Stimulating effect of nanobubbles on the reactive oxygen species generation inside barley seeds as studied by the microscope spectrophotometer

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Abstract

In recent years, nanobubbles (NBs) as a novel technology have attracted people’s great concern in both agricultural and engineering fields. It has been proved that the water containing NBs can accelerate the growth of plants, shellfish and yeast. Up to now, the explanation has not theoretically been given for these new scientific findings, especially to growth promotion and sterilization. On the other hand it is known that reactive oxygen species (ROS) play a vital role in growth by facilitating the required cell wall loosening for cell elongation. In our latest research we’ve reported that the water containing NBs can produce hydroxyl radicals in the water. Here, we've reported a direct evidence for the positive effect of NBs on the ROS production inside barley seeds.

The mechanism of NBs’ physiological activity promotion was investigated with germination tests and nitroblue tetrazolium (NBT) staining method. The germination rates of barley seeds dipped in water containing NBs were more than 10 percentage points greater than those of the seeds dipped in distilled water with the same concentration of dissolved oxygen (DO). 5 germinated seeds in each group were incubated in 1 mM nitroblue tetrazolium (NBT) in 10mM Tris-HCl buffer (pH 7.34) at room temperature for 30 minutes. Then the seeds were cut into 100μm slices with a cutting machine. ROS can be visualized as deposits of dark-blue insoluble formazan compound using microscope spectrophotometer.

ROS accumulated inside both the seeds dipped in the water containing NBs and distilled water after 12 hours dipping time. The accumulation of ROS was observed mainly in the embryo part. From the absorbance measurement, the amount of generated ROS in the seeds has positive correlation with the absorbance value around 560nm. The absorbance value around 560nm of germinating seeds in the water containing NBs were obviously larger than that in the distilled water at the same dipping time. Our results proved that NBs could not only produce the ROS in the water, but when the barley seeds were dipped in the water containing NBs, ROS production inside seed would also increase. Once fully understanding NBs’ promotion of living organism growth is achieved, the manipulation of NBs will develop a new technology in agricultural applications.

Keywords: nanobubbles, reactive oxygen species, microscope spectrophotometer, germination, barley seed

1 Introduction

Since the micro and nanobubbles (MNBs) technology found its favor in the scientific world, both new fundamental ideas and application expectations for it have come out (Liu et al.
In medicine and medical field, MNBs can be used as ultrasound contrast agents. They can also be used for the drug delivery and the gene delivery (HernotandKlibanov, 2008). In engineering fields, nanobubbles (NBs) are useful cleaning agents in the semiconductor processing industry. Moreover, they are effectively used in the flotation process (Liu et al. 2010) and in the ozonation process (Chu et al. 2008) for the wastewater treatment.

In recent years, MNBs technology has attracted people’s great concern in the biological field. It has been reported that MNBs can increase the physiological activity of living organisms. In the hydroponic field, Park found that under the similar dissolved oxygen (DO) concentration the fresh weights of the microbubble-treated lettuces were 2.1 times heavier than those of the normal bubble-treated lettuces. In the aerobic fermentation, Kurata et al. (2007) reported that the relative alkaline phosphatase activity of osteoblastic cell was significantly enhanced by microbubbles (MBs). And the alkaline phosphate is intimately related to the cell growth. In the fisheries field, Ohnari (2003) found that the blood flow of the scallop, when provided with micro bubbles, increased about twice as much as that when MNBs weren’t provided. Besides, MNBs also have physiological promotion effect on animals and humans. Ebina et al. (2013) reported that free oral intake of oxygen-NB distilled water significantly promoted the weight and length of mice compared with that of free oral intake of normal water. Mice drinking oxygen-NB water took higher dose of food. Up to now, the explanation has not theoretically been given for these new scientific findings. Although many reports attempted to describe why physiological activity was promoted, they have simply presented case studies without elaborating the mechanisms. Thus, there is a need to clarify these mechanisms.

