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**Research Article**

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# Optimizing a Hybrid Biofertilizer-Foliar Nutrition Strategy for Mexican Lime under Water Deficit: Insights into Water Status, Osmolytes, and Membrane Stability

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**Abstract**

Drought stress markedly limits citrus growth, highlighting the need for practical strategies to strengthen seedling resilience. Here, we assessed whether root inoculation with the plant growth-promoting bacterium Bacillus subtilis CS1, alone or combined with foliar potassium nitrate (0 and 2%), can mitigate water-deficit impacts in Mexican lime (*Citrus aurantiifolia*). Seedlings were exposed to three irrigation regimes representing 40, 70, and 100% evapotranspiration potential (ETP). Drought significantly depressed biomass accumulation, photosynthetic pigments, leaf area, and relative water content. Under severe stress (40% ETP), CS1 inoculation markedly enhanced plant water status, increasing relative water content by 48.2% and leaf area by 240.1% relative to untreated controls. Inoculated plants also maintained carbohydrate metabolism more effectively, exhibiting higher soluble sugars and reduced starch depletion during drought. Potassium nitrate spraying improved membrane stability, lowering electrolyte leakage, and promoted shoot and root fresh and dry weights under stress. Notably, the combined CS1+2% $KNO_3$ treatment under drought increased leaf area by 7.98% and proline by 249.4%, while reducing malondialdehyde by 49.3% compared with the control, indicating alleviated oxidative damage. Overall, CS1 outperformed $KNO_3$ alone and offers a sustainable, low-risk approach for improving drought tolerance in citrus cultivation.

**Keywords**: *Drought stress, Malondialdehyde, Relative water content, Photosynthetic pigments, Potassium nitrate, Water soluble carbohydrate*.

| Indexes | Abbreviation |
|---|---|
| 1-aminocyclopropane-1-carboxylate deaminase | ACC deaminase |
| Ammonium nitrate | $NH_4NO_3$ |
| Analysis of variance | ANOVA |
| Central composite design | CCD |
| Chlorophyll | CHL |
| Drought stress | DRS |
| Dry weight | DW |
| Electrolyte leakage | EL |
| Fresh weight | FW |
| Hydrochloric acid | HCl |
| Intended points | Adj $R^2$ |
| Leaf area index | LAI |
| Malondialdehyde | MDA |
| Molecular weight | MW |
| Plant growth-promoting bacteria | PGPB |
| Potassium Nitrate | $KNO_3$ |
| Reactive Oxygen Species | ROS |
| Real data | $R^2$ |
| Relative water content | RWC |
| Response Surface Methodology | RSM |
| Superoxide dismutase | SOD |
| Trichloroacetic acid | TCA |
| Turgid weight | TW |

## 1. Introduction

Climate change has emerged as one of the most critical challenges affecting agricultural sustainability worldwide. Recent climate models predict increasingly irregular precipitation patterns accompanied by more frequent droughts, floods, and rising temperatures, leading to the expansion of arid and semi-arid regions (Guzzetti et al., 2019). Among abiotic stresses, drought is considered one of the most severe constraints on plant growth, geographical distribution, and productivity, thereby posing a serious threat to global food security (Lobell et al., 2014).

The persistence and intensification of such climatic extremes have far-reaching consequences beyond yield reductions, substantially undermining ecosystem stability (Straffelini, Luo and Tarolli, 2024). Under these conditions, soil one of the core components of terrestrial ecosystems is profoundly affected, as hydrological extremes alter soil moisture regimes and environmental quality, thereby reshaping the structure and functioning of soil microbiomes. These microbially mediated changes indirectly disrupt biogeochemical cycling, reduce agricultural productivity, and ultimately exacerbate threats to global food security (Furtak and Wolińska, 2023).

Citrus species are particularly sensitive to water deficit and other abiotic stresses, which markedly influence their vegetative growth, reproductive development, and final yield (Amri and Shahsavar, 2010; Vand and Abdullah, 2012). Drought stress in citrus plants is commonly associated with reduced leaf area, decreased biomass accumulation, impaired photosynthetic efficiency, and disturbances in plant water relations. Consequently, mitigating the adverse effects of water stress, especially during the early post-planting seedling stage, represents a major priority for sustainable citrus production systems. In this context, improving seedling physiological quality and stress resilience is essential for successful orchard establishment and long-term productivity.

A deeper understanding of plant–soil–microorganism interactions and their regulatory roles in plant metabolic processes under stressful conditions is therefore crucial for producing healthy, high-quality seedlings in nurseries. The plant microbiome plays a pivotal role in enhancing plant growth and health, offering both economic and environmental benefits (Kusano et al., 2011). Among the microbial communities associated with plants, plant growth-promoting bacteria (PGPB) are particularly recognized for their ability to stimulate plant growth and development through both direct and indirect mechanisms (Vejan et al., 2016). Root inoculation with PGPB can enhance plant tolerance to abiotic and biotic stresses by improving nutrient acquisition, increasing water-use efficiency through the regulation of transpiration and stomatal conductance, and reducing the accumulation of reactive oxygen species (ROS) (Vejan et al., 2016).

Species of the genus *Bacillus* are among the most extensively studied PGPB due to their multifunctional traits, including the production of phytohormone precursors, phosphate solubilization, and siderophore synthesis, all of which contribute to enhanced plant growth. In addition, *Bacillus* species are capable of producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that lowers ethylene levels and alleviates oxidative stress, thereby improving plant stress tolerance (Gowtham et al., 2020; Kashyap et al., 2019). Previous studies have demonstrated that inoculation with *Bacillus subtilis* can improve plant growth, nutrient assimilation, and photosynthetic performance in both contaminated and non-contaminated soils (Nevhulaudzi, Kanu and Ntushelo, 2020; Nevhulaudzi, Ntushelo and Kanu, 2020).

Alongside biological approaches, the use of mineral fertilizers remains essential for achieving optimal growth and productivity in horticultural crops such as citrus (Gimeno et al., 2014). Potassium nitrate ($KNO_3$), a widely used fertilizer in Mediterranean regions, provides both potassium and nitrogen, two key macronutrients involved in plant growth and stress

tolerance (Quinones et al., 2009). Potassium plays a fundamental role in osmotic regulation, water balance, enzyme activation, and overall metabolic functioning, particularly under stress conditions (Marschner, 2011; Mengel and Kirkby, 2001). Foliar application of potassium nitrate has been reported to enhance drought tolerance by improving physiological and biochemical activities, increasing photosynthetic pigment content and potassium accumulation, and reducing sodium accumulation and lipid peroxidation, thereby alleviating oxidative damage (Bukhari et al., 2021; Fayez and Bazaid, 2014; Moaaz Ali et al., 2020).

Complementing these strategies, the role of plant growth-promoting bacteria (PGPB) in modulating drought stress and mitigating its damage to plants is increasingly recognized within the framework of soil microbiome dynamics under climate change. For instance, hydrological extremes such as droughts profoundly alter soil moisture regimes, reshaping microbial communities and disrupting biogeochemical cycles that PGPB rely on to enhance plant resilience through mechanisms including nutrient solubilization and hormonal regulation, ultimately threatening agricultural productivity (Furtak and Wolińska, 2023). Moreover, drought-induced ecosystem succession in alpine swamp meadows reduces soil multifunctionality, potentially diminishing the abundance of beneficial PGPB that support vegetation health and stress tolerance by influencing carbon and nutrient dynamics (Yang et al., 2023). Similarly, grazing and reclamation practices in alpine steppes induce microbiome shifts that affect organic carbon stability in soil aggregates, highlighting how such alterations might impair PGPB's ability to prevent drought damage through improved soil structure and microbial support (Hu et al., 2023). These insights underscore the need for strategies that preserve or enhance PGPB populations to safeguard plants against escalating drought risks.

Although the ability of plant growth-promoting bacteria (PGPB) to alleviate abiotic stresses is well documented in herbaceous crops, their performance and underlying mechanisms in woody perennials such as citrus especially at the nursery/seedling stage that largely determines

orchard establishment and later productivity remain insufficiently characterized, and much of the citrus literature has predominantly focused on mycorrhizal associations. In addition, most existing studies have evaluated biological and nutritional interventions separately, leaving a clear gap regarding how a beneficial bacterial inoculant may interact with a foliar nutrient treatment across a realistic drought gradient and how such interactions can be quantified in a predictive manner. Consequently, the potential synergy among drought intensity, KNO3 foliar feeding, and PGPB inoculation and its simultaneous effects on growth and plant water status versus membrane stability and oxidative-stress biomarkers has not been adequately resolved. The novelty of the present work lies in proposing and testing a dual "bio-nutritional" strategy to enhance drought resilience in Mexican lime seedlings, in which root inoculation with Bacillus subtilis CS1 is evaluated as a targeted biostimulant to modulate drought-tolerance responses (improved water status, better carbohydrate management with reduced starch depletion, and enhanced membrane integrity), while foliar KNO3 is assessed as a complementary osmotic–ionic regulator capable of reducing electrolyte leakage and oxidative damage. Importantly, beyond reporting single-factor effects, this study employs a CCD–RSM framework to quantify main effects and two- and three-way interactions among drought × KNO3 × PGPB and to derive an optimization-based management point, moving the outcomes toward a practical, transferable decision framework for drought management in citrus seedlings. Accordingly, the objective of this study is to evaluate and compare the individual and combined effects of Bacillus subtilis CS1 inoculation and potassium nitrate foliar application (0 and 2%) on the morphophysiological and biochemical responses of Citrus aurantifolia seedlings under three drought levels (40, 70, and 100% ETP), by tracking biomass and growth traits, RWC and LAI, photosynthetic pigments, electrolyte leakage, proline, MDA, soluble carbohydrates, and starch, and ultimately using RSM to identify conditions that maximize stress-tolerance indicators while minimizing oxidative damage markers.

## 2. Materials and Methods

Mexican lime seedlings are highly sensitive to water deficit, which reduces growth, leaf expansion, photosynthetic pigments, and plant water status, while increasing membrane damage and oxidative stress. This study addresses the need for a practical, low-risk mitigation strategy by evaluating whether Bacillus subtilis CS1 inoculation, alone or combined with foliar KNO3, can alleviate drought impacts and improve morphophysiological and biochemical performance under different evapotranspiration-based irrigation levels.

### *2.1. Sample Preparation and Greenhouse Conditions*

Mexican lime seedlings (*Citrus aurantiifolia*) were obtained from a certified commercial nursery in Dezful, Khuzestan Province, approved by the Agricultural Jihad Organization. The seedlings were transferred to the greenhouse of Shahid Chamran University of Ahvaz (31.3033° N, 48.6564° E, 22 m a.s.l.). Greenhouse conditions were maintained at 25 °C during the day and 19 °C at night, with a 14 h light/10 h dark photoperiod and relative humidity of 40–45%.

The seedlings were transplanted into 8 kg capacity pots filled with a 1:1:1 (v/v) mixture of agricultural soil, sand, and decomposed animal manure, which was autoclaved at 120 °C for 2 h prior to use. The experiment was arranged as a factorial design with three factors: bacterial inoculation (inoculated or non-inoculated), irrigation levels (40%, 70%, and 100% of evapotranspiration), and foliar application of potassium nitrate ($KNO_3$ at 0% or 2% w/v). Foliar spraying with $KNO_3$ was performed two weeks after the initiation of drought stress treatments. Each treatment combination was conducted with three replicates.

### *2.2. Drought Stress Treatments*

Irrigation treatments were determined using six reference pots. These pots were fully irrigated, and their weight was recorded after 24 h to obtain the initial (primary) weight. After five days, the pots were weighed again to determine the secondary weight. The difference between the two measurements was used to estimate water loss due to evapotranspiration, which was considered as 100% evapotranspiration (ET). Based on this reference value, irrigation volumes corresponding to 40% and 70% evapotranspiration were calculated and applied to the respective treatments.

### *2.3. Bacterial Preparation*

Bacillus sp. CS1 (accession number OR816114), obtained from the Soil Biology Group at Shahid Chamran University of Ahvaz, was cultured in nutrient broth at 28 °C for 18 h under continuous shaking at 130 rpm. This strain has been previously characterized for its ability to solubilize insoluble phosphate and zinc oxide, produce indole-3-acetic acid and hydrogen cyanide, and inhibit the growth of Fusarium culmorum (unpublished data). The bacterial suspension was applied to the soil at a concentration of $1.5 \times 10^6$ CFU, incorporated at a depth of approximately 2 cm. Figure 1 presents a schematic overview of the experimental setup and treatment application.

