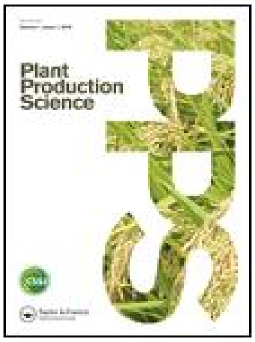

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SHORT REPORT

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Ultrafine bubbles effectively enhance soybean seedling growth under nutrient deficit stress

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ABSTRACT

Ultrafine bubbles (UFBs) exhibit a number of unique physical characteristics; however, reports on plant growth enhancement by UFB application are controversial. Here, we report experiments conducted to test whether UFB technology effectively promotes plant growth. We analyzed the effects of three experimental factors: water (UFB addition), hypoxia (aeration), and fluid movement (stirring) on the growth of soybean seedlings cultured hydroponically under low and high nutrition levels in a controlled environment room under artificial lighting for 8 days. When no nutrients were supplied, positive UFB water effects were evident, but low nutrition reduced UFB water-mediated growth enhancement and high nutrition totally obliterated any growth enhancement by UFB water. We concluded that UFB water-induced growth enhancement was effective and significant under nutrient deficit stress but no growth enhancement was observed, and even negative effects were evident, under favorable plant growth conditions.

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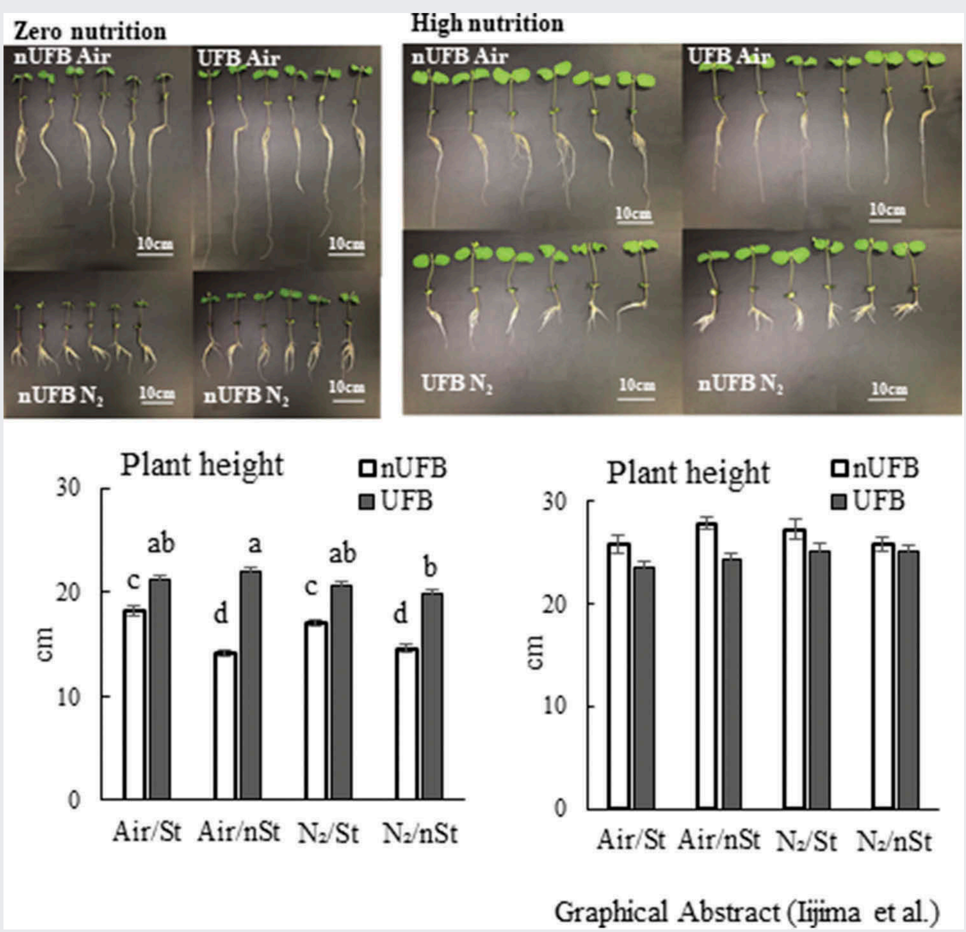

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Supplemental data for this article can be accessed here.

