Influence of the rhizosphere microbial community on root-borne disease suppression in the multiple parallel mineralization system

(MPM 法における根部病害抑止効果に影響を及ぼす根圏微生物生態系に関する研究)

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Summary

Rhizosphere microbial community inhabiting in a hydroponics system called multiple parallel mineralization (MPM) system can potentially suppress root-borne diseases. The suppression of the root-borne diseases is associated with microbe–pathogen interactions in the MPM system. In the Bacterial wilt disease caused by the bacteria *Ralstonia solanacearum*, disease symptoms did not appear, and the *R. solanacearum* was not detected from the microbial community of the plant rhizosphere. For a fungal pathogen, *Fusarium oxysporum* inoculated into the MPM system remained present within the microbial community of the plant rhizosphere, but disease symptoms did not appear. Potential bacterial antagonists were detected from the MPM system based on denaturing gradient gel electrophoresis profiles, but microbiological factors influencing *R. solanacearum* and *F. oxysporum* were moderated or lost when the rhizosphere microbial community underwent filtration, autoclaving, and dilution. Morphological characterization carried out on the *F. oxysporum* in the rhizosphere microbial community showed that rhizosphere microorganisms adhered to *F. oxysporum*, and the number of *F. oxysporum* cells formed chlamydospores which survive unfavorable conditions. The suppression against *F. oxysporum* was reproduced by several bacteria isolated from the rhizosphere microbial community. These results suggest that the rhizosphere microbiota including core bacterial interactions suppresses proliferation of *F. oxysporum* in the MPM system by converting to endurance organs and constructs an ecosystem consistent with with *F. oxysporum*.

Introduction

Most plants establish complex associations with microorganisms in soil. The rhizosphere is a key interface governing multiple interactions between plant roots and soil microorganisms. The dynamics of the rhizosphere play a critical role in establishing the resulting ecosystems between plants and microorganisms. Plant-microbial and microbial-microbial interplays in the rhizosphere can provide plants with physiological traits that cannot be acquired without microbes. For agriculturally important characteristics, among other things, the interactions contribute to supply of nutrients that would otherwise be in form inaccessible (Bais et al, 2006), protection of soil-borne phytopathogens (Innerebner et al. 2008), growth improvement (Ali et al. 2009), and environmental adaptation such as heat and salt tolerance (Castiglioni et al 2008; Zhang et al 2008). Current works addressing associations between microbiological functions and microbial diversity by the advanced DNA sequencing provides an opportunity to reveal the picture of the rhizosphere microbial community. The rhizosphere microbiota which contributes to nitrification in a natural soil was influenced by a host plant (Bulgarelli et al. 2012; Lundberg et al. 2012). A study in the rhizosphere microbiota related to protection against soil-borne diseases indicated the existence of core microbial community influencing disease control in the rhizosphere (Mendes et al. 2011). However, the massive information about microbial compositions in the rhizosphere from the advanced DNA sequences provides fundamental and comprehensive questions to be addressed. Understanding associations between plant-microbial interplays and their functional traits capable of answering how the rhizosphere microbial community generates its functional trait and how microbiological

diversity is correlated with its functional trait would be required.

Due to complex entities of microorganisms in soils, important aspects of the study in plant-microbial interplays are to use an experimental model in which microbial-induced functions can be repeatable. To determine the microbial factors influencing the establishment of functional abilities within plant-microbial ecosystem, one approach for such model is to use the multiple parallel mineralization (MPM) system, a novel form of hydroponics. The MPM system forges microbial activities leading to the mineralization of organic substances in the plant rhizosphere and to the development of plant–microbe ecosystems (Shinohara et al. 2011). The rhizosphere microbial community that develops in MPM system is responsible for two reproducible functions that are necessary for sustainable plant growth: high nutrient production efficiency and the control of root diseases. The former trait results from mineralization of the organic fertilizer used in the hydroponics solution by a unique process that we have called “multiple parallel mineralization” (Shinohara et al. 2011). This process promotes the transformation of organic nitrogen into nitrate nitrogen in the hydroponics solution as a result of two sequential microbial processes: ammonification and nitrification. To achieve this process, it is necessary to culture soil microorganisms in the hydroponics solution and develop a microbial community that is capable of mineralizing organic fertilizer into nitrate ions. This approach recreates a microbial environment that promotes coexistence of the plants and microbes in a hydroponics system in a manner similar to that which occurs in soils (Shinohara et al. 2011).