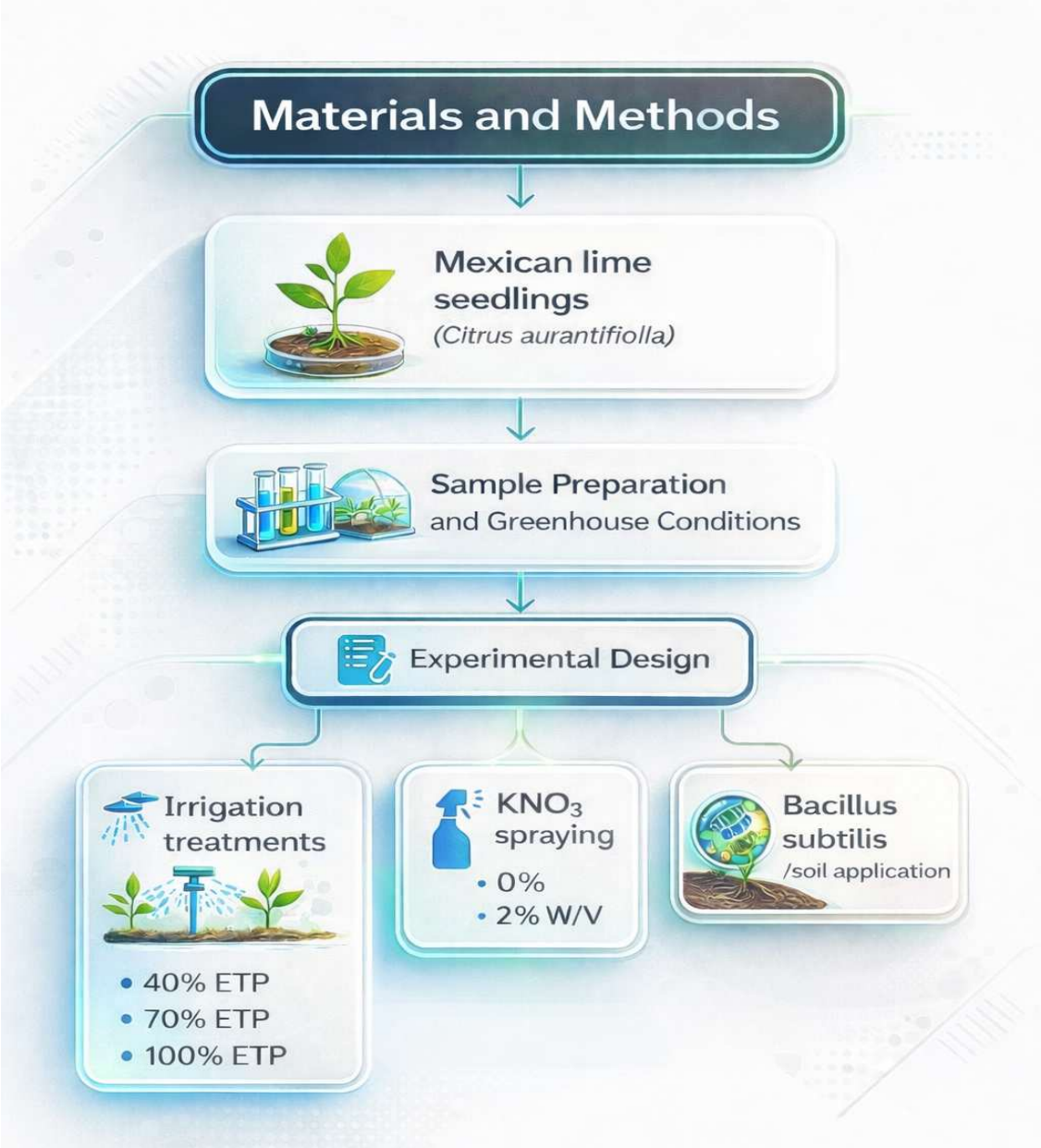



Fig. 1. Overview of the integrated bio-nutritional experimental framework used to assess drought stress mitigation in Mexican lime seedlings.

### *2.4. Plant Analysis*

#### *2.4.1. Morpho-Physiological Traits*

After 16 weeks of treatment, the seedlings were harvested, and the shoots and roots were carefully separated, thoroughly washed with distilled water, and weighed to determine fresh weight. Shoot and root dry weights (DW) were obtained after oven-drying the samples at 70

°C until a constant weight was achieved. Leaf area index (LAI) was measured using a leaf area meter (Delta-T Devices Ltd., UK). Relative water content (RWC) was determined according to the method described by (Udawat et al., 2016). Leaf samples were first weighed to obtain fresh weight, then immersed in deionized water overnight to reach full turgidity and weighed again to determine turgid weight. Subsequently, the samples were oven-dried at 70 °C and reweighed to obtain dry weight. Relative water content was calculated using Equation (1).

$$\mathrm{RWC}\ (\%) = 100 \times \left( FW - \frac{\mathrm{DW}}{\mathrm{TW}} - \mathrm{DW} \right) \quad (1)$$

### *2.4.2. Biochemical Traits*

Chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl a + Chl b), and carotenoid contents were determined following the method of (Lichtenthaler, 1987). Briefly, 0.1 g of fresh young leaf tissue was homogenized in 80% (v/v) acetone, and the absorbance of the extracts was measured at 663, 646 and 470 nm using a spectrophotometer (SmartSpec 3000, Bio-Rad). Pigment concentrations were calculated using standard equations and expressed on a fresh weight basis (mg $g^{-1}$ FW).

Electrolyte leakage (EL) was assessed according to the method described by (Lutts, Kinet and Bouharmont, 1996). Leaf discs were immersed in 10 mL of deionized water and incubated for 24 h on a floating shaker at room temperature, after which the initial electrical conductivity ($EC_1$) was recorded. The samples were then boiled for 20 min to release all electrolytes, cooled to room temperature, and the final conductivity ($EC_2$) was measured. Electrolyte leakage was calculated using Equation (2).

$$EL(\%) = \left( \frac{EC_1}{EC_2} \right) \times 100 \quad (2)$$

Proline content was determined following the method described by (Bates, Waldren and Teare, 1973) Frozen leaf samples (0.5 g) were homogenized in 5 mL of 95% ethanol, and the homogenate was centrifuged to collect the supernatant. The extraction of the leaf residues was repeated twice using 5 mL of 70% ethanol each time, and all supernatants were combined. An aliquot of 2 mL of the extract was reacted with 2 mL of ninhydrin reagent and 2 mL of glacial acetic acid. After incubation, 4 mL of toluene was added, and the mixture was vigorously vortexed, allowing the formation of a reddish-orange chromophore in the upper toluene phase. The absorbance of this phase was measured at 520 nm using a spectrophotometer. Proline concentration was quantified using a standard calibration curve and expressed on a fresh weight basis.

Total soluble carbohydrate content was determined according to the method described by (Irigoyen, Einerich and Sánchez‐Díaz, 1992). An aliquot of 100 µL of the ethanol extract obtained during the proline extraction was mixed with 3 mL of anthrone reagent. The mixture was heated in a boiling water bath for 10 min, then rapidly cooled to room temperature. Absorbance was measured at 625 nm using a spectrophotometer, and carbohydrate concentration was quantified using a standard calibration curve.

Starch content was determined according to the method described by (Marshall, 1986). After complete evaporation of ethanol from the residues remaining from the soluble carbohydrate extraction, 5 mL of 1.1% (v/v) HCl was added to each sample. The mixture was incubated in a water bath at 100 °C for 30 min to hydrolyze starch. After cooling, 10 mL of distilled water was added, and 1 mL of the extract was transferred to a 10 mL Falcon tube and cooled at 0 °C. Subsequently, 5 mL of anthrone reagent was added to each tube, and the samples were heated again in a boiling water bath for 11 min. After rapid cooling in an ice bath, absorbance was measured at 630 nm using a spectrophotometer. Starch concentration was quantified using a standard curve.

Malondialdehyde (MDA) content, as an indicator of lipid peroxidation, was measured following the method of (Heath and Packer, 1968). Fresh leaf tissue (0.1 g) was homogenized in 1.5 mL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 15,000 rpm for 15 min. A 0.5 mL aliquot of the supernatant was mixed with 2 mL of a reaction solution containing 20% TCA and 0.5% thiobarbituric acid (TBA). The mixture was heated at 95 °C for 30 min and then rapidly cooled in an ice bath. Absorbance was recorded at 532 and 600 nm, and MDA concentration was calculated using Equation (3). The biochemical traits evaluated in this study are schematically illustrated in Figure 2.

$$MDA = \frac{(A532 - A600) \times 155}{0.1} \quad (3)$$

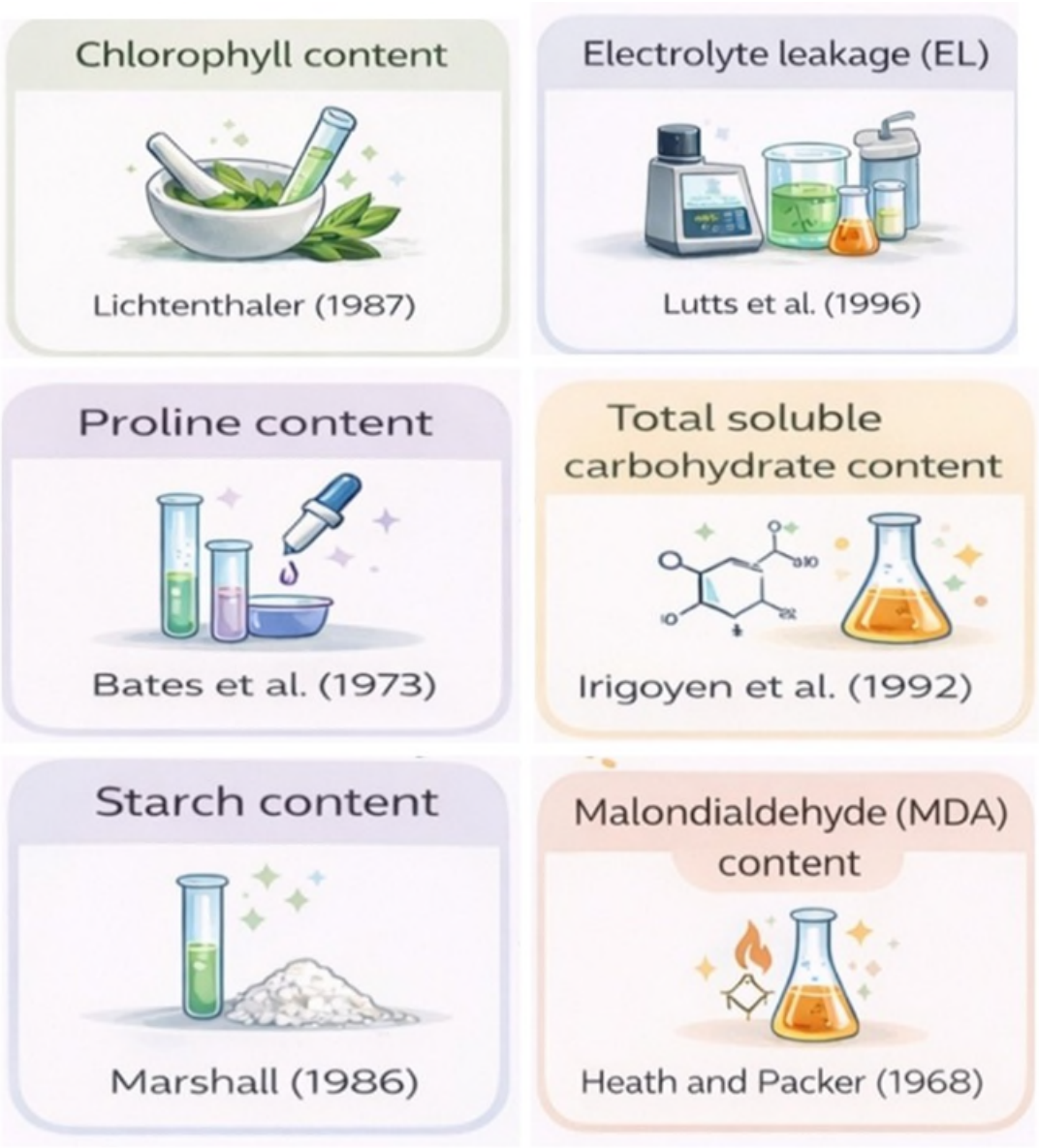



Fig. 2. Schematic overview of biochemical traits assessed in plant tissues.

