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# Precision nanobubble irrigation tailors plant physiology to drive sustainable lettuce growth with water savings

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ABSTRACT

Under accelerating climate-driven water scarcity, improving irrigation efficiency for high-value leafy crops has become an urgent challenge for global food security. Nanobubbles (NBs), gas-filled cavities < 500 nm in diameter, have emerged as a promising irrigation technology capable of enhancing root-zone processes and early plant development with minimal additional water inputs. However, their effects in soil-based systems remain poorly resolved, particularly for leafy vegetables grown in water-stressed regions. Here, we systematically evaluate the influence of four gas types ($O_2$, $CO_2$, $N_2$, and air), delivered as NBs at three dilution levels (100 %, 50 %, and 10 %; corresponding to $>10^8$ particles $mL^{-1}$ at 100 %), on early-stage growth and water productivity (WP) in lettuce (*Lactuca sativa,* var. 'Little Gem') grown in a peat moss, coconut coir, and vermiculite mixture. Our results reveal that moderately diluted $O_2$ NBs (10 %–50 %) accelerate germination, boost biomass accumulation, and improve water savings by up to ~23 %. In contrast, high concentrations (100 %) of $O_2$ NBs reduced overall performance and induced elongated but narrow leaf morphology, consistent with stress-related growth allocation. The use of $CO_2$ NBs, particularly at higher concentrations, stimulates root expansion and leaf area development, while moderate $N_2$ NBs concentration enhanced ammonium uptake and root elongation. Air NBs produce modest and variable effects, serving as a baseline but never outperforming pure gas NBs. Together, these results demonstrate that gas-specific NBs treatments can be strategically tuned to regulate distinct physiological pathways during early plant development, supporting the potential of NBs-based irrigation as a tool for water-efficient, climate-resilient leafy crop production.

## 1. Introduction

Increasing water scarcity is reshaping global agriculture and posing a major threat to food security, as agriculture accounts for approximately 70 % of global freshwater withdrawals. With irrigation water availability declining, crop yields are increasingly constrained at a time when global food demand is projected to rise between 2010 and 2050, driven by population growth toward 9 billion people (FAO, 2022; Shah et al., 2018; van Dijk et al., 2021). If agricultural land area remains constant, food productivity will need to increase by approximately 35 %–56 %, underscoring the urgent need to maximize crop output per unit of water. Consequently, improving water productivity (WP), which directly links marketable crop yield to water consumption, has become a central goal of sustainable agriculture (Fernández, 2023; Hatfield and Dold, 2019). While water-saving technologies such as drip irrigation, partial root-zone irrigation, and hydroponic systems have been developed to reduce water inputs (Barbosa et al., 2015; Du et al., 2017; Liang et al., 2013; Lu et al., 2016; Manos and Xydis, 2019; Sidhu et al., 2021), achieving high WP without compromising yield and produce quality remains a critical challenge, highlighting the need for continued innovation and integrated solutions.

Soil aeration plays a key role in WP and overall crop productivity. Oxygen ($O_2$) is vital for root respiration, supporting energy-intensive processes like nutrient and water uptake. Roots access $O_2$ either

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through air-filled soil cavities or dissolved oxygen (DO) in irrigation water. However, atmospheric air contains 20.9 % $O_2$ (equivalent to 274 mg $L^{-1}$ of $O_2$ at 1 atm and 25°C), while water under the same conditions holds only 8.3 mg $L^{-1}$ of $O_2$ at saturation. In addition, $O_2$ diffuses approximately 10,000 times slower in water than in air, hindering $O_2$ transport in substrates and its availability to roots (Yafuso and Fisher, 2017). When soils lack sufficient $O_2$, whether from poor aeration or waterlogging, root function deteriorates, resulting in reduced plant growth and lower yield potential. Additionally, aerobic microorganisms essential for nutrient cycling are inhibited in low-$O_2$ environments, disrupting soil fertility and plant health (Bai et al., 2023; Bhattarai et al., 2005). Although injecting gaseous $O_2$ directly into the soil may seem like a possible solution, this approach suffers from poor efficiency due to rapid gas dissipation and limited contact time with root surfaces. In contrast, delivering $O_2$ through water may provide more sustained root exposure, as the dissolved gas travels with the irrigation front and remains in closer proximity to root surfaces for longer durations.

Root-surface oxygenation can significantly improve crop yields through bubble injection in subsurface drip irrigation (Abuarab et al., 2013), venturi and fluidic oscillator aeration systems (Lei et al., 2016), bubbling in hydroponics (Abu-Shahba et al., 2021), and the addition of chemicals, such as hydrogen peroxide (Abd Elhady et al., 2021; Wang et al., 2024). However, these techniques are still inefficient in terms of gas-liquid mass transfer, resulting in significant gas losses to the environment and limited $O_2$ saturation in the liquid medium. Over the past decade, nanobubble-based oxygenation has emerged as a transformative solution to address these inefficiencies. Nanobubbles (NBs), also known as ultrafine bubbles, are defined as gas-filled cavities in liquids with diameters smaller than 500 nm that exhibit remarkable physicochemical properties (Atkinson et al., 2019; Jia et al., 2023). Unlike macro- and microbubbles, which are limited by buoyancy and rapid gas escape, at the nanoscale bubbles create metastable gas domains. NBs do not experience buoyancy forces and are dictated by Brownian motion when suspended in solution, which enhances NBs gas-liquid contact and promote uniform dispersion in aqueous media (Azevedo et al., 2016; Yaparatne et al., 2022). This allows NBs to remain suspended, continuously saturating solution for extended periods, often persisting for days, weeks or even months after generation in airtight containers (Soyluoglu et al., 2021). More importantly, NBs offer a surface area over 1000 times greater than conventional bubbles, resulting in significantly improved mass transfer, from around 0.05 % of gas usage with macrobubbles to as high as 60 % with NBs (Magdaleno et al., 2023).

The unique properties of NBs position the technology as a promising solution for future agricultural practices (Morón-López et al., 2023; Pal and Anantharaman, 2022). Research across a variety of crops, including lettuce, carrot, fava bean, tomato, spinach and barley, has shown that NBs may accelerate seed germination and enhance early plant growth (Ahmed et al., 2018; Liu et al., 2017; Xue et al., 2023). One proposed mechanism underlying these effects is the generation of highly reactive oxygen species (ROS) during the collapse of NBs (Liu et al., 2017, 2015; Sun et al., 2022). These ROS are speculated to break seed dormancy, loosen cell walls, and facilitate key cellular signaling and metabolic processes, ultimately contributing to improved seed sprouting (Considine and Foyer, 2021). In addition, it has been suggested that the negative ζ-potential of NBs may facilitate the immobilization of soil ions via ion exchange with $H^+$ and $OH^-$, thus enhancing nutrient bioavailability and improving fertilizer utilization (Wang et al., 2021; Wu et al., 2019; Xue et al., 2022).

Beyond direct plant–soil interactions, NBs have also been reported to influence soil structure and microbial communities, promoting conditions favorable for root development (Chen et al., 2023; Zhou et al., 2020). Importantly, NBs have been shown to effectively reduce biofouling and organic deposition in agricultural water distribution systems, thereby lowering the risk of emitter clogging during drip irrigation and improving system performance (Xiao et al., 2020). Collectively, these effects have been linked to improved plant yields, crop quality, and reduced water usage in greenhouse crop production (Liu et al., 2019; Ouyang et al., 2021).