The second trait means that the MPM system has the potential to control root-borne diseases by the actions of the microbial community that develops in the rhizosphere. We

have observed that some root-borne diseases that often damage several plant species in inorganic hydroponics systems, including lettuce, komatsuna, rice, cucumber, and pepper, did not occur in the MPM system, allowing cultivation of the plants without requiring fungicides or other antibiotics until they could be harvested (Shinohara 2006; Shinohara et al. 2011). These results revealed the importance of the microbial community that colonizes the plant rhizosphere in root-bone disease suppression.

To elucidate the mechanisms by which soil-borne disease suppression occurs by plant-microbe and microbe-microbe interactions, understanding of the interplay has been increased by studies of a variety of pathogens, symbionts, and mycorrhizal fungi, and the insights gained from this research strongly suggested that soil microorganisms are associated with plant growth and health (Bisseling et al. 2009; Lugtenberg and Kamilova 2009). The microbiological basis for suppression of pathogenesis by associations between a specific pathogen and microbial antagonist (Weller et al. 2002; Borneman and Becker 2007) or between plant hosts and specific rhizobacteria (Van Peer et al. 1991; Van Loon et al. 1998) has been well established. The components of suppression have been described for multiple pathosystems, especially for natural or inoculated microorganisms that were recruited as biocontrol agents (Mazzola 2004; Borneman and Becker 2007). The study of naturally occurring disease-suppressive soils has helped researchers to make considerable progress in identifying the biotic and abiotic attributes that contribute to suppression of root diseases caused by various plant pathogens (Weller et al. 2002; Mazzola 2004). Molecular analytical tools such as DNA-based methods and culture-based techniques have been applied to characterize the microbial components responsible for this suppression and reveal the structural and functional attributes of the soil microbial community (Cook et al.

1995; Levesque et al. 1998; Muyzer and Smalla 1998; Benitez and Gardener 2009; Mendes 2011).

Our interest in the root disease suppression obtained in MPM system led us to explore the properties of the rhizosphere microbial community that are capable of inducing the suppression of root-borne diseases. To discover the biological nature of the disease suppression and describe the possible factors influencing root-borne pathogens in MPM system, we used two approaches. The first approach originated from molecular profiling of microbial population structures. This population-based approach relies on the evaluation of ribosomal gene sequences extracted from the rhizosphere environment. We used the polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) method to characterize the major microbial population of the rhizosphere biofilms that develop in the MPM system (Muyzer et al. 1993). This approach aims to reveal the population structure of the rhizosphere microorganisms that control root diseases. The second approach explored the microbiological factors that underlie the suppression of root-borne diseases, and led us to investigate the interplays between rhizosphere microbes and root-borne pathogens in the MPM system. In this study, we tried to examine the potentials for controlling both bacterial and fungal soil-borne pathogens and related microbial properties. To explore the suppressive effects on bacterial root-borne diseases, we evaluated suppressive effects on bacterial wilt disease caused by *Ralstonia solanacearum*, a plurivorous phytopathogenic bacterium (Hayward 1994) and focused on the bacteria wilt disease of tomato, which causes significant damages on tomato plants in both soil and soilless culture (Schell 2000; Shimizu et al. 2007). For fungal soil-borne disease suppression, we used the fungal root pathogen *Fusarium oxysporum*, an important phytopathogenic fungus in many crops

(Armstrong and Armstrong 1981; Beckman 1987; Di Pietro et al. 2003). We focused on *F. oxysporum* f. sp. *lactucae*, which causes root rot and wilting of commercial lettuce plants and has become a significant problem in Japan (Fujinaga et al. 2003, 2005). In addition, we tried to examine the potentials for suppressive effects of the rhizosphere microbes isolated from the MPM system on *the F. oxysporum*. The success of these approaches will improve our understanding of microbial contributions to the suppression of root-borne diseases and provide insights into previously unknown biological phenomena that occur in the plant rhizosphere.

