



Synergistic and Antagonistic Effects of pH Interactions with Sodium Chloride on The In Vitro Growth of (*Medicago Sativa* L.)

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ABSTRACT

Salinity and medium pH are critical abiotic factors influencing plant growth, yet their interactive effects remain poorly understood. This study aimed to evaluate the primary and interacting effects of medium pH and sodium chloride (NaCl) on the in vitro development of *Medicago sativa* L. (cv. CUF 101). A 3 × 4 factorial design was employed to investigate pH levels of 4.5, 6.5, and 8.5 in conjunction with NaCl concentrations of 0, 60, 120, and 180 mM in a completely randomized design, with growth traits (root and shoot length, biomass, and leaf number) measured after 21 days of germination. This approach allowed us to capture responses across varying gradients of saline-pH stress. The effects of pH, salinity, and their interaction were all found to be highly significant ($p < 0.01$). Near-neutral pH (6.5) consistently maximized biomass and exhibited antagonistic effects by alleviating the growth inhibition caused by NaCl. Conversely, acidic conditions (pH 4.5) combined with NaCl produced synergistic effects, resulting in shoot elongation uncoupled from biomass gain. Under alkaline conditions (pH 8.5) with NaCl, the interaction also acted synergistically, severely inhibiting root elongation and indicating an early failure of root establishment. Salinity imposed a range of penalties on all traits, exhibiting significant reductions beyond approximately 120 mM and approaching a critical collapse at 180 mM. The correlations among traits indicated a trade-off in stress allocation: root length was positively correlated with total biomass and negatively correlated with shoot elongation and leaf number. These findings demonstrate that precise pH management is essential for optimizing salt tolerance in alfalfa with implications for cultivar screening under saline-alkaline stress.

Keywords: *Medicago sativa*; salinity (NaCl); pH (acidic-alkaline); in vitro culture; salinity-pH interaction.

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INTRODUCTION

Medicago sativa L. (alfalfa) is a valuable perennial forage legume highly valued worldwide for its high crude protein content, high digestibility, and ability to improve soil fertility by fixing nitrogen in a symbiotic manner [1]. [2] assert that resilient feed crops are essential for enhancing soil health and establishing sustainable livestock systems. Global salinization is highlighting the importance of these goods, as many farming areas are already harmed and could worsen [3].

Osmotic difficulties that limit water absorption and lead to ionic toxicity are the primary consequences of Na⁺ and Cl⁻ ion-induced salinity stress [4]. This damages membranes, alters nutritional balance, and lowers photosynthetic efficiency [5]. Recent physiological and transcriptomic investigations on *M. sativa* discovered that salt stress lowers biomass, changes membrane integrity, and inhibits photosynthesis [6-7-8]. Alfalfa can cause adaptive responses such as antioxidant enzyme production and osmolyte buildup. However, these reactions do not always stop the progression of salt damage [9, 10].

Plant stress responses are highly controlled by pH and salinity in the medium or soil [11]. Alkaline pH levels in *M. sativa* exacerbate NaCl-induced stress by lowering nutrient solubility, impeding root absorption, and impairing photochemistry of photosystem II (PSII) and photosystem I (PSI) [12,13]. Factorial research revealed that elevated alkalinity (pH 9.0) combined with high NaCl concentrations (200 mM) resulted in considerably higher reductions in root activity, nutritional absorption, and photosystem performance than neutral pH circumstances. [14]. Ion

homeostasis is a dynamic process that is essential for all living organisms, including plants. Physiological stability may be affected by fluctuations in the medium's pH [15]. [16] proposed that physiological stability can be influenced by changes in ion distribution patterns, despite the relative constancy of Na⁺ uptake, as a result of variations in medium pH [14, 17].

