ± 0.71 b	0.24 ± 0.01 b	3.84 ± 0.44 b	0.94 ± 0.33 c	1.91 ± 0.03 b
Liquid	Sucrose	6.02 ± 1.19 a	0.33 ± 0.04 a	2.25 ± 0.28 c	1.50 ± 0.13 a	3.37 ± 0.41 a
	Glucose	3.71 ± 0.52 b	0.26 ± 0.01 b	4.91 ± 0.26 a	0.72 ± 0.26 d	1.06 ± 0.19 c
Factor Means						
Culture System	Solid	4.60 ± 1.46	0.25 ± 0.03 b	3.11 ± 0.84	1.13 ± 0.41	1.59 ± 0.34
	Liquid	4.86 ± 1.49	0.29 ± 0.04 a	3.58 ± 1.42	1.11 ± 0.45	2.22 ± 1.25
Carbon Source	Sucrose	5.88 ± 1.05 a	0.30 ± 0.05 a	2.32 ± 0.29 b	1.41 ± 0.32 a	2.64 ± 0.81 a
	Glucose	3.59 ± 0.60 b	0.25 ± 0.01 b	4.37 ± 0.66 a	0.83 ± 0.30 b	1.16 ± 0.16 b
p						
Culture System		N.S.	*	N.S.	N.S.	N.S.
Carbon Source		***	**	***	***	***
Culture System × Carbon Source		***	**	***	**	***

Means followed by different lowercase letters within the same column indicate statistically significant differences according to Duncan's multiple range test ($p < 0.05$). Asterisks in the *p*-value section denote the level of statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; N.S.: Not significant. Standard error (±SE) is provided for each mean.

Correlation analysis revealed strong and meaningful relationships among the growth parameters, callus characteristics, and mineral nutrient concentrations (Figure 1). Plant length was significantly and positively correlated with the number of leaves (r = 0.60), callus width (r = 0.73 ***), callus height (r = 0.65 **), and SPAD values (r = 0.83 ***). Similarly, SPAD was strongly and positively correlated with the number of leaves (r = 0.88 ***), shoot multiplication (r = 0.85 ***), and callus-related traits, suggesting that chlorophyll content is strongly associated with both shoot proliferation and plant vigor. The shoot multiplication rate was also highly correlated with callus height (r = 0.90 ***) and callus width (r = 0.88 ***).

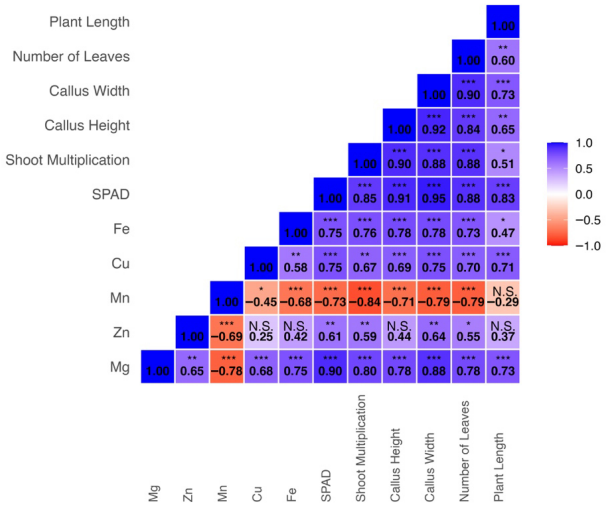


Figure 1. Pearson correlation heatmap showing relationships between morphological traits and mineral nutrient concentrations in chestnut in vitro culture (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; N.S.: Not significant).

Among the mineral nutrients, Fe and Mg showed strong positive correlations with growth parameters, particularly SPAD, shoot multiplication, and plant length. Cu also exhibited consistent positive correlations with most of the growth traits. Zn showed

moderate positive associations with growth parameters, suggesting a supportive but less dominant role. In contrast, Mn displayed significant negative correlations with nearly all growth parameters, including SPAD (r = −0.73 ***), shoot multiplication (r = −0.84 ***), callus width (r = −0.79 ***), and plant length (r = −0.29). Mn was negatively correlated with Fe and Mg.