Ultra-small-sized air bubbles (called ultrafine bubbles, hereafter UFBs) formed in water exhibit a number of unique physical characteristics. UFBs have been defined as gas in a medium enclosed by an interface with a volume-equivalent diameter of less than 1 μm ('ISO 20480-1: 2017 (en)'). Water containing UFBs (hereafter UFB water) shows antibacterial action and is often used for washing without any chemical compounds in industrial applications (Ushida, Hasegawa, Nakajima, Uchiyama & Narumi, 2012). In agriculture, Park and Kurata (2009) first indicated that UFB water promoted the production of lettuce biomass in a plant factory. Similarly, Ebina et al. (2013) showed that the growth of hydroponically cultured *Brassica campestris* was significantly promoted by UFB water after 4 weeks. In Liu, Kawagoe, Makino and Oshita (2013), Liu et al. (2015) showed that the germination rate of barley seeds significantly increased upon UFB water treatment. These studies showed that plant growth and/or productivity can be promoted by UFB water treatment. In contrast, while comparing the effects of air, O_2, CO_2, and N_2, as alternative sources of UFBs, Ahmed et al. (2018) reported that UFB water did not significantly promote germination or growth of lettuce, carrots, fava beans, or tomatoes when air was used as the test gas. Moreover, Minamikawa, Takahashi, Makino, Tago and Hayatsu (2015) reported that rice growth did not differ between plants irrigated with oxygen-UFB water and those irrigated with control water. These conflicting results indicated that precise growth experiments under a controlled environment are necessary to ascertain whether UFB water is always effective for promoting crop growth and productivity. The environmental factors affecting the production or growth of crop species cultured in UFB water have never reported so far. This study aimed to identify the environmental factors that control the growth-enhancement effects of UFB water. Thus, a simple experimental system based on hydroponic culture was used to evaluate, exclusively, the effect of UFB on the early growth of soybean seedlings under various environmental conditions.

Materials and methods

Our experimental model for the research described herein was soybean (*Glycine max* 'Fukuyutaka'), as it is a representative summer legume crop. Soybean plants were grown in a plant growth room at 28/23°C day/night temperature, under a 14-h photoperiod and 318 ± 2 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) at canopy top level (Iijima et al., 2017, in press). Average water temperature was 29.0°C and 26.1°C during daytime and at night over the plant growth period, respectively. Seeds were surface-sterilized five times with 2.5% (*v*/*v*) sodium hypochlorite for 3 min and then sown on paper towels wetted with deionized water in a Petri dish. Seeds were pre-germinated in a dark incubator at 30°C for 30 h. Next, seedlings with 20- to 25-mm radicles were selected and transferred to either zero, low, or high nutrient level in the hydroponic solution (Hoagland & Arnon, 1950). Separate experiments were conducted in the same growth chamber to test the nutrient solution treatments. The water in all treatments was changed three times, namely, at 2, 4, and 6 days after seeding. Zero nutrient level consisted in de-ionized water without any nutrients added during the whole 8-day experimental period; thus, in this case, soybean seedlings grew on the cotyledonary nutrient supply alone. We designated this treatment as 'nutrient stress condition.' Seedlings subjected to the low nutrition treatment were kept in deionized water only for the first 4 days of the experimental period; then they were transferred onto 1/4 strength Hoagland solution for 2 days, and then onto 1/2 strength Hoagland solution for 2 days; according to preliminary trials this treatment was designated as 'normal for soybean growth.' Finally, seedlings under high nutrition were grown in 1/4 strength Hoagland solution for the first 4 days and then in 1/2 strength Hoagland solution for the remainder of the test period. This treatment should supposedly provide an 'ideal nutrient supply,' i.e. no nutrient stress for soybean seedlings at all. The difference among zero, low, and high nutrition treatments in these experiments derived exclusively from the nutrient solution used, as all other environmental factors were under precise control (see Materials and methods in Iijima et al., 2017, in press). The lower parts of hypocotyl of each pre-germinated seedling were fixed through a hole on the plastic panel floating on the top of the plastic containers (300 × 210 × 50 mm) filled with the hydroponic solution. The gaps around seedling shoots and between the floating panel and the container were sealed with absorbent cotton and masking tape in order to keep aerated or non-aerated (N_2 gas) conditions. Six seedlings were grown in each container, and the effects of three factors were analyzed for each nutrient treatment: the main factor was UFB water (with/without), the second one was aeration (air/N_2 gas), and third was stirring (with/without). Stirring was selected as an environmental factor that may influence UFB effects on seedling roots because in the case of UFBs, Brownian motion predominates over buoyancy force (Alheshibri, Qian, Jehannin & Craig, 2016); therefore, the UFBs will stay in water long time, even more than months in some cases (Oh, Han & Kim, 2015). UFBs keep their characteristic enduring stability in water by a combination of surface tension, high internal air pressure, and repulsion due to electric charge (zeta potential), whereby they are not crushed by water pressure. Zeta potential of UFBs might affect ion-uptake characteristics of plant roots (Ueda et al., 2015), therefore, stirring of UFB water may be necessary due to the extraordinarily high stability of UFBs in water.

