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Nutrient Multi-factor Study: Nitrogen Plays an Important Role *in vitro* Shoot Proliferation of *Vitis labrusca* 'Concord'

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ABSTRACT

The composition of the culture medium, particularly its nutrient elements and plant growth regulators, plays a pivotal role in in vitro shoot proliferation. Understanding the individual and interactive effects of these factors on *in vitro* growth traits requires comprehensive and multifactorial experimentation. In this study, we employed a response surface methodology with 42 experimental runs to investigate the effects of six medium components, NH_4NO_3, KNO_3, mesos, micros, iron, and BAP, at various concentrations on the *in vitro* shoot proliferation of *Vitis labrusca* 'Concord'. The results revealed that KNO_3 had the most significant positive effect, while BAP exerted the most significant negative effect on the number of proliferated shoots. A reduction in NH_4NO_3 concentration promoted increased shoot length. Both KNO_3 and NH_4NO_3 were found to negatively influence leaf number and leaf coloration, underscoring the importance of nitrogen form and concentration in modulating morphological traits. Our findings highlight the critical role of precise nitrogen source management in optimizing *in vitro* shoot production. Moreover, significant interaction effects were observed between BAP and other components such as KNO_3, iron, and micros, further influencing shoot proliferation outcomes. Based on these results, we recommend using reduced concentrations of NH_4NO_3 and KNO_3 (within 0.5–2.5 MS medium) in combination with 2.25 mg L^{-1} BAP to enhance shoot production, elongation, and foliage quality in *Vitis labrusca* 'Concord'. These insights provide a valuable basis for refining *in vitro* protocols in *Vitis* species.

Abbreviation: 6-Benzylaminopurine (BAP or BA), Response surface methodology (RSM)

Introduction

In plant tissue culture, the culture medium is supplemented with various substances, including inorganic elements (macro- and micronutrients), organic compounds (such as vitamins), carbohydrates, and plant growth regulators, to create an artificial environment conducive to explant growth. These components serve different physiological and developmental purposes. Given the complexity of the medium and the difficulty of optimizing the concentrations of multiple components simultaneously, traditional statistical approaches are often insufficient. Consequently, more advanced analytical techniques are required to assess the effects of multiple factors concurrently. Response surface methodology (RSM), a multifactorial statistical tool, has recently been successfully applied in plant tissue culture studies (Niedz and Evens, 2007; Niedz et al., 2007; Reed et al., 2013; Niedz et al., 2014; Poothong and Reed, 2014; Akin et al., 2016; Kovalchuk et al., 2017; Karimpour et al., 2020a, b; Dagne et al., 2023; Ali and Aasim, 2024). *Vitis labrusca* L. (Vitaceae),

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commonly known as fox grape, is a wild American grape species with moderate cold tolerance (Zhang et al., 2012). It is widely utilized in the pharmaceutical and food industries and as an ornamental plant. Cultivars such as 'Concord' and 'Catawba' are believed to be natural hybrids between *V. labrusca* and *V. vinifera*, and are referred to as *Vitis* × *labruscana* (Walker et al., 2019; Huber et al., 2016). These cultivars were foundational to the development of the grape industry in the eastern United States (Hedrick et al., 1908).

Although hardwood cuttings of *V. labrusca* are typically difficult to root, this species is used as a rootstock for *V. vinifera* cultivars in regions where phylloxera aphids are prevalent (Walker et al., 2019). The Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) is commonly recommended for *in vitro* culture of *V. labrusca* (Carvalho et al., 2013; Bettoni et al., 2016). However, both the quantity and quality of proliferated shoots are often suboptimal. Elevated concentrations of cytokinins, particularly 6-benzylaminopurine (BAP), can further reduce shoot quality and increase shoot vitrification. Adjusting macro- and micronutrient concentrations in the culture medium has been shown to mitigate the adverse effects of high cytokinin levels (Preece, 1995). For instance, mesos components ($CaCl_2 \cdot 2H_2O$, KH_2PO_4, and $MgSO_4$), iron, and MS micronutrients (including B, Cu, Co, I, Mn, Mo, and Zn) significantly influenced shoot quality in *Pyrus* genotypes, while NH_4NO_3, iron, and mesos had stronger effects on the number of proliferated shoots (Reed et al., 2013). Similarly, NH_4NO_3 and iron affected shoot proliferation in pear cv. Shekari under a quadratic model (Karimpour et al., 2022b), and a positive linear relationship was observed between BAP concentration and shoot proliferation in apple cv. Abbasi (Karimpour et al., 2022a).