Principal component analysis (PCA) revealed a clear separation of variables based on their relationships and contributions to the overall variability (Figure 2).

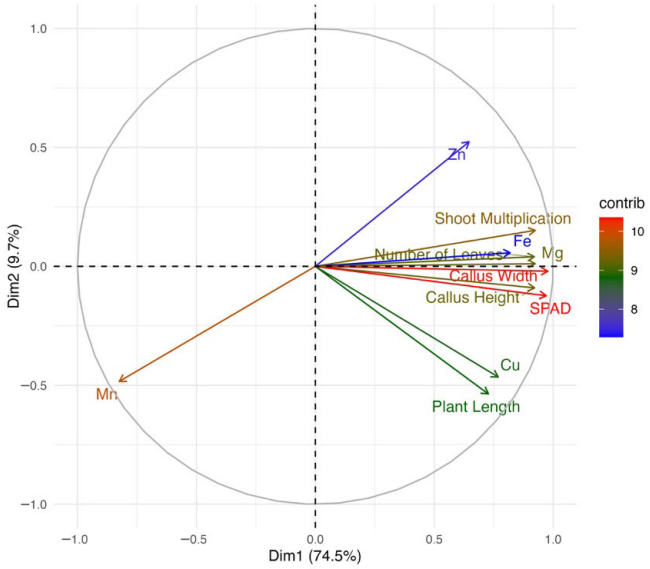


Figure 2. Principal component analysis (PCA) biplot showing the relationships between morphological traits and mineral nutrient concentrations in chestnut in vitro culture.

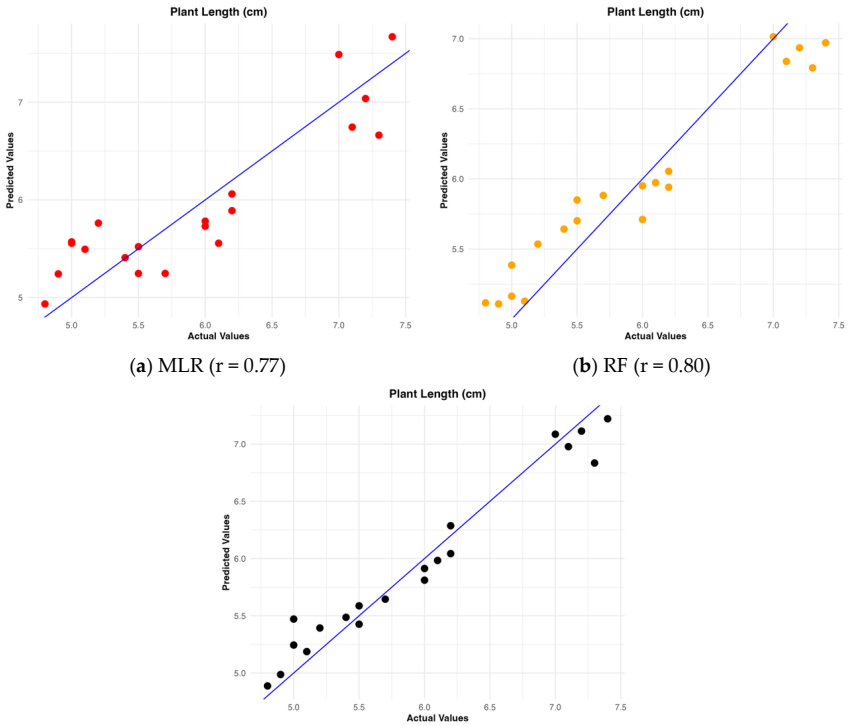
The first principal component (PC1) explained a substantial proportion of the total variance (74.5%). PC1 represents the main gradient of variation associated with plant growth and nutrient accumulation. Most growth-related parameters, including plant length, number of leaves, SPAD, shoot multiplication, callus width, and callus height, were positively and strongly associated with PC1 and clustered on the right side of the plot. Similarly, mineral nutrients such as Fe, Mg, Cu, and Zn were positioned in the same direction, indicating a strong positive relationship with plant growth and physiological performance. PC2, which explains 9.7% of the variance, further differentiates Zn from other nutrients. In contrast, Mn acts in opposition to this trend as observed in the correlation analysis.

