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Response surface for quantifying the effects of NaCl and pH on lettuce

Abstract – The objective of this work was to evaluate the response surface methodology (RSM), with a large sample size, to investigate the effects of NaCl and pH on the root elongation of lettuce (*Lactuca sativa*) in vitro. For this, 20 lettuce seeds were placed in sterile Petri dishes with two filter papers at the bottom and 3.0 mL of the sample being tested. A rotatable 2x2 factorial arrangement was used, with two factors and a two-level matrix, incorporating two center points and ±1.41 α axial points. Each treatment group consisted of 40 replicates, resulting in 400 experimental observations. Increasing NaCl concentration from 0.0065 to 2,700 g L^{-1} caused a 19.3% decrease in lettuce root elongation. When pH decreased from 7.0 to 1.5, there was a 68.8% decrease in root elongation, but, when it increased from 7.0 to 12.5, there was a 44.9% decrease. The analysis of combined effects showed that the increase in NaCl concentration reduced the ability of the plant to resist changes in pH. The RSM was effective in evaluating the statistical significance of the NaCl and pH factors, including their second-order effects and interactions, as well as in quantifying their effects on the root elongation of lettuce under the experimental conditions. Therefore, the RSM is a valuable tool for interpreting the results and evaluating the sensitivity of multi-factor research, even when dealing with a large sample size.

Index terms: factorial arrangement, interaction evaluation, multi-factor, quantification of effects, rotatable design.

Superfície de resposta para quantificação dos efeitos de NaCl e pH em alface

Resumo – O objetivo deste trabalho foi avaliar a metodologia de superfície de resposta (MSR), com grande tamanho de amostra, para investigar os efeitos do NaCl e do pH no alongamento radicular de alface (*Lactuca sativa*) in vitro. Para tanto, 20 sementes de alface foram colocadas em placas de Petri estéreis, com dois papéis de filtro no fundo e 3,0 mL da amostra a ser testada. Utilizou-se arranjo fatorial rotacional 2x2, com dois fatores e matriz com dois níveis, tendo-se incorporado dois pontos centrais e pontos axiais α de ±1,41. Cada grupo de tratamento consistiu em 40 réplicas, o que resultou em 400 observações experimentais. O aumento da concentração de NaCl de 0,0065 para 2.700 g L^{-1} causou redução de 19,3% no alongamento radicular da alface. Quando o pH diminui de 7,0 para 1,5, houve redução de 68,8% no alongamento radicular, mas, quando aumentou de 7,0 para 12,5, houve redução de 44,9%. A análise dos efeitos combinados indicou que o aumento da concentração de NaCl reduziu a capacidade da planta de resistir às mudanças de pH. A MSR foi eficaz em avaliar a significância estatística dos fatores NaCl e pH, com seus efeitos de segunda ordem e interações, bem como em quantificar seus efeitos no alongamento radicular de alface sob as condições experimentais. Portanto, a MSR é ferramenta valiosa para interpretar os resultados e avaliar a sensibilidade de pesquisa multifatorial, mesmo com grande tamanho de amostra.

Termos para indexação: arranjo fatorial, avaliação de interações, multifatorial, quantificação de efeitos, design rotativo.

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Introduction

Lettuce (*Lactuca sativa* L.), a leafy plant belonging to the Asteraceae family, is the most consumed and cultivated vegetable worldwide, with a total production of approximately 27.3 million tons in 2018 (Medina-Lozano et al., 2021). Native to the Mediterranean region, the species is widely cultivated for its edible leaves, commonly consumed raw in salads and various culinary dishes (Negrão et al., 2021).

The proper cultivation of lettuce requires a consistent growth medium, which can be influenced by several factors (Hossain & Ryu, 2017). Among these, the presence of NaCl and pH level can significantly affect lettuce growth and development (Anderson et al., 2017; Breś et al., 2022). These factors may also exhibit a combined effect, where the level of one variable can influence the impact of the other, resulting in a joint effect that is different from the sum of the individual effects (Mainardi & Bidoia, 2022).