241

242

243 ***2.5. Statistical Analysis***

244 The experiment was carried out with three replicates, and the data were analyzed using
245 Response Surface Methodology (RSM) implemented in Design-Expert software (version 11)
246 based on a Central Composite Design (CCD). The effects of three independent variables $KNO_3$
247 concentration, irrigation level, and bacterial inoculation on the measured responses were
248 investigated across 15 experimental runs. Model performance was evaluated using the adjusted

coefficient of determination (Adjusted $R^2$), predicted coefficient of determination (Predicted $R^2$), Adequate Precision, and the coefficient of variation (CV). Analysis of variance (ANOVA) was applied to assess the model's goodness of fit, predictive accuracy, and the level of agreement between predicted and experimental values. Adequate Precision values greater than 4 were considered indicative of an adequate signal, and the difference between Adjusted $R^2$ and Predicted $R^2$ was kept below 0.2 to confirm the reliability of the developed models (Table 1). Figure 3 illustrates the schematic overview of plant analysis procedures, including morpho-physiological measurements, biochemical assays, and statistical analysis based on response surface methodology (RSM).

Table. 1. The response surface approach involves the identification of independent variables and the determination of their respective levels.

| Factor | Name | Units | Minimum | Maximum | Mean | Std. Dev. |
|---|---|---|---|---|---|---|
| A | Drought | % | 40.00 | 100.00 | 70.00 | 25.21 |
| B | $KNO_3$ | % | 0.0000 | 2.00 | 1.000 | 0.8402 |
| C | PGPB | | Inoculation | Non-inoculated | **Levels:** | 2 |

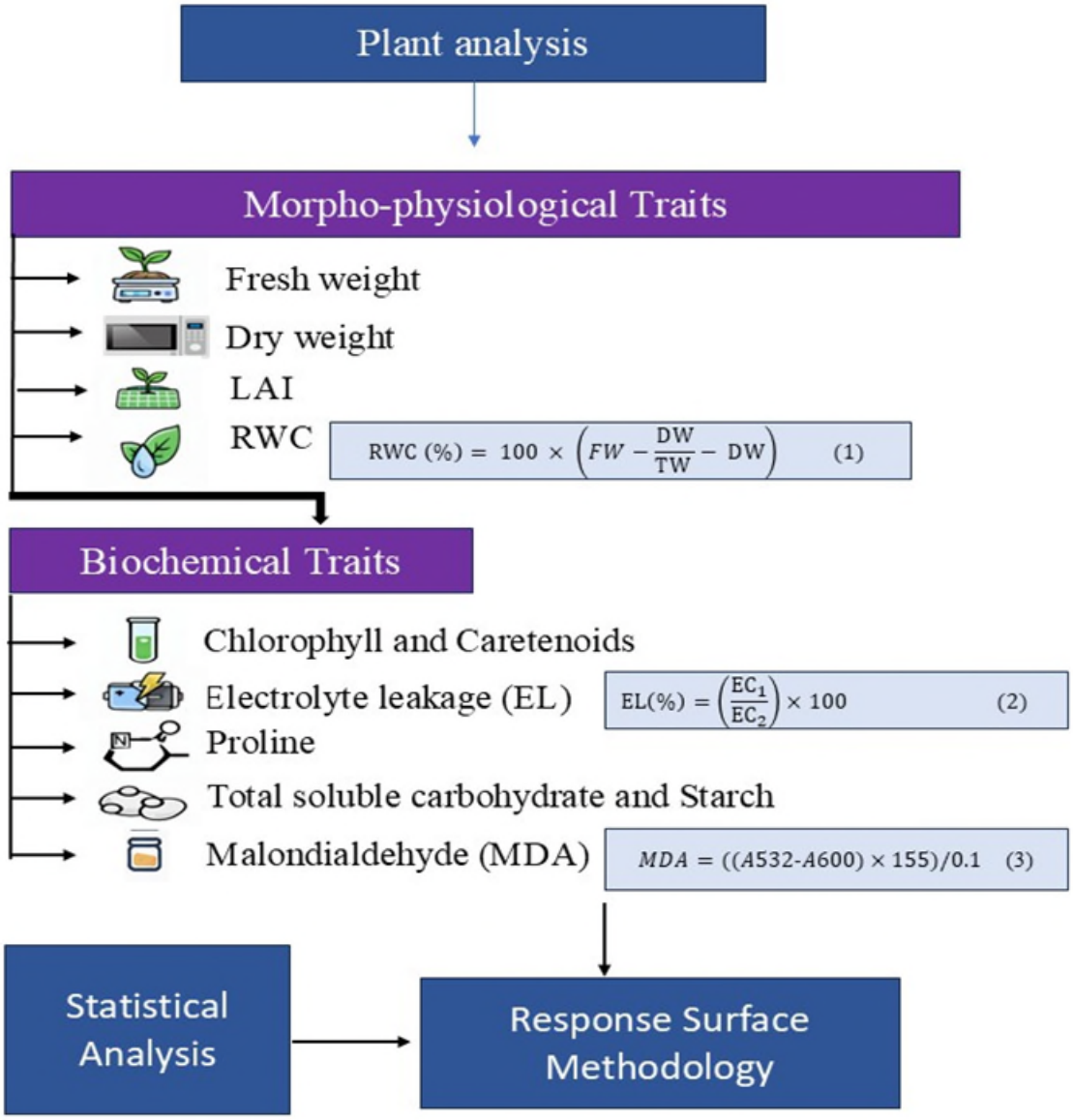



Fig. 3. Schematic overview of plant analysis procedures, including morpho-physiological measurements, biochemical assays, and statistical analysis using response surface methodology (RSM).

## 3. Results

The RSM-predicted mean responses presented in figure 4 summarize the behavior of all physiological, biochemical, and growth-related variables considered in the Central Composite Design. These responses represent the key plant indicators modeled and optimized in the RSM framework to evaluate drought tolerance and overall seedling performance. As shown, bacterial inoculation resulted in a consistent increase in

photosynthetic pigments, including chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids, indicating an improvement in photosynthetic efficiency and pigment stability under the studied conditions. Similarly, relative water content (RWC) and leaf area index (LAI) were markedly higher in inoculated plants, reflecting enhanced water status and canopy development. Growth-related responses, including fresh and dry weights of shoots and roots, also exhibited higher mean values under inoculation, demonstrating a strong positive effect of PGPB on biomass accumulation and carbon allocation. In parallel, metabolic indicators associated with stress tolerance, such as proline, starch, and total carbohydrates, were elevated in inoculated treatments, suggesting improved osmotic adjustment and energy storage capacity under drought stress. In contrast, stress-damage markers, namely electrolyte leakage (EL) and malondialdehyde (MDA), showed substantially lower predicted means in inoculated plants, confirming reduced membrane disruption and oxidative damage. Collectively, these modeled responses illustrate the integrated physiological response of Mexican lime seedlings to drought, nutrient supply, and microbial inoculation. The clear separation between inoculated and non-inoculated treatments across nearly all response variables highlights the dominant role of PGPB in modulating plant performance. These results further confirm the suitability of the selected response variables for RSM analysis and validate their effectiveness in capturing both stress intensity and mitigation mechanisms within the proposed experimental model.

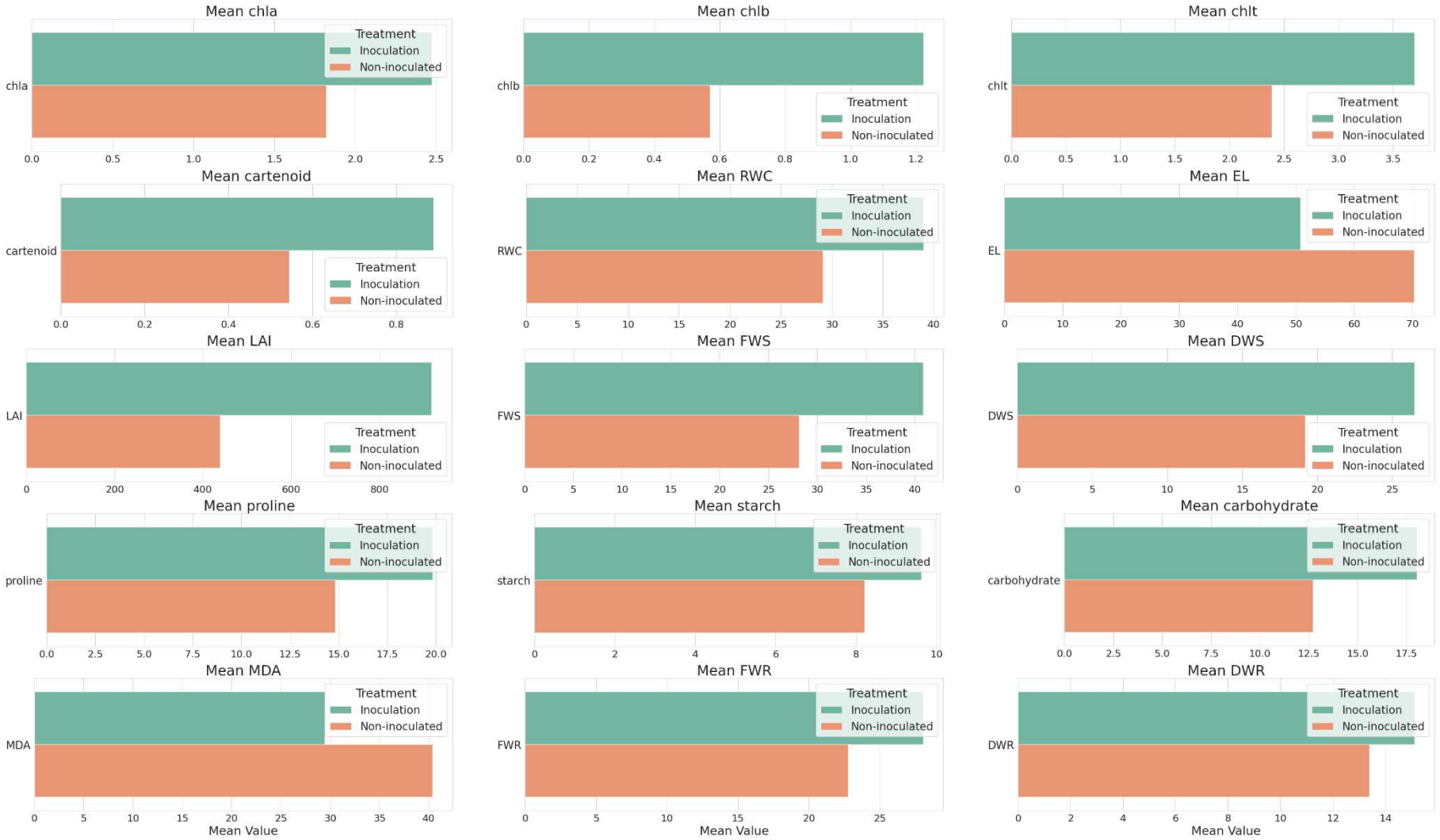



Fig. 4. RSM-predicted mean values of physiological, biochemical, and growth-related response variables under inoculated and non-inoculated conditions, illustrating the responses evaluated in the Central Composite Design model.

Figure 5 presents the correlation heatmap summarizing the relationships among all 15 physiological response variables measured in this study, showing how the responses co-vary with one another, consistent with the trends already observed in the previous figures. Strong positive correlations were observed among photosynthetic pigments (Chl a, Chl b, total chlorophyll, and carotenoids), leaf area index (LAI), relative water content (RWC), and biomass-related traits (FWS, DWS, FWR, and DWR), indicating that improved photosynthetic capacity, canopy development, and plant water status occur simultaneously with enhanced growth performance. In contrast, stress-related indicators such as electrolyte leakage (EL), malondialdehyde (MDA), and proline exhibited strong negative correlations with most growth- and performance-related traits, confirming that increased cellular damage and oxidative stress are associated with reduced physiological performance. Moderate to strong negative correlations between MDA and carbohydrates, as well as between proline and biomass traits, further highlight the shift in plant metabolism under stress conditions. Overall, this correlation analysis reinforces the integrated physiological response of Mexican lime seedlings to drought stress and mitigation treatments, demonstrating that all measured responses are interrelated and respond coherently, in agreement with the patterns previously discussed and illustrated in earlier figures.