In Persian walnut, doubling the NH_4NO_3 concentration in DKW medium resulted in longer shoots, while increasing KNO_3 to 1900 mg L^{-1} had no significant effect on shoot length but led to paler leaves (Zamani and Vahdati, 2001). Additionally, altering macronutrient concentrations in DKW medium improved both the quality and quantity of *in vitro* shoots in Persian walnut (Najafian Ashrafi et al., 2010). The present study was designed to investigate, for the first time, the relationships between inorganic nutrient concentrations and the plant growth regulator BAP on shoot proliferation in *V. labrusca* using response surface methodology. This approach aims to optimize culture conditions and enhance both the quality and quantity of *in vitro* shoot production in this economically and horticulturally important species.

Material and methods

Plant material

Current season shoots of *Vitis labrusca* 'Concord' in the middle of spring, year 2023, were collected from the collection orchard of Sabdiz company (37°24'05.7"N, 57°48'39.1"E) and transferred to the Biotechnology Laboratory, Shirvan Faculty of Agriculture, Bojnord University. After washing with tap water, the nodal segments were disinfected with sodium hypochlorite 1.5% (15 min) after soaking in 70% ethanol (60 s), then washed with sterilized water for three times (15 min). The shoots were introduced to MS medium with 1 mg L^{-1} BAP. The healthy and similar micro-shoots were transferred to media with different treatments (Table 1) after 4 weeks. MS vitamins, sucrose (30 g L^{-1}) and agar (8 g L^{-1}) were added for all medium, and pH was adjusted at 5.8±0.2. All media and jars were autoclave-sterilized. The cultures kept in photoperiodic lighting (16 h of light: 8 h of dark, 80 $\mu Mm^{-2}s^{-1}$ irradiance by solar LED lights) at 21±2 ℃.

Design of experiment

Six factors that include A) NH_4NO_3, B) KNO_3, C) mesos, D) micros, E) Fe-EDTA, and F) BAP were investigated using an RSM D-optimal design generated using Design Expert® ver. 13 software (Statease, MN, USA) (Table 2). The design included 42 combinations of the six investigated factors (Table 1). Each treatment contained 10 replications (with 5 micro-shoots). A replication included 10 jars to estimate the response for each treatment. Jars were randomly placed in the growth shelves under the same conditions. Data were collected on proliferated shoot number (PSN, shoot size ≥ 0.5 cm), shoot length (SL, cm), leaf number (LN), leaf color (LC; 1= white-yellow, 2= yellow, 3= acceptable green, and 4= dark green), lateral shoot number (LSN), shoot browning (SB, %) and root number (RN, root size ≥ 0.3 cm) were recorded after 4 weeks of cultivation.

Statistical analysis

The response data represent the means of individual explants. Model selection was based on the highest-order polynomial for which additional terms were statistically significant ($P \leq 0.05$). Data were analyzed using analysis of variance (ANOVA). Both observed responses at design points and predicted responses at untested points were visualized graphically.

Results

The results of the statistical analysis indicated that the evaluated factors had significantly effects on all measured responses across the quadratic, two-factor interaction (2FI), and reduced linear models (Table 3). The fitted response surface models illustrating the effects of the studied factors on each response are presented in Figures 1–4.

Table 1. Six-factor design including 42 model points used for *in vitro* shoot proliferation of *Vitis labrusca* 'Concord'.