The predictive performance of MLR, RF, and SVR models for plant length and SPAD values is summarized in Table 4.

Table 4. Prediction performance of machine learning models for plant length and SPAD.

Parameters	ML Models	R^2	RMSE	MAE
Plant Length (cm)	MLR	0.60	0.548	0.472
	RF	0.64	0.544	0.499
	SVR	0.74	0.451	0.375
SPAD	MLR	0.69	2.889	2.274
	RF	0.85	2.164	1.654
	SVR	0.87	1.943	1.416

For plant length prediction, the SVR model exhibited the highest predictive performance (R^2 = 0.74), outperforming both RF (R^2 = 0.64) and MLR (R^2 = 0.601) (Figure 3). Also, it had the lowest error metrics (RMSE = 0.451; MAE = 0.375).

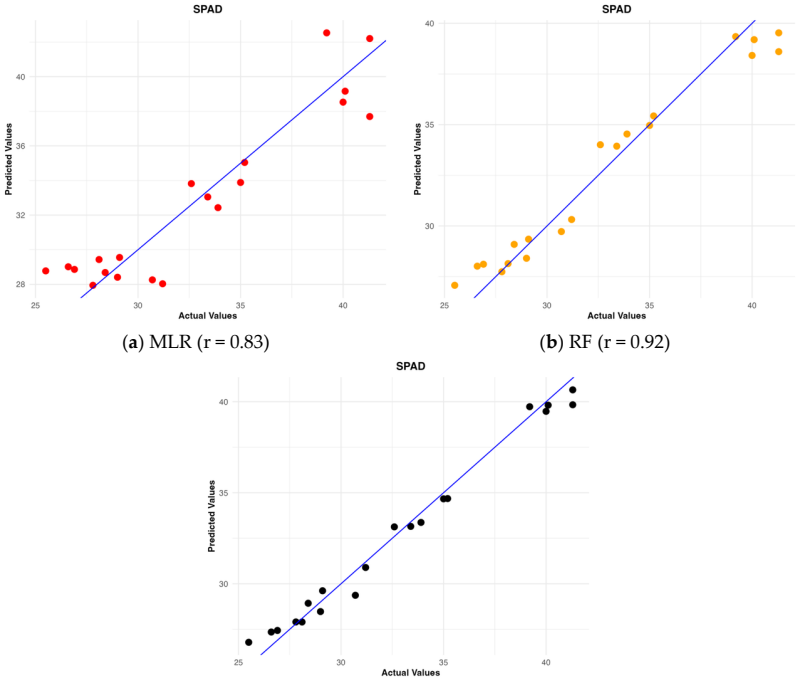


(**a**) MLR (r = 0.77) (**b**) RF (r = 0.80)

(**c**) SVR (r = 0.86)

Figure 3. Comparison of actual and predicted plant length (cm) using different machine learning models with correlation coefficients (r).

For SPAD values, both the RF and SVR models showed substantially improved performance compared to MLR. The SVR model achieved the highest predictive accuracy (R^2 = 0.871). This was followed by RF (R^2 = 0.862), while MLR showed a lower performance (R^2 = 0.699) (Figure 4).



(**a**) MLR (r = 0.83) (**b**) RF (r = 0.92)

(**c**) SVR (r = 0.93)

Figure 4. Comparison of actual and predicted SPAD values using different machine learning models with correlation coefficients (r).