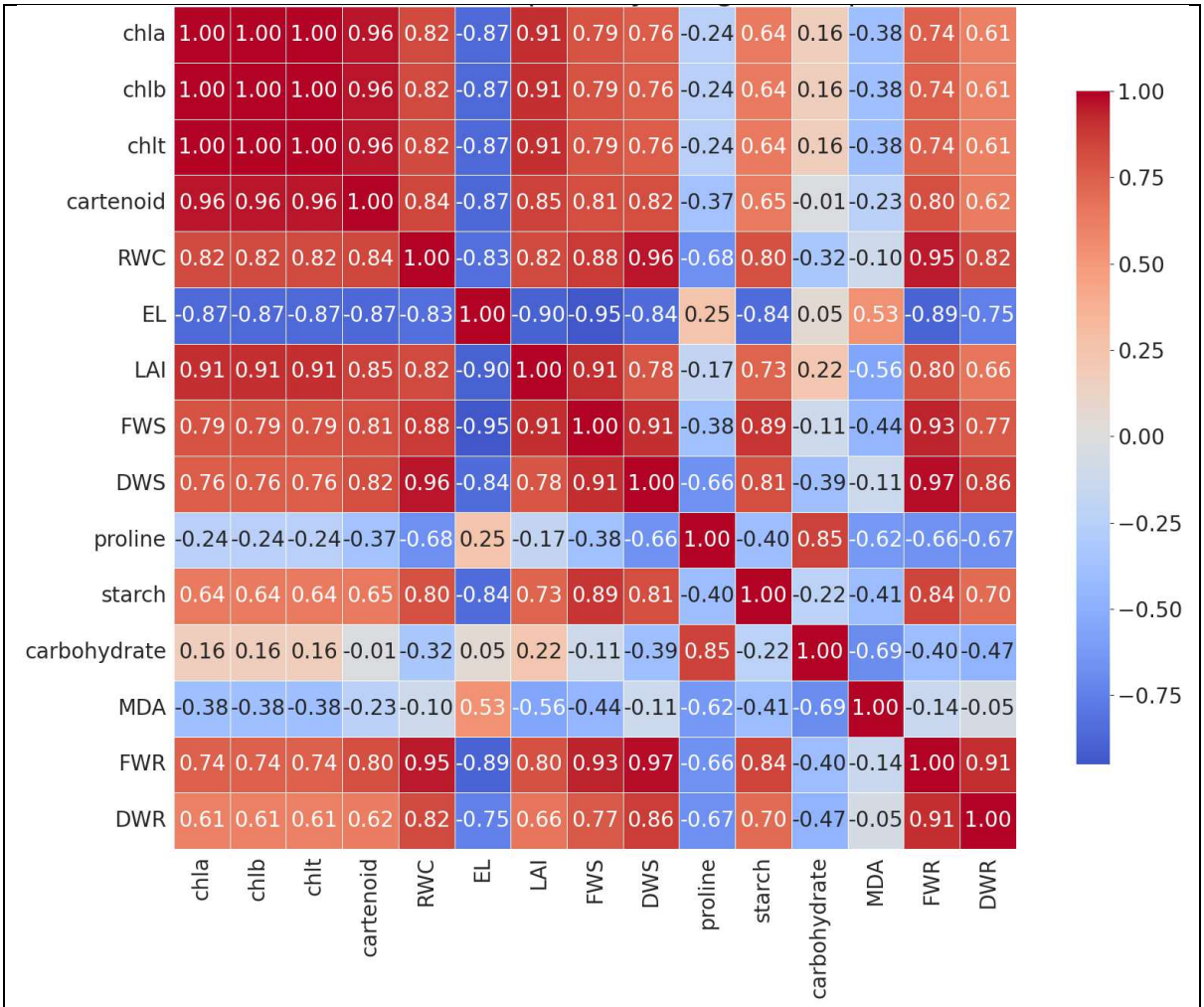



Fig. 5. Pearson correlation heatmap showing the relationships among all measured physiological and growth-related response variables in Mexican lime seedlings.

Morpho-Physiological Reactions to Treatment Interactions ANOVA revealed that drought stress (DRS), potassium nitrate ($KNO_3$) foliar application, and plant growth-promoting bacteria (PGPB) inoculation all had significant main effects as well as interaction effects on the fresh weight (FW) and dry weight (DW) of shoots and roots. The interactions DRS × $KNO_3$ and DRS × PGPB had a substantial effect on the FW and DW of both shoots and roots. Additionally, the $KNO_3$ × PGPB interaction significantly affected the FW of both shoots and roots ($p \leq 0.01$). The relative water content (RWC) of leaves was significantly influenced by DRS, PGPB inoculation, and their interaction (DRS × PGPB) ($p \leq 0.01$). The triple interaction of DRS × $KNO_3$ × PGPB also significantly impacted the leaf area index (LAI) (Table 2).

315

Table. 2. An ANOVA will be conducted to examine the effects of a CCD design on growth parameters and RWC.

| Sources | df | Mean squares | | | | | |
|---|---|---|---|---|---|---|---|
| | | FWS | DWS | FWR | DWR | LAI | RWC |
| Model | 7 | $176.50^{**}$ | $0.0876^{**}$ | $101.79^{**}$ | $31.54^{**}$ | $1.221E+05^{**}$ | $0.2443^{**}$ |
| A-Drought | 1 | $403.72^{**}$ | $0.6890^{**}$ | $890.89^{**}$ | $89.90^{**}$ | $1.556E+05^{**}$ | $1.36^{**}$ |
| B- $KNO_3$ | 1 | $66.87^{**}$ | $0.0621^{**}$ | $61.39^{**}$ | $3.19^{**}$ | $348.16^{ns}$ | $0.0045^{ns}$ |
| C-PGPB | 1 | $283.16^{**}$ | $0.0585^{**}$ | $67.07^{**}$ | $8.09^{**}$ | $3.068E+05^{**}$ | $0.3140^{**}$ |
| AB | 1 | $17.29^{**}$ | $0.0247^{**}$ | $0.1794^{*}$ | $26.31^{**}$ | $535.36^{*}$ | $0.0011^{ns}$ |
| AC | 1 | $172.38^{**}$ | $0.0246^{**}$ | $11.12^{**}$ | $2.10^{**}$ | $44854.02^{**}$ | $0.0283^{**}$ |
| BC | 1 | $2.29^{**}$ | $0.0019^{ns}$ | $0.6775^{**}$ | $0.2269^{ns}$ | $317.88^{ns}$ | $0.0035^{ns}$ |
| ABC | 1 | $0.1375^{ns}$ | $0.0015^{ns}$ | $0.0953^{ns}$ | $0.0525^{ns}$ | $694.82^{*}$ | $0.0007^{ns}$ |
| CV (%) | | 0.8986 | 2.22 | 0.5946 | 1.39 | 0.9978 | 0.9472 |
| $R^2$/Adj $R^2$ | | 0.99/0.99 | 0.99/0.98 | 0.99/0.99 | 0.99/0.99 | 0.99/0.99 | 0.99/0.99 |

ns, *,**- not significant, significant at 5 and 1% level of probability, respectively.

316

317 ***3.1. Shoot and Root Fresh Weight and Dry Weight***

318 Figure 6 illustrates the three-dimensional response surface plots of shoot fresh weight (FWS)
319 and dry weight (DWS) as influenced by drought severity and foliar $KNO_3$ application under
320 non-inoculated and PGPB-inoculated conditions. Increasing drought stress (reduction of ETP
321 from 100% to 40%) markedly reduced shoot biomass in non-inoculated plants, highlighting the
322 high sensitivity of Mexican lime shoot growth to water limitation. Foliar application of $KNO_3$
323 significantly mitigated these adverse effects. Under severe drought stress (40% ETP), $KNO_3$
324 treatment increased FWS and DWS by 71.7% and 111.7%, respectively, compared with
325 untreated controls. Under non-stress conditions (100% ETP), the maximum shoot biomass was
326 observed at 2% $KNO_3$, indicating that potassium and nitrogen availability enhanced biomass
327 accumulation even under adequate water supply. PGPB inoculation exerted a stronger
328 stimulatory effect than $KNO_3$ alone. At 40% ETP, inoculated plants exhibited increases of

162.7% in FWS and 98.8% in DWS relative to non-inoculated controls, demonstrating the pronounced role of Bacillus subtilis CS1 in sustaining shoot growth under water deficit. This improvement is likely associated with enhanced root development, hormonal regulation, and improved water and nutrient acquisition. Notably, the combined application of $KNO_3$ and PGPB showed a synergistic effect, resulting in a 120.3% increase in shoot fresh weight compared with untreated plants, confirming the effectiveness of integrating biological and nutritional strategies to alleviate drought-induced growth suppression.

Figure 7 presents the response surface plots of root fresh weight (FWR) and dry weight (DWR) as affected by drought stress, $KNO_3$ foliar spray, and bacterial inoculation. In non-inoculated plants, increasing drought severity substantially reduced root biomass, reflecting impaired root development under limited water availability. Foliar application of $KNO_3$ partially alleviated this reduction. At 40% ETP, $KNO_3$ treatment enhanced FWR and DWR by 29.5% and 28.4%, respectively, compared with untreated controls, suggesting that potassium-mediated osmotic regulation and improved metabolic activity contributed to better root growth under stress conditions. GPB inoculation resulted in a more pronounced improvement in root biomass than $KNO_3$ alone. Under severe drought stress (40% ETP), inoculated plants showed increases of 41.1% in FWR and 30.8% in DWR relative to non-inoculated plants. This indicates that Bacillus subtilis CS1 effectively enhanced root proliferation and biomass accumulation, thereby strengthening the plant's capacity to extract water and nutrients from the soil. The combined application of $KNO_3$ and PGPB consistently produced the highest root biomass across drought levels, confirming a synergistic interaction between microbial inoculation and mineral nutrition. Overall, the results in Figure 7 suggest that improvements in shoot performance under drought stress are closely linked to enhanced root growth and functionality induced by the combined treatments.