Reactive oxygen species (ROS) have long been regarded only as damaging compounds. However, they have recently emerged as key players in plant physiology. ROS play a vital role in growth by facilitating the required cell wall loosening for cell elongation (Passardi et al. 2004). Meanwhile, it has been reported that the ROS, such as hydrogen peroxide, can enhance germination and release residual dormancy of barley seeds (Mabuchi 1994; Ishibashi et al. 2010). Besides, hydroxyl radical was generated in the cell wall during radicle elongation and weakening of endosperm of cress seeds (Muller et al. 2009). Once it is proved that NBs can produce ROS, the production of ROS caused by NBs will offer a reasonable explanation for both NBs’ physiological promotion effect and sterilization effect.

Up to now, there have already been some reports about the production of hydroxyl radicals by MBs. A series of articles from the research group of Takahashi reported that free radicals produced during the collapse of MBs in the absence of dynamic stimulus, such as ozone (Takahashi et al. 2012), strong acid (Takahashi et al. 2007) and copper (Li et al. 2009). Just recently, the present authors reported that not only MBs, but also the NBs can produce ROS in the water, and oxygen-NBs can continuously produce ROS for more than 24 hours (Liu et al. 2014).

In this study, we intend to further clarify whether the water with NBs can increase the amount of ROS inside barley seed cell. The accumulations of ROS inside barley seed cell are compared between the germinated seeds dipped in the water with and without NBs at the same DO concentrations.

2 Materials and methods

2.1 Seed germination

Barley seeds (Hordeum vulgare L.) were harvested in 2012 and were obtained from the University of Ehime, Japan. Germination tests were performed with a pair of seed groups composed of 50 barley seeds each. One group was dipped in a 1-L beaker filled with distilled water containing NBs and the other in a beaker filled with distilled water without NBs. The germination tests were done under two DO levels. For the first DO level, the distilled water was used as the control. The DO concentration of distilled water was approximately 8 mg/L. Then the mixed gas of nitrogen (purity 99.99995%) and air (CO₂<1ppm, THC<1ppm) was introduced into the distilled water through a micro-bubble generator (OM4-GP-040, Aura Tec Co. Ltd., Japan) for 1 hour at the constant temperature of 20°C to obtain the “water containing NBs”. The dissolved oxygen (DO) concentration of the water containing NBs was ad-
justed to be the same as that of the distilled water through a mixed gas flow regulator (Log MIX-D100A-0050 and Log MIX-D100A-0052).

For the second DO level, the MNBs formed with air. The DO concentration of water containing MNBs was about 12mg/L. Then the distilled water was aerated with oxygen to adjust the DO concentration to be the same as that of the water containing air MNBs.

2.2 Cutting process

After 12 hours dipping time, 5 geminated seeds in each group were incubated in 1 mM nitroblue tetrazolium (NBT) in 10mM Tris-HCl buffer (pH 7.34) at room temperature for 30 minutes. Then all the stained seeds were washed with ultrapure water and frozened below -100 °C. In order to compare the amounts of ROS in the same location of seeds dipped in the water with and without NBs. All the seeds were cut vertically into 100μm slices continuously from the tip of sprout part with a cutting machine (Leica CM1950, Leica Biosystems Co. Ltd., Japan). The section areas for the first sliced sample and second sliced sample were about 0.1μm² and 0.25 μm², respectively.

2.3 Distribution of ROS inside barley seed cell

ROS (superoxide radical) can be visualized as deposits of dark-blue insoluble formazan compound using microscope spectrophotometer (MSV-5000, JASCO Co. Ltd., Japan). The microscope spectrophotometer gave the UV-visible spectra, and spectral analyses were done on the small area of the samples. The distribution of ROS inside barley seed cell was determined with fixed wavelength measurements. The difference of absorbance values at wavelength 560nm and 700nm represented the amount of ROS at each location. The absorbance value at 560nm can be used to represent the amount of dark-blue formazan. What's more, the spectral background influence caused by impurities or radiation scattering can be reduced by way of obtaining the absorbance difference at wavelengths 560nm and 700nm. In order to know the distribution of ROS in the sample, the measurement covered the entire sample. For the first sliced sample and the second sliced sample, the numbers of measurement dots were about 100 and 325 respectively. The diameter of each measurement dot was 30μm.