Despite recent advances in NBs applications for plant growth, critical uncertainties remain regarding their effects in soil-based systems, particularly for leafy crops. Previous studies have explored various bubble sizes within the nano- and microbubble range, tested with different gases such as $O_2$, carbon dioxide ($CO_2$), nitrogen ($N_2$), and air (Ahmed et al., 2018; Iijima et al., 2020; Liu et al., 2015), and examined distinct bubble concentrations and saturation levels (Iijima et al., 2021; Xue et al., 2023; Yan et al., 2023). Researchers have also delved into the NBs mechanisms and key plant-soil interactions, including ROS generation, seed germination, nutrient uptake, and yield improvement across diverse crop species (Liu et al., 2016; Sun et al., 2022; Yan et al., 2023). However, this body of work remains highly fragmented, often relying on limited sample sizes, single-gas treatments or narrow concentrations ranges, and rarely targeting leafy vegetables. In lettuce, one of the most widely consumed leafy vegetables worldwide, experimental evidence remains particularly scarce, with existing studies typically including lettuce only as one of several test species rather than as a dedicated model crop. As a result, meaningful cross-study comparisons are challenging and, more critically, potential trade-offs associated with NBs irrigation remain poorly resolved. For leafy crops, enhanced early growth or biomass accumulation does not necessarily translate into improved market quality, as it may be accompanied by adaptive morphological and physiological responses in leaves, such as altered shape or uniformity (Yang et al., 2025). Because most previous studies prioritize yield-related endpoints, these morphology-related drawbacks have largely been overlooked.

Moreover, studies specifically addressing the impact of NBs on WP are notably scarce (Liu et al., 2019; Zhou et al., 2019a). This gap is especially critical given that over half of the global expansion of irrigated agriculture in the past 25 years has occurred in regions experiencing water stress (Mehta et al., 2024). A representative example is the lower Colorado River basin, including Yuma, Arizona, which supplies the majority of winter lettuce consumed in the United States while simultaneously facing severe water scarcity and increasing competition for limited water resources (Abdelmohsen et al., 2025; Barbosa et al., 2015). In such systems, even modest improvements in WP can have substantial implications for agricultural sustainability, underscoring the urgent need to rigorously assess NBs irrigation strategies that enhance productivity while conserving water.

In this study, we address these gaps by conducting a statistically robust evaluation of NBs irrigation effects on early-stage lettuce growth. We systematically compare four gases ($O_2$, $CO_2$, $N_2$, and air) delivered as NBs (~100 nm) across three different concentration levels (100 %, 50 %, and 10 % NB water solutions), based on a large experimental dataset ($n = 75$ plants per treatment). Focusing on seed germination and early seedling development (up to 10 days), a critical stage influencing subsequent plant performance and yield, this study pursues four main objectives: (i) to quantify the effects of gas type and NBs concentration on germination dynamics and early seedling growth; (ii) to evaluate NBs-induced changes in seedling morphology, with particular emphasis on leaf traits; (iii) to assess how NBs irrigation influences biomass accumulation and WP; and (iv) to examine changes in nutritional composition, providing mechanistic insight into the physiological responses underlying observed growth, morphological, and biomass outcomes. Together, these objectives enable a comprehensive assessment of both the benefits and trade-offs of NBs irrigation during early lettuce development, with direct relevance to water-efficient and commercially viable crop production.

## 2. Material and methods

### 2.1. Nanobubble generation and characterization

All NBs solutions were generated using a Gaia Ultra-Fine Bubble generator (UFB-75) equipped with each specific gas source to produce ultrafine gas bubbles with diameters less than 500 nm. High-purity gases (≥99 %, provided by Arizona State University Gas Services) of oxygen ($O_2$), carbon dioxide ($CO_2$), nitrogen ($N_2$) and air were used for these experiments. The NBs generator operated by continuously injecting pressurized gas into 1 L of ultrapure water (Elga Lab Water, resistivity > 18.2 MΩ cm at 25 °C) at controlled flow rates (1 L $min^{-1}$). A sparging pressure of 10 psi was maintained to ensure consistent NBs production. The stability and concentration of NBs in the solution were characterized immediately after generation and at intervals of 5, 10, 20, 30, 45, and 60 min post-sparging.

Different NBs concentration (100 %, 50 %, and 10 %) were prepared by diluting NBs solution water with ultrapure water. It is important to note that absolute NBs concentrations and size distributions can vary substantially depending on the water matrix, gas type, and NBs generator, even when identical percentage dilution levels are applied; therefore, the NBs solution percentages reported in this study should be interpreted as relative dilution levels and benchmarked against the quantitative characterization data provided in Fig. 1. In this study, bubble size and distribution were measured by a nanoparticle tracker analyzer (NTA, NanoSight Pro, Malvern Panalytical), while ζ-potential was measured using NanoBrook 90 Plus PALS (Brookhaven Instruments). Dissolved oxygen (DO) levels were quantified with a DO meter (YSI ProDSS). The solution pH, temperature, and conductivity were also monitored to ensure consistent experimental conditions. All produced NBs solutions were stored in airtight Pyrex® glass bottles without headspace until use. Storage was limited to two days to ensure the stability and effectiveness of the NBs for experimental use. Control water consisted of ultrapure water without NBs.

### 2.2. Seed germination tests

Germination assays were conducted using lettuce seeds (*Lactuca sativa*, var. 'Little Gem') purchased in January 2024. Prior to the experiment, viable seeds were selected by rinsing and soaking in deionized water for 2 h. The germination system consisted of Petri dishes lined with sterile filter paper to evaluate radicle emergence and hypocotyl development. For each treatment, 60 seeds were placed in Petri dishes, which were sealed with Parafilm to reduce evaporation. Dishes were incubated at 25 ± 2°C under a 16:8 h light:dark photoperiod, using LED grow lights (photosynthetic photon flux density of 180 μmol $m^{-2}$ $s^{-1}$, GPS 4′ Clone Light, GrowPros) inside a greenhouse grow tent (Gorilla Grow Tent). Germination progress was monitored daily for 4 d, and seeds were considered germinated upon hypocotyl emergence. Germination rate (%) and mean germination time ($t$) were calculated for each treatment.

### 2.3. Seedling development and growth

Soil-based plant growth experiments were performed using the same *Lactuca sativa* (var. 'Little Gem') seeds in seedbeds filled with horticultural soil mix (Seed Starting Mix – Jiffy), composed of peat moss, coconut coir, and vermiculite. Prior to sowing, the seedbeds were premoistened for 24 h with the respective NBs treatments or NBs-free control solution. A total of 300 seeds per treatment (3 seeds per pot) were sown at a depth of 1 cm, and subsequently irrigated daily with 3 mL of the corresponding treatment solution. Pots were maintained in a greenhouse (Gorilla Grow Tent) at 25 ± 2 °C under a 16:8 h light:dark photoperiod. Air recirculation was provided by fan aeration, and lighting was supplied by LED lights (photosynthetic photon flux density of 180 μmol $m^{-2}$ $s^{-1}$, GPS 4′ Clone Light, GrowPros). The experiment was conducted over a 10-day period to assess early growth responses.