Run	Factor A*: NH_4NO_3	Factor B: KNO_3	Factor C: Mesos	Factor D: Micros	Factor E: Fe-EDTA	Factor F: BAP
	× based on MS concentration					(mg L^{-1})
1	0.5**	0.5	2	0	1.5	1.25
2	2.5	2.5	0.5	0	1.5	2.25
3	2.5	2.5	2	0	0.25	2.25
4	1.5	0.5	0.5	1.5	0.25	2.25
5	2.5	0.5	0.5	0.75	0.25	0.25
6	0.5	2.5	0.5	1.5	0.875	2.25
7	0.5	1.5	0.5	0	0.25	0.25
8	1.5	2.5	0.5	1.5	1.5	0.25
9	2.5	2.5	2	1.5	1.5	2.25
10	2.5	0.5	0.5	0.75	1.5	2.25
11	2.5	1.5	2	1.5	0.25	0.25
12	0.5	2.5	1.25	1.5	0.25	0.25
13	2.5	2.5	0.5	0	0.25	1.25
14	0.5	2.5	2	0	1.5	2.25
15	2.5	0.5	2	0	1.5	0.25
16	0.5	0.5	2	0.75	0.25	2.25
17	2.5	0.5	2	0	1.5	2.25
18	0.5	2.5	0.5	0	1.5	1.25
19	2.5	2.5	0.5	1.5	0.25	2.25
20	1.5	0.5	2	0	0.25	0.25
21	0.5	1.5	2	1.5	1.5	0.25
22	0.5	0.5	0.5	0	0.875	2.25
23	1.5	0.5	2	0	0.25	0.25
24	2.5	0.5	1.25	1.5	1.5	0.25
25	1.5	1.5	1.25	0.75	0.875	1.25
26	0.5	0.5	1.25	1.5	1.5	2.25
27	0.5	0.5	0.5	0	1.5	0.25
28	0.5	2.5	2	0	0.875	0.25
29	0.5	2.5	0.5	0	0.25	2.25
30	1.5	1.5	1.25	0.75	0.875	1.25
31	2.5	0.5	2	1.5	0.25	2.25
32	0.5	0.5	0.5	1.5	0.25	1.25
33	0.5	0.5	1.25	1.5	1.5	2.25
34	0.5	0.5	0.5	1.5	0.25	1.25
35	2.5	2.5	0.5	1.5	0.25	0.25
36	0.5	0.5	0.5	1.5	1.5	0.25
37	2.5	1.5	0.5	0	1.5	0.25
38	2.5	0.5	1.25	0	0.25	2.25
39	0.5	2.5	2	1.5	0.25	1.25
40	2.5	2.5	2	0.75	1.5	0.25
41	0.5	0.5	2	1.5	0.875	0.25
42	2.5	0.5	1.25	0	0.25	2.25

*The amounts used for factors A to E are based on the amount of the same salts in the MS medium (mg L^{-1}): NH_4NO_3: 1650, KNO_3: 1900, Mesos ($CaCl_2$. $2H_2O$: 440; KH_2PO_4: 170; $MgSO_4$. $7H_2O$: 370), Micros (H_3BO_3: 6.2; $MnSO_4$. H_2O: 16.9; $ZnSO_4$. $7H_2O$: 8.6; KI: 0.83; $NaMoO_4$. $2H_2O$: 0.25; $CoCl_2$. $6H_2O$: 0.025; $CuSO_4$. $5H_2O$: 0.025), Fe-EDTA ($FeSO_4$. $7H_2O$: 27.8; Na_2EDTA: 37.3).

Table 2. The six factors used to construct the six-dimensional design space for *in vitro* shoot proliferation of *V. laborusca*.

Factors	**Details**	**Range (based on MS concentration)**
Factor A	NH_4NO_3	0.5 - 2.5×
Factor B	KNO_3	0.5 - 2.5×
Factor C	Mesos ($CaCl_2.2H_2O$; KH_2PO_4; $MgSO_4$)	0.5 - 2.0×
Factor D	Micros (MS salts of B, Cu, Co, I, Mn, Mo, and Zn)	0 - 1.5×
Factor E	Fe-EDTA	0.25 - 1.5×
		Range (mg L^{-1})
Factor F	BAP	0.25 - 2.25

Results

The results of the statistical analysis indicated that the evaluated factors had significant effects on all measured responses across the quadratic, two-factor interaction (2FI), and reduced linear models (Table 3). The fitted response surface models illustrating the effects of the studied factors on each response are presented in Figures 1–4.