2.4 Quantitative determination of ROS

After determining the location of maximum amount of ROS in the sample by fixed wavelength measurements, the spectrum measurements were also done ranging from 400nm to 850nm at the location with maximum amount of ROS. The correction of spectral background was done by Origin 8.0. The diameter of each measurement dot was 10μm. For each sample, we measured more than 10 microscopic sample dots.

2.5 DO concentration and pH

Dissolved oxygen (DO) concentration was measured in both control water and water containing NBs at 20°C using a DO metre (SG6, Mettler Toledo GmbH, Switzerland). The pH was measured by a pH meter (D-55, Horiba Ltd., Japan). The measurement range of this pH meter is from 0 to 14 with accuracy of ±0.01. The pH meter was set to compensate automatically the pH value at 20 °C.

3 Results and Discussions

3.1 The promotion effect of NBs on the germination process of barley seed

Seed germination is a crucial process in the seed plant life cycle and is important for plant establishment in the natural and agricultural ecosystem as well (Karin, W. et al., 2011). Dur
The germination, the seeds quickly and physically recover from maturation drying, resume a sustained intensity of metabolism, complete essential cellular events to allow for the embryo to emerge, and prepare for subsequent seeding growth (Hiroyuki, N. et al., 2010). As a result, the germination test is a very appropriate method to verify the physiological promotion of water containing NBs.

As shown in Fig. 1, four repetitive germination experiments showed that the germination rates of barley seed dipped in the water containing NBs were higher than those in the distilled water which clearly verified the NBs’ physiological effect. Moreover when the DO concentration increased, the germination rates in the water both with and without NBs increased. These results were coincident with the results we got last year with the seed harvested in 2011. The mechanism of germination promotion effect of NBs was further studied by NBT staining method.

3.2 The distribution on the reactive oxygen species production inside barley seed

The ROS accumulation in the germinating barley seed was very heterogeneous. It was observed mainly in the embryo part (data not shown). In order to compare the amount of ROS in the same location of seeds dipped in the water containing NBs and distilled water, after 12 hours dipping time, the germinated seeds in each group were cut from the tip continuously. The thickness of the sliced sample was 100 μm. From the third sliced sample on, the accumulation of ROS was hardly observed.

Figure 2 and Figure 3 show the fixed wavelength measurement results of representative seeds in each group. Figure 2(A)-(D) and 3(A)-(D) show the microscopic images of representative samples in each measurement condition. The microscopic magnification is 16 times. Figure 2(E)-(H) and 3(E)-(H) show the results of fixed wavelength measurements. The X and Y axis show the location of sample, and the color bar shows the value of absorbance difference at wavelength 560nm and 700nm. As can be seen in Figure 2 and 3, the 3-D images from the spectra value can very precisely quantify the amount of ROS at different locations of samples.

At the same location of seeds, the amount of ROS in the seeds dipped in the water containing NBs was obviously larger than that in the distilled water at two different DO concentrations. While at higher DO concentration (DO about 12.4mg/L), the accumulation of ROS was markedly higher than that under the lower DO concentration. From the 3-D images result, we can also conclude that the ROS do not randomly distribute on the sample. They are mainly accumulated in the edge part.
Figure 2: Images of ROS produced after imbibitions in the sprout part of barley seeds. Dark blue staining indicates the ROS production. DO concentrations of water containing NBs and distilled water were 8.57mg/L and 8.28mg/L respectively. The color bars showed the difference absorbance value at 560nm and 700nm.