To ensure uniformity, only the first seedling to emerge in each pot was considered germinated; any subsequent seedlings were removed, maintaining one plant per pot for the duration of the experiment. Although 100 pots were initially established per treatment, not all

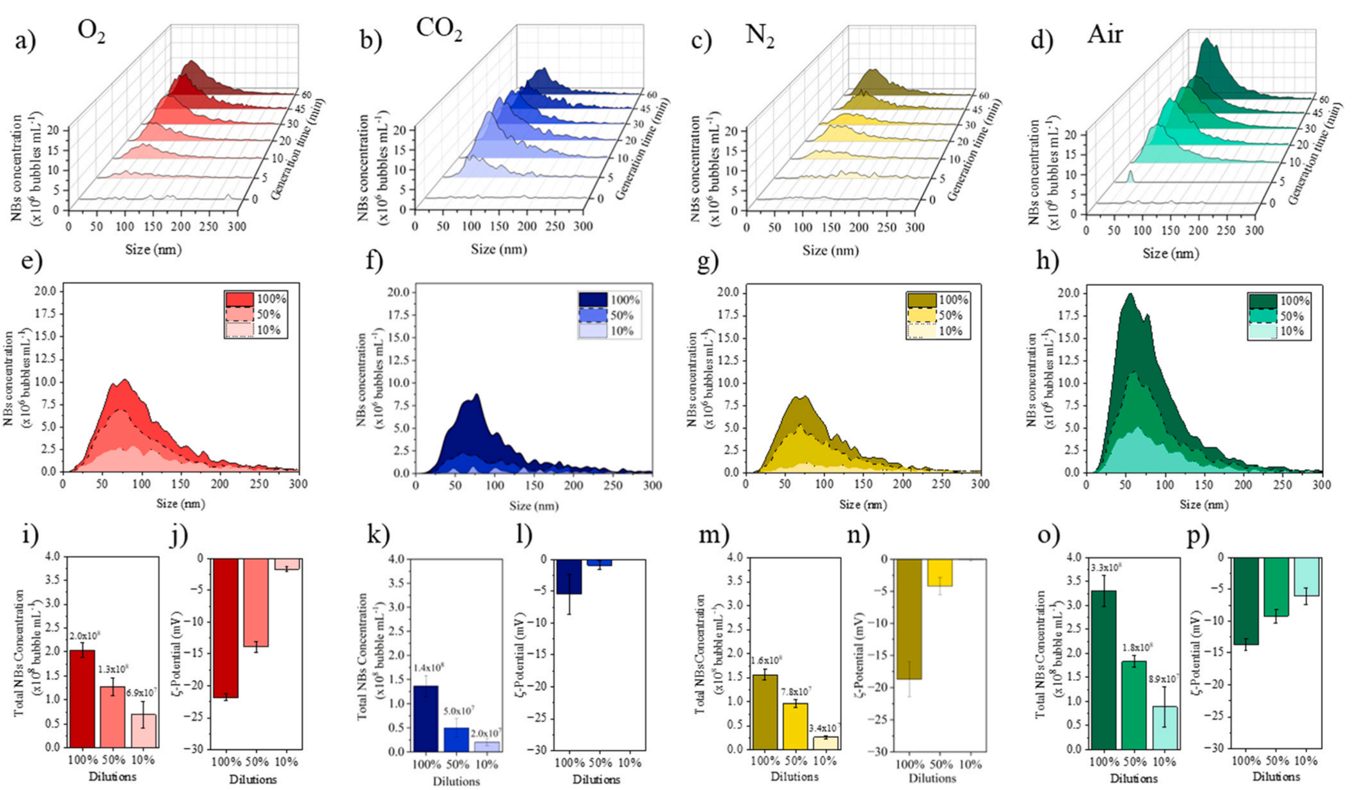


**Fig. 1.** Characterization of NBs generated with $O_2$ (red), $CO_2$ (blue), $N_2$ (yellow), and air (green), across various dilution ratios (undiluted [100 %], 50 %, and 10 %): (a–d) NBs concentration and size distribution over generation time; (e–h) NBs concentration and size distribution at different dilution levels; (i, k, m, o) Total NBs concentration across dilutions; and (j, l, n, p) corresponding ζ-potential measurements.

seedlings developed properly. Therefore, to standardize the evaluation across treatments, only the first 75 healthy and fully developed plants per treatment were assessed at the end of the 10-day period. This duration was selected based on preliminary one-month trials (data not shown) and guidelines for optimal seedling transplanting in hydroponic systems. It is important to note that no nutrients other than those present in the soil were added to the plants. On day 10, leaf chlorophyll content was measured using a SPAD meter (Minolta SPAD-502) as a proxy for plant stress.

Plants were irrigated daily with 2 mL of the respective water treatment solutions. On day 10, plant growth metrics, including leaf dimensions (length and width), stem height, root length, and total biomass, were measured. Leaf area was estimated by multiplying leaf length and width and applying a correction factor to account for variations in leaf shape. A correction factor of 0.45 was used, which was derived using ImageJ software based on measurements from 10 randomly selected plants.

### 2.4. *Analysis of biomass, soil conditions and water productivity*

Biomass was assessed by measuring the fresh weight of 75 entire plants (leaves, stems, and roots) after thorough root cleaning and rinsing. The samples were then oven-dried at 80 °C for 3 days to determine dry weight. Seedbeds subjected to various NBs treatments were photographed on day 10 of the experiment using a thermal camera (E5 Pro, Flir) to measure soil temperature. The camera was calibrated with a temperature range of 19°C to 27°C. Images were captured 24 h after the final irrigation at a height of approximately 1 m. Post-capture image processing and analysis were performed using the Flir software. Additionally, soil pH was monitored daily using a soil-specific pH meter (BlueLab) to ensure consistent environmental conditions across treatments. Water consumption was recorded throughout the experiment by monitoring the daily irrigation volume per plant. Water Productivity (WP) was calculated and normalized as the ratio of total dry biomass (kg) to total water consumed (L $day^{-1}$).

### 2.5. *Characterization of Mineral Content and Nutrient Composition*

Nutrient analysis of lettuce samples was conducted by collecting, dehydrating, then grinding plants by mortar and pestle. All plants within each treatment were combined to obtain sufficient biomass. A mass of approximately 150 mg of the ground samples were then digested in plasma pure $HNO_3$ by boiling the samples for approximately 1 h. The digestate was then allowed to cool, diluted with ultrapure $H_2O$, and analyzed by inductively coupled plasma mass spectroscopy (ICP-MS). ICP-MS analysis was conducted on a Perking Elmer NexION 1000 model, via a 5-point calibration with excellent linear correlations ($R^2 > 0.999$) for each analyte.

The quantification of N-species was conducted via extraction process. Approximately 150 mg of the dried and ground lettuce samples were dispersed in 30 mL of 1 M $H_2SO_4$ and placed on a table shaker at 100 rpm, for 24 h. Following agitation, samples were centrifuged and the supernatant was then diluted by a factor of 2X. Nitrate ($NO_3^-$), nitrite ($NO_2^-$), and ammonium ($NH_4^+$) concentrations were quantified using a HACH DR6000 UV–vis spectrophotometer. Briefly, $NO_3^-$ was determined by differential absorbance at 220 and 275 nm after acidification, $NO_2^-$ by the Griess assay at 540 nm, and $NH_4^+$ by the salicylate method at 655 nm. Total inorganic nitrogen (TN) was calculated as the sum of $NO_3^-$–N, $NO_2^-$–N, and $NH_4^+$–N concentrations. Additional methodological details are reported in Flores et al., (2025).

