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Disinfection by Ozone Microbubbles Can Cause Morphological Change of *Fusarium oxysporum* f. sp. *melonis* Spores

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To investigate the difference in the disinfectant efficiency of ozone microbubbles (O_3MB) and ozone millibubbles (O_3MMB), the morphological change of the treated *Fusarium oxysporum* f. sp. *melonis* spores was observed with scanning and transmission electron microscopies (SEM and TEM). The disinfectant efficiency of O_3MB on *F. oxysporum* f. sp. *melonis* spores was greater than that of O_3MMB. On observation with SEM, it was revealed that morphological change of *F. oxysporum* f. sp. *melonis* spores was caused by O_3MB and O_3MMB, and damage to the spore surfaces by O_3MB occurred sooner than that by O_3MMB. On observation with TEM, it was furthermore confirmed that *F. oxysporum* f. sp. *melonis* spores treated with O_3MB induced wavy deformation of cell membrane and the intracellular change different from that with O_3MMB. Therefore, the greater disinfection efficiency of O_3MB was suggested to be caused due to the function of the MB in addition to the oxidative power of O_3.

Keywords : disinfection, *Fusarium oxysporum* f. sp. *melonis* spores, ozone microbubbles

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Hydroponic culture is a plant cultivation technique in which plants are grown in a nutrient solution without soil. It has the potential for high crop productivity in a small area. However, if plant pathogens enter the solution, they can spread rapidly throughout the hydroponic culture facility and cause catastrophic damage. For this reason, disinfection of the solution is essential, although direct administration of pesticides into the solution has been prohibited at law (Ministry of Agriculture, Forestry and Fisheries, 2010). Therefore, it is desired to establish safe and effective alternative disinfection methods, and the various disinfection treatments have been investigated, including UV light, heat, the titanium dioxide photocatalytic reaction, and ozone (O_3) (Bando et al., 2008; Dannehl et al., 2016; Ehret et al., 2001; Igura et al., 2004; Koohakan et al., 2003; Ohtani et al., 2000; Runia, 1995). However, these treatments still are not put into any practical use in terms of the efficiency, treatment time, and running cost. Particularly, O_3 gas is effective disinfectant due to its strong oxidation power, although it is difficult to use in hydroponic cultures because of its extremely low solubility in water (0.105 g 100 ml^{-1} (0°C)).

Recently, tiny bubbles less than 50 μm in diameter, called microbubble (MB), have been studied and used in many fields. They rise more slowly in water than millibubbles (MMB), which have diameters in the mm to cm range (Takahashi et al., 2003; Takahashi, 2005), and possess additional properties such as the interface charge, long stagnation, slow buoyancy, the shrinkage and the generation of free radicals by their collapsing other than dissolving power (Li and Tsuge, 2006; Li et al., 2009; Zheng et al., 2015). Previously, we focused on long retention time in water and the dissolving power of MB in addition to the strong oxidative power of O_3, and found that O_3MB were more effective than O_3MMB for the disinfection of *Fusarium oxysporum* f. sp. *melonis* spore and *Pectobacterium*

carotovorum subsp. *carotovorum* in nutrient solution for hydroponic culture (Kobayashi et al., 2011a, 2011b, 2011c, 2012). Furthermore, effective disinfection by O_3MB has been reported by other researchers (Chuajedton et al., 2015; Inatsu et al., 2011), although it is still not clear about the exact mechanism of disinfection by O_3MB. In this study, the morphological change of *F. oxysporum* f. sp. *melonis* spores by O_3MB and O_3MMB was therefore observed by using scanning and transmission electron microscopies (SEM and TEM), and the difference was discussed.