Experimental Procedures

Hydroponics solution and microbial biofilm

The inorganic nutrient solution was prepared, using inorganic fertilizers (4.8 g of Otsuka House TM No. 1 and 2.7 g of Otsuka House TM No. 2; Otsuka Chemical, Osaka, Japan) to supply 200 mg L^{-1} of nitrate ions. MPM solution, which is a nutrient solution used in MPM system, was prepared by a procedure that we have called “multiple parallel mineralization,” as previously described by Shinohara et al. (2011). We prepared 15 L of water containing 150 g of nursery soil as a microbial inoculum (Nae-ichiban; Sumirin Agro-Products, Aichi, Japan). We then added 4 g of corn steep liquor (Nature Aid; Sakata Seed, Yokohama, Japan) containing 120 mg N daily for 7 days, followed by adding 150 g of oystershell lime (Ryujyoseruka; Urabe Sangyo, Hiroshima, Japan) to the water as a supplement to provide minor nutrients, according to the previous report (Shinohara et al. 2011). The water was aerated with an aeration pump and held at room temperature for more than 2 weeks, until

daily tests using an RQ-Flex Plus Analyzer (Merck, Frankfurt, Germany) revealed the presence of at least 200 mg L^{-1} nitrate. Soils added to the water were removed when more than 5 mg L^{-1} of nitrate began to be detected. We used MPM solution that contained 200 mg L^{-1} of nitrate ions generated through this procedure as hydroponics solution.

The MPM solution was centrifuged at 10,000 × *g* for 5 min, and then the microbial pellet was resuspended in sterile distilled water and centrifuged again using the same rate and duration. This washing step was repeated three times and the resulting microbial solution was used as the stock biofilm solution.

Microscopic observations

Tomato (*Solanum lycopersicum* L. cv. Ponderosa) seeds were sown in vermiculite soil and grown in a greenhouse (about 16 hours of light and 10-30 °C). At 2 weeks after seeding, four seedlings were transplanted into MPM solution, and cultivated in a greenhouse. At 4 days after transplanting, the roots were observed with a light microscope (BX50; Olympus, Tokyo, Japan) and a digital microscope (VHX-1000 with VHX-D500/D510 lens; Keyence, Osaka, Japan). We also fixed 5-mm sections of the roots twice for 2 h in 2% (v/v) glutaraldehyde at room temperature and dehydrated the sections through a graded ethanol series (20 to 100%), followed by immersion in 100% acetone for complete drying. Samples were coated with a thin gold layer using a JEE-400 vacuum evaporator (JEOL, Tokyo, Japan) and observed using a JSM-5800 scanning electron microscope (JEOL).

PCR–DGGE and data analysis

Rice (*Oryza sativa* L. *japonica* cv. Koshihikari), komatsuna (*Brassica rapa* L. var.

perviridis cv. Komatsuna), and tomato (*S. lycopersicum* L. cv. Ponderosa) seeds were sown in vermiculite soil and grown in a greenhouse (about 16 hours of light and 10-30 °C). At 2 weeks after seeding, 16 seedlings of each species were transplanted into MPM solution. Seedlings were then cultivated in a greenhouse.

Individual plant roots (n = 1 per plant) were harvested from two plants at 1, 2, 3, 7, and 14 days after transplanting. The root samples were stored in 15-mL tubes containing sterilized water. After the roots were gently washed to remove any microbes or biofilms, the water was centrifuged at 10,000 × g for 5 min, and the resulting bacterial pellet was used for the DGGE analysis.