### 2.6. *Statistical analysis*

All statistical analyses were performed in R version 4.4.1. Data were LOG-transformed to meet the assumptions of normality and homoscedasticity prior statistical analyses. An analysis of variance (ANOVA) was then conducted to assess significant differences in plant trait variables across treatment concentration and metal groups, followed by Tukey's Honest Significant Difference (HSD) post-hoc test for pairwise comparisons. A Principal Component Analysis (PCA) was subsequently carried out to explore patterns in nutrient absorption under varying treatment concentrations. All analyses were performed using the *stats* package from base R, and all plots were generated using the *ggplot2* package. Statistical hypotheses were tested at 99.9 %, 99 % and 95 % level of significance.

## 3. Results and discussion

### 3.1. *Nanobubble generation and stability*

The agricultural potential of NBs stems largely from their unique physicochemical properties, particularly their size distribution which allows for enhanced gas delivery to liquid solutions (Atkinson et al., 2019; Favvas et al., 2021; Xu et al., 2023). These factors are tightly interdependent, as NBs form in gas-supersaturated environments, and their presence may enhance gas retention in water. This relationship is particularly important in the context of plant irrigation, where both the physicochemical profile of NBs and the degree of gas enrichment can influence plant growth and soil processes, especially through their effects on microbial activity and nutrient cycling (Chen et al., 2023; Xue et al., 2022). Our results show that NBs concentrations increased rapidly, reaching approximately $10^8$ particles $mL^{-1}$ within 10 min for $O_2$, $CO_2$, $N_2$, and air (Fig. 1a-d). Concurrently, bubble size distributions evolved from initially broad and heterogeneous during the first 10–20 min of generation to progressively narrower and more uniform distributions between 30 and 60 min, indicating stabilization of the NBs size distribution over time. After one hour of generation, the maximum concentrations achieved were $3.3 \times 10^8$, $2.0 \times 10^8$, $1.6 \times 10^8$, and $3.3 \times 10^8$ particles $mL^{-1}$ for air, $O_2$, $CO_2$, and $N_2$, respectively (Fig. 1i, k, m, o), and the mean bubble size was consistent across all gas types, measuring $68 \pm 10$ nm (Fig. 1e-h). These results are in line with previous studies showing NBs sizes typically around 100 nm (Favvas et al., 2021; Nirmalkar et al., 2018; Ushikubo et al., 2010).

NBs generation altered the ζ-potential of the solution, resulting in −13.7 mV for air, −21.8 mV for $O_2$, −5.5 mV for $CO_2$, and −18.7 mV for $N_2$. According to DLVO (Derjaguin–Landau–Verwey–Overbeek) theory, more negative ζ-potential values enhance electrostatic repulsion and are generally associated with increased colloidal stability, whereas values close to neutrality (±10 mV) indicate reduced electrostatic stabilization. The gas-dependent differences observed here are consistent with previous reports showing that $O_2$ and $N_2$ bubbles generally acquire a more negative surface charge through preferential adsorption of hydroxide ions at the gas–water interface, promoting greater stability (Karimi et al., 2024; Ma et al., 2022). In contrast, $CO_2$ NBs undergo partial dissolution that forms carbonic acid and releases protons, lowering local pH and shifting the ζ-potential toward less-negative or near-neutral values, which is expected to reduce electrostatic stabilization (Antonio Cerrón-Calle et al., 2022). Dilution of the suspensions to 50 % and 10 % lowered NBs concentrations proportionally without noticeably altering bubble size (Fig. 1e–h) (Arias-Sanchez et al., 2026). Yet the ζ-potential became progressively less negative with dilution (Fig. 1j, l, n, p), supporting the view that NBs behave as colloidal entities whose electrostatic stabilization depends on surface charge density and ionic strength (Jadhav and Barigou, 2021; Yadav et al., 2024). Reducing the number of bubbles, and thus the total interfacial area, diminishes the overall surface charge in the system, compresses the surrounding electrical double layer, and leads to a measurable decrease in ζ-potential (Fig. 1j, l, n, p). Together, these observations underscore the pivotal role of gas chemistry and colloidal interactions in governing NBs stability and reactivity in agricultural solutions.

Gas saturation levels varied depending on the gas injected as NBs (Figure S1 in the supplementary material). The DO levels increased rapidly to 35 mg $L^{-1}$ within 5 min following the generation of $O_2$ NBs, whereas air NBs maintained DO below the typical saturation levels observed in water, i.e. approximately 8.2 mg $L^{-1}$. In contrast, the injection of $CO_2$ and $N_2$ NBs led to a sharp decline in DO concentration within 5 min due to $O_2$ displacement. This observation highlights that $CO_2$ and $N_2$ NBs solutions were effectively depleted in DO, emphasizing that both gas enrichment and gas displacement effects (i.e. anoxic solutions) must be considered when irrigating with NBs-enriched water containing gases other than $O_2$ or air (Xue et al., 2022). Additionally, the generation of $CO_2$ NBs caused a notable pH reduction, with values dropping below starting pHs, while other gases showed no measurable effect on pH. This result aligns with the ζ-potential data, reinforcing the idea that gas-specific chemical properties influence both interfacial charge and solution chemistry. Gas supersaturation persisted across all conditions until the end of the experiment. In diluted NBs treatments, the addition of NBs-free water led to proportional decreases in gas saturation; however, saturation levels remained consistently higher than in the NBs-free control across all dilution levels (Table S1). These findings underscore the ability of NBs to maintain elevated gas concentrations in solutions, even after significant dilution, which may have important implications for controlled delivery of gases in agricultural systems.

### 3.2. Impact of nanobubbles on seed germination rates

The positive effect of NBs on seed germination has been consistently reported across various plant species, including lettuce, carrot, barley, spinach, tomato, and fava bean (Ahmed et al., 2018; Liu et al., 2016, 2015; Singh et al., 2024). This enhancement has been associated to radical species weakening cell walls and increasing water permeability, thereby facilitating germination (Liu et al., 2017; Sun et al., 2022). In our study, $O_2$ and air NBs increased germination on day 1 across all dilutions (Fig. 2). The strongest response was observed with 50 % $O_2$ NBs, which boosted germination by 28.3 % on day 1. This aligns with previous findings showing that the high ROS levels from $O_2$ and air NBs improved germination in several species (Ahmed et al., 2018), with oxidative capacities of NBs estimated to be equivalent to 0.5 and 0.3 mM of hydrogen peroxide (Liu et al., 2015).

In our results, the next most effective treatments after 50 % $O_2$ NBs were 50 % air NBs and 100 % $N_2$ NBs, which increased germination rates by 20 % and 16.7 %, respectively. The influence of NBs concentration, modulated through dilution, has also been reported for spinach and carrot (Liu et al., 2016), and our findings support this pattern in lettuce. The presence of $N_2$ NBs have also been shown to enhance germination and hypocotyl elongation in lettuce and tomato, but not in carrot (Ahmed et al., 2018; Xue et al., 2023). Results presented here also support this observation in lettuce, further suggesting that the mechanisms underlying NBs-induced germination may be crop-specific. Notably, undiluted (100 %) $CO_2$ NBs, characterized by both a lower pH and a higher concentration of NBs (Fig. 1), also led to improved germination compared to the control. This finding is consistent with previous studies indicating that $CO_2$ NBs may enhance seed germination (Ahmed et al., 2018; Singh et al., 2024), but it further demonstrates that such effects are dependent on sufficiently high NBs concentrations. These findings imply that the germination-promoting effects observed may be primarily attributed to the physical presence and properties of NBs, aligning with the emerging hypothesis that ROS may contribute to the observed physiological responses (Liu et al., 2016, 2015).