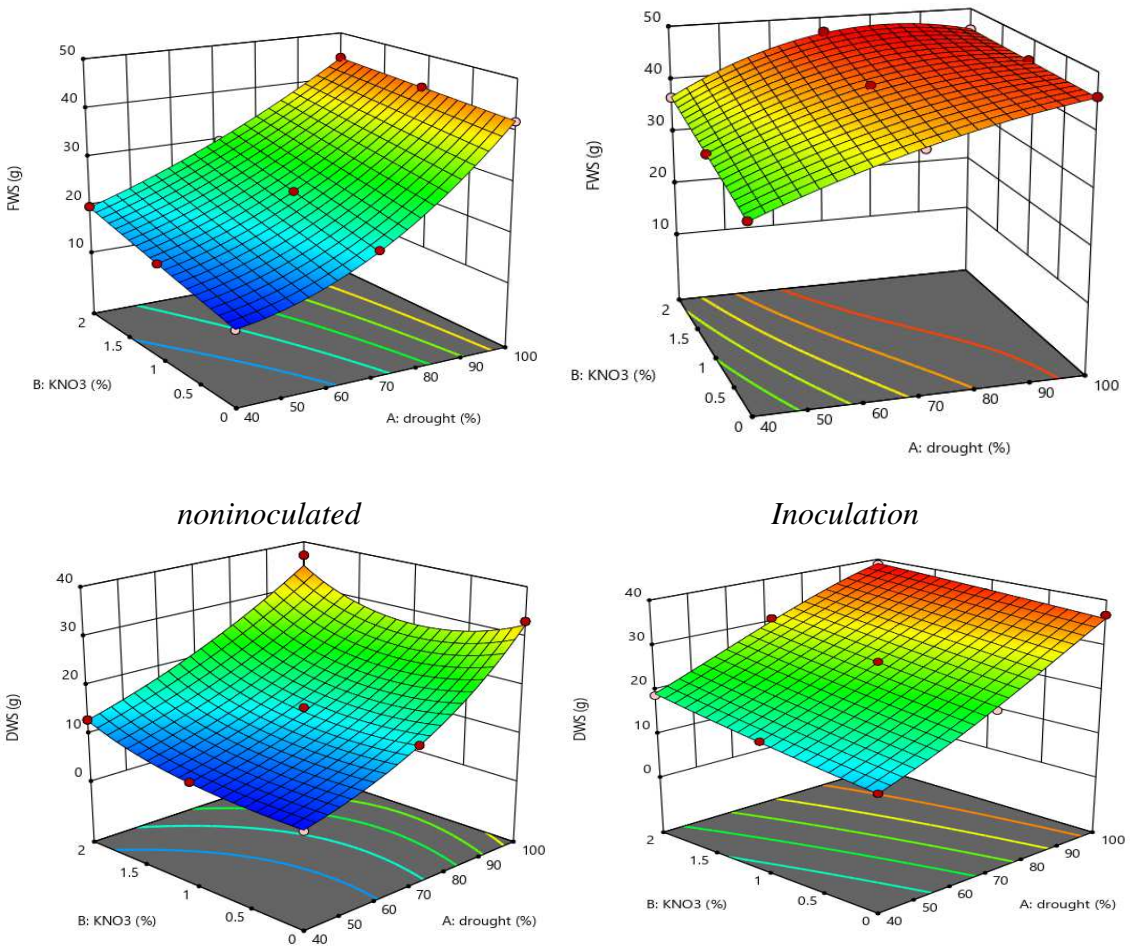
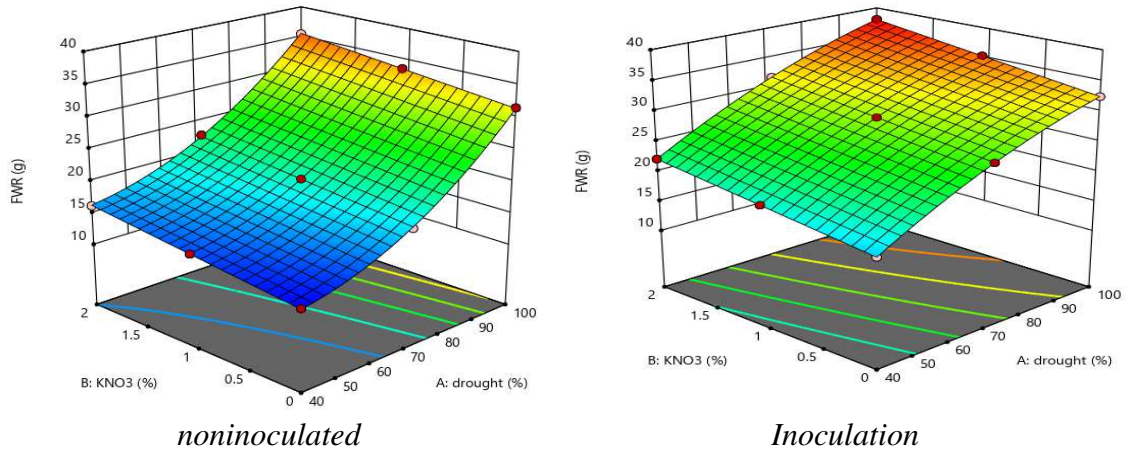
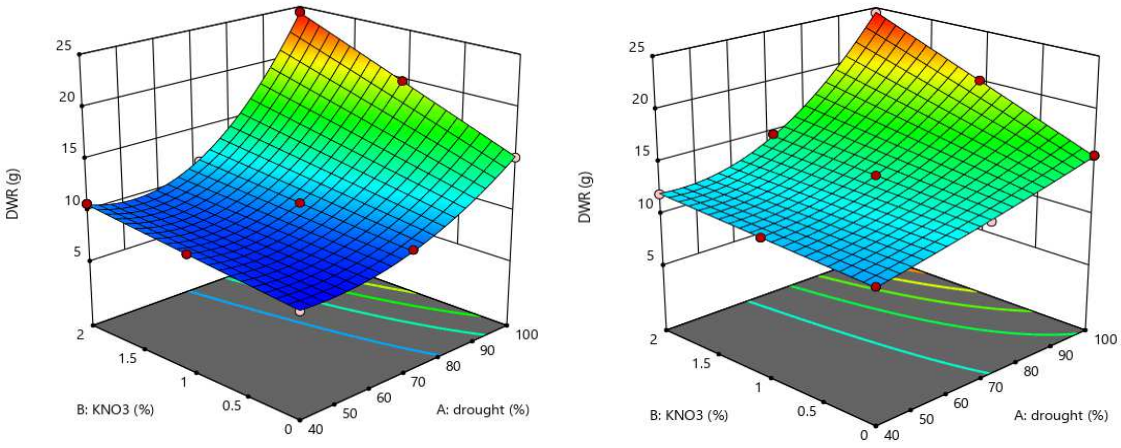



*Fig. 6. Three-dimensional (3D) response surface plots are created to visualize the impact of the main components found in CCD on the FW and DW of shoot.*

*CCD: Central Composite Design, FWS: Fresh weight of shoot, DWS: Dry weight* of *shoot.*






*noninoculated* *Inoculation*

*Fig. 7. Three-dimensional (3D) response surface plots are created to visualize the impact of the main components found in CCD on the FW and DW of roots.*

*CCD: Central Composite Design, FWR: Fresh weight of root, DWR: Dry weight of root.*

### *3.2. Leaf Relative Water Content and Leaf area index*

Figure 8 shows that drought stress significantly reduced leaf relative water content (RWC) as evapotranspiration potential decreased from 100% to 40%. However, PGPB inoculation consistently improved RWC across all drought levels. The beneficial effect was most pronounced under severe drought stress, where inoculated plants exhibited a 48.2% higher RWC at 40% ETP compared to non-inoculated plants. The increasing separation between inoculated and non-inoculated trends with rising drought severity indicates that Bacillus subtilis CS1 effectively enhances plant water retention and drought tolerance rather than merely improving water status under optimal conditions.

Figure 9 illustrates that leaf area index (LAI) declined markedly with increasing drought stress in non-inoculated plants, reaching its minimum value (225.2 $cm^2$) at 40% ETP without $KNO_3$ application. In contrast, PGPB inoculation significantly enhanced LAI at all irrigation levels, with further improvement observed following $KNO_3$ foliar application. The maximum LAI (1090 $cm^2$) was achieved under non-stress conditions (100% ETP) with combined PGPB inoculation and 2% $KNO_3$ spray. These results indicate a synergistic interaction between

370 microbial inoculation and potassium nutrition in maintaining leaf expansion and canopy
371 development under water-limited conditions.
372

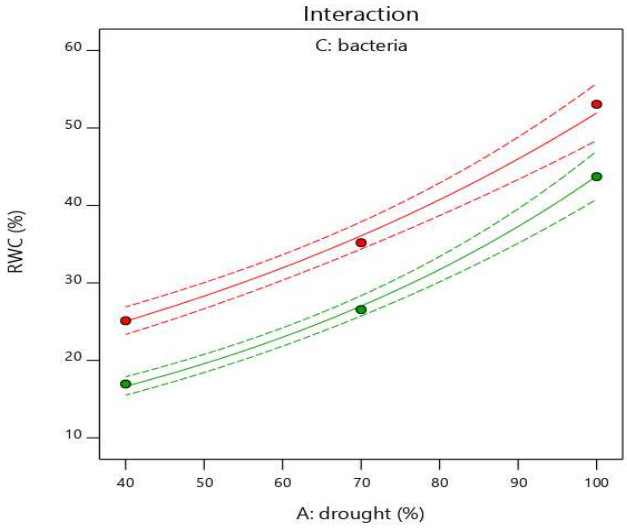
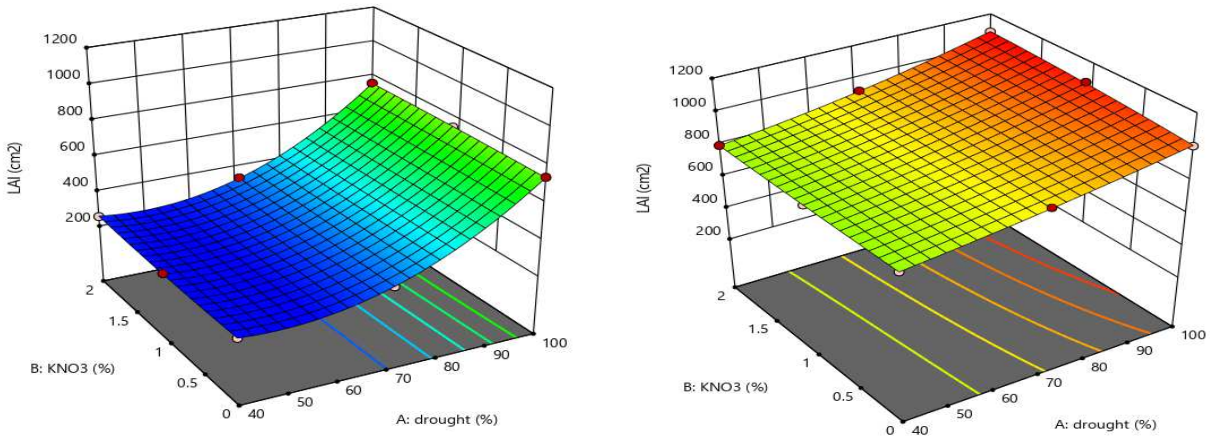



*Fig. 8. Interaction Effect of the drought levels and bacterial inoculation on the RWC*




*non-inoculation* *Inoculation*

Fig. 9. Three-dimensional (3D) response surface plots are created to visualize the impact of the main components found in CCD on LAI.

CCD: Central Composite Design, LAI: Leaf Area Index

373

374 ***3.3. Photosynthetic Pigments and Electrolyte Leakage***

ANOVA results indicated significant effects of DRS, KNO3, and PGPB on chlorophyll a (Chl a), total chlorophyll, and carotenoid content. Additionally, DRS and PGPB significantly influenced chlorophyll b (Chl b). The DRS × PGPB interaction significantly affected Chl a, Chl b, total chlorophyll, and carotenoids. For electrolyte leakage (EL), significant effects were observed for the main factors and the interactions DRS × KNO3, DRS × PGPB, and KNO3 × PGPB (Table 3).

Table. 3. ANOVA of CCD for photosynthetic pigments and EL.

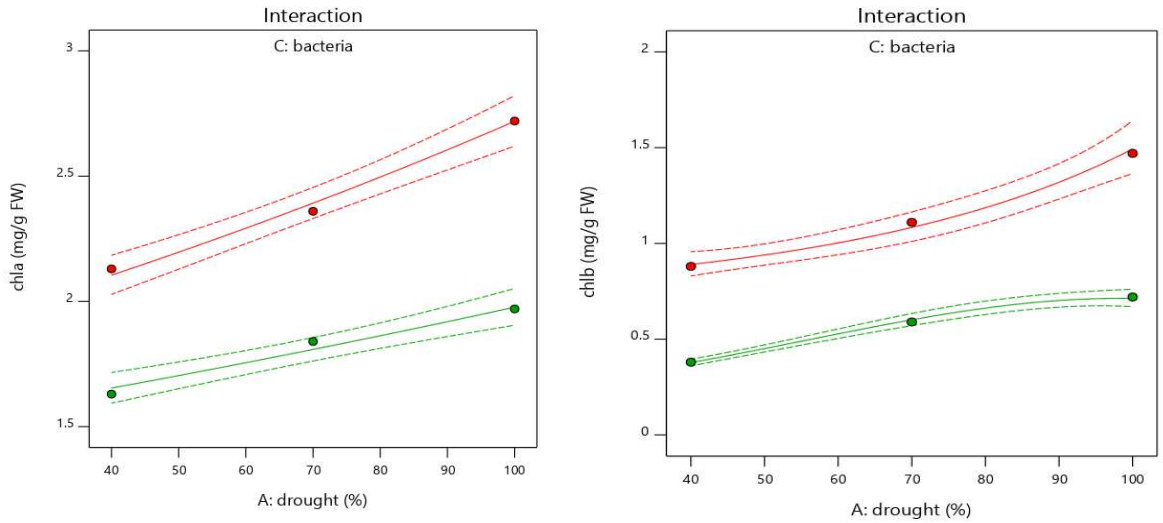
| Sources | df | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | | Total chl | Chla | Chlb | Carotenoid | EL |
| Model | 7 | $0.1308^{**}$ | $0.0161^{**}$ | $0.1040^{**}$ | $0.1516^{**}$ | $0.1018^{**}$ |
| A-Drought | 1 | $0.2652^{**}$ | $0.0325^{**}$ | $0.1341^{**}$ | $0.4370^{**}$ | $0.2618^{**}$ |
| B-$KNO_3$ | 1 | $0.0045^{*}$ | $0.0005^{*}$ | $0.0015^{ns}$ | $0.0147^{*}$ | $0.1296^{**}$ |
| C- PGPB | 1 | $0.6289^{**}$ | $0.0781^{**}$ | $0.1105^{**}$ | $0.5339^{**}$ | $0.3118^{**}$ |
| AB | 1 | $0.0018^{ns}$ | $0.0002^{ns}$ | $0.0011^{ns}$ | $0.0000^{ns}$ | $0.0028^{*}$ |
| AC | 1 | $0.0115^{**}$ | $0.0009^{*}$ | $0.0350^{**}$ | $0.0631^{**}$ | $0.0037^{**}$ |
| BC | 1 | $0.0029^{ns}$ | $0.0003^{ns}$ | $0.0013^{ns}$ | $0.0120^{ns}$ | $0.0028^{*}$ |
| ABC | 1 | $0.0006^{ns}$ | $0.0000^{ns}$ | $0.0001^{ns}$ | $0.0002^{ns}$ | $0.0000^{ns}$ |
| CV (%) | | 1.65 | 2.99 | 1.26 | 6.94 | 0.3701 |
| $R^2$/Adj $R^2$ | | 0.99/0.98 | 0.99/0.99 | 0.99/0.99 | 0.97/0.96 | 0.99/0.99 |
| Predicted $R^2$ | | 0.97 | 0.98 | 0.97 | 0.93 | 0.99 |

ns, *,**- not significant, significant at 5 and 1% level of probability, respectively

