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Ultrafine bubbles alleviated osmotic stress in soybean seedlings

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ABSTRACT

Growth promotion through application of ultrafine bubbles (UFBs) was observed in several crop species grown under suboptimal conditions. In the current study, mitigation of osmotic stress through polyethylene glycol 6000 (PEG6000) was analyzed in soybean seedlings to assess the effects of UFB on plants under soil drought stress. In no-nutrient conditions, growth suppression due to osmotic stress was increasingly mitigated by UFB application as stress intensity increased. Shoot biomass of UFB-treated plants (at all examined PEG6000 concentrations) exceeded to that of the absolute control. Production of superoxide radicals (O_2^-) under osmotic stress was 1.5-fold higher than that in the control, whereas that in UFB-treated plant showed a similar level as the absolute control. The reduction of O_2^- through treatment with UFB may help mitigate osmotic stress in soybean seedlings.

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KEYWORDS

Drought stress; nanobubbles; nutrient stress; osmotic stress; soybean; superoxide radical; ultrafine bubble

Introduction

Ultrafine bubbles (UFB), which are defined as gas in a medium surrounded by an interface with a volumeequivalent diameter of less than 1 µm (ISO 20480-1: 2017(en), 2021), were predicted to be of use for various industrial applications. Their potential use in agriculture was also suggested; however, practical applications have not been widely implemented so far. Therefore, quantitative basic knowledge on the effects of UFB water on crop growth under controlled conditions is required. Previous studies on the effects of UFB on plant growth indicated both positive (e.g. Ebina et al., 2013; Park & Kurata, 2009) but also not positive (e.g. Ahmed et al., 2018; Minamikawa et al., 2015) effects on growth. To elucidate the effects of UFB water on promotion or suppression of crop growth under various conditions, we previously cultivated several food crop species in hydroculture under controlled conditions in a growth room. Growth promotion was not consistent under highnutrition conditions, and some traits in fact suggested growth suppression (lijima et al., 2020). Moreover, interspecific differences occurred among cereal and leguminous species under UFB concentrations that promoted growth (lijima et al., 2021). So far, the growth-promoting effect was predominantly observed in plants growing under suboptimal conditions such as a lack of nutrients and/or anaerobic conditions. This suggests that the growth-promoting effect may be more pronounced during stress. For example, if UFB can alleviate soil drought stress, which is the most common type of stress in largescale field cultivation, their use may help improve staple food crop production. In this study, basic experiments were conducted in hydroculture under controlled conditions to assess the effects of UFB on soil drought stress mitigation. In soil drought studies, various factors of soil biological, chemical, and physical characteristics must be controlled in addition to the water environment. In this study, to reduce the effects of other environmental factors as much as possible, we induced osmotic stress through the hydroponic solution as a substitute for soil drought stress and aimed to clarify the effects on crop growth.

Materials and methods

The experiment was conducted under the same conditions as described previously (lijima et al., 2020, 2021). Briefly, plants were grown in a plant growth room at 28/ 23°C day/night temperature, under a 14-h photoperiod, and 318 \pm 2 µmol m⁻² s⁻¹ of photosynthetically active radiation at the top of the canopy (lijima et al., 2017). Soybean (*Glycine max* 'Fukuyutaka') was used as previously reported (lijima et al., 2020). Pre-germination was performed at 30°C in the dark for 28 h, and plants were grown for nine days with either no nutrients or

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PEG 2000 control).

under full nutrient conditions. The no-nutrient treatment was used to induce severe nutrient stress by growing plants in de-ionized water without any nutrients added during the entire experimental period. The full-nutrient treatment represents ideal nutrient supply condition where plants were grown in 1/4-strength Hoagland solution (Hoagland & Arnon, 1950) for the first four days, after which the concentration was increased to 1/ 2-strength Hoagland solution for the remaining growth period. To simplify the environmental conditions, aeration (2.0 L air min⁻¹ 5 L container⁻¹) and stirring (1,500 rpm) using a magnetic stirrer were continuously executed during the experimental period. The solution was replaced every two days. As observed previously (lijima et al., 2020), UFB treatment did not affect the pH, electrical conductivity, and dissolved oxygen content.