Here, UFB water was made by an Ultra-fine Bubble Generator (EAT-SWHI, Eatech Co. Ltd., Kumamoto, Japan), and bubble size distribution was profiled by nanoparticle tracking analysis using NanoSight LM10 (Malvern Panalytical, Tokyo, Japan). A combination of both the resonance foaming method and the vacuum cavitation method (U.S. Patent No. 10,500,553, 2019) enabled us to produce 2.7 × 10^7 UFBs per ml with average diameter of 226 nm (Figure 1), which is presumably a normal and effective UFB density, according to results reported previously (Ueda, Tokuda, Shigeto, Nihei & Oka, 2013; Ueda, Tokuda & Zushi, 2014).

Deionized water (G-10, ORGANO Co. Ltd., Japan) aired at a flow of 3.0 L min^{-1} was supplied to the generator at a rate of 30.0 L min^{-1}, causing a gas pressure of −0.025 MPa. In the aerated nutrient solution, aeration was continuous at the rate of 2.0 L min^{-1} to each container, and in the non-aerated nutrient solution, N_2 gas (purity >99.99%) was supplied for 6 h day^{-1} at a rate of 2.0 L N_2 min^{-1} to each container. Stirring of the nutrient solution was done using a magnetic stirrer (HS-4AN, AS ONE Co. Ltd., Japan) at 1500 rpm, whereas for the non-stirring condition, no stirring was done at all except for the aeration treatment and changing solutions at 2, 4, and 6 days after seedling transplant. pH, electrical conductivity (EC), and dissolved oxygen (DO) concentration were measured by a pH meter (LAQUA twin, Horiba, Japan), an EC meter (CT-27112B, DKK-TOA, Japan), and a DO sensor (Multi3410, WTW CoLtd, Germany), respectively. pH of the 0, 1/4, and 1/2 strength Hoagland solutions varied in the range 5.5–6.5, 5.3–5.8, and 5.1–5.7, respectively, during the experimental period. As for EC during soybean growth, it varied in the ranges 0.01–-0.02, 0.47–0.51, and 1.03–1.06 (dS/m), respectively, while DO values in N_2 treatment and air varied in the range of 0.61–1.89 and 7.31–7.51 (mg/L), respectively. DO values were not significantly different between treatments with and without stirring during the whole experimental period. UFBs formation did not change either pH or EC values, as reported previously (Park & Kurata, 2009); neither were DO values affected by UFB treatment. Most probably, the DO sensor used did not detect the oxygen gas contained in the tiny sized UFBs; further, an air-bubbling inlet tube was set at the bottom of the container.

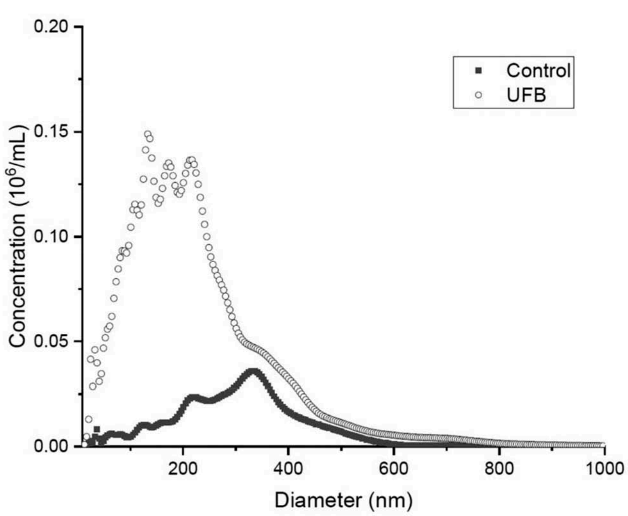

Figure 1. Bubble size distribution in control water (closed squares) and in water containing UFBs (open circles). The tracking motion picture was recorded for 30 s and distributions were calculated by Nanosight LM10. Bubble size distribution was measured three times under each condition and averaged values were plotted.