3.3. Changes in Mineral Nutrient Concentrations of Culture Media

The mineral nutrient concentrations in the culture medium were significantly affected by the culture system, carbon source, and sampling time. Across all treatments, a clear decline in nutrient concentrations was observed after 4 weeks, indicating substantial uptake by the explants (Table 5).

Table 5. Changes in Fe, Cu, Mn, Zn, and Mg concentrations (ppm) in solid and liquid culture systems under different carbon sources before and after 4 weeks of in vitro culture.

Culture System	Carbon Source	Sampling Time	Fe (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)	Mg (ppm)
Solid	Glucose	Initial	3.08 ± 0.22 b	0.21 ± 0.01 b	6.80 ± 0.32 a	1.14 ± 0.08 b	2.15 ± 0.12 b
Liquid			5.12 ± 0.28 a	0.35 ± 0.02 a	6.68 ± 0.34 a	1.76 ± 0.08 a	2.72 ± 0.15 a
Solid	Sucrose		3.01 ± 0.25 b	0.23 ± 0.02 b	6.61 ± 0.29 a	1.12 ± 0.07 b	2.11 ± 0.09 b
Liquid			5.01 ± 0.31 a	0.33 ± 0.02 a	6.60 ± 0.27 a	1.79 ± 0.07 a	2.77 ± 0.14 a
Solid	Glucose	After 4 weeks	1.80 ± 0.11 c	0.15 ± 0.01 d	3.98 ± 0.41 bc	0.68 ± 0.09 e	1.76 ± 0.10 c
Liquid			2.87 ± 0.22 b	0.21 ± 0.02 c	3.62 ± 0.29 c	1.04 ± 0.05 c	2.11 ± 0.12 b
Solid	Sucrose		1.03 ± 0.12 e	0.12 ± 0.01 e	4.26 ± 0.38 b	0.60 ± 0.06 e	1.44 ± 0.05 d
Liquid			1.46 ± 0.05 d	0.14 ± 0.01 ef	4.31 ± 0.25 b	0.83 ± 0.06 d	1.76 ± 0.11 c
	p		***	***	***	***	***

Means followed by different lowercase letters within the same column indicate statistically significant differences according to Duncan's multiple range test ($p < 0.05$). Asterisks in the *p*-value section denote the level of statistical significance: *** $p < 0.001$. Standard error (±SE) is provided for each mean.

At the initial stage, liquid culture systems consistently exhibited higher concentrations of Fe, Cu, Zn, and Mg than solid media. With respect to Fe, the highest depletion was observed in sucrose treatments, particularly in the liquid cultivation system supplemented with sucrose (from 5.01 ± 0.31 ppm to 1.46 ± 0.05 ppm; 70.86% decrease), followed by the solid cultivation system supplemented with sucrose (3.01 ± 0.25 ppm to 1.03 ± 0.12 ppm; 65.78% decrease). In contrast, glucose treatments showed lower Fe depletion, with reductions of 41.56% (solid–glucose) and 43.95% (liquid–glucose). Cu concentrations also declined significantly across all treatments. The greatest reduction was recorded in the liquid sucrose treatment (0.33 ± 0.02 ppm to 0.14 ± 0.01 ppm; 57.58% decrease), followed by the solid cultivation system supplemented with sucrose (47.83%). Glucose treatments exhibited comparatively lower Cu depletion, with 40.00% (liquid–glucose) and 28.57% (solid–glucose) reductions. Mn showed a different trend compared to other elements. Although Mn concentrations decreased across all treatments, the highest depletion was observed in the glucose treatments, with 45.81% (liquid–glucose) and 41.47% (solid–glucose) reductions. In contrast, sucrose treatments showed lower Mn depletion (35.55% in solid–sucrose and 34.70% in liquid–sucrose). Zn concentrations decreased substantially in all treatments, with reductions ranging from 40.35% to 53.63%. The highest depletion was observed in the liquid cultivation system supplemented with sucrose treatment (53.63%). It was followed by the solid cultivation system supplemented with sucrose (46.43%). Glucose treatments showed slightly lower but still considerable reductions (40.35–40.91%). Mg exhibited the lowest overall depletion among all elements, with reductions ranging from 18.14% to 36.46%. The lowest decrease was observed in the solid cultivation system supplemented with glucose treatment (18.14%). On the other hand, the highest decrease was recorded in the liquid cultivation system supplemented with sucrose (36.46%).