Table 3. Summary of the statistical analysis results for the effects of five media components on four shoot multiplication traits of *Vitis labrusca* 'Concord' for *in vitro* conditions.

Response (Model)	*P value*			R^2	Adj R^2	Pred R^2
	Model	Factors*	*Lack of fit*			
Proliferated shoot number; PSN (Quadratic)	3.05803E-07	A: 0.0121 B: 7.35306E-05 C: 0.042 F: 6.56583E-06 BC: 0.0434 BF: 0.0097 CF: 0.0012 F²: 0.0210	0.1568	0.8426	0.7670	0.6352
Proliferated shoot length; PSN (Quadratic)	0.0001	A: 0.0005 B: 0.0123 F: 0.0055 AE: 0.0345 BC: 0.0024 A²: 0.0108	0.4613	0.6208	0.5197	0.3789
Leaf number; LN (Reduced Linear)	4.81889E-05	A: 0.0004 B: 0.0012	0.0184	0.4243	0.3924	0.3204
Leaf color; LC (2FI)	0.0001	A:0.0011 B: 3.4364E-05 E: 0.0332 AB: 0.0100 DF: 0.0103 EF: 0.0399	0.2333	0.6151	0.5125	0.3315
Lateral shoot number; LSN (2FI)	0.0025	A: 0.0401 AB: 0.0006 AC: 0.0084 AE: 0.0229 DF: 0.0078 EF: 0.0377	0.9206	0.6316	0.4616	0.1381
Shoot browning; SB (2FI)	0.0012	A: 0.0009 E-Fe:0.0595 BD: 0.0409 BF: 0.0058 CE: 0.0142 DF: 0.0025	-	0.6560	0.4973	0.0844
Root number; RN (Reduced Linear)	0.0026	D: 0.0284 F: 0.0041	1.41043E-18	0.3741	0.3005	0.1784

*Factors A: NH_4NO_3, B: KNO_3, C: Mesos, D:Micros, E: Fe-EDTA, F:BAP.

Proliferated shoot number (Quadratic model)

KNO_3 and BAP had the most significant effect on the proliferated shoot number of *V. labrusca* 'Concord' following a quadratic model with negative and positive effects, respectively (Table 3). The largest amount of PSN was obtained when KNO_3 was at its lowest concentration (×0.5) and BAP was at 2.25 mg L^{-1} (Fig. 1). Increasing the KNO_3 level (from ×0.5 to ×2.5) led to a decrease in proliferated shoot number from 3.3 to 2.1. Conversely, increasing BAP concentration led to an increased shoot number. The interaction between these two main factors was also significant ($P \leq 0.0097$), but not as effective as their individual effects.

Proliferated shoot length (Quadratic model)

The proliferated shoot length trait of *Vitis labrusca* 'Concord', was best described by a quadratic model,

with NH_4NO_3 being the most influential factor in a negative manner. Other factors, such as KNO_3 and BAP, along with some interaction effects, showed less significant effects on this trait (Table 3). The optimal concentration of NH_4NO_3 was ×1.2 the concentration in the MS medium. Deviations from this point led to decreased shoot length (Fig. 2).

Leaf number (Reduced Linear model)

The main factors exerting the most control over the leaf number of *Vitis labrusca* 'Concord' were NH_4NO_3 and KNO_3, according to a reduced linear model (Table 3). The maximum number of leaves per explant, reaching up to four, was achieved when NH_4NO_3 and KNO_3 concentrations were 1.5 times their concentration in MS medium (Fig. 3).

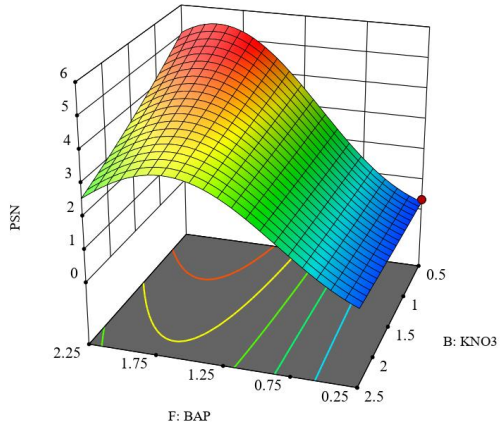

Fig. 1. Surface response plot of KNO_3 and BAP effect on proliferated shoot number (PSN) of *Vitis labrusca* 'Concord'.