F. oxysporum f. sp. *melonis* NBRC6385 suspension (approximately 1.0×10^7 spores ml^{-1}) was prepared in a manner similar to a previous report (Kobayashi et al., 2011a). For each experiment, 15 l of tap water were collected in a plastic cylindrical container (28 cm dia. × 48 cm height) and kept for 24 h at room temperature to remove chlorine. Water quality test paper (Nissan aquacheck 3; Nissan Chemical Industries, Ltd., Tokyo, Japan) was used to confirm that no residual chlorine remained. O_3 was generated by using an O_3 generator (ED-OG-A10, Ecodesign Co. Ltd., Saitama, Japan) at a flow rate of 2.5 l min^{-1}. O_3MB and O_3MMB were generated by using a decompression type MB generator (20NEDO4S, Shigen-Kaihatsu Co., Ltd., Kanagawa, Japan) and a commercial air pump, respectively. The concentration of dissolved O_3 in both O_3MB and O_3MMB waters was set to 1.5 ppm at 15°C. The pH of O_3MB and O_3MMB waters was the same at 6.8 and was not changed before and after O_3MB and O_3MMB generation. *F. oxysporum* f. sp. *melonis* spores were added to 100 ml of the O_3MB and O_3MMB waters with final concentrations of 1.0×10^3-1.0×10^4 cfu ml^{-1}. Aliquots of the treated waters were collected after 0, 15, 30, 45, 60 and 120 s. Aliquots of 0.1 ml of the collected waters were plated on potato dextrose agar (Difco, Becton Dickinson, Flanklin Lakes, NJ, USA) plates, and the plates were incubated at 30°C for 48 h. After incubation, the numbers of surviving spores were measured by counting the colonies formed on the plates. The detection limit was 10 cfu ml^{-1}. Each experiment was performed in duplicate.

F. oxysporum f. sp. *melonis* spores were collected from 5 ml of the suspension by filtration with a cartridge filter (Anotop 10, GE Healthcare UK Ltd., Buckinghamshire, UK). The sample on the cartridge filter was pre-fixed with 2.5% glutalaldehyde solution diluted with a phosphate buffer solution (PBS, pH 7.0). A filter ejected by decomposing the cartridge filter was washed with PBS (pH 7.0), post-fixed with 2% OsO_4 solution for 1 h, and then serially dehydrated for 20 min each in 50%, 70%, 80%, 90%, 95%, 99.5% and dehydrated ethanol. SEM observations were performed as follows: The dehydrated sample was

immersed in a mixture of t-butyl alcohol and dehydrated ethanol (1:1) for about 10 min, transferred to 100% t-butyl alcohol, freeze-dried with a freeze drier (ES-2030, Hitachi High Technologies Co., Tokyo, Japan), and OsO_4-coated with a OsO_4 coater (HPC-1SW, Vacuum device Inc., Mito, Japan) (the thickness of the coating was adjusted to 3 nm). Then the sample was observed with an SEM (JSM-6700F, JEOL Ltd., Akishima, Japan) operated at 3.0 kV. Nine *F. oxysporum* f. sp. *melonis* spores in the SEM photographs were selected at random and the widths was measured with a scale. Significant differences were evaluated by the ANOVA and Fisher's LSD using the Ekuseru-Toukei 2012 for Window statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan) ($P < 0.05$). TEM observations were performed as follows: The dehydrated samples were serially immersed for 2 h each in 1:1, 2:1, and 3:1 mixtures of Quetol-651 (Cosmo Bio Co., Ltd., Tokyo, Japan) and ethylene glycol diglycidyl ether, and then embedded in 100% Quetol-651 at 60°C. Ultra-thin sections (thickness 70-100 nm) were made from the embedded samples with an ultramicrotome (ULTRA CUT UCT, Leica Microsystems, Wetzlar, Germany). The ultra-thin sections were doubly electron-strained using 4% uranyl acetate for 12 min and lead nitrate for 5 min, and then observed with a TEM (JEM-2010, JEOL Ltd.) operated at 140 kV.

The survival rates of *F. oxysporum* f. sp. *melonis* spores in water treated with O_3MB and O_3MMB are shown in Fig. 1. The disinfectant efficiency of O_3MB on *F. oxysporum* f. sp. *melonis* spores was greater than that of O_3MMB, because the numbers of surviving spores after treatment with O_3MB and O_3MMB reached the detection limit at 45 s and 60 s, respectively. The result agreed with our previous study (Kobayashi et al., 2011a). Amount of hydroxyl
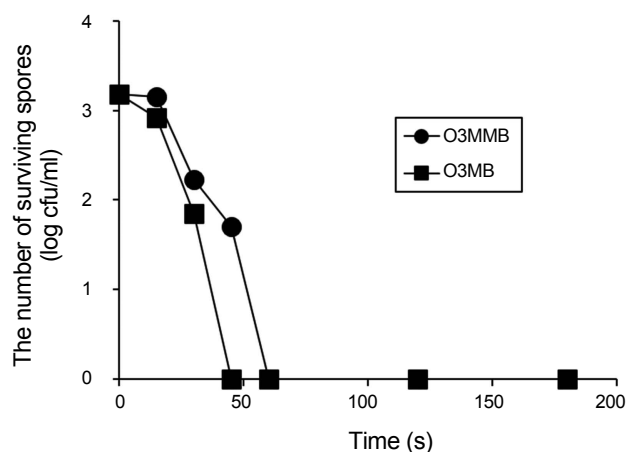