The target of this study is to assess the primary and interactive effects of culture-medium pH (from acidic to alkaline) and NaCl salinity on the growth performance of *M. sativa* under controlled *in vitro* conditions. This study targets a critical gap in understanding how pH regulates plant responses to salinity across realistic selection and agronomic management in salt – affected culture medium. In other words, it seeks to understand how pH variations influence salt stress across realistic NaCl gradients, providing mechanistic insights to guide cultivar selection and agronomic strategies for salt-affected environments

2. Materials And Methods

2.1. Plant Material and Preparation

Certified seeds of *Medicago sativa* L. (alfalfa), cultivar [CUF 101], which is a non-dormant / low dormancy alfalfa cultivar, were released in 1976 by UC / USDA in California. The seed was obtained from Phoenix Desert Seeds (Seed & Agricultural Supply Store) Phoenix, Arizona, USA. Seeds were first cleaned of dust. They were then subjected to surface-sterilization by immersion in 70% (v/v) ethanol for 1 minute. This was followed by disinfection in a 1% (w/v) sodium hypochlorite solution containing two drops of Tween-20 / 100 mL for ten minutes, with gentle agitation to ensure uniform contact. Sterilized seeds were rinsed thoroughly three times with sterile distilled water to remove all traces of disinfectant. Germination was carried out on hormone-free Murashige and Skoog (MS) basal medium under aseptic conditions. Once the seedlings reached a uniform size, hypocotyl and shoot segments of consistent length were excised and immediately transferred to treatment media.

2.2. Preparation of MS Medium

The basal medium was prepared following the formulation of Murashige and Skoog [18] and supplemented with 3% (w/v) sucrose. Agar was added at a concentration of 0.7% (w/v) as a gelling agent. Medium pH was adjusted to 4.5, 6.5 (considered near-optimal for *M. sativa* growth *in vitro*), or 8.5 using either 1 N KOH or 1 N HCl before autoclaving at 121 °C for 20 minutes. After sterilization, the medium pH was rechecked and adjusted to the desired level for the study. When the medium cooled to approximately 40 °C, it was poured into sterile 10 cm disposable Petri plates, with 30 mL of sterilized medium in each plate [19].

The pH readjusting step after MS medium autoclaving is essential in physiological studies especially that investigate the influence of pH on plant growth and development, because the autoclaving process alters medium pH through chemical reactions such as sucrose hydrolysis and phosphate - ammonium interactions. These reactions can shift the pH by ±0.3 - 0.7 and up to 1 unit sometimes, leading to inaccurate experimental pH treatments. Maintaining the needed pH (4.5, 6.5 and 8.5) after sterilization ensures that plants are exposed to the true intended hydrogen-ion [19, 20].

2.3. Experimental Treatments: Combined pH and NaCl

A 3 × 4 factorial experimental design was employed to evaluate the interaction between pH and salinity stress. The factors were three pH levels (4.5, 6.5, 8.5) and four NaCl concentrations: 0.0 mM (Control), 60 mM, 120 mM, and 180 mM. Sodium chloride was incorporated into the medium prior to autoclaving at the target concentrations. Each pH level was tested in combination with each NaCl level, resulting in a total of 12 treatment combinations. For each treatment, ten replicates (one explant per replicate) were prepared. The entire experiment was conducted in three independent runs to account for variability across culture batches [20].

2.4. Growth Conditions and Monitoring

Cultures were incubated in a controlled-environment growth chamber set at 24 ± 2 °C, with a 16-hour light/8-hour dark photoperiod. Illumination was provided by cool-white fluorescent tubes delivering 80–100 μmol m⁻² s⁻¹ at the culture surface. Relative humidity was maintained at 60–70%. Cultures were inspected weekly for signs of microbial contamination, and any contaminated samples were removed and recorded.

2.5. Data Collection After 21 days of culture, morphological and physiological measurements were taken, including fresh weight, dry weight, shoot length, root length, and leaf number. The plant samples fresh weight was directly measured after seedlings harvest in lab. Then the plants harvested samples were dry for 24 h. at 60°C. after drying the samples, they were moved to a desiccation jar and the weighing process immediately done inside the hood at a Lab temperature of 25 ± 2.

2.6. Statistical Analysis

Data were analyzed using SPSS version 26 (IBM Corp., Armonk, NY, USA). The Shapiro–Wilk test was used to assess the normality of the distribution, while Levene's test was employed to evaluate the homogeneity of variances. A factorial design facilitated the use of two-way ANOVA to examine the main effects of pH, NaCl concentration, and their interaction. When significant differences were identified ($p \leq 0.05$), means were compared through Duncan's multiple range test [21]. Additionally, Pearson correlation coefficients were calculated to investigate the relationships among the measured traits.