### *3.3.1. Photosynthetic Pigments*

Figure 10 shows that drought stress clearly impaired the photosynthetic pigment system in Mexican lime leaves, particularly in plants that were not inoculated with PGPB. As water availability declined from 100% to 40% ETP, chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents all decreased noticeably, indicating progressive damage to the

photosynthetic apparatus under water deficit conditions. In contrast, inoculation with Bacillus sp. CS1 substantially alleviated these negative effects of drought. Even under severe drought stress (40% ETP), inoculated plants maintained markedly higher pigment concentrations than non-inoculated ones. At this stress level, Chl a, Chl b, total chlorophyll, and carotenoid contents reached 2.13, 0.88, 3.01, and 0.51 mg $g^{-1}$ FW, respectively, whereas the corresponding values in non-inoculated plants were only 1.63, 0.38, 2.01, and 0.41 mg $g^{-1}$ FW. The difference was particularly pronounced for chlorophyll b and total chlorophyll, suggesting that bacterial inoculation effectively protected the light-harvesting components of the photosynthetic system. Overall, the consistently higher pigment levels observed in inoculated plants across all drought levels indicate that Bacillus sp. CS1 helps preserve chloroplast functionality and slows drought-induced pigment degradation. This protective effect likely stems from improved plant water status, reduced oxidative damage, and enhanced metabolic stability, enabling inoculated plants to sustain photosynthetic capacity more effectively under drought stress.



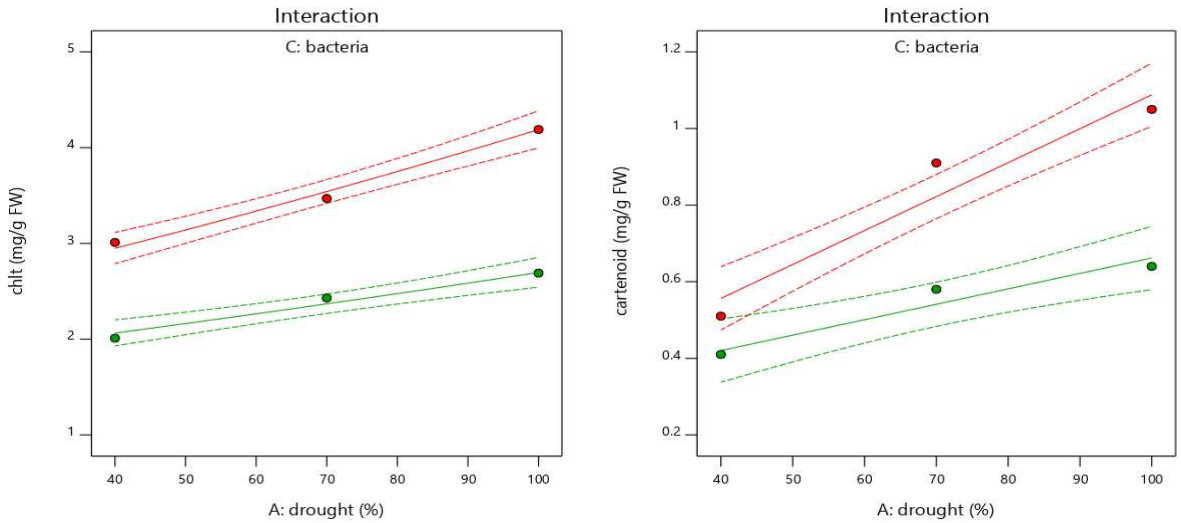


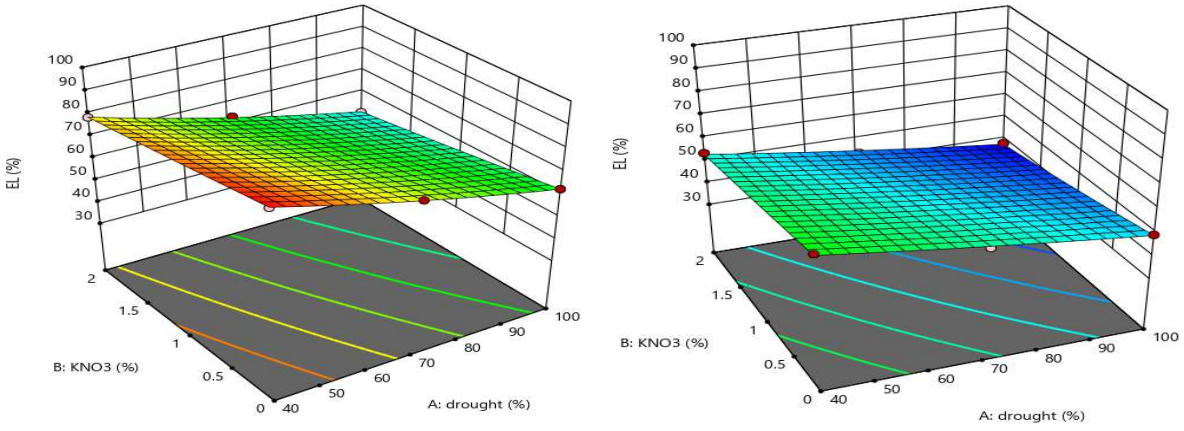

*Fig. 10. Effect of the drought on the Chl a, Chl b, total Chl and carotenoids of the PGPB C1(green)-noninoculated- C2(red)-inoculation*

401

402 ***3.3.2. Electrolyte Leakage (EL)***

403 KNO3 foliar application dramatically decreased EL by 8.9%, 15.1%, and 19.7% at 40%,
404 70%, and 100% ETP, respectively, while increasing drought stress increased EL (Figure 11).
405 In comparison to plants that were not inoculated, PGPB inoculation decreased EL by 27.6%,
406 26.5%, and 21.6% at 40%, 70%, and 100% ETP, respectively (Figure 11).
407 When compared to controls without KNO3 or PGPB, the combined KNO3× PGPB treatment
408 reduced EL by 42.6%, which was more effective than KNO3 alone (Figure 11).

409



*noninoculated* *Inoculation*

*Fig. 11. Three-dimensional (3D) response surface plots are created to visualize the impact of the main parameters revealed in CCD on electrolyte-leakage.*

CCD: Central Composite Design

410

411 **3.4. Biochemical Responses**

412 ANOVA results indicated significant effects of DRS, KNO3, and PGPB on proline,
413 malondialdehyde (MDA), soluble carbohydrates, and starch content. The triple interaction
414 (DRS × KNO3 × PGPB) significantly affected proline and MDA, while the DRS × PGPB and
415 KNO3 × PGPB interactions influenced carbohydrate and starch levels (Table 4).

416 ***3.4.1. Proline Content***

417 Proline content increased with drought stress, peaking at 30.41 µmol/g FW in PGPB-
418 inoculated plants treated with KNO3 at 40% ETP. The lowest proline content (7.95 µmol/g
419 FW) was observed in non-inoculated plants at 100% ETP without KNO3 (Figure 12).

420

Table. 4. ANOVA for CCD design for biochemical characteristics.

| Sources | df | Mean squares | | | |
|---|---|---|---|---|---|
| | | Proline | MDA | Carbohydrate | Starch |
| Model | 7 | $88.48^{**}$ | $96.82^{**}$ | $24.84^{**}$ | $6.38^{**}$ |
| A-Drought | 1 | $763.89^{**}$ | $51.68^{**}$ | $119.07^{**}$ | $41.33^{**}$ |
| B-$KNO_3$ | 1 | $1.27^{**}$ | $53.34^{**}$ | $16.53^{**}$ | $1.24^{**}$ |
| C- PGPB | 1 | $10.37^{**}$ | $69.63^{**}$ | $20.43^{**}$ | $0.1925^{*}$ |
| AB | 1 | $0.5895^{**}$ | $4.69^{*}$ | $0.0002^{ns}$ | $0.0633^{ns}$ |
| AC | 1 | $74.70^{**}$ | $398.93^{**}$ | $8.04^{**}$ | $14.18^{**}$ |
| BC | 1 | $0.2441^{*}$ | $0.0025^{ns}$ | $0.6429^{*}$ | $0.5455^{**}$ |
| ABC | 1 | $0.7193^{**}$ | $7.71^{**}$ | $0.0623^{ns}$ | $0.0548^{ns}$ |
| CV (%) | | 0.9907 | 1.86 | 1.51 | 1.95 |
| $R^2$/Adj $R^2$ | | 0.99/0.99 | 0.99/0.99 | 0.99/0.98 | 0.99/099 |
| Predicted $R^2$ | | 0.99 | 0.97 | 0.98 | 0.96 |

*ns, *,**- not significant, sinificab=nt at 5 and 1% level of probability, respectively.*

### *3.4.2. Malondialdehyde Content (MDA)*

Figure 12 illustrates the three-dimensional response surfaces describing the combined effects of drought severity and $KNO_3$ application on key biochemical stress indicators under non-inoculated and PGPB-inoculated conditions. In general, drought stress induced marked biochemical alterations in Mexican lime seedlings; however, these responses were strongly modulated by bacterial inoculation and foliar nutrition. Proline content increased progressively with increasing drought severity, reflecting its role as a compatible osmolyte under water deficit. This accumulation was substantially enhanced by PGPB inoculation, particularly when combined with $KNO_3$ application. Under severe drought stress (40% ETP), inoculated plants treated with $KNO_3$ exhibited the highest proline levels, indicating improved osmotic adjustment and stress tolerance. In contrast, non-inoculated plants showed a comparatively weaker proline response, suggesting limited adaptive capacity under drought conditions.

Malondialdehyde (MDA), an indicator of lipid peroxidation and oxidative damage, increased markedly under drought stress in non-inoculated plants, confirming the occurrence of drought-induced oxidative stress. However, PGPB inoculation, especially in combination with $KNO_3$, significantly reduced MDA content at 40% ETP. This reduction indicates that the combined treatment effectively mitigated oxidative damage, likely through enhanced antioxidant activity and reduced accumulation of reactive oxygen species (ROS).

### *3.4.3. Carbohydrate Content*

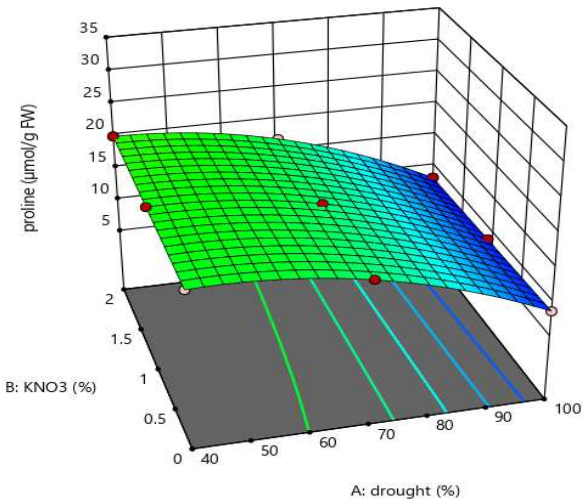
Carbohydrate content showed a contrasting trend. In PGPB-inoculated plants, soluble carbohydrate levels increased by 26.8%, 32.4%, and 41.6% at 100%, 70%, and 40% ETP, respectively, compared to non-inoculated plants. The highest carbohydrate content (23.17 mg $g^{-1}$ FW) was observed under severe drought stress (40% ETP) in inoculated plants, whereas the lowest value (11.86 mg $g^{-1}$ FW) occurred in non-inoculated plants under non-stress

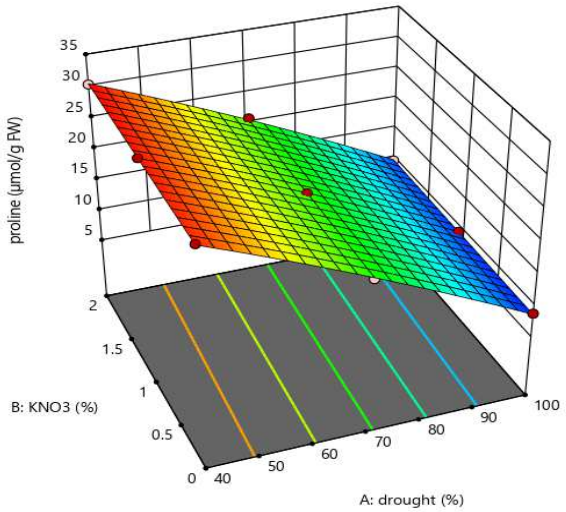
conditions. These results suggest that PGPB inoculation promotes carbohydrate accumulation under drought, contributing to osmotic regulation and metabolic stability. At full irrigation (100% ETP), carbohydrate levels increased in both inoculated and non-inoculated plants regardless of $KNO_3$ application, indicating a reduced influence of foliar nutrition under optimal water supply.