Fig. 1. Disinfection of *F. oxysporum* f. sp. *melonis* spores by O_3MB and O_3MMB. The data presented was the mean of duplicate.
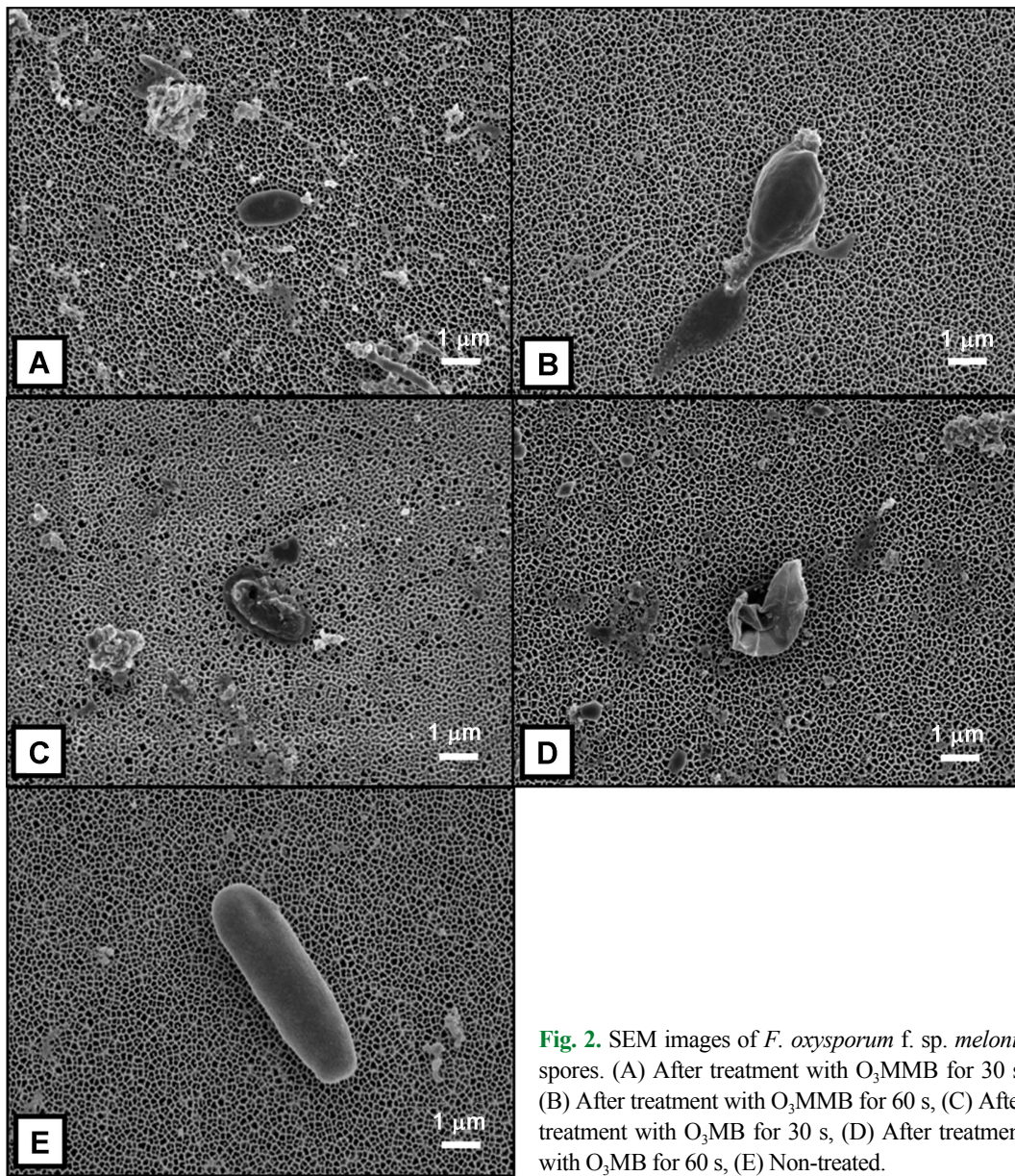
Fig. 2. SEM images of *F. oxysporum* f. sp. *melonis* spores. (A) After treatment with O₃MMB for 30 s, (B) After treatment with O₃MMB for 60 s, (C) After treatment with O₃MB for 30 s, (D) After treatment with O₃MB for 60 s, (E) Non-treated.