Seedlings were harvested at 8 days after transplant and shoot and root fresh weight, plant height, and taproot length were measured. All statistical analyses were performed using Excel Statistics Version 2012 software (SSRI).

Results and discussion

In this study, we used soybean young seedlings as a model to examine the environmental factors affecting plant growth enhancement by UFB water. Three sets of experiments under different nutrient levels were performed. Relative to zero nutrition, the average total fresh weight under low and high nutrition treatments was 20% and 40% greater, respectively. The effects of UFB water on plant growth depended highly on nutrient level (Table 1). In the case of zero nutrient supply, i.e. stressful environment for plant growth, UFB water positive effects were evident (supplemental Fig. S1), consistently with previous studies (e.g. Ebina et al., 2013; Park & Kurata, 2009). However, under low nutrition, overall growth enhancement by UFB water was very much reduced, as total fresh weight and taproot length did not show any significant differences between water treatments (Non-UFB vs UFB), and furthermore, root fresh weight was significantly reduced in UFB water. Lastly, under high nutrition, UFB water did not show any statistical growth enhancement effect on shoot growth, whereas root fresh weight was again reduced in this case. These findings agreed with two previous studies (Ahmed et al., 2018; Minamikawa et al., 2015). The overall trends described indicated that UFB water was effective in promoting soybean seedling growth under nutrient stress but not under favorable growth conditions. Liu, Oshita, Kawabata, Makino and Yoshimoto (2016) concluded that reactive oxygen species (ROS) can be continuously produced in UFB water and that ROS played an important role in enhancing

Table 1. Three-way ANOVA for six agronomic traits. Non-UFB, deionized water; UFB, ultra-fine bubble water. *, 5% significance level. **, 1% significance level. ***, 0.1% significance level. ns, not significant.

(a) Zero nutrition

	Total fresh weight (g plant^{-1})	Shoot fresh weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Plant height (cm)	Taproot length (cm)	Shoot/root ratio
Water						
Non-UFB	2.72	1.98	0.75	16.0	22.2	3.00
UFB	3.30	2.45	0.85	21.0	30.5	3.26
Gas flow						
Air	3.34	2.30	1.04	19.0	40.3	2.22
N_2	2.67	2.12	0.55	18.1	12.4	4.04
Fluid movement						
Stirring	3.12	2.27	0.85	19.3	27.3	2.89
Non-stirring	2.90	2.15	0.75	17.7	25.5	3.38
ANOVA						
Water	***	***	**	***	***	ns
Gas flow	***	*	***	**	***	***
Fluid movement	ns	ns	*	***	ns	**
W*G	ns	ns	ns	ns	***	ns
W*F	ns	ns	ns	***	ns	ns
G*F	ns	ns	ns	ns	ns	**
W*G*F	ns	ns	**	**	ns	*

(b) Low nutrition

	Total fresh weight (g plant^{-1})	Shoot fresh weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Plant height (cm)	Taproot length (cm)	Shoot/root ratio
Water						
Non-UFB	3.53	2.81	0.72	19.5	22.0	4.01
UFB	3.68	3.10	0.58	22.5	22.9	5.69
Gas flow						
Air	3.84	3.06	0.77	21.8	35.3	5.70
N_2	3.38	2.85	0.53	20.2	9.7	4.07
Fluid movement						
Stirring	3.65	2.98	0.67	21.3	23.5	4.64
Non-stirring	3.56	2.94	0.63	20.6	21.4	5.13
ANOVA						
Water	ns	**	***	***	ns	***
Gas flow	***	*	***	**	***	***
Fluid movement	ns	ns	ns	ns	ns	ns
W*G	ns	ns	ns	***	ns	ns
W*F	ns	ns	ns	**	ns	ns
G*F	ns	ns	*	ns	ns	*
W*G*F	ns	ns	ns	ns	ns	*