The analysis of the relationship between variables, such as NaCl concentration, pH, and lettuce growth, can demand significant resources due to research complexity and time requirements (Mason et al., 2003; Montgomery, 2017). Additionally, considering inherent biological variability, the use of a sufficiently large sample size is crucial to ensure precise and reliable conclusions (Lenth, 2001; Biau et al., 2008).

For that analysis, the conventional approach consists in conducting one-factor-at-a-time experiments, where a response variable is analyzed by varying each factor while keeping all others constant, which can be time consuming, labor intensive, and may not adequately quantify the interactions between factors and their combined effects (Mainardi & Bidoia, 2022). This method also requires additional testing to estimate the experimental error of the response variable across the experimental region (Weissman & Anderson, 2015; Díaz-Cruz et al., 2019).

To overcome these limitations, alternative experimental designs, such as the response surface methodology (RSM), have been recommended (Mainardi & Bidoia, 2022). These approaches, which generally employ factorial arrangements with all possible treatment combinations, enable the simultaneous consideration of multiple variables, providing a more comprehensive understanding of their interactions (Fisher, 1936). Specifically, the RSM methodology can be used to express the relationships between variables in polynomial equations, simplifying the interpretation of empirical results through response surface plots (Mainardi & Bidoia, 2022). According to Mason et al. (2003) and Montgomery (2017), the response surface can be used to evaluate important characteristics of the experiments, such as the sensitivity of factors, their interactions, and their combined effects. Biau et al. (2008) and Onyiah (2008) added that the RSM approach can also be effectively applied to a large sample size to improve precision and reduce the bias of random variation, leading to an increased statistical power and a better representation of the obtained results.

The objective of this work was to evaluate the RSM, with a large sample size, to investigate the effects of NaCl and pH on the root elongation of lettuce in vitro.

Materials and Methods

To quantify lettuce development, root elongation was measured during germination under controlled in vitro conditions. The lettuce seeds used in the experiment came from a commercial source free of agrochemicals, and the NaCl was obtained from Vetec Química Fina Ltda. (Duque de Caxias, RJ, Brazil). The growth medium consisted of 25 mL deionized water, which was added to giant test tubes with varying NaCl concentrations and pH values. The pH values of the growth medium were determined using the DMPH-2 calibrated pH meter (Digimed, São Paulo, SP, Brazil), with buffer solutions of pH 4.0 and 7.0.

The experiment was conducted following the method described by Araújo & Monteiro (2005). For this, 20 lettuce seeds were placed in sterile Petri dishes with two filter papers at the bottom and 3.0 mL of the sample being tested. After incubation, at 21°C, for 120 hours in complete darkness, the root elongation of each plant was measured using a ruler. In the analysis, non-germinated seeds were considered to have zero elongation. In addition, two control treatments were performed: a positive control, with a zinc sulfate solution of 0.05 mol L^{-1}; and a negative control, with sterile deionized water. The experiment was duplicated.

The central composite rotatable design was used for the RSM, consisting in a rotatable 2x2 factorial arrangement, with two factors and a two-level factorial matrix with two center points and ±1.41 α axial points (Box & Hunter, 1957). The pH values of the treatment points in the matrix were adjusted using 1.0 mol L^{-1} hydrochloric acid and sodium hydroxide solutions. Each treatment group consisted of 40 replicates, resulting in 400 experimental observations. Other details of the adopted design, including the coded and real values of the treatments, are shown in Table 1. The obtained responses refer to the root elongation of lettuce and were fit to the following quadratic equation, which describes the relationship between NaCl concentration, pH factors, and the root elongation of the plant:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2,$$

where y represents the dependent variable; x_1 and x_1 are the predictor variables; β_0 is the model intercept; and β_1, β_2, β_{12}, β_{11}, and β_{22} are the coefficients of the regression equation. The outliers were identified as standardized residuals greater than ±2 (Montgomery, 2017) and were removed before the statistical analysis was conducted.

The regression model obtained from the RSM was used to quantitatively estimate the contribution of the independent variables in the prediction of the dependent ones (Montgomery, 2017), through the following equation:

$$RE = -0.44 - 1.08x + 0.40x^2 + 1.74y - 0.11y^2 - 0.06xy,$$

where RE is the percentage of the root elongation of lettuce, x is the concentration of NaCl (g L^{-1}), and y is pH value.