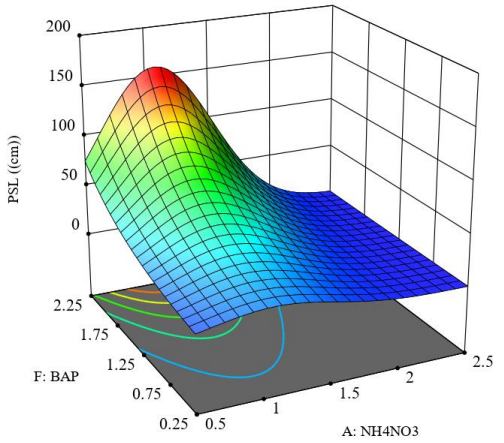

Fig. 2. Surface response plot of NH_4NO_3 and BAP effect on proliferated shoot length (PSL) of *Vitis labrusca* 'Concord'.

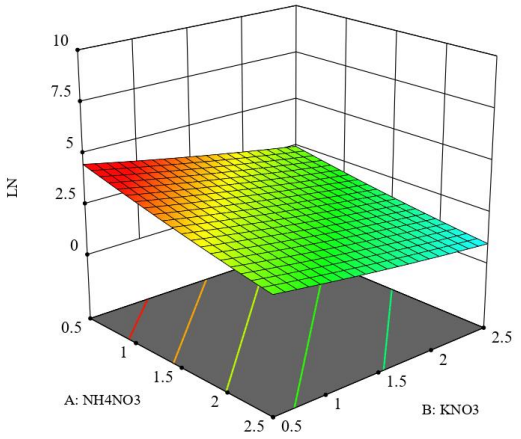

Fig. 3. Surface response plot of NH_4NO_3 and KNO_3 effect on leaf number (LN) of *Vitis labrusca* 'Concord'.

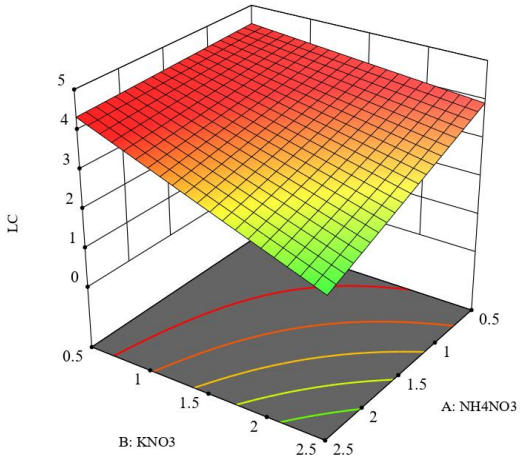

Fig. 4. Surface response plot of NH_4NO_3 and KNO_3 effects on leaf color (LC) of *Vitis labrusca* 'Concord'.

Leaf color (2FI model)

Leaf color trait was best represented by a 2FI model, with NH_4NO_3 and KNO_3 identified as the primary factors (Table 3). NH_4NO_3 and KNO_3 had a negative effect on the leaf color (Fig. 4). An acceptable green color for leaves (value= 3) appears to be associated with NO_3^-, which can be provided by NH_4^+ or K^+. To achieve the desired leaf color, an increase in one compound necessitates a reduction in the other (Fig. 4). Iron was also an influential factor, but its impact was less significant than that of the previous two factors (Table 3).

Lateral shoot number, shoot browning and root number

The 2FI models were applied to analyze lateral shoot number and shoot browning, whereas a reduced linear model was used for root number. However, all these models proved inadequate due to their substantially low predictive R^2 values (Table 3).