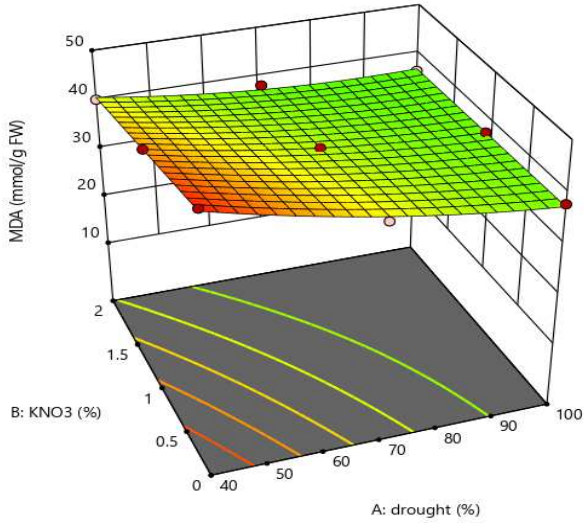
### *3.4.4. Starch Content*

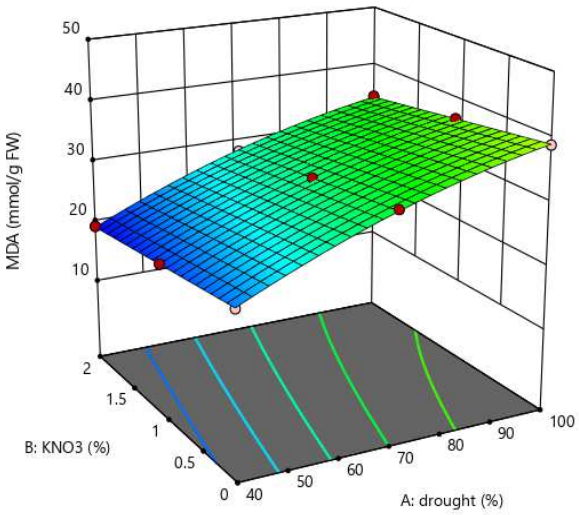
Starch content was strongly reduced by drought stress in non-inoculated plants, with a 60.8% decrease at 40% ETP compared to 100% ETP, reflecting enhanced starch degradation to meet energy demands under stress. In contrast, PGPB-inoculated plants exhibited only a 14.9% reduction in starch content under the same conditions, demonstrating a greater capacity to preserve carbon reserves. The highest starch content was recorded at 100% ETP with PGPB inoculation, and the combined application of $KNO_3$ and PGPB increased starch content by 14.8% compared to untreated controls.

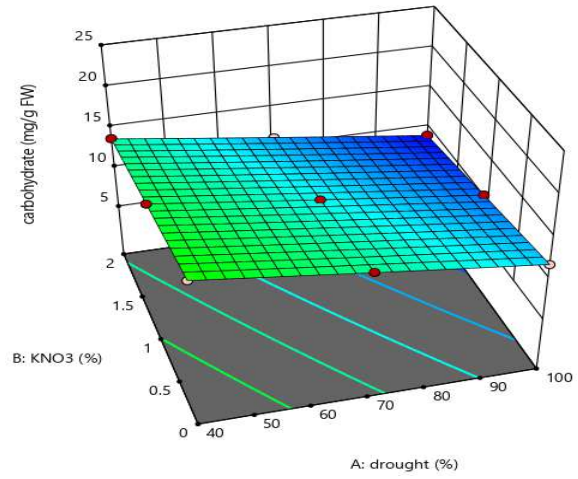
Overall, Figure 12 demonstrates that PGPB inoculation, particularly when combined with $KNO_3$ foliar application, shifts plant biochemical responses toward improved osmotic adjustment, reduced oxidative damage, and enhanced carbon reserve stability under drought stress. These coordinated biochemical adjustments underpin the improved drought tolerance observed in Mexican lime seedlings.

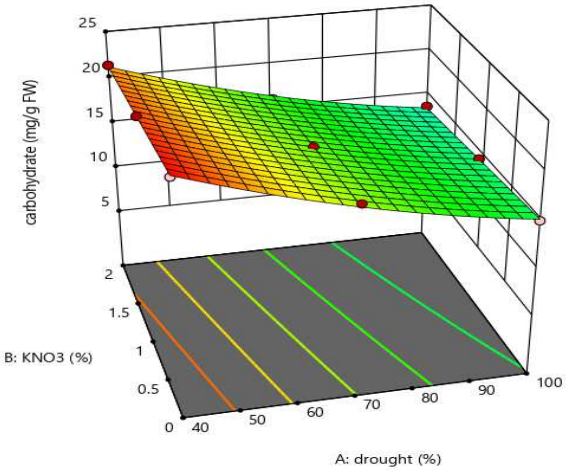

proline (µmol/g FW)
35
30
25
20
15
10
5
B: KNO3 (%)
2
1.5
1
0.5
0
40
50
60
70
80
90
100
A: drought (%)

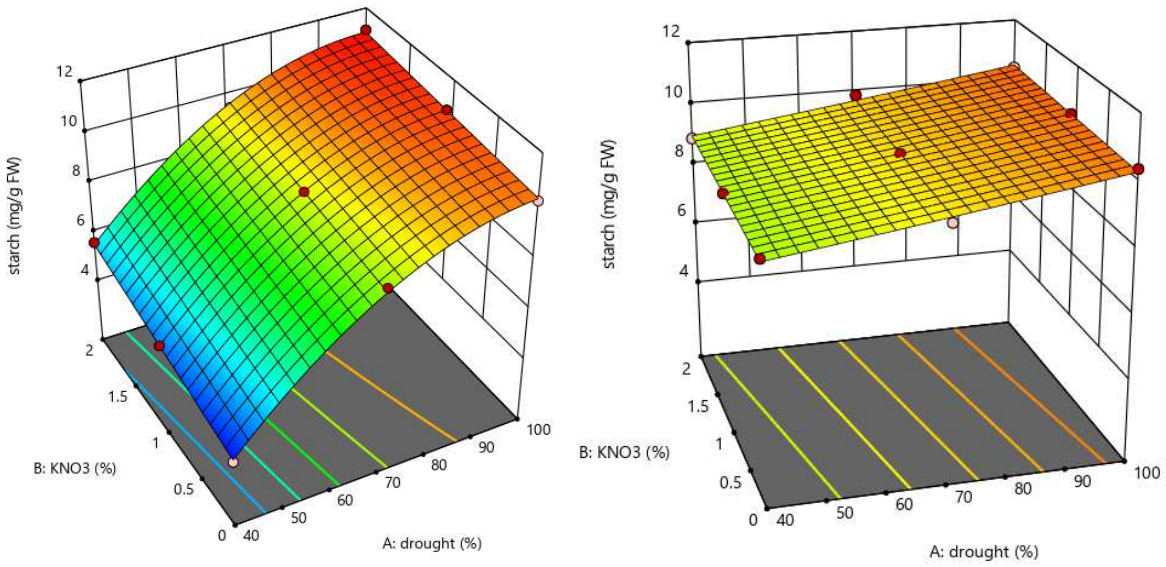

proline (µmol/g FW)
35
30
25
20
15
10
5
B: KNO3 (%)
2
1.5
1
0.5
0
40
50
60
70
80
90
100
A: drought (%)


MDA (mmol/g FW)
50
40
30
20
10
B: KNO3 (%)
2
1.5
1
0.5
0
40
50
60
70
80
90
100
A: drought (%)


MDA (mmol/g FW)
50
40
30
20
10
B: KNO3 (%)
2
1.5
1
0.5
0
40
50
60
70
80
90
100
A: drought (%)


carbohydrate (mg/g FW)
25
20
15
10
5
B: KNO3 (%)
2
1.5
1
0.5
0
40
50
60
70
80
90
100
A: drought (%)


carbohydrate (mg/g FW)
25
20
15
10
5
B: KNO3 (%)
2
1.5
1
0.5
0
40
50
60
70
80
90
100
A: drought (%)




*noninoculated* *Inoculation*

*Fig. 12. The major variables found in CCD that influence proline, MDA, carbohydrates, and starch are shown by three-dimensional (3D) response surface plots.*

CCD: Central Composite Design, MDA: Malondialdehyde

467

468

469 ***3.5. Optimization***

470 Using the Central Composite Design (CCD) coupled with response surface methodology,
471 the optimal management conditions were identified at 93.08% evapotranspiration potential
472 (ETP), 1.15% (w/v) $KNO_3$ foliar application, and PGPB inoculation. As illustrated in Figure
473 13 which depicts the optimization of input parameters of the model for enhancing plant
474 resistance under stress conditions, these optimized conditions resulted in pronounced
475 improvements in morpho-physiological and biochemical traits. Under the identified optimum,
476 photosynthetic pigments reached their predicted maxima (Chl a = 2.75, Chl b = 1.48, total
477 chlorophyll = 4.26 mg $g^{-1}$ FW, carotenoids = 1.10 mg $g^{-1}$ FW), accompanied by improved plant
478 water status (RWC = 49.20%) and canopy development (LAI = 1028.26 $cm^2$). Biomass
479 production was simultaneously maximized, with shoot fresh and dry weights of 46.17 g and
480 35.49 g, and root fresh and dry weights of 34.49 g and 18.79 g, respectively. In addition,
481 carbohydrate (14.78 mg $g^{-1}$ FW), starch (10.22 mg $g^{-1}$ FW), and proline (11.89 µmol $g^{-1}$ FW)

contents were enhanced, while stress-damage indicators were minimized, including electrolyte leakage (43.35%) and malondialdehyde content (35.28 nmol $g^{-1}$ FW). These results confirm the strong synergistic effect of moderate water supply, optimized $KNO_3$ nutrition, and PGPB inoculation in maximizing drought tolerance and physiological performance of Mexican lime seedlings.