The RSM generated the following polynomial equation:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2,$$

which was used to create two-dimensional line graphs illustrating the individual effects of the NaCl and pH variables, including their curvature (Figure 1 A), as well as to draw a three-dimensional response surface plot that depicted the combined effect of the studied factors (Figure 1 B).

A Pareto diagram (Figure 2 C) was developed to simplify the identification of the statistically significant factors in the experimental design (Mainardi & Bidoia, 2022).

Nonsignificant variables with a p-value>0.01 were excluded from the estimation of factor effects, equations, and plots. The statistical analysis was performed using the 14.0.1 trial version of the Statistica software (TIBCO Software Inc., Palo Alto, CA, USA).

Table 1. Treatment runs of the response surface methodology matrix, coded and real values of the studied factors, mean values, standard deviations (SD), and ±95% confidence limits (CL) for the experimental results obtained for the root elongation (RE) of lettuce (*Lactuca sativa*)[(1)].

Treatment	NaCl (g L^{-1})	pH	RE (cm)	RE SD (cm)	n	-95% CL (cm)	+95% CL (cm)
1	(-1.00); 0.4000	(-1.00); 3.00	4.03	0.76	33	3.76	4.30
2	(-1.00); 0.4000	(1.00); 11.00	4.49	0.56	40	4.31	4.66
3	(1.00); 2.3000	(-1.00); 3.00	3.94	0.41	39	3.80	4.07
4	(1.00); 2.3000	(1.00); 11.00	3.97	0.33	25	3.83	4.11
5	(-1.41); 0.0065	(0.00); 7.00	5.70	0.70	36	5.46	5.94
6	(1.41); 2.6935	(0.00); 7.00	4.02	0.35	40	3.91	4.13
7	(0.00); 1.3500	(-1.41); 1.34	0.01	0.03	40	0.00	0.02
8	(0.00); 1.3500	(1.41); 12.66	0.97	0.16	40	0.92	1.02
9 (C)	(0.00); 1.3500	(0.00); 7.00	4.81	0.50	74	4.69	4.92
Mean	-	-	3.55	0.42	367	3.41	3.69

[(1)]Values between parentheses indicate the coded values of the matrix.

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Results and Discussion

The RSM, based on a two-factor two-level central composite rotational design matrix, was successfully used to quantify the effects of NaCl concentration and pH on the root elongation of lettuce under the experimental conditions. The statistical design, with a large sample size of 400, allowed of using a trimming method to remove standardized residuals greater than ±2 (Christensen, 1996; Montgomery, 2017), which were identified as outliers resulting from natural biological variability. The remaining 367 data points, accounting for 91.75% of the total measurements, provided a