Optimization

According to the optimal model for the composition of the culture medium for the maximum amounts in traits including proliferated shoot number (5), proliferated shoot length (2 mm), and leaf number (10.5), it was predicted that the reduced amounts of NH_4NO_3 (×0.528) and KNO_3 (×0.500) along with the increased concentrations of mesos (×2.000) and micros (×1.500) and the average amount of iron (×1.109) with the application of BAP at 1.587 mg L^{-1} can produce desirable result (Desirability= 0.841). In this case, values of 4.62, 13.80 (mm), 4.48, 3.03, 0.37, 1.40 (mm), 0.01 (%), and 0.00 were predicted for the attributes of proliferated shoot number, proliferated, shoot length, leaf number, leaf color, lateral shoot number, lateral shoot length, shoot browning, and root number, respectively.

Discussion

Nitrogen, a macro-element, is usually taken up by plants in two inorganic forms, NO_3^- and NH_4^+, via specific transporters. For *in vitro* conditions, NH_4NO_3 and KNO_3 are common nitrogen sources, which also supply nitrogen in the MS medium. Amino acids and proteins are composed primarily of nitrogen, along with other key biological macro-molecules. Nitrate signaling activates gibberellin production and reduces DELLA protein stability, leading to enhanced plant growth and development (Camut et al., 2021). Moreno et al. (2020) discovered that NO_3^- has been shown to play a regulatory role in shoot growth by modulating cell size and endo-replication. *In vitro* studies have widely investigated the complex interactions among the essential nutrients NH_4^+, NO_3^- and K^+ (Niedz et al., 2014). The effect of NH_4NO_3 or KNO_3 on the shootadd the number was reported in other studies with various results that confirms its dependence on species/genotype. Shoot number of different pear genotypes was controlled by NH_4NO_3, Fe, or in rare cases by mesos, but not by KNO_3 (Reed et al., 2013). Similarly, the negative effect of KNO_3 was reported by Zamani and Vahdati (2001) on Persian walnut. The increased activity of K+ absorption leads to increased competition with NH_4^+, which can have significant implications on various biological processes. When the amount of KNO_3 is constant, NH_4NO_3 can have negative effects on shoot multiplication rate in *Pyrus communis* cv. Shekari based on a quadratic model (Karimpour et al., 2020a). However, in red raspberry genotypes, shoot number was influenced by KNO_3 and Fe. In some of them, it was by NH_4NO_3 (Poothonga and Reed, 2014). In a wild type Kazakhstan apricot, *Prunus armeniaca*, shoot number was affected by Ca $(NO_3)_2$, while NH_4NO_3 was non-significant (Kovalchuk et al., 2017). The multiplication rate of OHF *Pyrus* rootstocks was affected by some minerals such as

NH_4^+ and NO_3^- and/or PGRs based on mono-objective GA optimized MLPNN and RBFNN-based models (Jamshidi et al., 2016 and 2019). In a similar modelling research, it was found that the decreasing amount of NH_4^+ enhanced the quality of produced micro-shoots of G × N15 *Prunus* rootstock (Arab et al., 2019). Cytokinins play a crucial role in modulating cell proliferation, particularly in shoots. Additionally, they are important for regulating leaf phyllotaxy (Besnard et al., 2014), female gametophyte development (Cheng et al., 2013), root architecture (Chang et al., 2013), and other aspects of plant growth and development. The primary role of cytokinin's *in vitro* is to trigger shoot induction, a process that relies on the regulation of auxin synthesis (Di et al., 2016; Yan, et al., 2017; Šmeringai, et al., 2023), because a crucial factor for the initiation of new shoots is the availability of auxin in particular cell types. In a RSM study, Karimpour et al. (2020b) reported that proliferated shoot number of *Malus domestica* cv. Abbasi was positively affected by BAP concentration in a linear model.

Previous studies have shown that shoot length is primarily controlled by iron and mesos, although micros, iron, NH_4NO_3, and KNO_3 have also shown significant effects in certain genotypes. In line with the current results, NH_4NO_3 has been found to be a driving factor for shoot length in several other plants, such as Persian walnut (Zamani and Vahdati, 2001), red raspberry genotype 'Willamette' (Poothong and Reed, 2014), wild Kazakhstan apricot (Kovalchuk et al., 2017) and pear cv. 'Shekari' (Karimpour et al., 2019). The negative effect of NH_4NO_3 on shoot length is probably due to the disturbance of the nitrate to ammonium ratio. With increasing NH_4NO_3, the nitrate to ammonium ratio increases due to the presence of KNO_3 (fixed) in the medium. The reduced K concentration and NH_4^+ caused longer *in vitro* shoots in Persian walnut (Najafian Ashrafi et al., 2010).