Bacterial DNA extraction was conducted using the standard procedure (Sambrook and Russell 2001). The DNA fragments extracted from the bacterial samples were amplified using the GC-341f (CCT ACG GGA GGC AGC AG) and GC-534r primer pair (ATT ACC GCG GCT GCT GG) (Muyzer et al. 1993). Electrophoresis was performed with a Dcode DGGE complete system (Bio-Rad, Hercules, CA, USA), using 8% polyacrylamide gel for the PCR products. We used 12 h at 100 V in a linear 25 to 65% denaturant gradient for the electrophoresis conditions. DGGE bands were excised and reamplified using the same primers. The reamplified PCR products underwent the DGGE, and the procedure for the DGGE was repeated a few times, allowing for detecting a single DGGE band that represents a single bacterium.

DNA sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3130x1 Genetic Analyzer System (Applied Biosystems) using the standard methods (Sambrook and Russell 2001). The sequences have been deposited in the DDBJ/EMBL/GenBank databases under

accession numbers AB605770 to AB610410. The sequences were compared with the NCBI DNA database sequences using the BLAST software (Altschul et al. 1997). Based on the nucleotide sequences of the 16S ribosomal DNA, the phylogenetic relationships among 29 isolates were analyzed using the neighbor-joining method provided by version 2.10 of the DNASIS Pro software (Hitachi Software Engineering Co., Tokyo, Japan). The stability of groups was assessed using 1000 bootstrap replications of the data sets. Clustering analysis was performed using an unweighted pair-group method with arithmetic mean (UPGMA) based on Bray–Curtis indices to evaluate the percentage similarity among the samples using the vegdist function of the vegan package for the R statistical software (Cleary et al. 2012). For this analysis, each DGGE band was assumed to be operational taxonomic units (OTUs) and assigned to a bacterial phylum. A permutation test was performed based on the values in the Bray–Curtis distance matrix using the adonis function provided by the vegan package (Oksanen 2011). The number of permutations was set at 999; all other parameters used the default values set in the function.

Bacterial and Fungal pathogen inoculum

We used *R. solanacearum* strain MAFF301487 (Yamazaki et al. 2000). The strain was cultured in 100 mL of CPG medium (0.1% casamino acid, 1% peptone, and 0.5% glucose, pH 7.0) in a flask with shaking (180 strokes/min) at 32°C for 3 days in the dark. The resulting cell suspension was centrifuged at 6,500 rpm for 5 min at 15°C. The supernatant was decanted, and the cell pellet was washed with sterilized water. After final centrifugation, the cell pellet was resuspended in 10 mL of sterilized water, and the final concentration was adjusted to approximately 1×10^5 cells mL^{-1} of sterile distilled water for

use as inoculum.

For a fungal pathogen, we used *F. oxysporum* f. sp. *lactucae* strain H111; its red fluorescent protein (dsRed; Takara Bio, Shiga, Japan) expression transformant, H111-dsRed; and *F. oxysporum* f. sp. *radicis-lycopersici* strain LS89-1-1 (Yamauchi et al. 2005). We constructed the dsRed expression vector pTEFRFP, which is driven by the *Aureobasidium pullulans* TEF promoter of pTEFEGFP (Vanden Wymelenberg et al. 1997), using the primers Ptef-RFP, Tgla-RFP, RFP-F, and RFP-R (Table 1) and the In-Fusion dry-down PCR cloning kit (Clontech, Mountain View, CA, USA). Protoplast preparation and the cotransformation of *F. oxysporum* strain H111 were performed as previously described (Iida et al. 2008). Transformants carrying *hph* were selected on regeneration medium containing hygromycin B (Wako Pure Chemicals, Osaka, Japan) at 100 μg mL^{-1} (Iida et al. 2008).

Table 1. Primers used in transformation of *F. oxysporum* [a].

Name	Sequence (5' → 3')
Ptef-RFP	CCT CGG AGG AGG CCA TGT TTG ACG GTG ATG TAT GGA
Tgla-RFP	CCA CCT GTT CCT GTA GAC AAT CAA TCC ATT TCG CTA
RFP-F	*ATG* GCC TCC TCC GAG GAC GT
RFP-R	*CTA* CAG GAA CAG GTG GTG GC

[a] The primers have 16 bases (underlined) that are identical to the sequences at the ends of the dsRed gene. The initiation and termination codons are indicated in italics.