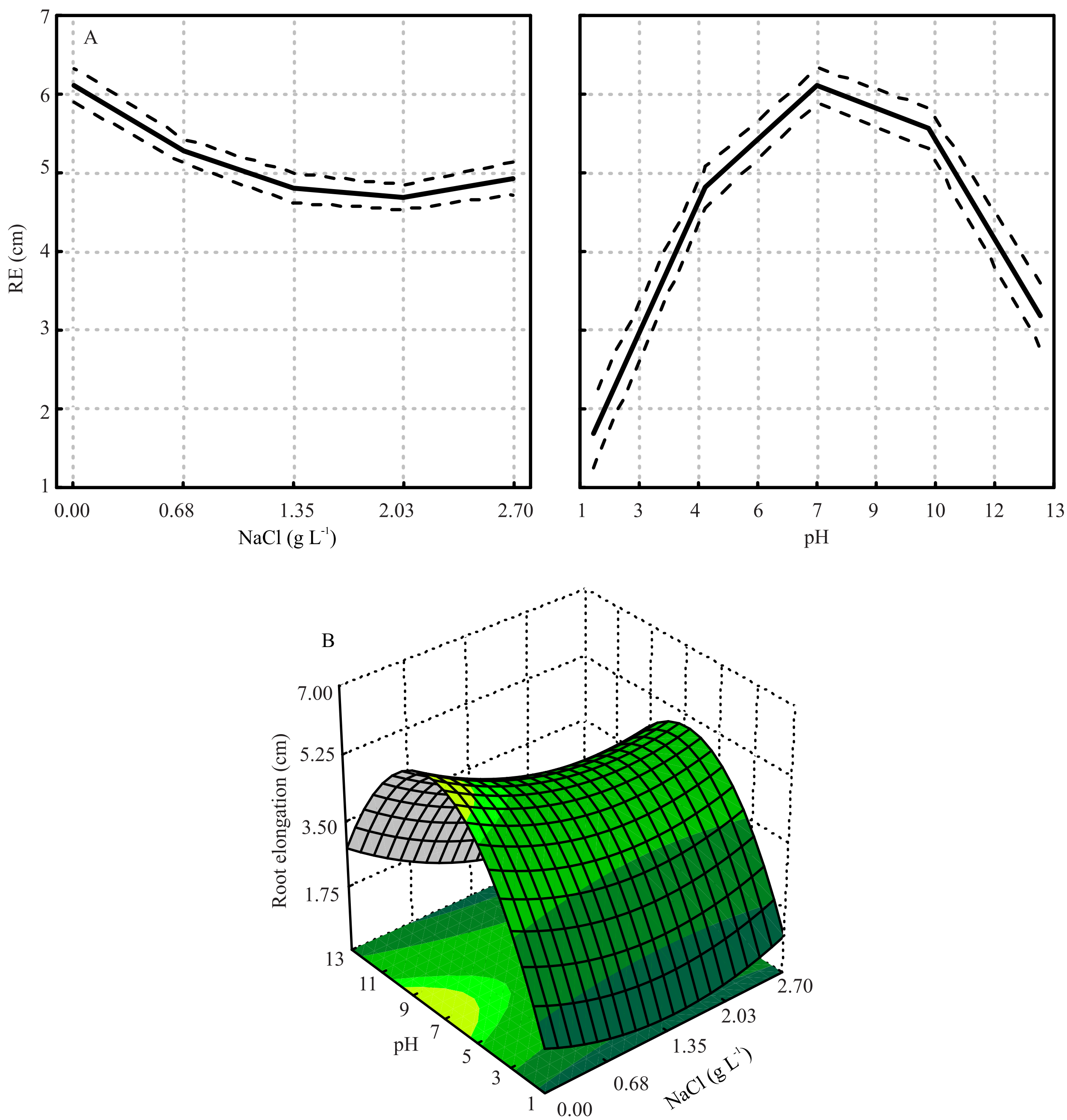

Figure 1. Line graph illustrating the effects of NaCl and pH on lettuce (*Lactuca sativa*) root elongation, in which bands represent a 95% confidence interval (A), as well as the response surface plot showing the relationship between the studied factors and the root elongation of the plant (B).

reliable estimation of tendencies for all factorial treatment runs. The overall mean of root elongation obtained for the 367 runs was 3.55 cm, with a standard deviation of 0.42 cm (Table 1). The eliminated data points are shown in the typical residual plots in Figure 2 A and B.

