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Emil Joakim Wolff Anthony
MSc in Plant Science

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Emil Joakim Wolff Anthony
MSc in Plant Science
Norwegian University of Life Sciences
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Abstract

Nanobubbles (NBs) are minuscule gas bubbles with distinct properties that have shown promise for application in hydroponic horticulture. This study aimed to evaluate the effect of O_2 NBs on plant growth and tipburn injury of crispy lettuce (*Lactuca sativa* var. *crispa*) cultivated in a commercial nutrient film technique (NFT) system. Seedlings were transplanted to NFT channels that were irrigated with a O_2 NB super-oxygenated nutrient solution or a non-aerated control. At harvest (4–5 weeks after transplanting), there were no significant differences between the two treatments in terms of shoot fresh weight, leaf length, number of leaves, or tipburn severity and incidence. In conclusion, NBs may not promote growth of certain crops in systems where plant roots are not continuously submerged or exposed to oxygen- or nutrient-limiting conditions.

1 Introduction

Nanobubbles (NBs; also known as bulk NBs or ultrafine bubbles) are tiny gaseous bubbles generally considered to be < 1 µm in diameter (Alheshibri et al., 2016; Tsuge, 2014). Due to their minuscule size, NBs possess distinct properties in water when compared to ordinary macrobubbles (Tsuge, 2014; Ahmed et al., 2018b). Similar to microbubbles (between 10 µm and 100 µm in diameter; Tsuge, 2014), NBs have very long residence time in water, due to their low buoyancy and large surface:volume ratio which also allows for high gas dissolution, as well as a negative surface charge (ζ potential; Tsuge, 2014; Ahmed et al., 2018a). This negative charge cause NBs to not attract each other, thus providing resistance to coalescence and formation of larger bubbles, but instead attract positively charged materials and ions like Ca and Mg (Takahashi, 2005; Ushikubo et al., 2010; Park et al., 2010). Furthermore, NBs gradually decrease in size and ultimately collapse creating shock waves which, in turn, generate reactive oxygen species (ROS; mainly hydroxyl radicals) that may, under certain conditions, stimulate plant physiological activity (Ushikubo et al., 2010; Ahmed et al., 2018b).

In past studies, the application of micro- and nanobubbles in hydroponic horticulture to deliver dissolved gas into liquid solutions have yielded promising results on the promotion of plant growth (e.g., Park et al., 2010; Ebina et al., 2013; Ahmed et al., 2018b; Tsutsumi et al., 2020). Park and Kurata (2009) first reported that the fresh weight of leaf lettuce treated with microbubbles in a Deep Flow Technique (DFT) system was 2,1-fold larger than those treated with macrobubbles. Importantly, the authors found that there were no differences in pH, electrical conductivity (EC) and dissolved oxygen (DO) levels between the two treatments. Nevertheless, the mechanisms of how micro- and nanobubbles promote growth are still uncertain and results vary based on, among others, nutrient status, crop species and the concentration of micro- and nanobubbles used (Iijima et al., 2020, 2021).

Hydroponic lettuce production is commonly troubled with quality and yield loss caused by the physiological disorder of tipburn characterised by necrotic leaf tips and margins (Saure, 1998; Samarakoon et al., 2020). This disorder is thought to be caused, in large part, by a physiological deficiency of Ca as it is an element that cannot be mobilized within the plant (Saure, 1998; Frantz et al., 2004).

As most of the previous studies on the application of micro- and nanobubbles were carried out in DFT or Deep Water (DWC) cultures, the primary objective of this study was to evaluate if nanobubbles could improve hydroponic nutrient film technique (NFT) production of crispy lettuce, which is widely used in Norway. Particularly, the aim was to investigate the effect of irrigation with a super-oxygenated nutrient solution injected with pure O_2 NBs on plant growth, development, and tipburn injury of crispy lettuce 'Frillice' in a commercial NFT system.