4. Discussion

4.1. Integrated Effects of Carbon Source and Culture System on Growth and Mineral Nutrition

The findings of this study demonstrate a synergistic interaction between the liquid culture system and sucrose. This interaction plays an important role in both morphological development and regenerative capacity in chestnut explants. While the liquid system provides a better physical environment, sucrose seems to play a major role in physiological processes. Carbon sources provide carbon skeletons, and osmotic potential for shoot proliferation, root induction, embryogenesis, and organogenesis [42]. Also, sucrose induces osmotic stress and sugar signaling by reprogramming cells and influencing processes like somatic embryogenesis and callus regeneration [43]. It is believed that sucrose is a better carbon source due to its role not only as an energy source but also in regulating osmotic balance, ROS levels, and hormonal signaling [44,45]. In a study conducted on the 'Marsol' chestnut cultivar similar results were also obtained. In that study, 30 g L^{-1} sucrose maximized plant length, leaf number, SPAD values, callus dimensions, and multiplication rates, while glucose had similar but lower values [20]. Gago, Bernal, Sánchez, Aldrey, Cuenca, Christie, and Vidal [13] reported that reducing sucrose levels particularly reduced shoot length and leaf quality under continuous submersion. Similarly, in plum and willow, liquid/bioreactor systems supplemented with sucrose produced longer shoots, larger leaves, and higher pigment concentrations [46,47]. In another study conducted on *Vernonia condensata*, the liquid/bioreactor + high sucrose treatment significantly increased the number of shoots, biomass, leaf nutrient content, and pigments [17]. In *Justicia gendarussa,* sucrose has been associated with larger calluses that have a high regenerative potential [48]. A study conducted on *Agapanthus praecox* found that sucrose induces a unique hormonal/ROS profile that enhances differentiation capacity [44]. On the other hand, in datura and rosa, glucose was found to be a better carbon source [26,49]. This also shows that the effect of the carbon source can vary according to the plant species. Interestingly, the lowest multiplication rate was recorded in the liquid system supplemented with glucose. This contrast to the generally growth-promoting nature of liquid systems may be linked to the synergy between a liquid environment and glucose, which potentially induces a localized osmotic stress or triggers hyperhydricity-related physiological responses. Some studies show that a high level of carbon source concentrations can negatively affect plant growth by causing osmotic stress [50–52].

In addition, the carbon source is an important factor in element accumulation and related morphological development. In plants grown in a sucrose-containing medium, the accumulation of Fe, Cu, Zn, and Mg increased, while glucose promoted the accumulation of Mn. Strong and positive correlations were observed between the increased Fe and Mg levels and the SPAD value, plant length, and shoot proliferation. Similarly, increasing micronutrient mixtures significantly increased shoot length and leaf number in both stevia and hazelnut [53,54]. This supports the fundamental roles of these elements in chlorophyll synthesis and photosynthetic activity. Indeed, while Fe deficiency or inadequate transport can cause chlorosis and reduced photosynthesis, Mg is critical to plant development because it serves as the central atom of the chlorophyll molecule and acts as a cofactor for photosynthetic enzymes [32,55–57]. In addition, the positive correlation between increased Cu and Zn levels and growth parameters in sucrose treatments could be from the impact of these micronutrients on shoot quality and proliferation. It has been shown that Zn and Cu exhibit strong positive correlations with chlorophyll content, leaf area, and yield in Nagpur mandarin [58]. This relationship can be further explained by the physiological roles of Zn and Cu in plant metabolism. Zn is essential for the biosynthesis of tryptophan, a precursor of the growth hormone indole-3-acetic acid (IAA), which directly promotes shoot elongation [59]. Meanwhile, increased Cu accumulation contributes to improved

tissue firmness and shoot quality by acting as a cofactor for enzymes involved in cell wall lignification, such as laccases [60]. Therefore, the enhanced uptake and efficient utilization of these micronutrients under sucrose treatments provide a plausible explanation for the superior morphogenetic development observed compared to glucose treatments.