UFB water was prepared using an ultra-fine bubble generator (EAT-SWHI, Eatech Co. Ltd., Japan). Bubble size distribution was measured by nanoparticle tracking analysis using a NanoSight LM10 device (Malvern Panalytical, Tokyo, Japan). The concentration of UFB was $0.7 \times 10^8 \text{ mL}^{-1}$, and the average diameter of UFB was 215 nm. The osmotic stress treatment was performed using polyethylene glycol 6000 (PEG6000; H(OCH₂CH₂)nOH; Fujifilm Wako Pure Chemical Industries, Ltd., Japan) to simulate soil drought stress. PEG6000 is a macromolecule and therefore does not enter the root apoplast (Carpita et al., 1979); it is a suitable osmotic agent to remove water from cells and is non-toxic in well-aerated environments (Verslues et al., 1998). Thus, soil drought stress can be simulated under hydroponic culture conditions (Ogawa & Yamauchi, 2006). PEG6000 was dissolved in de-ionized water at the time of changing the hydroponic solution on the fourth day after sowing, and osmotic stress was induced at four levels, i.e. using 0%, 10%, 15%, or 20% PEG6000 dissolved in the hydroponic solution. According to Ogawa and Yamauchi (2006), the water potential (ψ) at 10% PEG6000 was -0.21 MPa and -0.41 MPa at 20% PEG6000. In major cereal and leguminous staple food crop species, such levels of osmotic stress are equivalent to severe and/or very severe soil drought stress (lijima & Kato, 2007). Three factors were assessed, i.e. no - and full-nutrient treatment, presence or absence of UFB, and four levels of osmotic stress (only two levels were tested under full-nutrient conditions), and six replicates were used. Plants were harvested nine days after sowing, and shoot and root fresh weight, plant height, and tap root (the oldest and longest root) length were measured as agronomic parameters. The dry weight of plants was not

Effects of UFB on plant growth may include production of reactive oxygen species (ROS; Liu et al., 2017, 2016). The exogenously applied ROS by UFB water stimulated the generation of endogenous superoxide radical (O_2^{-}) inside the seed and played an important role in seed germination (Liu et al., 2016). In the present study, the effects of UFB water on ROS generation were analyzed by quantifying superoxide radical (O₂⁻) concentrations in the roots. We focused on the no-nutrient treatment that showed significant differences in growth response in previous studies (lijima et al., 2020, 2021). The effects of UFB on plants treated with no PEG6000 (no osmotic stress) and 15% PEG6000 (osmotic stress) were compared using eight replicates. O₂⁻ was quantified using the method of Kuzniak et al. (1999). After sampling, the roots were immediately cut into 5-mm segments, which were incubated in 10 mM potassium phosphate buffer containing 10 mM NaN3 and 0.05% nitro blue tetrazolium (NBT) for 24 h at 25°C in the dark. Production of O₂⁻ was assessed by the ability to reduce NBT in the medium, and absorbance was measured at 580 nm wavelength to calculate the amount of generated O2⁻. A three-way analysis of variance (ANOVA) was performed for all treatments, and a two-way ANOVA was performed for no-nutrition and high-nutrition treatments using Excel Statistics 2015 software (Social Survey Research Information Co., Ltd., Japan). Differences between means of two groups were tested using a t-test. Significance is reported at p < 0.05.

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Results and discussion

Table 1 shows the results of a three-way ANOVA of the effects of nutrient conditions, osmotic stress, and UFB application on soybean grown for nine days. All parameters associated with biomass production (shoot and root fresh weights) and elongation growth (plant height and tap root length) were affected by the nutrient conditions. Regarding osmotic stress (0% vs. 15% PEG6000), there was a significant difference in biomass production but not in elongation growth. UFB application produced significant effects on all traits except root biomass. Regarding interactions between factors, there was no significant interaction effect of nutrient and osmotic stress on any trait, whereas nutrient stress and UFB application showed interaction effects on all traits. Similarly, osmotic stress and UFB application produced interaction effects on biomass production but not on elongation growth. Interaction effects of all three factors were significant only plant height. These results

Table 1. Three-way ANOVA of the effects of nutrient and osmotic stresses, and ultrafine bubble applications on nine-days-old sovbeans.

`	Shoot	Poot frach	Dlant	Tap root
	frech	NOUL HESH	Fidilit	longth
	iresn	weight	neight	length
	weight (g)	(g)	(cm)	(cm)
No nutrient				
0% nUFB	1.56	1.06	9.5	21.6
UFB	2.06	1.15	12.2	42.9
15% nUFB	1.21	0.64	9.0	20.3
UFB	1.98	0.94	13.4	41.9
Full nutrient				
0% nUFB	3.92	1.39	15.5	38.4
UFB	3.91	1.15	16.7	46.0
15% nUFB	3.21	1.14	15.7	39.9
UFB	3.29	1.06	16.1	43.4
ANOVA				
Nutrient (N)	**	**	**	**
Osmotic (O)	**	**	ns	ns
UFB (U)	**	ns	**	**
N*O	ns	ns	ns	ns
N*U	**	**	**	**
O*U	*	*	ns	ns
N*O*U	ns	ns	*	ns