3. Results And Discussion

Two-way ANOVA (Table 1) revealed that pH had a significant effect on all traits ($p < 0.01$). Across pH levels, 6.5 consistently delivered the highest root length (4.82 cm), shoot weight (8.16 g), and plant weight (2.55 g), as in Table 1, whereas 4.5 depressed biomass but produced the longest shoots (0.488 cm) and the most significant leaf number among the tested pH values. Root growth was absent or undetectable at pH 8.5 in the compiled summary, indicating potent inhibition of primary root elongation under alkaline conditions. Together, these patterns point to a growth optimum near neutrality, with acidity favoring axial elongation at the expense of mass, and alkalinity suppressing root establishment [22, 23]. Mechanistically, neutrality likely optimizes ion activity and nutrient availability (especially P, Fe, and micronutrients) and stabilizes membrane transport [24], whereas alkalinity reduces solubility of Fe and P and may introduce bicarbonate-induced dysfunction that constrains root metabolism [25, 26]. Shoot elongation enhanced by acid is linked to the concept of acid growth, where auxin prompts the activation of the plasma membrane H^+ -ATPase. This activation results in the loosening of the cell wall, thereby decoupling the process of extension growth from the accumulation of biomass [27 - 28]. As is shown in Table 1, salinity imposed a strong, graded penalty on every endpoint ($p < 0.01$). Root length decreased by approximately (24% at 60 mM), (31% at 120 mM), and (76% at 180 mM NaCl), compared to the control group. Over the same steps, plant weight decreased by approximately (24%, 40%, and 86%), whereas shoot weight decreased by approximately (4%, 24%, and 89%). Shoot length and leaf number similarly reduced, while shoot length showed minor non-monotonicity at (180 mM), indicating stress-induced elongation that does not result in mass gain. These values imply that 120-180 mM exceeds the physiological buffering capability of the examined substance. The trajectory is similar to canonical salinity syndromes, with an early osmotic component (reduced water potential, curtailed cell expansion) followed by ionic toxicity (Na^+/Cl^- accumulation, K^+ displacement, ROS burst), which depresses photosynthesis and growth [5, 29]. Recent alfalfa work confirms that PSII/PSI photochemistry and nutrient acquisition deteriorate with rising salt, even when gross growth appears partly maintained at moderate salinity (≈ 100 mM) [30] and is further evidenced by proline-/antioxidant-mediated buffering and multi-omics signatures under (saline-alkali) stress [31].

Table 1. Effect of pH values and salt concentrations on plant characteristics

	Overall mean				
	Root length (cm)	Plant weight (g)	Shoot weight (g)	Shoot length (cm)	Leaf number / explant
Mean	4.081	1.198	4.752	.227	1.045
$\pm$ SE	0.104	0.052	0.16	0.007	0.019
	Effect of pH				
	Root length (cm)	Plant weight (g)	Shoot weight (g)	Shoot length (cm)	Leaf number / explant
pH					
4.5	3.346 b	.041c	.025 c	.488 a	2.791 a
6.5	4.816 a	2.545 a	8.160 a	.074 b	.042 b
8.5	-	.942 b	6.510 b`	.086 b	.057 b
$\pm$ MSE	0.15	0.094	0.288	0.013	0.034
p value	0.001 **	0.001 **	0.001 **	0.001 **	0.001 **
	Effect of Concentration				
	Root length (cm)	Plant weight (g)	Shoot weight (g)	Shoot length (cm)	Leaf number / explant
Concentration					
0.0 mM NaCl control	6.06 a	1.791 a	6.238 a	.266 a	1.168 a
60 mM NaCl	4.610 b	1.366 b	6.015 ab	.221 bc	1.043 a
120 mM NaCl	4.183 b	1.072 b	4.731 b	.197 c	.976 b

180 mM NaCl	1.462 c	.244 c	.658 c	.226 bc	.969 b
± MSE	0.203	0.09	0.29	0.221	0.98
<i>p</i> value	0.001 **	0.001 **	0.001 **	0.001 **	0.001 **

All traits exhibited significant pH × NaCl interactions ($p < 0.01$), indicating that medium pH influences the actual impact of NaCl. The best-performing cell was the combination pH 6.5 × 0 mM, whereas the worst was pH 4.5 × 180 mM for root length (near-abolition of elongation). Importantly, even at moderate salinity levels (60–120 mM), neutral pH buffered losses relative to both acidity and alkalinity, supporting the view that appropriate pH management can partially offset salt stress *in vitro*. The reported absence of root length data at pH 8.5 underscores the sensitivity of root growth to alkaline settings across the salinity gradient.