(c) High nutrition

	Total fresh weight (g plant^{-1})	Shoot fresh weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Plant height (cm)	Taproot length (cm)	Shoot/root ratio
Water						
UFB	4.27	3.48	0.79	22.1	20.9	4.57
Non-UFB	4.08	3.36	0.72	22.1	20.4	4.81
Gas flow						
Air	4.39	3.52	0.87	22.2	31.3	4.07
N_2	3.96	3.32	0.64	22.0	10.0	5.31
Fluid movement						
Stirring	4.22	3.44	0.78	22.3	22.1	4.54
Non-stirring	4.13	3.40	0.73	21.9	19.2	4.85
ANOVA						
Water	ns	ns	*	ns	ns	ns
Gas flow	***	ns	***	ns	***	***
Fluid movement	ns	ns	ns	ns	ns	ns
W*G	ns	ns	ns	**	ns	ns
W*F	*	*	ns	ns	ns	ns
G*F	ns	ns	ns	ns	ns	ns
W*G*F	ns	ns	ns	ns	ns	ns

seed germination. Thus, most likely, ROS production in UFB water might be involved in the observed growth enhancement of soybean plants.

The shoot/root ratio (SR ratio) showed different responses under the three nutrient conditions tested; it was not statistically affected by UFB water under zero or high nutrition levels, which indicated that total carbon (either cotyledonary or photosynthetic) partitioning did not change significantly. The response of SR ratio to low nutrition is not clear. It is possible that UFBs might accelerate carbon translocation to the shoot under optimal shoot/root balance. Under low nutrition, taproot elongation growth was not affected by UFB water, however, root fresh weight was significantly lower in UFB water than in the control treatment. This result implies that lateral root development was reduced by UFB water. Lateral root development is known to be promoted by a number of environmental factors, such as soil mechanical impedance to root growth, whereby the growth of the main root axis is reduced and branching is promoted (Iijima, Kono, Yamauchi & Pardales, 1991; Iijima, Morita, Zegada-Lizarazu & Izumi, 2007). Although root elongation growth was not reduced in UFB water, root branching was reduced by an unknown mechanism.

Agronomic traits that showed interaction effects among the three experimental factors (i.e. water, gas flow, and fluid movement, W*G*F in Table 1) were further analyzed by multiple comparison tests (Figures 2 and 3). Fluid