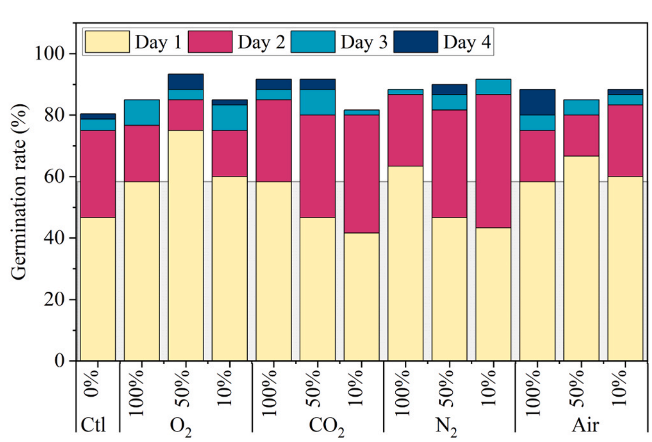


**Fig. 2.** Effect of different gases and dilutions on the germination rate (%) of lettuce measured in Petri dishes.

### 3.3. Effect of Different Nanobubbles Gases on Seedling Growth and Leave Morphology

Fig. 3 illustrates the growth performance of seedlings ($n = 75$) after 10 days of exposure to various NBs solutions. Seedlings treated with NBs exhibited accelerated growth, characterized by earlier leaf emergence compared to the control group. Notably, irrigation with $O_2$ and $CO_2$ NBs significantly ($p < 0.001$) increased leaf production, with treated plants producing 4 %–18 % more leaves than the control (Fig. 4). The observed benefits of $O_2$ NBs may be linked to their influence on soil structure and rhizosphere microbial dynamics (Chen et al., 2023; Zhou et al., 2020). Increased $O_2$ availability in the root zone may improve nutrient uptake, stimulate root development, and enhance overall plant health, ultimately promoting leaf formation and greater resilience to stress (Niu et al., 2012; Xue et al., 2023; Yan et al., 2023). For instance, Singh et al., (2024) reported a significant increase in the number of leaves in tomato plants exposed to $CO_2$ NBs. The authors suggested that NBs act as efficient carriers of nutrients and water, thereby promoting more vigorous plant growth.

In contrast, irrigation with $N_2$ and air NBs did not result in a statistically significant increase in leaf number during germination (Fig. 4). These findings are consistent with previous studies in bean and carrot crops, where $O_2$ and $CO_2$ NBs treatments promoted leaf production, but $N_2$ and air NBs did not (Ahmed et al., 2018; Xue et al., 2023). While $N_2$ NBs did not result in an immediate increase in leaf number in our study, other studies have indicated that they can improve soil $N_2$ availability, potentially by increasing $NH_4^+$ levels and reducing $PO_4^{3-}$ release (Xue et al., 2022). Ahmed et al., (2018) did report enhanced leaf production in tomato plants irrigated with $N_2$ NBs observable after 37 days of growth, suggesting that the effects of $N_2$ may become evident over longer exposure periods. Induced shifts in soil nutrient dynamics may contribute to plant growth and leaf development over extended periods, which warrants further investigation in the future.

NBs treatments not only induced changes in leaf counts, but also significant ($p < 0.05$) improvements in terms of width, length and area of the leaves (Fig. 3 and Figure S2). The most striking effects were observed under high-concentration $CO_2$ NBs (100 % and 50 %), which increased leaf area by 48 % and 68 % ($p < 0.001$), respectively. This result may be partially explained by a "chimney effect" (Abuarab et al., 2013), in which $CO_2$ released from the soil diffuses upward and temporarily enriches the air around the aerial part of the plant. Elevated local $CO_2$ availability can stimulate photosynthetic demand, prompting plants to increase light interception to maximize carbon assimilation. This coordinated response may involve upregulation of Calvin cycle enzyme activity and expansion of leaf area, ultimately improving overall photosynthetic performance (Pal and Anantharaman, 2022; Pan et al., 2020). Low-concentration treatments with 10 % NBs of $O_2$, $N_2$, and air also significantly promoted leaf development, resulting in leaf area increases of 52 % ($p < 0.001$), 69 % ($p < 0.001$), and 31 % ($p < 0.01$), respectively. These findings are consistent with previous studies in tomato, carrot, and bean crops, where $N_2$ NBs preferentially enhanced leaf dimensions, followed by $CO_2$ NBs (Ahmed et al., 2018). Our results also suggest that air NBs may still positively influence leaf dimensions, as reported in other studies in *Brassica campestris* (Ebina et al., 2013),