In contrast, Mn showed a strong negative relationship with growth parameters and SPAD values. Moreover, its interactions, particularly with Fe and Mg, are consistent with the mineral nutrition literature and could suggest the presence of a possible element antagonism. The homeostasis of Fe, Zn, and Mn is regulated by mutual interactions and the fact that high Mn levels can inhibit Fe uptake and chlorophyll synthesis [31,55,56]. Increasing Mg levels in sweet orange has been reported to reduce Mn uptake in roots and stems, whereas an increased Mn concentration negatively affects photosynthetic performance [56]. It has been clearly demonstrated in both classical nutritional physiology studies and research on hyperaccumulator plants and soybeans that Mn can inhibit chlorophyll synthesis and growth by competing with Fe, Mg, and other microelements [61–65]. Considering the Fe-Mg-Zn/Cu synergy and potential antagonistic interactions with Mn described in the literature [31,55–58], it can be stated that the selection of a carbon source in chestnut in vitro cultures is a critical factor that influences not only carbon nutrition but also mineral nutrient balance and the associated morphogenetic processes. The preferential accumulation of Fe, Mg, and Zn under sucrose supplementation, as opposed to Mn accumulation under glucose, can be explained by differences in the metabolic efficiency of these carbon sources. It can hypothesized that active nutrient uptake is an ATP-dependent process, and sucrose metabolism provides a more efficient energy supply for the operation of H^+-ATPase pumps [66], thereby facilitating the uptake of beneficial micronutrients. In contrast, the accumulation of Mn under glucose may further exacerbate its antagonistic effects on Fe and Mg uptake, as high Mn levels are known to inhibit Fe acquisition and chlorophyll biosynthesis [67]. In this context, the observation of high Mn accumulation accompanied by low SPAD values and poor shoot proliferation supports the conclusion that Mn exerts an inhibitory effect when present at elevated levels.

The PCA results further demonstrate these relationships, showing that in vitro chestnut growth is positively correlated with Fe, Mg, Cu, and Zn. In particular, the fact that PC1 explains 74.5% of the total variance indicates that the primary variability in the system is concentrated along the same axis as the vegetative growth and micronutrient accumulation. These findings are also consistent with studies conducted on other species, such as citrus and grapevines. In these studies, PC1 was identified as the primary component representing the co-variation in chlorophyll content, leaf area, and yield with nutrient elements such as Fe, Zn, and Mg [58,68,69]. These findings are also consistent with previous studies, which have demonstrated the critical roles of Mg, Fe, and Zn in growth through deficiency experiments and variance decomposition analyses in species such as Eucalyptus and Epimedium [70,71]. Furthermore, the partial separation of Zn by PC2 is consistent with findings suggesting that while Zn supports growth in conjunction with Fe-Mg-Cu, it may also exert more specific effects on secondary metabolism and quality traits [58,71,72].

Predicting plant traits such as SPAD values and plant length is crucial for optimizing plant tissue culture protocols and improving productivity. The findings of the study indicate that non-linear modeling approaches are more effective in capturing the relationships between mineral composition and plant length and are particularly powerful in predicting SPAD. The actual and predicted plots supported these findings, showing that SVR and RF predictions were more closely aligned with the 1:1 reference line (Figures 3 and 4). On the other hand, the MLR model exhibited greater dispersion and reduced accuracy. SVR provided superior predictions for plant length and SPAD. Numerous studies demonstrate that RF and SVR generally achieve higher predictive accuracy than traditional linear models

such as MLR, especially when dealing with non-linear or complex biological data [73]. The robustness of RF against overfitting makes it particularly suitable for high-dimensional datasets common in remote sensing or image-based phenotyping applications [74]. Meanwhile, SVR's strength lies in its effectiveness with smaller datasets or when kernel-based modeling is advantageous [73].