Figure 3: Images of ROS produced after imbibitions in the sprout part of barley seeds. Dark blue staining indicates the ROS production. DO concentrations of water containing NBs and distilled water were 12.36mg/L and 12.40 mg/L respectively. The color bars showed the difference absorbance value at 560nm and 700nm.

Figure 4: The distribution of amount of ROS in the seeds dipped in the water containing NBs and distilled water. DO concentrations of water containing NBs and distilled water were 8.57mg/L and 8.28mg/L respectively. The absorbance values showed the difference absorbance at 560nm and 700nm.
700 nm. (A) show the first sliced sample, (B) show the second sliced sample. The error bar shows the standard error of five seeds in each group.

Figure 5 The distribution of amount of ROS in the seeds dipped in the water containing NBs and distilled water. DO concentrations of water containing NBs and distilled water were 12.36 mg/L and 12.40 mg/L respectively. The absorbance values showed the difference absorbance at 560 nm and 700 nm. (A) show the first sliced sample, (B) show the second sliced sample. The error bar show the standard error of five seeds in each group.

The proportional distributions of ROS inside barley seed in each group were further calculated and showed in Figure 4 and Figure 5. As can be seen in Figure 4, the proportion of low concentration (absorbance value in the range of 0.05-0.3) of ROS in barley seed dipped in the water without NBs was higher than that in the water containing NBs. While, for the proportion of medium concentration (absorbance value in the range of 0.3-0.8) of ROS in the barley seed dipped in the water containing NBs was statistically higher than that in the water without NBs. For the high concentration of ROS (absorbance value above 0.9), it is hard to observe in the seeds dipped in the water without NBs.

Figure 5 shows the ROS accumulation under the higher DO concentration. When the DO concentration increased, the germination process also became faster. Compare to Figure 4, it can be concluded that both the seeds dipped in the water with NBs and without NBs, the ROS production inside barley seeds increased. The embryo is covered by both living endosperm and dead testa. For the sake of successful germination of barley seeds, ROS is necessary for the cell wall loosening and the covering envelopes weakening (Muller et al. 2009). The amount of ROS inside the barley seeds has positive correlation with the germination speed.

3.3 The effect of NBs on the production of ROS inside the barley seed cell

After determining the location of maximum amount of ROS in the sample by fixed wavelength measurements, the spectrum measurements were also done from 400 nm to 850 nm at the location with maximum amount of ROS. As can be seen in Fig. 6, the absorbance values around 560 nm of seeds germinating in the water containing NBs were obviously larger than those in the water without NBs at the same dipping time.

The DO concentrations of NBs water and the water without NBs were adjusted to be the same. Thus only NBs themselves resulted in the increase of ROS inside barley seeds, although the ROS generated by NBs was in vitro. Exogenously supplied H₂O₂ can promote the germination of cereal plants, such as barley, wheat, rice and zinnia elegans seeds (Naredo et al. 1998; Ogawa K and Iwabuchi M 2001). From our results, it can be confirmed that the existence of NBs can increase the ROS production inside barley seeds. The generation of ROS by NBs could play a positive role both in the defense system of plants against pathogenic organisms and promotion of physiological activity of plants.
Figure 6 Spectra of formazan formed as a result of ROS production in germinated barley seeds. For each group we measured 5 seeds. Each curve represents the mean value of more than 50 microscopic sample dots. (A) and (C) were the first sliced samples (B) and (D) were the second sliced samples. (A) and (B) DO concentration about 8.4 mg/L. (C) and (D) DO concentration about 12.4 mg/L

4 Conclusions

In summary, the direct evidence is provided that NBs increase the production ROS inside barley seeds during the germination process. NBs could not only produce the ROS in the water, they could also increase the ROS production inside seed. From our research above comes out a novel mechanism for NBs’ promotion effect on physiological activity of living organisms. Once fully understanding NBs’ effect on living organism growth is achieved, the manipulation of NBs will develop a new technology in agricultural applications.

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6 References


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