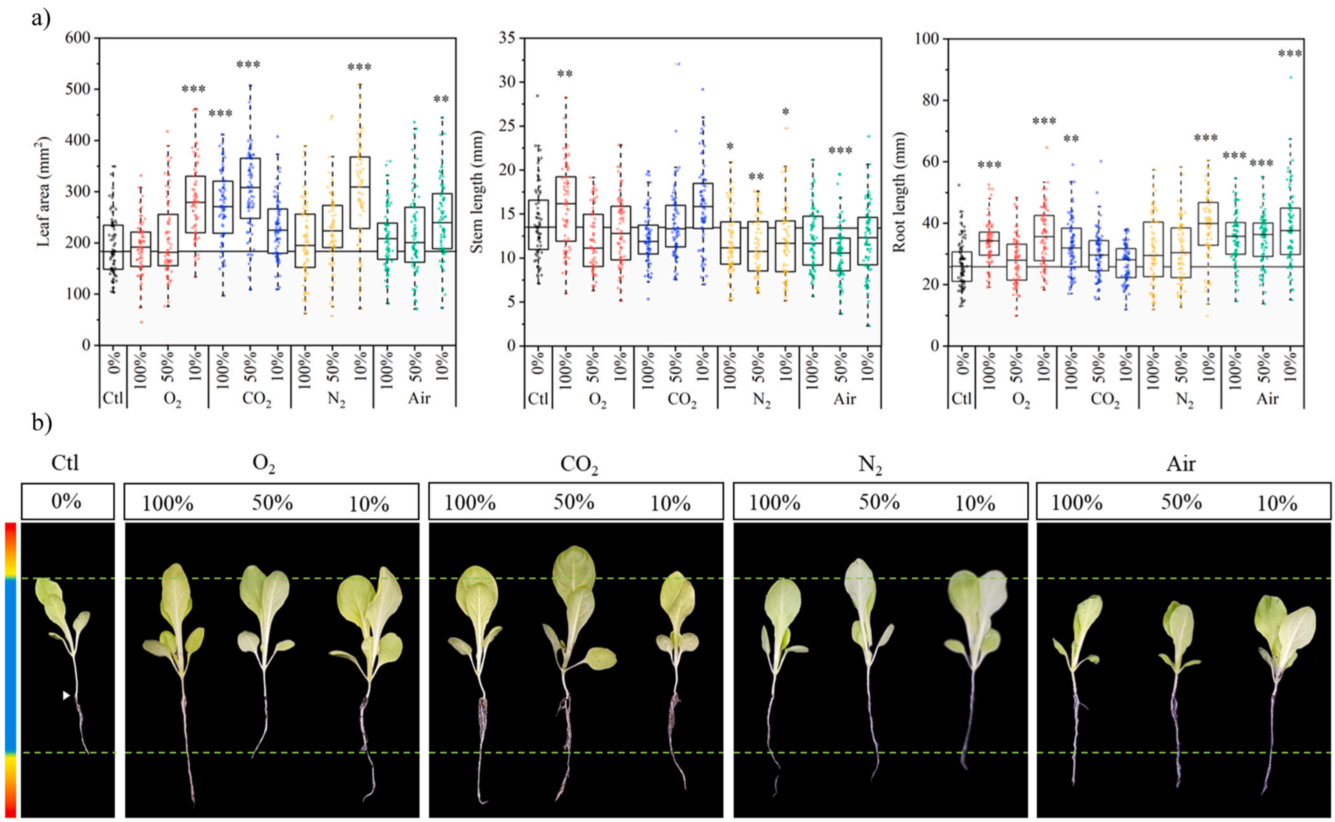


**Fig. 3.** Influence of gas type and NB dilution on (a) leaf area, stem length, and root length of lettuce seedlings. (b) Representative images of lettuce growth phenotypes under different NB treatments compared to the control. The arrow indicates the crown of the lettuce, marking the same reference point for all plants shown. Significant differences are presented with *p*-values representing $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$(***).

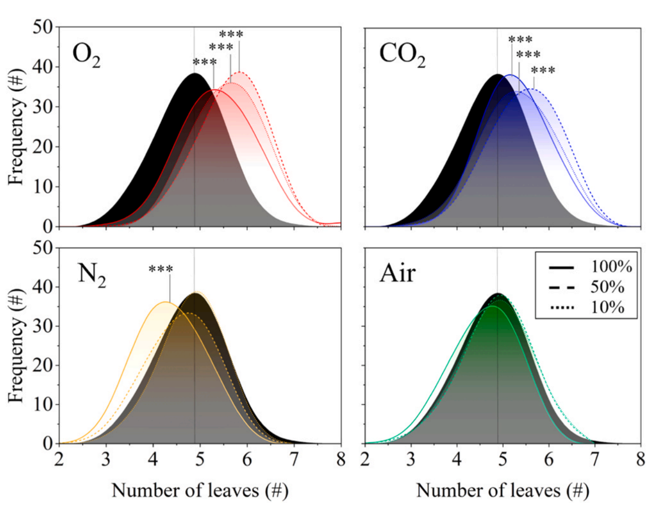


**Fig. 4.** Influence of gas type and NB dilution on the frequency of the number of leaves per plant. Significant differences are presented with *p*-values representing $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$(***).

although to a lesser extent than other gas types.

A notable morphological response was observed in seedlings treated with 100 % $O_2$ NBs, where leaves exhibited a distinctively narrow shape (Fig. 3b and Figure S2). These leaves showed a significant increase of 18 % ($p < 0.001$) in length compared to the control but the smallest recorded width ($p < 0.01$), highlighting the nuanced effects of $O_2$-enriched NBs irrigation on leaf morphology. Such responses are consistent with physiological stress induced by hyperoxic conditions in the root zone, which can disrupt normal tissue expansion. Elevated $O_2$ availability may also promote photorespiration, competitively inhibiting photosynthetic $CO_2$ fixation and reducing carbon-use efficiency, ultimately contributing to narrower and less robust leaves (Ku and Edwards, 1977). Supporting this interpretation, studies on tomato and maize reported that DO levels above 20 mg $L^{-1}$ did not enhance plant growth (Xue et al., 2023; Zhou et al., 2019a), while high NBs concentrations have been linked to excessive oxidative stress and reduced antioxidant capacity (Yan et al., 2023). The fact that the more robust increases in leaf area observed under lower NBs concentrations of $O_2$ and $N_2$ suggests that moderate NBs exposure favors healthy development, whereas extreme concentrations of these gases may impose physiological stress.

Irrigation with NBs also influenced stem and root growth, with responses varying by gas composition. Treatments with 100 % $O_2$ resulted in significantly increased stem elongation ($p < 0.01$). The combination of elongated stems and the notably narrow leaf morphology observed under this treatment suggests a resource allocation trade-off, whereby plants prioritize vertical growth over foliar development. This pattern is consistent with a stress-related response, potentially mediated by ROS-induced signaling and altered auxin distribution (Considine and Foyer, 2021). Conversely, treatments with $N_2$ and air NBs consistently produced significant shorter stems in lettuce, with a maximum reduction of 24 % ($p < 0.001$) in plants treated with 50 % air NBs, in line with previous studies in bean and tomato (Ahmed et al., 2018; Xue et al., 2023).

The NBs treatments also stimulated root elongation, with root length increasing up to 54 % compared to the control ($p < 0.01$) (Fig. 3). While previous studies have shown that improved water oxygenation via $O_2$ NBs promotes root growth in maize, tomato, cucumber, and spinach (Ikeura et al., 2018; Zhou et al., 2019a, 2019b), our results demonstrate that $CO_2$, $N_2$, and air NBs can also enhance root elongation, indicating that this response is not exclusive to $O_2$ delivery. At the physiological level, NBs-induced root stimulation has been linked to the activation of growth-related genes and hormone-regulated signaling pathways (Wang et al., 2021). Root architectural plasticity is tightly regulated by ROS-mediated signaling, which enables plants to adjust water and nutrient acquisition in response to environmental constraints (Ramachandran et al., 2024). Our findings suggest that NBs treatments promote such adaptive responses when applied at moderate concentrations, where ROS function as regulatory signals rather than stressors (Mittler, 2017). In contrast, excessive gas exposure is likely to disrupt redox homeostasis, leading to ROS accumulation associated with abnormal growth (Huang et al., 2019). Among the treatments, $CO_2$ NBs produced the most pronounced effect, stimulating extensive secondary root proliferation (Fig. 3b). This enhanced root architecture suggests that plants irrigated with $CO_2$ NBs were transitioning to more advanced vegetative stages. Together, these findings demonstrate that the type and concentration of NBs play a critical role in shaping plant morphology and physiology, highlighting their potential to enhance growth through targeted manipulation of gas compositions.

### 3.4. Biomass and Water Productivity

The observed improvements in leaf area, stem length, and root development (Figs. 3 and 4) translated into substantial gains in both fresh and dry weights following just 10 days of irrigation with NBs water is shown in Fig. 5. Most NBs treatments significantly enhanced the fresh weight of seedlings, with increases of up to 38 %, 41 %, and 54 % ($p < 0.001$) for plants irrigated with 50 % NBs of $O_2$, $CO_2$, and $N_2$, respectively. Additionally, air NBs significantly enhanced fresh weight by 24 % at 50 % NBs concentration and by 35 % at 10 % of NBs solution ($p < 0.001$). Prior hydroponic studies using microbubbles in lettuce have suggested that the surface area and negatively charged surfaces of bubbles may enhance nutrient uptake by attracting positively charged ions in solution (Park and Kurata, 2009). Given their enhanced surface properties and stability, NBs may further amplify these mechanisms in our experiments, contributing to the observed gains in plant growth and biomass. Interestingly, thermal imaging captured on day 10 revealed that high $O_2$ NBs concentrations (100 % and 50 %) raised soil temperature, likely indicating increased microbial activity, while $CO_2$ NBs lowered it, possibly due to shifts in microbial communities and enhanced soil wettability (Figure S3).