2 Materials and Methods

Plant material and experimental growth conditions. *Lactuca sativa* var. *crispa* 'Frillice' plants were seeded in peat-filled pots in polystyrene trays and germinated in a glass greenhouse under supplementary high-pressure sodium (HPS) lamps at approx. 18°C for around two weeks. Following this, seedlings were transplanted to NFT grow channels of a O_2 NB treatment or a control treatment for the remainder of the culture period, which varied from 28–37 days after transplanting. Here, the plants ($n = 40$ per treatment) were maintained in a glass greenhouse compartment with horizontal air flow fans under supplementary HPS lamps at an average daily temperature of 19,1 ± 1,1°C with elevated CO_2-levels of 1100 ppm and a mean water vapour pressure deficit of 0,73 ± 0,12 kPa (mean relative humidity of 73 ± 12%) and a mean daily light integral of 13,8 ± 3,8 mol m^{-2} d^{-1} in 20 h photoperiods. On average, irradiance was supplemented with 10,6 ± 7,3 h of 145,5 ± 15,4 µmol m^{-2} s^{-1} (SpectroSense 2, Skye Instruments Ltd, Powys, UK) from the HPS lamps.

The NFT channels on the NB treatment table were irrigated with nutrient solution from a NB oxygen-supersaturated reservoir while the control table was irrigated with nutrient solution from the regular irrigation system. Both tables were irrigated identically in terms of frequency and duration following the regular operation plan. Electrical conductivity of the nutrient solutions were maintained at approx. 2,2 dS/m and pH at 5,8.

The experiment was carried out in a commercial system at a greenhouse grower in Lier, Norway from February 2021 until May 2021 and from September 2021 until November 2021. Plants for the NB treatment and the control were sowed, transplanted, and harvested on the same days.

Nanobubble generation and measurements of dissolved oxygen. O_2 NBs were generated continuously from injection of pure oxygen (Nippon Gases Norge, Oslo, Norway) through a ceramic membrane under a flow of approx. 0,05 L min^{-1} into the NB nutrient solution reservoir ensuring concentrations of around 21,1 ± 4,8 mg O_2 L^{-1} at all times. The reservoir was refilled continuously with complete nutrient solution directly from the regular fertilizer mixer system that also irrigated the control.

DO concentrations and water temperatures of the nutrient solutions were measured regularly with a handheld oxygen meter (Handy Polaris 2, Oxyguard International A/S, Farum, Denmark) at various points in the irrigation cycle from the fertilizer mixer to the nanobubble nutrient solution reservoir as well as to the drip and return for both treatments by sampling 10 mL of solution with a syringe. DO concentration in the nanobubble nutrient solution reservoir were also monitored continuously with a dissolved oxygen transmitter (IXIAN-DO, Atlas Scientific, Long Island City, NY, USA) connected to the climate computer.

Plant growth measurements. Five plants from each of the treatment and the control were systematically sampled from the irrigation drip side to the return side of the grow channels (i.e., sampling the head closest to the drip first and the remaining heads while nearing the return) at the end of the culture period. For each plant, the shoot fresh weight (FW), total number of true leaves (greater than 1 cm in length), and length of the longest leaf were recorded.

Evaluation of tipburn injury. Outer and inner tipburn injury were evaluated on a whole-head basis for each sample on a scale of 0-5, where 0 = no visible symptoms, 1 = few necrotic patches on the margins of one-to-few leaves, 2 = necrotic patches on most margins of few-to-several leaves, 3 = few-to-several margins are mostly necrotic, 4 = large necrotic patches on one-to-few leaves, and 5 = severe necrotic patches throughout several leaves. For each sample, the frequency of tipburn injury were measured as the fraction of tipburn injured leaves of the total number of leaves.

Data analysis. Data was analysed using GraphPad Prism 9.3.0 (GraphPad Software, San Diego, CA, USA) for macOS. Variations in dissolved oxygen levels were tested by a one-way analysis of variance and a subsequent *post-hoc* Tukey's multiple comparison test was used to identify significant differences between the sample points ($p < 0{,}05$). Linear regression models were fitted to each measured growth and tipburn parameter for the separate treatments and subsequently tested for significant differences using analyses of covariance (ANCOVA; $p < 0{,}05$).