Leaf greenness (leaf color) serves as an indicator of chlorophyll content, particularly chlorophyll *a*, within the leaf that was negatively controlled by NH_4NO_3 and KNO_3 and positively by Fe in our results. In pear trees, mesos concentration influenced leaf color, while similar to our finding, in the "Hang Pa Li" genotype, NH_4NO_3 concentration had a negative impact (Reed et al., 2013). The chlorophyll *a* content in *in vitro Pyrus communis* cv. Shekari leaves was influenced by NH_4NO_3 (negatively) and Fe (positively) concentrations in the medium (Karimpour et al., 2020a). In some red raspberry genotypes, KNO_3, mesos and iron significantly affected leaf color (Poothong and Reed, 2014), while NH_4NO_3, $Ca(NO_3)_2$, K_2SO_4, and micros played a role in determining leaf color for the wild Kazakhstan apricot (Kovalchuk et al., 2017).

Conclusions

Unlike *Vitis vinifera* species, *V. labrusca* produces low-quality and low-quantity micro-shoots under *in vitro* conditions using a common medium composition for *Vitis* commercial cultivars, i.e., MS medium with cytokinin BAP. The composition of the culture medium, comprising macro- and micro-elements, vitamins, carbohydrates, and growth regulators, significantly influences the quality and quantity of *in vitro* produced shoots. The response surface method enables the simultaneous investigation of several factors. In this study, we aimed to investigate the interaction between NH_4NO_3, KNO_3, mesos, micros, iron, and BAP in order to optimize the *in vitro* proliferation of *Vitis labrusca* 'Concord' shoots. Nitrogen, either independently or in interaction with other components, demonstrated the most significant impact on all shoot production traits. The form of the nitrogen source also played a key role. KNO_3 was found to be effective for shoot number production, while NH_4NO_3 exhibited a driving effect on shoot length. The number and color of leaves were regulated by both nitrogen sources. The regulatory effect that nitrogen has on all these traits showed a negative relationship with salt (KNO_3, NH_4NO_3) concentration. Supplementation of N, P, and K minerals in *vitro* can have adverse effects on explant growth, if levels of these minerals are excessive and exceed the adequate range required for optimal plantlet development (Judd et al., 1996). In this experiment, the best shoot proliferation response (Desirability= 0.841) for *Vitis labrusca* 'Concord' was predicted by applying ×0.50 concentrations of the values in MS medium for factors NH_4NO_3 and KNO_3, and a ×2.00 and ×1.50 increase in the values of mesos and micro with the same concentration of iron with application of BAP at 1.587 mg L^{-1}.

Another influencing factor was BAP, which had a positive controlling effect on the number of produced shoot. BAP appears to interact significantly with various media minerals, including KNO_3 for proliferated shoot number, micros for leaf number, and both micros and iron for leaf color. These interactions suggest that the interplay between BAP and these factors could play a crucial role in determining the overall growth and development of shoots. Further investigation into these interactions may contribute to optimizing *in vitro* culture conditions for *Vitis labrusca* 'Concord'. In addition to factors related to the composition of the medium, it is recommended to investigate external factors such as the quantity and quality of light on the *in vitro* cultures and their interaction with the medium composition factors. Combining blue and red light-emitting diodes (LEDs) during *in vitro* cultures can significantly enhance growth and promote the development of functional stomata and

photosynthetic activity in explants, thus improving the overall effectiveness of the *in vitro* culture system (Saeedi et al., 2023; Saeedi et al., 2024).

Author Contributions
SK; directed the experiment, fulfilled the project, designed the experiment, analyzed the data, improved the manuscript, and drafted the manuscript.

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Conflict of Interest
The authors indicate no conflict of interest in this work.

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