4.2. Carbon Source-Dependent Nutrient Uptake and Depletion Dynamics

Nutrient depletion percentages clearly demonstrated that sucrose, particularly in liquid culture systems, significantly enhances the uptake of Fe, Cu, Zn, and Mg. However, Mn uptake is relatively higher under glucose treatments, indicating a differential and element-specific nutrient utilization pattern. At the end of the four-week period, the decrease in nutrient concentrations across all treatments showed a medium level of depletion and active uptake, as described in the literature [30,75]. In particular, the presence of higher initial concentrations of Fe, Cu, Zn, and Mg in liquid systems and the observation of the highest depletion levels of these elements in sucrose-containing liquid media can be explained by the more efficient mass transfer and increased nutrient uptake which could be associated with the high growth rate metabolically supported by sucrose [18,30,75]. This indicates that liquid culture systems not only enhance physical accessibility but also increase metabolic activity, thereby accelerating nutrient consumption. Similarly, in Petunia in vitro culture, it has been reported that Fe, Mg, and Mn uptake increase along with pigment content under appropriate combinations of Fe form and pH, and that nutrient uptake is highly sensitive to environmental conditions [76]. In Curcuma bioreactor studies, a direct relationship between sucrose consumption and biomass increase has been highlighted. It has been emphasized that the mineral balance of the medium strongly shapes growth responses [18]. In contrast, the depletion of Mn in the medium with the highest glucose content in this study is consistent with the literature, which indicates that this element has a transport mechanism that is distinct from those of other micronutrients. It has been reported that in sugarcane, Cu and Zn are taken up via the same carrier system, whereas Mn is transported via a more independent and metabolically controlled system [77]. Furthermore, the fact that Mn uptake is less affected despite competitive inhibition by Zn and Cu supports the existence of a separate regulatory mechanism for Mn [77]. In this context, the fact that glucose increases Mn uptake in chestnut while not promoting Fe, Cu, and Zn uptake as much as sucrose can be explained by nutrient preference and transport regulation linked to the carbon source [30,78,79].

4.3. Study Limitations

Despite the significant insights it provides into the interaction between carbon sources and culture systems, this study has several limitations that should be acknowledged. First, the experiments were conducted using a single hybrid chestnut cultivar ('Akyüz') and given the high genotype-dependency of chestnut micropropagation, the results may not be directly applicable to all *Castanea* species. Second, while the machine learning models (SVR and RF) showed high predictive performance, the dataset size is characteristic of in vitro studies and remains relatively small compared to large-scale agricultural datasets. This may limit the models' generalizability across broader environmental conditions. Furthermore, the study focused on a specific four-week subculture period and a limited set of mineral elements (Fe, Cu, Mn, Zn, and Mg). Long-term effects on rooting and acclimatization stages were not evaluated, which are critical for the commercial success of micropropagation protocols. Future research should incorporate a wider range of genotypes and longer-term physiological assessments to validate these findings.

5. Conclusions

This study demonstrates that the interaction between the culture system and carbon source plays a critical role in regulating both morphogenetic responses and mineral nutrient dynamics in chestnut in vitro cultures. Among the tested treatments, the liquid culture system supplemented with sucrose consistently produced superior results in terms of plant growth, chlorophyll content, callus development, and shoot proliferation. Sucrose not only functions as an energy source but also significantly enhances the accumulation of essential micronutrients. In contrast, glucose treatments led to increased Mn accumulation, which could exhibit an antagonistic interaction with Fe and Mg and showed a clear inhibitory effect on plant growth parameters. These results indicate that mineral balance, rather than individual nutrient concentration, appears to be a significant factor for in vitro propagation success. These findings provide valuable insights for improving large-scale propagation protocols and contribute to a better understanding of the physiological and nutritional mechanisms underlying in vitro plant development.

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