3 Results

Dissolved oxygen concentrations in the nutrient solutions. The DO concentration of the NB nutrient solution reservoir was significantly greater than the DO level of the solution used to irrigate the control (Figure 1; Tukey's multiple comparison, $p < 0{,}05$). However, DO levels declined significantly already from the NB reservoir to the irrigation drip and even more so to the end of the NFT channel at the return (Tukey's multiple comparison, $p < 0{,}05$). In contrast, there were no differences in DO concentrations at any cycle points for the control. The water temperatures were consistent throughout the cycle points.

Plant growth and development. Treatment of crispy lettuce plants in NFT channels with a O_2 NB-supersaturated nutrient solution for the duration of the production cycle after transplanting did not result in significant differences in shoot FW, total number of leaves or length of the longest leaf when compared to a control (Figure 2A, B, C; ANCOVA, $p < 0{,}05$). In addition, there were no significant differences in any of the measured parameters between plants sampled closest to the irrigation drip and those sampled closest to the return in either treatment (data not shown).

Tipburn incidence and severity. In general, tipburn injury was more common and more severe on older, outer leaves than on younger, inner leaves. Overall, the mean frequency of

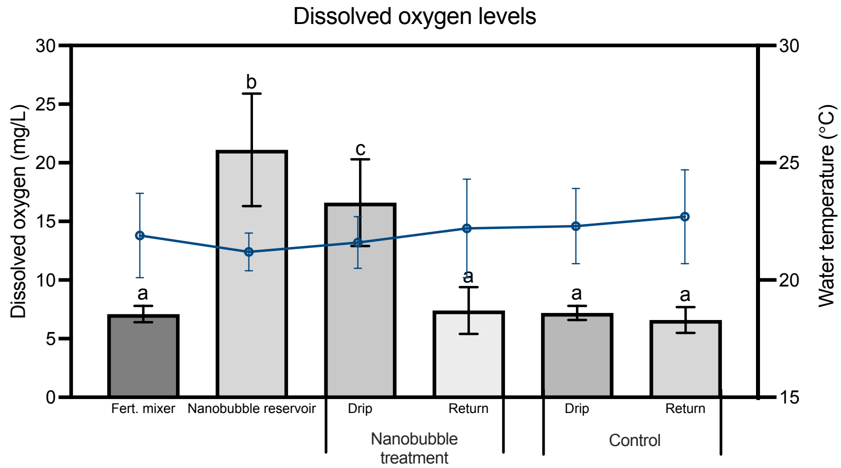

Figure 1: Dissolved oxygen concentrations (bars, left y-axis) and water temperatures (dots, right y-axis) at various points in the irrigation cycle for nanobubble treatment and the control. Nutrient solution from the fertilizer mixer was used for directly irrigating the control and in the nanobubble reservoir that was then supersaturated with oxygen and used for irrigating the treatment. Errors bars indicate ±1 SD ($n = 11$–19). Different letters denote significant differences (Tukey's multiple comparison, $p < 0{,}05$).

tipburn injured leaves was 23% of the total number of leaves, but there were no significant differences between the NB treatment and the control (Figure 2D; ANCOVA, $p < 0{,}05$).

In regards to inner tipburn injury, slopes of both the NB treatment and the control were not significantly different from zero (Figure 2E; simple linear regression, $p < 0{,}05$), which was caused by low incidence of inner tipburn at all points of sampling. While outer tipburn injury was more common than inner tipburn, the regression line slopes did also not differ significantly between the NB treatment and control (Figure 2F; ANCOVA, $p < 0{,}05$).