These interactions align with factorial studies in alfalfa, where a higher pH (≥ 9) exacerbated the depressive effects of salt on root activity, NPK uptake, and PSI/PSII function [32]. At low to moderate salt concentrations (100 mM), alfalfa can maintain photochemistry at near control levels; however, raising the pH shifts the balance toward photoinhibition and nutrient limitation [12]. The physiology is intuitive: alkalinity reduces Fe and P availability and impairs apoplastic pH control; together with Na⁺ load, this compromises H⁺-coupled transport, K⁺/Na⁺ selectivity, and antioxidant homeostasis [33]. Across pooled treatments, root length correlated positively with plant weight (0.511) and shoot weight (0.405), consistent with the notion that root system development underpins carbon gain. In contrast, root length correlated negatively with shoot length (0.161) and leaf number (0.206), while shoot length and leaf number were strongly positive (0.820). This pattern indicates a plastic allocation switch: when faced with suboptimal pH and/or salinity levels, plants prioritize resources for axial extension and leaf initiation (morphological persistence), even as biomass accumulation stagnates. This interpretation aligns with established principles of stress ecophysiology, where extension growth can become decoupled from overall mass [34]. It is supported by recent multi-omics studies examining responses to saline-alkali stress [35].

Table 2. Correlation coefficients between the studied characters

	Root length (cm)	Plant weight (g)	Shoot weight (g)	Shoot length (cm)	Leaf number / explant
Root length	1	.511**	.405**	-.161**	-.206**
Plant weight	.511**	1	.689**	-.475**	-.568**
Shoot weight	.405**	.689**	1	-.609**	-.720**
Shoot length	-.161**	-.475**	-.609**	1	.820**
Leaf number	-.206**	-.568**	-.720**	.820**	1