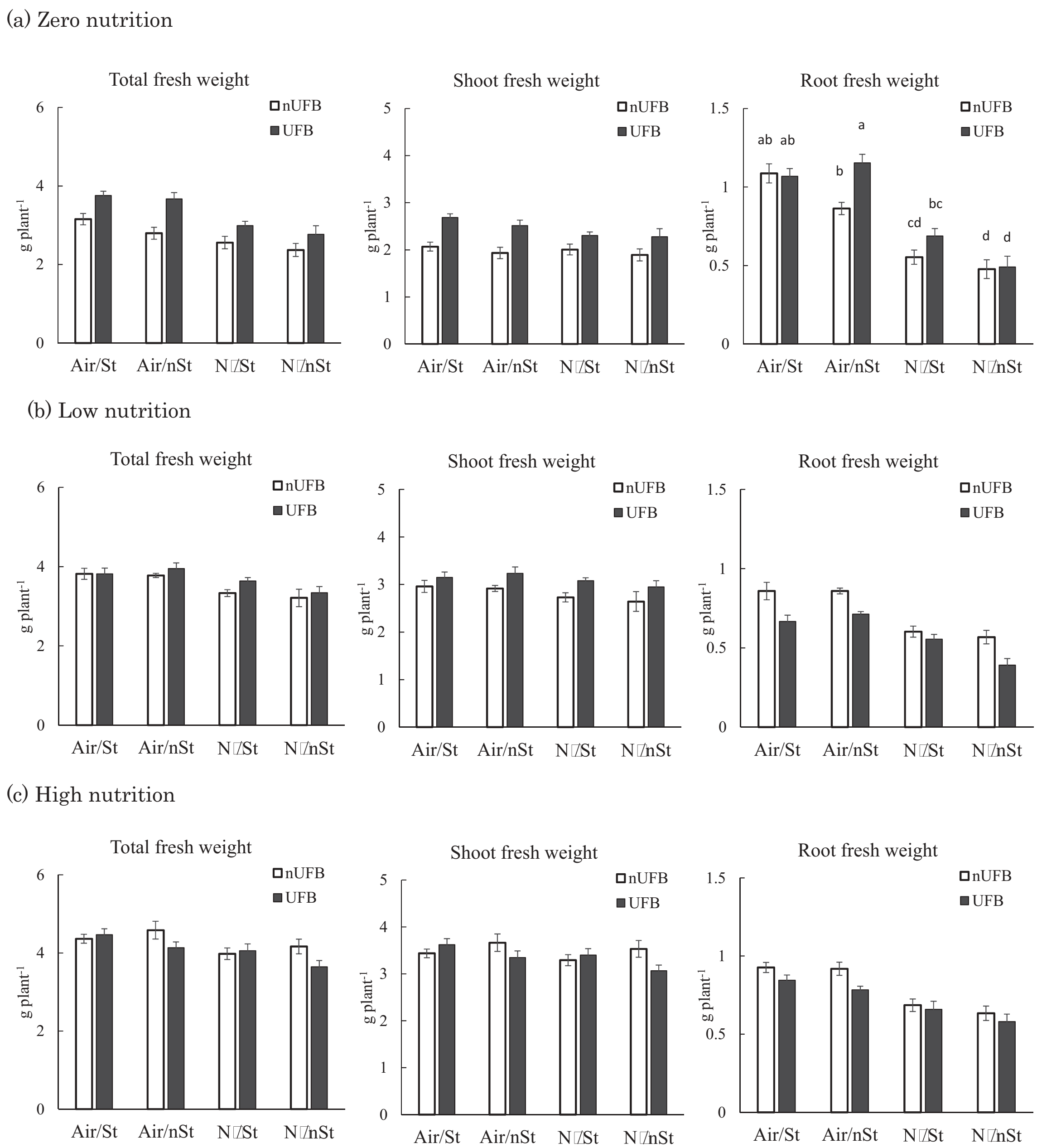

Figure 2. Total, shoot, and root fresh weights of soybean seedlings growing under different nutrition, water, gas flow, and fluid movement conditions. UFB, UFB water; nUFB, deionized water; Air, airflow; N_2, N_2 gas flow; St, stirring; nSt, non-stirring. Tukey–Kramer multiple comparison tests were conducted on traits showing interaction effects among the three experimental factors (Water*Gas flow*Fluid movement) in three-way ANOVA in Table 1. Means followed by the same lowercase letter were not significantly different at $P < 0.05$, according to Tukey–Kramer multiple comparison test. (a) Zero nutrition; (b) Low nutrition; (c) High nutrition.