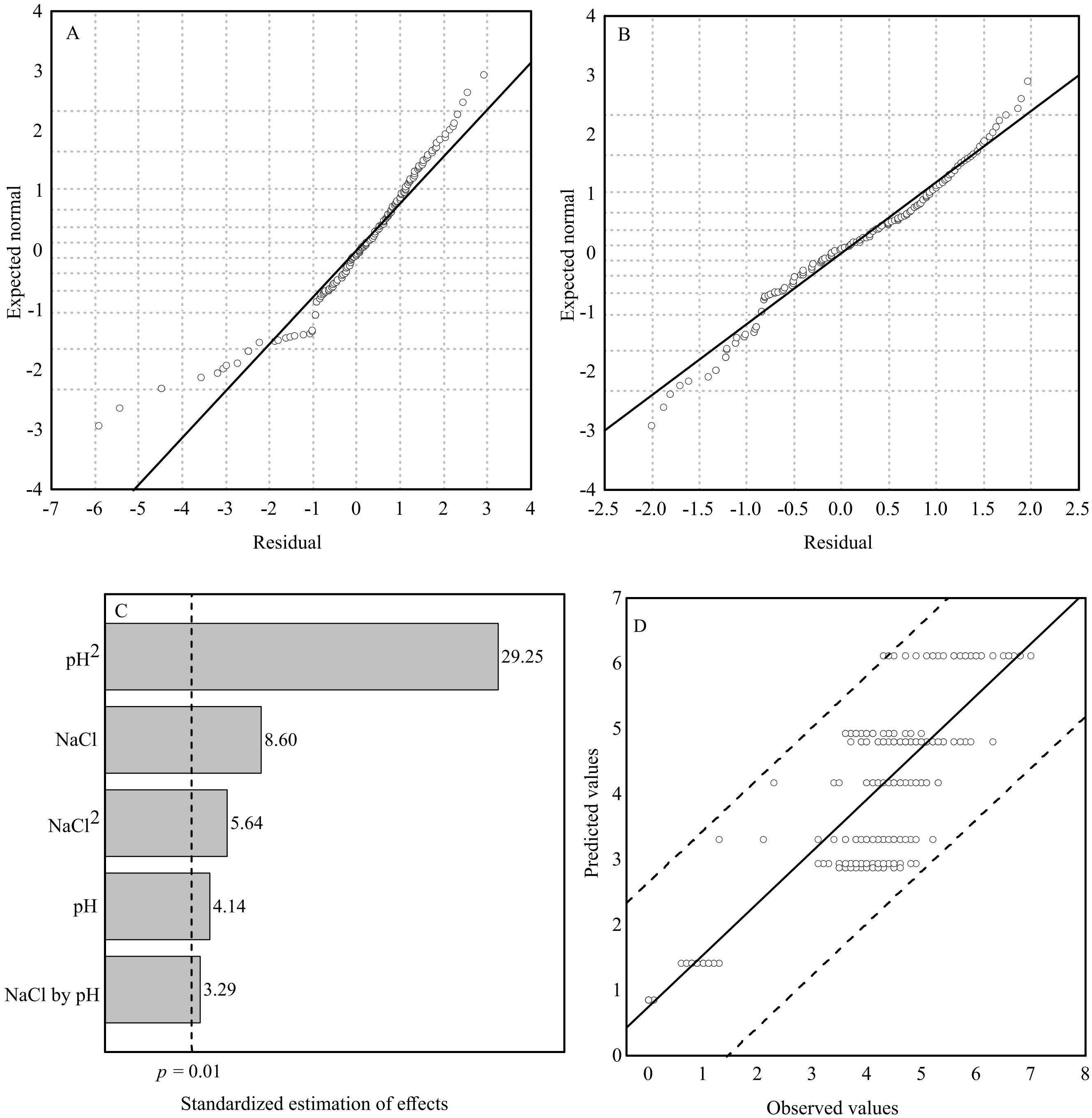

Figure 2. Normal residual plot before (A) and after (B) the trimming procedure to remove data values with standardized residuals greater than ±2.0 (outliers), as well as the generated Pareto chart showing the effects (C) and predicted vs. observed plots of the statistical response surface methodology (D). pH^2, second-order effect of pH; NaCl, effect of NaCl concentration on the response variable; NaCl^2, second-order effect of NaCl concentration; pH, effect of pH level on the response variable; and NaCl by pH, interaction effect between NaCl concentration and pH.

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The analysis of variance used in the RSM allowed of evaluating the statistical significance of the NaCl and pH factors, including their second-order effects and interactions (Table 2). According to the analysis, both NaCl and pH showed significant effects on root elongation under the experimental conditions, as evidenced by their respective F-calc and p-values (Mainardi & Bidoia, 2022). NaCl had an F-calc of 74.0252, with a p-value less than 0.01, indicating a highly significant effect on root elongation. Furthermore, pH also showed a significant effect, with an F-calc of 17.1153 and a p-value less than 0.01.

The second-order terms, represented as $NaCl^2$ and pH^2, also caused significant effects on root elongation. The $NaCl^2$ and pH^2 variables showed F-calc values of 31.7753 and 855.3600 respectively, with p-values less than 0.01. This suggests that the relationship of NaCl concentration and pH with root elongation was not purely linear but followed a quadratic trend (Montgomery, 2017).