**, Correlation is significant at the 0.01 level (2-tailed).

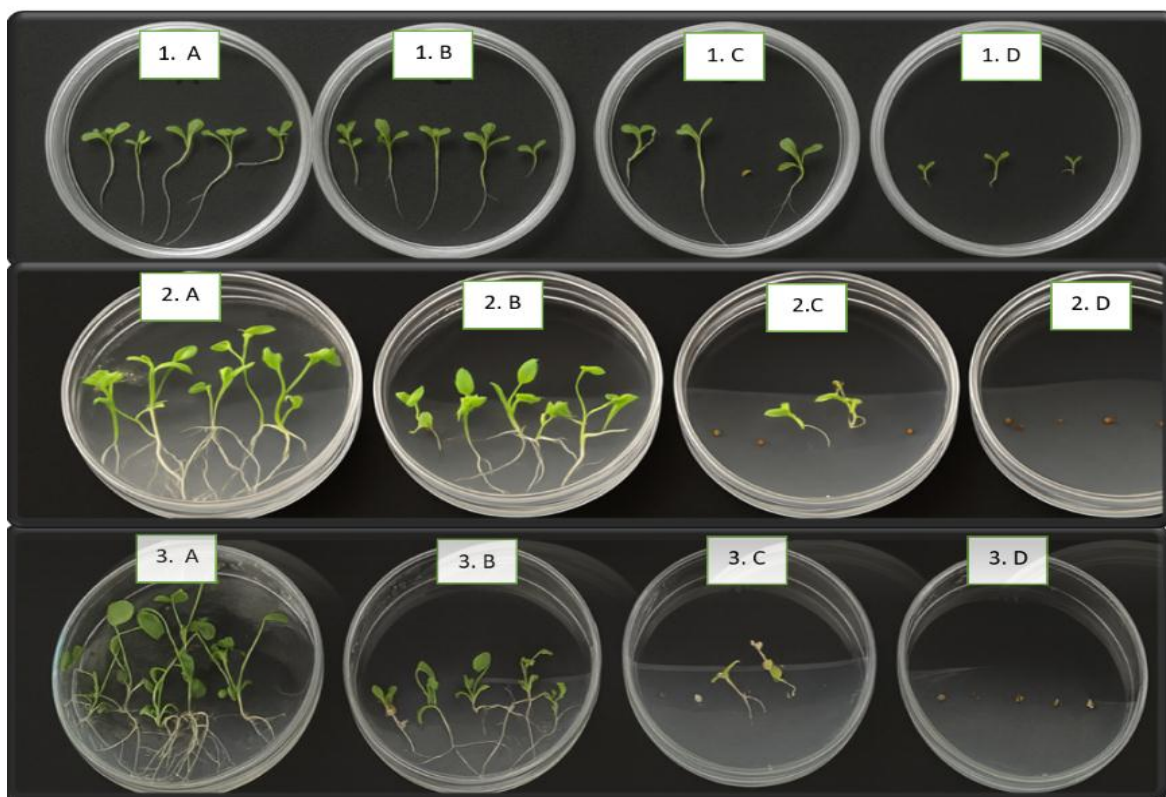


Figure 1: The interplay impact of pH and NaCl on the growth parameters of alfalfa after 21 days of germination in MS medium. The numbers 1, 2 and 3 are referred respectively to the pH treatment of 4.5, 6.5 and 8.5. The letters A, B, C and D are referred respectively to the Salt concentration of 0 mM control, 60mM, 120mM and 180mM.

The factorial results collectively indicate that pH control is not simply a background condition; rather, it serves as a crucial factor influencing salinity outcomes in *M. sativa*. Practically speaking, maintaining the culture media at a near-neutral pH (~6.5) optimizes growth and mitigates the effects of salinity, even at moderate NaCl levels. From a biophysical perspective, a neutral pH supports H^+ -ATPase-driven transport, preserves the balance of K^+/Na^+ , and keeps the availability of Fe/P within an optimal range. Conversely, acidic conditions can exacerbate cell-wall loosening, while reducing biomass production; alkaline circumstances cause bicarbonate/Fe difficulties that can initially impede root growth. These findings provide support for integrated salinity-pH management Approaches in field and hydroponic applications, including buffering, root-zone acidification, and Fe chelation. Furthermore, breeding efforts should cover both alkaline and salt conditions, not only salinity. [3, 14]. This can be further improved by combining AMF/bio-stimulants with targeted gene modules. [36, 37]. Alkaline soil pH commonly co-occurs with salinity and intensifies salt stress by restricting micronutrient availability, disrupting Na^+/K^+ selectivity, and aggravating ionic toxicity, thereby imposing constraints beyond those of salinity alone. Accordingly, effective breeding must target combined alkaline - saline tolerance via coordinated modulation of gene networks governing ion transport, cellular pH homeostasis, and stress signaling (e.g., SOS and HKT pathways). The integration of these genetic traits with arbuscular mycorrhizal fungi (AMF) and bio-stimulants can further enhance stress resilience by promoting rhizosphere acidification, nutrient uptake, and stress-responsive gene expression, improving plant performance under complex alkaline-saline soil conditions.

Conclusion

Across all pH levels studied, the near-neutral pH (6.5) consistently produced the most biomass, while an acidic pH of 4.5 increased shoot and root length while decreasing dry weight. Meanwhile, an alkaline pH of 8.5 decreased the main root's growth rate. Plant development slowed as salinity increased, particularly above 120 mM NaCl, with growth practically halting at 180 mM. These findings indicate that pH is an important factor in defining the conditions under which plants may efficiently manage salt-related stress. Maintaining a near-neutral pH ensures that nutrients are available and absorbable by the plant. In contrast, more acidic or alkaline environments require the plant to expend more energy simply to exist. Interestingly, several features tended to alter in tandem, for example, root growth

frequently mirrored total biomass, and longer shoots were associated with an increase in leaf number, even when stressed. This shows that the plants were changing shape rather than growing larger. This indicates that the plants primarily underwent morphological adjustment rather than an increase in overall biomass accumulation. From a practical standpoint, successful farming of alfalfa under salty conditions requires managing both pH and salinity carefully. It's also important to test different cultivars under combined saline and alkaline conditions. Traits like root length and total plant mass offer reliable ways to assess performance. Lastly, 120 mM of NaCl appears to be a useful cutoff point when comparing varieties or applying research findings to real-world farming.