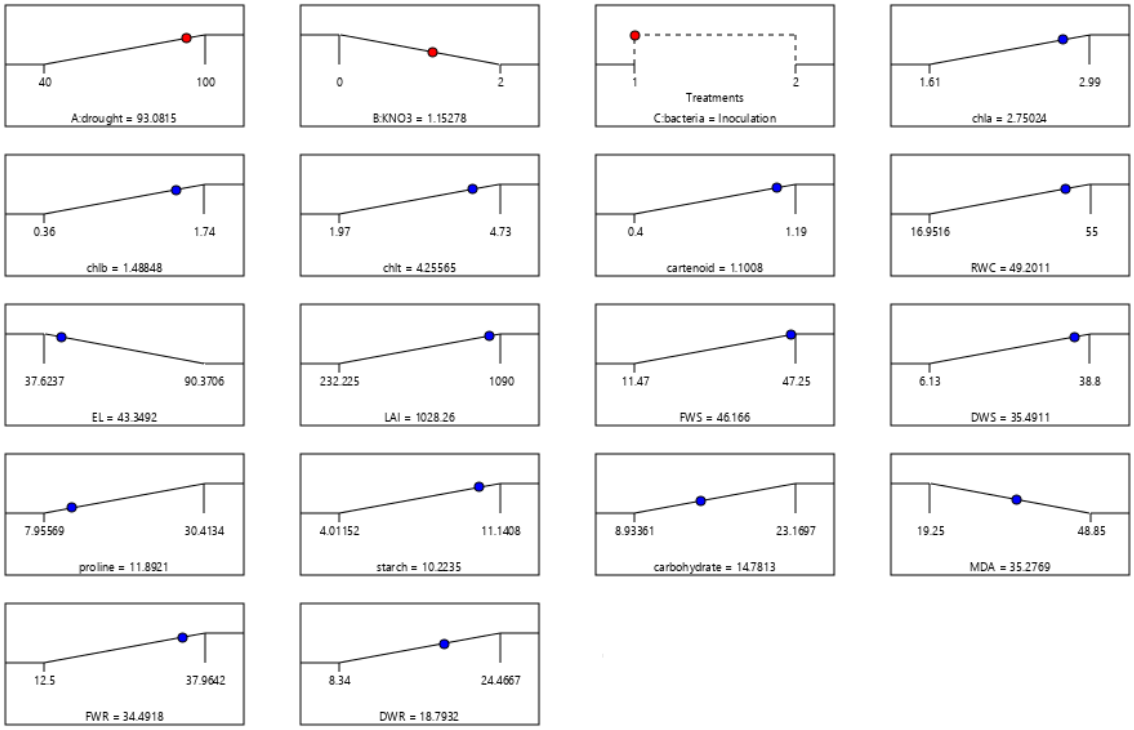



*Fig. 13. Optimization of input parameters of the model for plant resistance in stress conditions*

## *4. Discussion*

Water stress altogether disables citrus tree efficiency by compounding cellular harm through limited nutrient accessibility. Ideal potassium uptake and root colonization by plant growth-promoting microbes (PGPB) have been shown to moderate the unfavorable impacts of dry spell push (Manoj et al., 2020; Rajkumar, Bruno and Banu, 2017). In this study, both bacterial inoculation with Bacillus sp. CS1 and foliar application of potassium nitrate (KNO3)

alleviated drought stress in lemon trees, with their combined application yielding a more pronounced effect. Under drought conditions, *Bacillus* sp. CS1 inoculation significantly enhanced root growth and density, thereby increasing plant biomass. Comparable discoveries were detailed by (Saad and Abo-Koura, 2018), who watched improved biomass in sorghum vaccinated with PGPB beneath dry spell stretch, ascribing this to bacterial-mediated hormonal direction. Particularly, PGPB enhance phytohormone generation, such as auxins, which advance root and shoot advancement, in this manner maintaining biomass collection beneath push conditions(Gamalero and Glick, 2022).Also, foliar KNO3 application expanded dry weight in Citrus macrophylla beneath dry season stretch (Gimeno et al., 2014) and improved stem weight and plant length in spinach (Bukhari et al., 2021). Chlorophyll (Chl) substance is an imperative degree of plant resistance to stretch and photosynthetic capacity. Here, drought stress clearly reduced Chl content in lemon seedlings, possibly as a consequence of oxidative damage to chloroplasts. Under photosynthesis, electrons from chlorophyll can react with oxygen to form reactive oxygen species (ROS) like hydrogen peroxide ($H_2O_2$), disturbing metabolic homeostasis and inducing membrane injury (Gill and Tuteja, 2010), (Mignolet-Spruyt et al., 2016). In line with research on pistachio, tomato, and olive plants (Chitarra et al., 2016; Evelin et al., 2019; Nath et al., 2016; Shamshiri and Fattahi, 2014), Bacillus sp. CS1 inoculation mitigated these effects. Bacterial generation of phytohormones (such as cytokinins), siderophores, or high concentrations of auxins and 1-aminocyclopropane-1-carboxylate (ACC) deaminase can be the reason for these benefits. These compounds enhance thylakoid layer granule arrangement and increment the production of photosynthetic pigments, counting chlorophyll and carotenoids. These parameters too control the expression of qualities related with photosynthesis and the movement of photosynthetic proteins (Cortleven and Schmülling, 2015; Talla et al., 2016).

519 Drought stress enhanced electrolyte leakage (EL) in plants, mainly because of free radical
520 generation and peroxidation of lipids, disrupting lipid-protein interactions within membranes
521 and modifying ion transport (Ansari, Jabeen and Ahmad, 2021). Inoculation of PGPB greatly
522 diminished EL, in line with other plant species (Akhtar et al., 2021; Hussain et al., 2019). PGPB
523 enhance membrane integrity through alteration of the composition of fatty acids and restoration
524 of metabolic processes under drought stress (Batool et al., 2020; Singh and Jha, 2017). Foliar
525 KNO3 application also decreased EL, possibly because potassium plays a part in membrane
526 stabilization, improved osmotic adjustment, and organic salt synthesis.
527 This study also demonstrated that bacterial inoculation and KNO3 application increased
528 leaf area index (LAI) and relative water content (RWC) under drought stress, enhancing plant
529 resilience. Although the mechanisms by which PGPB increase RWC are not fully elucidated,
530 they likely involve the production of osmoprotectants, such as proline, which improve
531 membrane integrity, reduce EL, and minimize water loss. Additionally, PGPB-derived
532 hormones, such as auxins, stimulate root growth and lateral root development, enhancing water
533 and nutrient uptake (Cohen et al., 2015; de Araújo et al., 2020). Potassium supplementation
534 further increased LAI, dry matter production, and water retention, as previously reported
535 (Lindhauer, 1985).
536 Oxidative damage and lipid peroxidation, evaluated through malondialdehyde (MDA)
537 levels, were significantly reduced by Bacillus sp. CS1 inoculation under all levels of drought
538 stress. this reduction is most likely the result of enhanced antioxidant mechanisms protecting
539 cellular membranes, since PGPB enhance plant tolerance to environmental stresses (Sadeghi
540 and Khodakaramian, 2020). In addition, the combined application of KNO3 and PGPB
541 enhanced proline content, which, together with increased superoxide dismutase (SOD) activity,
542 improved membrane stability, osmotic adjustment, and ROS scavenging, alleviating drought
543 stress impacts (Ma et al., 2017; Rajkumar, Bruno and Banu, 2017). Foliar KNO3 application

544 also increased concentrations of organic salts, further facilitating osmotic balance (Soliman et
545 al., 2012).

546 PGPB inoculation increased the content of soluble sugars and free amino acids, thereby
547 triggering drought tolerance, as reported in previous studies (Kour and Yadav, 2022; Ngumbi
548 and Kloepper, 2016). In this study, seedlings inoculated with PGPB had a higher carbohydrate
549 and soluble starch content under drought stress compared to non-inoculated controls. The
550 combined application of PGPB and plant growth regulators has been found to preserve the
551 photosynthetic effectiveness of chickpea through increased soluble sugar accumulation, which
552 actuates the expression of qualities related to photosynthesis (Cabuslay, Ito and Alejar, 2002;
553 Cho et al., 2006; Nemeskéri et al., 2010). These sugars also act as osmoprotectants and
554 signaling atoms, controlling physiological forms essential for dry spell resistance (Sandhya et
555 al., 2010). Moreover, PGPB enhance nutrient uptake and soil structure through
556 exopolysaccharide generation, driving to expanded starch aggregation, which bolsters vitality
557 supply and stretch resistance (Fadiji et al., 2022). Potassium further promotes starch synthesis
558 by improving the action of chemicals such as sucrose-phosphate synthase and adenosine
559 diphosphate glucose pyrophosphorylase, basic for changing over sucrose into starch, in this
560 manner supporting plant development beneath stretch conditions (Dai, Yin and Wang, 2009).
561 Table 5 summarizes the key physiological and biochemical responses of Mexican lime to
562 drought stress and the mitigating roles of *Bacillus subtilis* CS1 and $KNO_3$ application. The
563 results indicate that PGPB predominantly improves root growth, water status, carbohydrate
564 metabolism, and antioxidant protection, while $KNO_3$ mainly enhances osmotic balance and
565 membrane stability. Their combined application shows a synergistic effect, leading to
566 improved growth performance and reduced oxidative damage under drought conditions.
567

Table 5. Summary of drought stress effects, mitigation mechanisms, and supporting evidence in Mexican lime.

| Supporting references | Aspect | Effect of drought stress | Effect of *Bacillus subtilis* CS1 | Effect of $KNO_3$ foliar application | Proposed mechanism |
|---|---|---|---|---|---|
| Rajkumar et al., 2017; Saad & Abo-Koura, 2018; Gimeno et al., 2014 | Plant growth and biomass | Reduced shoot and root growth due to limited nutrient uptake and cellular damage | Significant increase in root growth, density, and biomass under drought | Increased shoot and root dry weight under drought | Hormone-mediated root stimulation (auxins), improved nutrient acquisition |
| Gill & Tuteja, 2010; Shamshiri & Fattahi, 2014; Cortleven & Schmülling, 2015 | Photosynthetic pigments | Decrease in chlorophyll and carotenoids due to oxidative damage to chloroplasts | Mitigation of pigment loss and higher Chl and carotenoid content | Partial improvement of pigment stability | Enhanced phytohormone production, improved thylakoid organization, reduced ROS |
| Ansari et al., 2021; Batool et al., 2020; Singh & Jha, 2017 | Membrane stability (EL) | Increased electrolyte leakage due to lipid peroxidation | Strong reduction in EL under all drought levels | Decreased EL via potassium-mediated membrane stabilization | Fatty acid composition adjustment, osmotic regulation, membrane repair |
| de Araújo et al., 2020; Lindhauer, 1985 | Plant water status (RWC, LAI) | Reduced leaf area and relative water content | Significant increase in RWC and LAI | Further improvement in LAI and water retention | Osmoprotectant accumulation, enhanced root water uptake |
| Sadeghi et al., 2020; Ma et al., 2017 | Oxidative stress (MDA, ROS) | Elevated MDA due to ROS accumulation | Marked reduction in MDA under drought | Synergistic reduction when combined with PGPB | Enhanced antioxidant defense (SOD), ROS scavenging |
| Rajkumar et al., 2017; Soliman et al., 2012 | Osmolyte accumulation (proline) | Stress-induced increase | Strong stimulation of proline synthesis | Further enhancement in combined treatment | Osmotic adjustment, membrane protection |
| Ngumbi & Kloepper, 2016; Fadiji et al., 2022; Dai et al., 2009 | Carbohydrate metabolism | Excessive starch depletion and reduced soluble sugars | Higher soluble sugars and preserved starch reserves | Promotion of starch synthesis | Sugar signaling, energy buffering, enzyme activation |

## 5. Conclusion

The present study quantitatively demonstrated that drought stress substantially deteriorates the growth and physiological performance of Mexican lime (Citrus aurantifolia) seedlings. Under severe water deficit (40% evapotranspiration potential, ETP), shoot and root biomass, leaf area index (LAI), relative water content (RWC), and photosynthetic pigment concentrations were markedly reduced, while electrolyte leakage (EL) and malondialdehyde (MDA) increased, indicating pronounced membrane damage and oxidative stress.

Inoculation with Bacillus sp. CS1 significantly alleviated drought-induced damage, increasing shoot fresh and dry weights by 162.7% and 98.8%, respectively, and enhancing RWC by 48.2% under severe drought compared with non-inoculated controls. Leaf area was also markedly improved, showing a 240.1% increase at 40% ETP, reflecting enhanced canopy development and water retention. Biochemically, PGPB inoculation increased soluble carbohydrate content by up to 41.6% and limited starch depletion to 14.9%, compared with a 60.8% reduction in non-inoculated plants under the same stress level. Foliar application of 2% $KNO_3$ further contributed to drought mitigation by increasing shoot dry weight by 111.7% and reducing electrolyte leakage by 8.9–19.7% across drought levels, highlighting its role in membrane stabilization and osmotic regulation. Notably, the combined application of PGPB and $KNO_3$ produced the strongest synergistic response under severe drought, increasing leaf area by 7.98% and proline accumulation by 249.4%, while decreasing MDA content by 49.3% relative to untreated controls.

Optimization using response surface methodology identified 93.08% ETP, 1.15% (w/v) $KNO_3$ foliar application, and PGPB inoculation as the optimal management conditions for maximizing growth parameters, RWC, LAI, photosynthetic pigments, carbohydrate and starch reserves, and proline content, while minimizing EL and MDA. Collectively, these quantitative findings confirm that Bacillus sp. CS1 inoculation particularly when integrated with moderate

$KNO_3$ foliar nutrition provides an effective, sustainable, and numerically validated strategy for enhancing drought tolerance in citrus seedlings under water-limited conditions.

## Disclosure Statement

The creators pronounce no potential clashes of intrigued.

## Competing Interests Declaration

All authors of this manuscript declare that they have no competing interests. None of the authors have any financial, personal, or professional relationships with organizations, companies, or individuals that could potentially influence (or be perceived to influence) the content of the manuscript, the interpretation of results, or the decision to submit for publication. There are also no funding sources, indirect support, or other benefits that could be construed as a conflict of interest. There are no competing interests to declare.

## Data Availability Statement

The information supporting the conclusions of this article are accessible from the comparing creator, on ask at sensible take note.


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