After desiccation, total plant weight decreased substantially, with weight reductions of 94–96 % (Figure S4), consistent with the typical water content of healthy Romaine lettuce. Analysis of dry weights showed that high concentrations (100 %) of $O_2$, $N_2$, and air NBs did not outperform the control, supporting the notion that excessive NBs concentration or extreme DO levels, whether too high or too low, may not favor biomass accumulation. Nonetheless, our findings support previous reports that observed biomass accumulation with NBs irrigation (Ahmed et al., 2018; Xue et al., 2023; Yan et al., 2023), as lower concentrations (50 % and 10 %) of $O_2$, $N_2$ and air NBs did significantly increase dry weight ($p < 0.05$), with $O_2$ and $N_2$ NBs achieving gains up to 30 % (Fig. 5). The use of $CO_2$ NBs, however, showed a distinct trend, with significant increases of 14 % and 27 % at the highest (100 % and 50 %) concentrations ($p < 0.001$), but only a 7 % improvement with 10 % NBs treatment. This contrasting trend may be attributed to enhanced $CO_2$ volatilization at higher concentrations and higher photosynthesis activity.

A WP analysis, normalized by estimated lettuce yield, indicated that NBs-treated plants reduced water consumption during germination by 62–690 L per kg of dry weight (Fig. 6). The greatest water savings (~23 %) were observed in plants watered with 10 % $O_2$ and $N_2$ NBs, followed by treatments with 50 % $CO_2$ NBs. As observed with morphological and biomass metrics (Figs. 3 and 5), treatments with 100 % $O_2$, $N_2$, and air NBs showed no substantial improvements in the WP. These results indicate that, while certain NBs treatments may improve water savings by promoting efficient water-to-biomass ratios of growth, excessive gas concentrations may disrupt this balance, ultimately impairing the water footprint. This highlights the need to optimize NBs concentrations for effective irrigation strategies, particularly during early growth stages.

### 3.5. Changes in ion content and nutrient composition

Elemental profiling of lettuce seedlings revealed substantial alterations in $N_2$ species and micronutrient concentrations across NBs treatments compared to the control (Fig. 7). Notably, seedlings exposed to higher concentrations of $O_2$ NBs exhibited increased TN accumulation, characterized by a marked elevation in $NH_4^+$ levels and a slight increase in $NO_3^-$ content. This pattern suggests that elevated $O_2$ saturation may play a dual role by enhancing nitrification in the root zone and subsequently facilitating $NO_3^-$ uptake by the plant. Once absorbed, $NO_3^-$

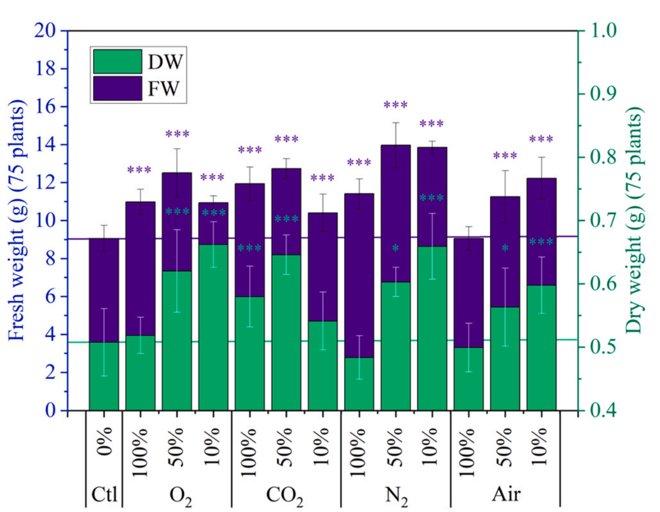


**Fig. 5.** Effect of gas type and NB dilution on the fresh (FW) and dry weight (DW) of lettuce seedlings. Values represent the average weight per set of 25 plants (75 plants in total) after 10 days of irrigation. Significant differences are presented with $p$-values representing $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$(***).

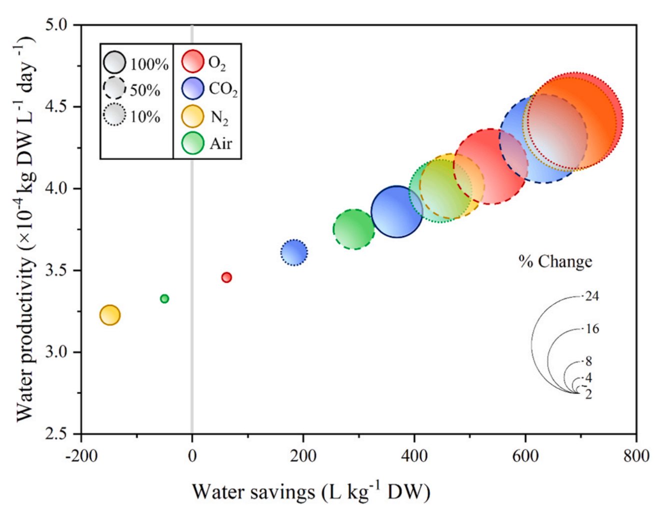


**Fig. 6.** Water productivity (WP) of plants irrigated with different gas types and NBs dilutions during the germination stage. The vertical line at zero on the x-axis represents the control treatment. Values to the left indicate higher water consumption per unit dry weight (DW) than the control (negative water savings), whereas values to the right indicate reduced water consumption per unit DW relative to the control (positive water savings).

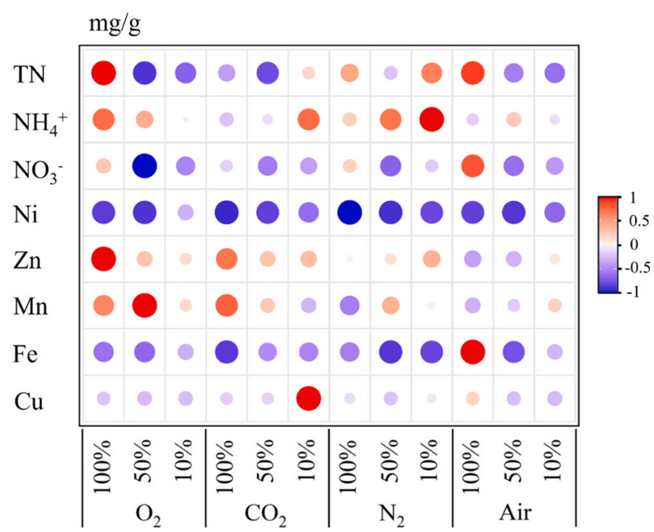


**Fig. 7.** Heatmap showing normalized z-scores of $N_2$ species and micronutrient concentrations in lettuce seedlings under different gas types and NBs dilutions. Red indicates increased values; blue indicates decreased values relative to control.

is reduced to $NH_4^+$ within the plant and assimilated into organic compounds such as amino acids and proteins. The elevated $NH_4^+$ levels observed under high $O_2$ NBs conditions may therefore reflect both increased nitrification in the soil and stimulated $N_2$ assimilation within the plant, driven by high DO availability. Previous studies have reported that $O_2$ NBs improve soil structure and nutrient availability by stimulating ammonia-oxidizing bacteria and increasing TN in soil (Chen et al., 2023; Zhou et al., 2020). This reinforces the idea that $O_2$ NBs facilitate both abiotic and biotic mechanisms contributing to $N_2$ enrichment. A similar trend in TN accumulation was observed in seedlings treated with increasing concentrations of air NBs. However, in this case, the increase was primarily due to higher $NO_3^-$ levels, with no corresponding rise in $NH_4^+$. This indicates that air NBs, containing ambient levels of $O_2$, may support nitrification in the rhizosphere but do not enhance $NH_4^+$ assimilation within plant tissues to the same extent as pure $O_2$ NBs.