No nutrient, deionized water culture; Full nutrient, Hoagland solution culture. Percentages (0%–15%) indicate the respective polyethylene glycol 6000 (PEG6000) concentration. UFB, ultrafine bubble application; nUFB, non-UFB application. Values are means of six replicates. * and ** indicate significant interactions at 5% and 1% levels, respectively. indicated that nutrient stress significantly affected all traits, whereas osmotic stress had no significant effect on elongation growth. Regarding interaction with UFB applications, nutrients showed highly significant results. Based on the above, a detailed analysis was performed for each nutritional condition. Under the no-nutrient condition (Figure 1), biomass growth suppression due to osmotic stress was found to be relieved to a greater extent by UFB application as stress intensity increased (with increasing PEG6000 concentrations). Shoot biomass under UFB application (at all examined PEG6000 concentrations) exceeded to that of the absolute control without any osmotic stress (0% PEG6000, control water). The 20% PEG6000 treatment ($\psi = -0.41$ MPa) was equivalent to severe drought stress in annual food crops (lijima & Kato, 2007), and it reduced shoot biomass by 71% in plants without UFB application. Under such severe stress conditions, shoot biomass production in the UFB treatment was 1.1-fold higher than that of the absolute control without any osmotic stress. In contrast, under full nutrient conditions and with 15% PEG6000, no significant improvement in any trait by UFB application



Figure 1. Effects of ultrafine bubble application on nine-days-old soybean growth under no-nutrient and osmotic stress conditions. UFB, ultrafine bubble application; nUFB, non-UFB application. Vertical error bars indicate standard errors of means of six replicates. * and ** indicate significant differences at 5% and 1% levels, respectively, using a t-test.

was observed (Figure 2). UFB thus did not ameliorate osmotic stress under full-nutrient conditions, which was in line with previous studies, indicating that growth promotion is not consistent under low – and high-nutrient conditions (lijima et al., 2020, 2021).

Superoxide radical (O_2^{-}) content in roots was investigated under no-nutrient conditions (Figure 3). A twoway ANOVA of the effects of osmotic stress and UFB application indicated significant effects of each factor on O_2^- content as well as interaction effect (p < 0.01). Under the control conditions (0% PEG6000), O₂⁻ content was not significantly affected by the UFB treatment. In contrast, under osmotic stress conditions (15% PEG6000), O₂⁻ content was significantly decreased after UFB application. In plants without UFB, O₂⁻ content was 1.5-fold higher than that in the absolute control (non-UFB, no PEG6000), and UFB-treated plants showed a similar value as the control (with a smaller mean value). $O_2^$ content in roots under osmotic stress was thus reduced by UFB application. Previous studies (Liu et al., 2017, 2016) suggested that exogenous reactive oxygen species (ROS) produced by UFB application would be related to the promoting effects of plant physiology. They have used the germinated seeds of barley for the estimation of ROS. In the present study, lower O_2^- content in roots of soybean seedling may be related to alleviate osmotic stress through UFB application. It is well known that pretreatment with a low concentration of ROS before stress treatments increases the activities of the antioxidant enzymes, thereby alleviating the damage under stress conditions (Uchida et al., 2002; Yamane et al. 2004). UFB application, which is a producer of ROS, could enhance the activities of antioxidant enzymes, such as superoxide dismutase, resulting in the lower O_2^- content in roots under osmotic stress conditions.

In addition, exogenous ROS produced by UFB application could be related to the growth promotion under the control conditions (0% PEG). A small amount of ROS, which is associated with growth promotion, can be generated by plasma membrane NADPH oxidase (Rodríguez et al., 2002). However, treatment with diphenyleneiodonium (DPI) – which inhibits NADPH oxidase, thereby decreasing H₂O₂ production – reduced the concentration of apoplastic H₂ O₂ and resulted in the elongation suppression of the young leaf blade in maize (Rodríguez et al., 2002). Similar



Figure 2. Effects of ultrafine bubble application on nine-days-old soybean growth under full-nutrient and osmotic stress conditions. UFB, ultrafine bubble application; nUFB, non-UFB application. Vertical error bars indicate standard errors of means of six replicates. * and ** indicate significant differences at 5% and 1% levels, respectively, using a t-test.



Figure 3. Effects of ultrafine bubble (UFB) application on production of superoxide radical (O_2^-) in roots of nine-days-old soybeans under no-nutrient and osmotic stress condition. nUFB, no UFB application. Vertical error bars indicate standard errors of means of eight replicates. * and ** indicate significant differences at 5% and 1% levels using an ANOVA or t-test, respectively.

mechanisms could come together to promote the growth under control conditions. Further detailed investigation of ROS production is necessary to clarify the effects of UFBs on various environmental stresses.

In conclusion, the present study quantitatively showed that UFB water can help reduce osmotic stress in soybean seedlings. Soil drought stress can be described as osmotic stress caused by a reduction in the absolute amount of soil water, which in turn reduces the water potential. Thus, the results of this study suggest that UFB water may reduce soil drought stress in plants. Soil drought stress is one of the most impacting factors which alter seriously the plant physiology, finally leading to the decline of the crop productivity (Kapoor et al., 2020). If UFB water increases yield in large-scale farm fields, it can be employed in drip irrigation, which is used in dryland agriculture. The effects of UFB water on mitigating soil drought stresses should thus be further examined in an experimental system where biological, chemical, and physical properties of soil are all properly highlighted actual field soils.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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