radicals generated from O_3 is not enough to have a disinfectant effect (Cho et al., 2003). However, O_3MB may generate more hydroxyl radicals than O_3MMB, because the oxidation-reduction potential and iodine liberation is higher with O_3MB than with O_3MMB (Chuajedton et al., 2015). Furthermore, the use of MB enhances the formation of hydroxyl radicals due to more rapid O_3 decomposition (Tsuge et al., 2009), and the hydroxyl radicals generated from O_3MB accelerate the oxidative power (Chu et al., 2008). The high oxidative power of O_3MB contributes to the oxidative power of O_3, the reactivity of the hydroxyl radicals, the substantivity, ζ surface potential, mass-transfer coefficient, and use efficiency (Zheng et al., 2015). Therefore,

the greater disinfectant efficiency of O_3MB than O_3MMB is likely be due to these synergistic effects.

The SEM images of *F. oxysporum* f. sp. *melonis* spores treated with O_3MB and O_3MMB are shown in Fig. 2. The spores treated with O_3MMB for 30 s showed no obvious surface injury, although spores treated with O_3MMB for 180 s were deformed. On the other hand, spores treated with O_3MB showed obvious surface injury after 30 s and the spores were completely destroyed after 180 s. The widths of *F. oxysporum* f. sp. *melonis* spores treated with O_3MMB for 30 s were lower than those of non-treated spores, and then the spores swelled after 180 s (Fig. 3). However, the widths of spores treated with O_3MB for 30
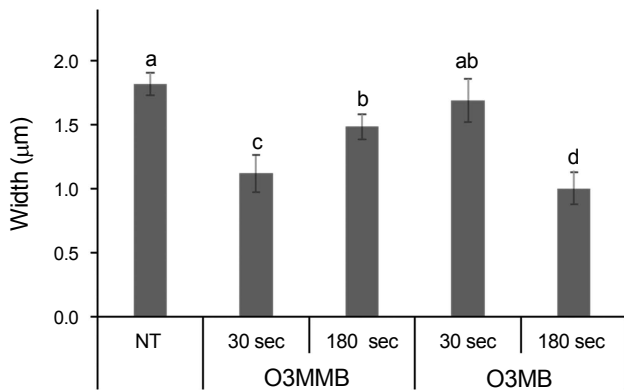
Fig. 3. The widths of *F. oxysporum* f. sp. *melonis* spores in SEM images. NT: Non-treated. The results indicate the means with standard deviation of 9 spores.