Recommendation

Overall, these findings highlight that medium pH serves as a crucial factor influencing the degree of salt tolerance in alfalfa, shaping both synergistic and antagonistic interactions with salinity. Integrating pH control with salinity management and screening genotypes under combined saline–alkaline regimes must be studied to refine cultivar selection better and inform soilless and field practices.

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التأثيرات التآزرية والتضادية لتداخلات الأس الهيدروجيني (pH) مع كلوريد الصوديوم على النمو في الزراعة النسيجية لنبات البرسيم الحجازي (*Medicago sativa* L.)

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الخلاصة

تُعدّ الملوحة (*Salinity*) ودرجة حموضة الوسط (*pH*) من العوامل اللاحيوية الرئيسة التي تتحكم في نمو النبات وتطوره، إلا أن التداخلات التفاعلية بينهما لا تزال غير موصوفة بشكل كافٍ. هدفت هذه الدراسة إلى تحليل التأثيرات الفردية والتفاعلية لكل من *pH* الوسط الغذائي وتركيز كلوريد الصوديوم (*NaCl*) في نمو وتطور نبات *Medicago sativa* L. الصنف (*CUF 101*) تحت ظروف الزراعة النسيجية. استُخدم تصميم عاملي (3×4) لدراسة ثلاث مستويات من *pH* (4.5، 6.5، و8.5) بالتزامن مع أربعة تراكيز من *NaCl* (0، 60، 120، و180 ملي مول)، ضمن تصميم عشوائي كامل، حيث تم تقييم صفات النمو المورفولوجية (طول الجذر وطول المجموع الخضري، الكتلة الحيوية، وعدد الأوراق) بعد 21 يوماً من الإنبات. أتاح هذا النهج رصد استجابات النبات عبر تدرجات متباعدة من إجهاد الملوحة-الأس الهيدروجيني.

أظهرت النتائج أن التأثيرات الرئيسة لكل من الحموضة والملوحة، إضافة إلى تفاعلها الداخلي، كانت عالية المعنوية إحصائياً ($p < 0.01$) فقد دعم الوسط ذو الحموضة شبه المتعادل (6.5) أعلى تراكم للكتلة الحيوية، وأظهر تأثيراً تضادياً تمثل في التخفيف من حدة التثبيط النمائي الناتج عن الملوحة. في المقابل، أدت الظروف الحامضية (4.5) عند اقترانها بالملوحة إلى تأثيرات تآزرية تجلت في زيادة استطالة المجموع الخضري دون زيادة متناسبة في الكتلة الحيوية. أما تحت الظروف القلوية (8.5) مع الملوحة، فقد كان التفاعل أيضاً تآزريراً لكنه أسفر عن تثبيط حاد في استطالة الجذور، مما يشير إلى فشل مبكر في تأسيس النظام الجذري.

فرضت الملوحة انخفاضات تدريجية على جميع صفات النمو المدروسة، مع تراجع معنوي واضح بعد تركيز يقارب 120 ملي مول، واقترب من حد الانهيار الفسيولوجي عند 180 ملي مول. كما كشفت معاملات الارتباط بين الصفات عن وجود مفاضلة في تخصيص الاستجابة للإجهاد؛ إذ ارتبط طول الجذر ارتباطاً موجباً مع الكتلة الحيوية الكلية، وارتباطاً سالباً مع كل من استطالة المجموع الخضري وعدد الأوراق. تؤكد هذه النتائج أن الضبط الدقيق لدرجة حموضة الوسط يُعدّ عاملاً حاسماً في تحسين تحمل الملوحة في البرسيم الحجازي، مع دلالات تطبيقية مهمة في برامج غربلة الأصناف تحت ظروف الإجهاد الملحي - القلوي.

الكلمات المفتاحية: *Medicago sativa*، الملوحة، درجة الحموضة، زراعة خارج الجسم الحي.