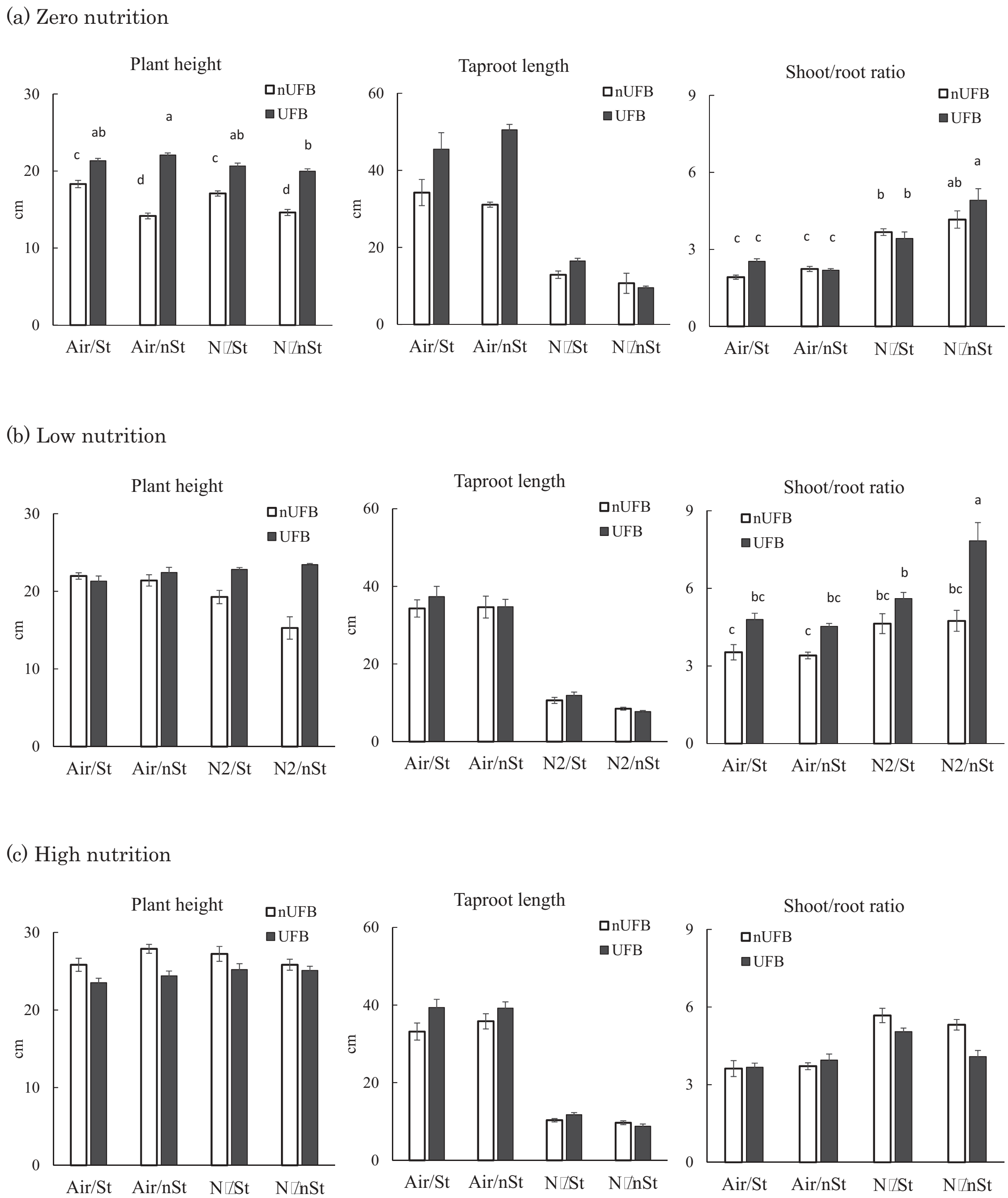

Figure 3. Plant height, tap-root length, and shoot/root ratio of soybean seedlings under different nutrition, water, gas flow, and fluid movement conditions. UFB, UFB water; nUFB, deionized water; Air, airflow; N_2, N_2 gas flow; St, stirring; nSt, non-stirring. Tukey–Kramer multiple comparison tests were conducted on traits showing interaction effects among the three experimental factors (Water*Gas flow*Fluid movement) in three-way ANOVA in Table 1. Means followed by the same lowercase letter were not significantly different at $P < 0.05$, according to Tukey–Kramer multiple comparison test.

movement (stirring) enhanced UFB water effects on root fresh weight (Figure 2) under hypoxia (N_2) but did not influence its effects on plant height (Figure 3) in the zero nutrition treatment. No-stirring under hypoxia may be stressful for lateral root development; however, UFB water seemed to sustain lateral root growth under aerated, non-stress conditions (Figure 2). In UFB water-treated seedlings, plant height under both aerated and non-aerated conditions was similar, except between the two non-stirring gas treatments, which implies UFB water supported shoot elongation under the stressful environment (Figure 3). Shoot/root ratio (Figure 3) was significantly higher under N_2/non-stirring (stressful environment) among UFB water treatments as well, implying carbon partitioning preferentially to the shoot. Significant interaction effects between UFB water and either of the other two factors (Table 1, W*G or W*F) are summarized in supplemental Fig. S2. The results confirmed that UFB water was effective under stress conditions but not under favorable conditions, in which case it proved even harmful. Plant height of UFB water-treated seedlings growing under non-stirring and/or hypoxia remained unchanged in either the zero or the low nutrient condition. Taproot elongation of UFB water-treated seedlings under aerated, non-hypoxic conditions was significantly higher in the zero nutrition treatment. Conversely, shoot fresh weight of UFB water-treated seedlings was significantly reduced by non-stirring in the high nutrition treatment (non-stress condition). Total fresh weight tended to follow the same trend, although no statistical significance was detected. As for shoot elongation growth (plant height) in UFB water-treated seedlings, it tended to be reduced in air (favorable conditions) and increased in N_2 (stress conditions).

In conclusion, UFB water effectively and significantly enhanced plant growth under the stress environment tested. Overall, UFB water maximized both shoot and root growth under nutrient deficit stress. In contrast, under favorable nutrient supply (i.e. an ideal environment for plant growth), shoot growth enhancement by UFB water was not apparent and root biomass was actually reduced in this case. Additional research is necessary to analyze other factors, such as light intensity, temperature, and humidity, among others, to fully understand the environmental factors affecting plant growth and productivity enhancement by UFB water.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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