The interaction between NaCl and pH was also significant, with an F-calc of 10.8434 and a p-value less than 0.01. This result is an indicative that the combined effect of NaCl and pH on root elongation was not simply additive, but involved interaction effects that contributed to the overall variability in root elongation (Mason et al., 2003). According to the carried-out analysis, the sum of squares error was significantly smaller than the total sum of squares, which suggests that the model with the NaCl and pH factors explained a substantial portion of the variability in root elongation (Montgomery, 2017).

The equation of the used regression model, according to the coefficient of determination and the adjusted coefficient of determination, exhibited a degree of fit of approximately 80%, indicating the adequacy between the observed and predicted values (Mainardi & Bidoia, 2022). The plot of the predicted versus observed values of the statistical model is shown in Figure 2 D.

According to the same equation, the maximum root elongation index of lettuce was 6.11 cm and occurred at a NaCl concentration of 0.0065 g L^{-1} and a neutral pH of 7.0. However, decreases of 19.3, 68.8, and 44% were observed in the root elongation of the plant with an increase in NaCl concentration from 0.0065 to 2,700 g L^{-1}, pH values from 7.0 to 1.50, and pH values from 7.0 to 12.50, respectively (Table 3).

Both plots drawn by the polynomial equation generated by the RSM proved to be useful tools for

Table 2. Results of the analysis of variance for the response surface methodology[(1)].

Source of variation	DF	Sum of squares	Mean square	F-calc	p-value
NaCl	1	50.368	50.368	74.025	0.000000*
$NaCl^2$	1	21.621	21.620	31.775	0.000000*
pH	1	11.646	11.646	17.115	0.000044*
pH^2	1	582.003	582.003	855.360	0.000000*
NaCl by pH	1	7.378	7.378	10.843	0.001089*
Residual error	361	245.631	0.680		
Total	366	1,191.402			

[(1)]DF, degrees of freedom; F-calc, F-value in the test statistics; NaCl, effect of NaCl concentration on the response variable; pH, effect of pH level on the response variable; $NaCl^2$, second-order effect of NaCl concentration; pH^2, second-order effect of pH; and NaCl by pH, interaction effect between NaCl concentration and pH.

Table 3. Estimates of the effect of the NaCl and pH factors on lettuce (*Lactuca sativa*) root elongation (RE).

NaCl concentration (g L^{-1})	RE (cm)	Reduction in RE (%)[(1)]	pH value	RE (cm)	Reduction in RE (%)[(2)]
0.0065	6.11	0.0	1.50	1.91	68.8
0.338	5.66	7.4	2.88	3.61	40.9
0.675	5.28	13.6	4.25	4.88	20.2
1.013	5.00	18.2	5.63	5.71	6.5
1.350	4.81	21.4	7.00	6.11	0.0
1.688	4.70	23.1	8.38	6.08	0.6
2.025	4.69	23.3	9.75	5.61	8.3
2.363	4.76	22.1	11.13	4.70	23.0
2.700	4.93	19.3	12.50	3.37	44.9

[(1)]Reduction in RE from the highest to the lowest NaCl concentration. [(2)]Reduction in RE from the highest to the lowest pH level.

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interpreting the obtained results and visually evaluating the sensitivity of the observed response, in alignment with Rodrigues & Iemma (2014). Furthermore, the obtained plots can be used to identify regions where the response was maximized or minimized, aiding in the determination of optimal conditions.

Conclusions

1. The response surface methodology (RSM) enables a comprehensive analysis of the relationships between NaCl concentration, pH, and lettuce (*Lactuca sativa*) root elongation, effectively capturing nonlinear relationships and interactions between NaCl concentration and pH.
2. The RSM accurately evaluates the significance of factors, including their second-order effects and interactions, within determined confidence intervals, providing a reliable data interpretation.
3. The RSM expresses variable relationships through polynomial equations, facilitating their visualization in 2D and 3D response surface plots, which allows determining optimal conditions.
4. The RSM provides a comprehensive matrix for analysis of large sample sizes, which makes it suitable for large-scale research.

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