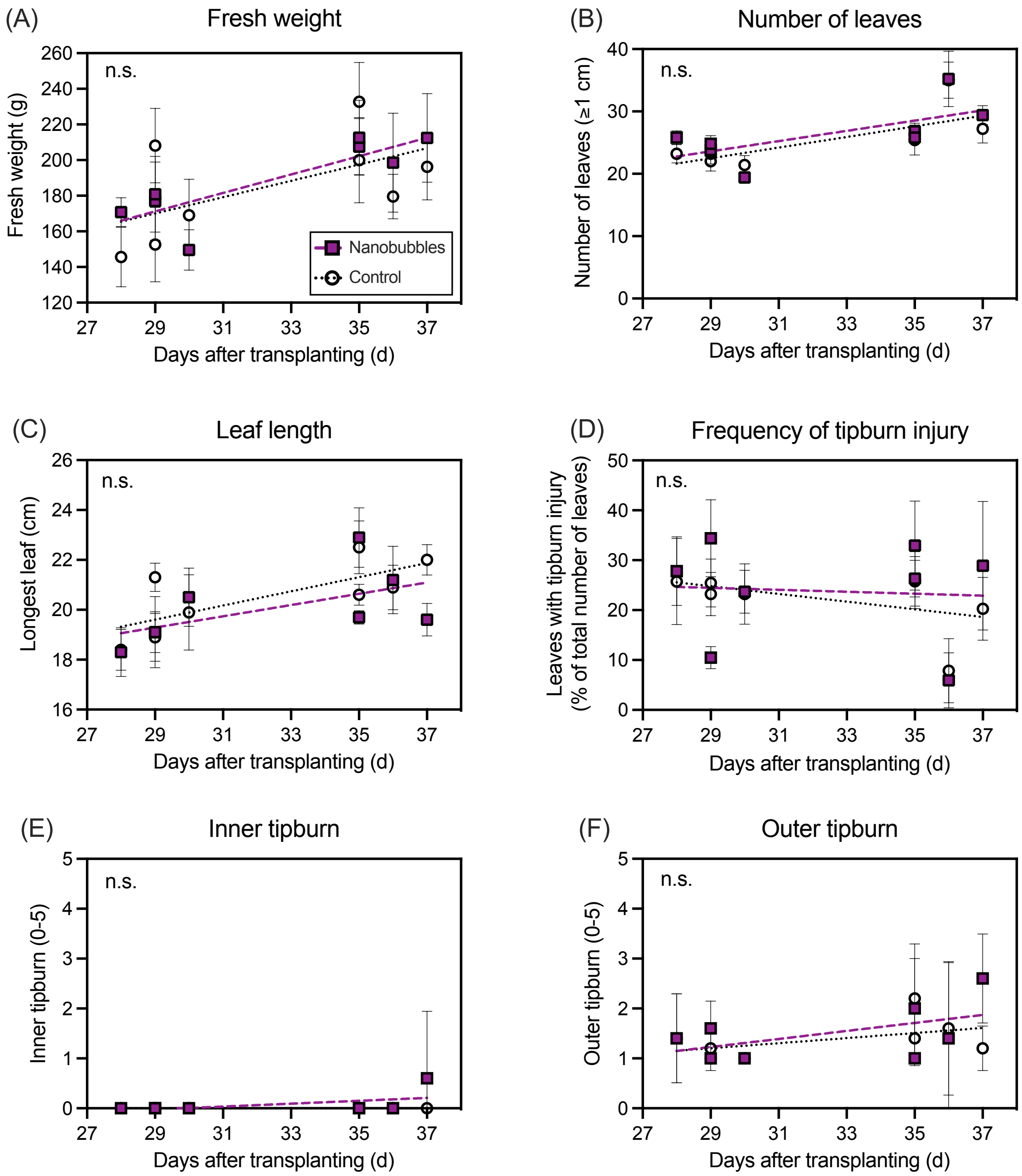

Figure 2: Plant growth measurements (A, shoot fresh weight; B, number of true leaves; C, length of the longest leaf) and tipburn injury (D, frequency of tipburn injury; E, inner tipburn severity; F, outer tipburn severity) of crispy lettuce 'Frillice' cultivated in a NFT system irrigated with O_2 nanobubble-supersaturated nutrient solution plotted against corresponding days after transplanting to the NFT channels. Dotted lines represent the linear regression models of the respective treatments. Each dot indicate mean value of five replicates and errors bars indicate ± 1 SD ($n = 40$ in total per treatment). n.s. denote that slopes of the regression models did not differ significantly between the NB treatment and the control (ANCOVA, $p < 0{,}05$).