Conversely, seedlings treated with $CO_2$ and $N_2$ NBs displayed an inverse pattern. While $NO_3^-$ concentrations remained unchanged or slightly decreased, $N_2$ NBs treatments led to a marked increase in $NH_4^+$ accumulation (Fig. 7). This pattern aligns with previous findings indicating that $N_2$ NBs may enhance soil $N_2$ availability by promoting $NH_4^+$ release (Xue et al., 2022). In the case of $CO_2$ NBs, a notable increase in $NH_4^+$ was observed only at the lowest concentration tested. At higher $CO_2$ NBs concentrations, this effect was diminished. This could be more attributable to reduced $O_2$ availability in the soil, which may suppress nitrification and limit $NO_3^-$ uptake by the plant. Under such conditions, any increase in $NH_4^+$ may result from greater assimilation capacity associated with the observed enhanced biomass production, though this likely depends on the presence of at least minimal $O_2$ to sustain certain metabolic and microbial activity in the root zone.

On the other hand, distinct patterns in micronutrient concentrations were observed across NBs treatments, particularly for Zn and Mn (Fig. 7). Seedlings exposed to high concentrations of $O_2$ and $CO_2$ NBs showed notable enrichment in these two metals. Interestingly, even lower concentrations of $N_2$ NBs, and therefore certain $O_2$ availability, also resulted in slightly higher Zn and Mn levels compared to the control. These findings align with a consistent pattern reported across NBs studies, in which NBs irrigation enhances micronutrient mobility and uptake (Singh et al., 2024; Xue et al., 2022; Yan et al., 2023). Within this framework, Zhang et al., (2024) showed that $O_2$ NBs irrigation increased Zn accumulation in plant roots while reducing Zn availability in the surrounding soil, supporting the idea that NBs facilitate nutrient translocation from the soil into plant tissues rather than simply increasing bulk solubility. Enhanced uptake of micronutrients such as Mn and Zn can elicit positive physiological responses, as Mn is essential for photosynthesis, enzyme activation, and overall metabolism, while Zn is involved in hormone production, chlorophyll synthesis, and enzymatic function (Alejandro et al., 2020; Hamzah Saleem et al., 2022). However, the elevated accumulation of these micronutrients under high NBs concentrations may have unintended consequences. Excess Zn and Mn can induce oxidative stress, disrupt enzyme systems, and impair overall physiological performance. Our results therefore indicate that the reduced performance of plants treated with 100 % $O_2$ NBs is likely driven by disruptions in metal uptake caused by the NBs (Figs. 3, 5 and 6).

In contrast, most NBs treatments were associated with reduced levels of Ni, Fe, and Cu relative to the control. Unlike Mn and Zn, these elements are generally less responsive to short-term rhizosphere perturbations, as Fe readily forms insoluble oxides under oxidizing conditions and requires reduction or chelation to become plant-available, Cu strongly complexes with soil organic matter, and Ni, despite being relatively mobile, has a low plant demand and can be readily excluded or displaced by competing cations (Ju et al., 2019; Shahzad et al., 2018; Shi et al., 2018). Because NBs irrigation did not significantly alter soil pH in this study (Table S2), the observed shifts in micronutrient profiles are unlikely to be driven by pH-mediated solubility changes. Instead, these patterns are consistent with gas-specific rhizosphere effects, although potential microbial contributions were not directly evaluated and warrant further investigation.

Overall, the observed micronutrient shifts highlight that NBs treatments exert multifaceted effects, not only on plant physiological processes and biomass accumulation, but also on nutrient acquisition and elemental balance. The interplay between NBs gas type, concentration, and rhizosphere responses underscores the importance of tailoring NBs gas strategies to optimize early-stage plant development. At the same time, these findings indicate that inappropriate gas selection or excessive NBs dosing may disrupt micronutrient homeostasis, emphasizing the need for careful control and system-specific optimization when deploying NBs technology. Future work should therefore evaluate NBs irrigation across later developmental stages, diverse soil types, and real-world irrigation systems, while also assessing economic feasibility, energy demand, and scalability. With such advances, strategically tuned NBs irrigation has the potential to enhance physiological performance and WP while supporting the sustainable intensification of lettuce cultivation and other leafy crops.

## 4. Conclusion

This study demonstrates that NBs irrigation can be strategically tailored to enhance early lettuce growth and WP through gas- and dose-specific effects. Moderate $O_2$ NBs concentrations (10–50 %) consistently improved germination, biomass accumulation, and WP, whereas fully saturated $O_2$ treatments induced altered leaf morphology and reduced performance, indicating physiological stress under hyperoxic conditions. $CO_2$ NBs at higher concentrations (50–100 %) promoted leaf expansion, root development, biomass production, and WP, suggesting that elevated $CO_2$ availability can stimulate early growth demand during germination. In contrast, $N_2$ NBs primarily enhanced early $NH_4^+$ uptake and seedling vigor at low to moderate concentrations, while higher concentrations produced variable responses, underscoring the need for careful optimization. Air NBs resulted in comparatively modest effects, highlighting the advantage of using targeted pure-gas treatments to achieve more pronounced physiological responses. Overall, these findings emphasize the importance of jointly optimizing NBs gas composition and concentration to match early developmental stages, enabling growth stimulation and improved WP while avoiding physiological and morphological stress. Extending this work across the full plant life cycle will be an important next step to evaluate the persistence and agronomic relevance of these responses. From an agricultural perspective, NBs systems could be readily integrated into existing irrigation infrastructures (*e.g.*, drip or fertigation systems) to enable gas- and dose-specific water treatments, particularly in water-limited environments where improvements in WP and early growth may translate into enhanced yield potential and resource sustainability. Future studies incorporating rhizosphere microbial characterization would further strengthen understanding of the mechanisms underlying NBs–plant interactions.

## CRediT authorship contribution statement

**Jesús Morón-López:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Aislinn Varela:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Renato Montenegro-Ayo:** Validation, Methodology, Investigation, Formal analysis. **Andrea Maya:** Validation, Methodology, Investigation, Formal analysis. **Mariana Hernandez-Molina:** Validation, Investigation, Formal analysis. **Kenneth Flores:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Emily E. Matula:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **John Graf:** Writing – review & editing, Validation, Methodology, Conceptualization. **Onur Apul:** Writing – review & editing, Visualization, Validation, Formal analysis, Conceptualization. **Sergi Garcia-Segura:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agwat.2026.110149.

## Data availability

Data will be made available on request.

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