s were the same as those of non-treated spores and diminished in size by 180 s. Furthermore, the TEM images indicated the appearance of liquid foam in the mid-regions of the *F. oxysporum* f. sp. *melonis* spores treated with O$_3$MMB for 180 s (Fig. 4). Then, the spores had swelled by O$_3$MMB for 180 s, as water entered the cells through the damaged cell wall. Cho et al. (2010) reported that disinfection of bacterial cells by O$_3$ was due to injury of the cell wall. Zhang et al. (2011) showed that disinfection of *Pseudomonas aeruginosa* by O$_3$ was due to increase in cell membrane permeability and coagulation of the intracellular substrate. Thanomsub et al. (2002) concluded that disinfection by O$_3$ was caused due to the destruction of the cell wall and leakage of the intracellular substrate, followed by
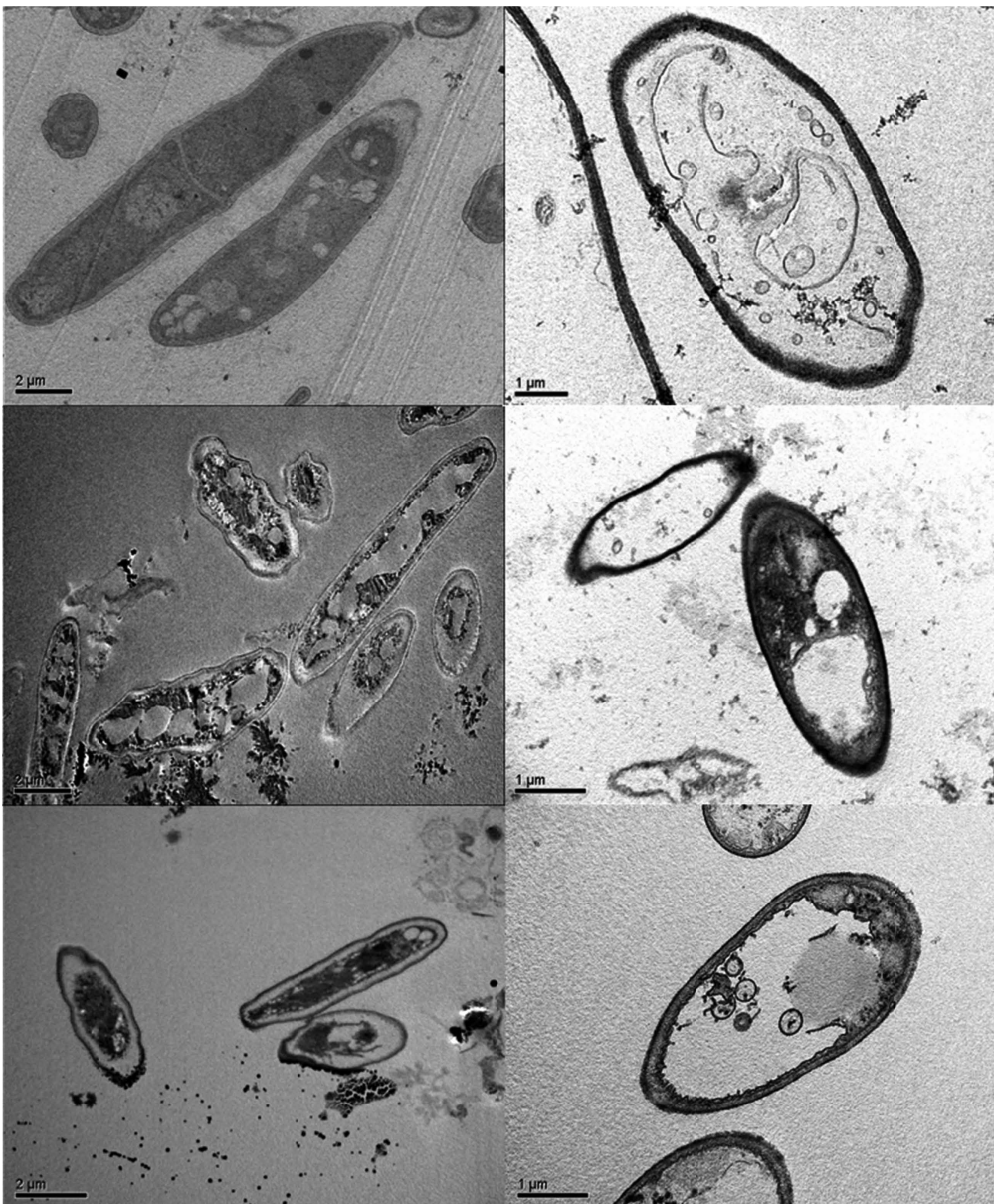

Fig. 4. TEM images of *F. oxysporum* f. sp. *melonis* spores. Top: Non-treated, Middle: After treatment with O$_3$MMB for 180 s, Bottom: After treatment with O$_3$MB for 180 s.

cell lysis. Therefore, it is possible that the *F. oxysporum* f. sp. *melonis* spores treated with O₃MMB for 30 s initially shrank due to leakage of the intracellular substrate through the damaged cell wall. On the other hand, the TEM images of the *F. oxysporum* f. sp. *melonis* spores treated with O₃MB for 180 s showed the wavelike deformation of cell membrane and appeared to have a space between the cell membrane and/or wall and the cytoplasm. Diao et al. (2004) confirmed that hydroxyl radicals generated by the Fenton reaction induced the injury of *E. coli* cell membranes greater than O₃ by the observation with SEM. Therefore, it appears that hydroxyl radicals generated from the O₃MB induces the wavy injury of cell membrane of *F. oxysporum* f. sp. *melonis* spores. Furthermore, it was considered that coagulation of the substrate within the spores and leakage of the substrate through the damaged cell membrane were induced by the higher amounts of hydroxyl radicals generated from the tiny O₃MB penetrated into the spores. The result may lead to cell death due to the leakage and/or coagulation of the intracellular substrate, followed by the lysis of the spore.

These results show that disinfection efficiency of O₃MB on *F. oxysporum* f. sp. *melonis* spores is higher than that of O₃MMB and may be due to the action of MB in combination with the high oxidative power of the O₃. Therefore, it is considered that disinfection of *F. oxysporum* f. sp. *melonis* spores by O₃MB causes the leakage and/or coagulation of intracellular components associated with damage to the cell membrane and/or cell wall, and subsequently leads to lysis of the spore.

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