4 Discussion

In contrast to a range of previous studies on micro- and nanobubbles in hydroponic horticulture, the present study did not find any significant effect of O_2 NBs in the nutrient solution on plant growth or tipburn injury of crispy lettuce cultivated in a NFT system. However, previous studies were largely carried out in DFT or DWC systems (e.g., Park and Kurata, 2009; Ebina et al., 2013; Ahmed et al., 2018b; Tsutsumi et al., 2020) in which the root system is constantly submerged in nutrient solution — unlike a NFT system in which the roots are only periodically submerged, and then, often only partially.

In DFT or DWC systems, plants are more dependent on the ability of the solution, where oxygen is much less diffusible than in air, to provide oxygen to the roots in order to sustain various metabolic activities, including respiration, nutrient absorption and translocation, and biosynthesis, particularly during periods of darkness (Ikeura et al., 2018; Tsutsumi et al., 2020). In fact, sub-saturated concentrations of dissolved oxygen in the water can cause hypoxia of the roots leading to abnormal phytohormone synthesis and decline in metabolism ultimately inducing root rot and inhibition of plant growth (Ikeura et al., 2018).

Although uncertain, it is thought that NBs easily adhere to the roots and efficiently increase the supply of oxygen directly to the positively charged root surfaces thus stimulating root and whole plant growth (Park & Kurata, 2009; Ahmed et al., 2018b). The high O_2 solubility of such a micro- and nanobubble solution essentially provides an alternative source of oxygen to the root system during night when the shoot cannot supply internal oxygen as photosynthesis ceases (Tsutsumi et al., 2020).

Additionally, the negative surface charges of NBs adsorp and accumulate dissolved ions, preferentially ions with a valency of +2 or greater (Takahashi, 2005). This property could help attract nutrients to the plant roots, such as Ca, K, and Mg, and, consequently, stimulate plant growth (Park & Kurata, 2009; Kobayashi & Yamaji, 2021). However, as the roots of plants in NFT channels are not continuously submerged, it is possible that they are not only less likely to be limited by oxygen supply to the roots but also may adsorp fewer NBs and attached nutrients than plants in other hydroponic cultures. Future studies may benefit from including an analysis of the mineral content in shoots of treated plants.

The formation of exogenous ROS by shock waves created from the collapse of NBs is thought to induce the formation of endogenous ROS in plants, which may affect plant physiological activity (Iijima et al., 2021). While excessive ROS can cause large damage, moderate levels may have beneficial effects on, among others, growth-related signaling molecules and cell wall loosening and cell elongation leading to increased growth (Ahmed et al., 2018b; Kobayashi & Yamaji, 2021). The toxic thresholds of endogenous ROS may, however, differ between species (Ahmed et al., 2018b; Iijima et al., 2021). It is therefore possible that a high level of NBs in the nutrient solution may induce the formation of an unfavourable, excessive level of exogenous ROS that could negatively affect crispy lettuce growth.

Lastly, a study found that the growth of soybean plants were only promoted by the addition of NBs when the plants were exposed to nutrient deficit stress, while there was no effect on shoot FW under high nutrition levels (Iijima et al., 2020). Similarly, the same authors found that the growth-promoting effect of NBs may be limited, especially under DFT/DWC conditions where sufficient nutrients are always available (Iijima et al., 2021). Ultimately, the promotive or suppressive effect of NBs can also depend on the specific species as well as on interactions between NB concentration and nutrient status (Iijima et al., 2021).

In conclusion, the addition of a high concentration of NBs to the nutrient solution of a commercial NFT system did not improve crop growth or tipburn injury in the present study. While NBs may act in several ways, the addition of NBs may improve growth mainly in systems where oxygen or nutrients are limiting (e.g., hydroponic cultures with continuous submersion of plant roots) or when a specific species benefits from increased levels of endogenous ROS.

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