Strains were routinely maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA) at 4 °C. PDA agar blocks (3 mm in diameter) carrying mycelia were inoculated into 100 mL of potato dextrose broth (PDB; Difco) in a flask and cultured on an orbital shaker (120 rpm) at 25 °C for 4 days in the dark. Subsequently, the culture was filtered through four layers of sterile gauze, and the filtrate, which contained bud cells, was centrifuged at 10,000 × *g* for 5 min. The cell pellet was resuspended in 50 mL of sterile distilled water and centrifuged three times at the same settings. The cell pellet was resuspended with sterile distilled water, and the final concentration was adjusted to 1×10^4 cells mL^{-1} of sterile distilled water for use as inoculum.

Inoculation test

Boston lettuce (*Lactuca sativa* L. cv. Saradana) and tomato (*S. lycopersicum* L. cv. Ponderosa) were used for the inoculation test. Seeds were sown in vermiculite soils, and 2 weeks-grown seedlings were transplanted to hydroponics system. For bacterial pathogen, twelve seedlings of tomato plants were transplanted into separate 15-L plastic containers filled with MPM solution or inorganic hydroponics solution. At 4 days after transplanting, each of the seedlings was inoculated with cell suspension of *R. solanacearum* at a final density of 0.9×10^5 to 1.59×10^5 cfu mL^{-1}. Experiments were performed at 32 °C with a 12-h photoperiod and done three times (three planters with twelve plants each) for each plant species–solution combination. The density of *R. solanacearum* in infected plant roots and in the experimental hydroponic solutions at the end of the study period (12 days after inoculation) was estimated using Hara-Ono medium (Hara and Ono 1983).

For a fungal pathogen, four seedlings of each species were transplanted into separate 1.5-L plastic pots filled with MPM solution or inorganic hydroponics solution. At 4 days after transplanting, each of the seedlings was inoculated with a bud cell suspension of *F. oxysporum* at a final density of 1×10^4 cells mL^{-1}. There were three replicates (three pots with four plants each) for each plant species–solution combination. All the experiments were performed at 32 °C with a 12-h photoperiod. The density of *F. oxysporum* in infected plant roots and in the experimental hydroponic solutions at the end of the study period (12 days after inoculation) was estimated using Komada's selective medium (1.0 g $Na_2B_4O_7\cdot10H_2O$, 1.0 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4\cdot7H_2O$, 0.01 g Fe-Na-EDTA, 2.0 g L-asparagine, 20.0 g D-galactose, 15.0 g agar, 1.0 g pentachloronitrobenzene, 0.5 g sodium cholate, and 0.3 g L^{-1} streptomycin sulfate) supplemented with hygromycin B at 100 μg mL^{-1} (Komada 1975).

A test for growth suppression of *R. solanacearum* and *F. oxysporum*

R. solanacearum strain MAFF301487 was added to a test tube filled with 5 mL of MPM solution at a final density of 1×10^4 cells mL^{-1}. To test whether the suppression resulted from diffusible substances produced by microbes in the solution or by the microbes themselves, the MPM solution was filtered through a 0.2-μm membrane (Millipore, Billerica, MA, USA) or autoclaved for 20 min at 121 °C. The inorganic solution was prepared and inoculated similarly. The inoculated solutions were cultured in the dark at 32 °C on a shaker (170 rpm). From each replicate, solution samples were collected 14 days after the experiment started and incubated at 32 °C for 3 days on Hara-Ono medium.

F. oxysporum strain H111-dsRed was added to a test tube filled with 5 mL of MPM solution at a final density of 1×10^2 cells mL^{-1}. To test whether the suppression resulted from diffusible substances produced by microbes in the solution or by the microbes themselves, the MPM solution was filtered through a 0.2-μm membrane (Millipore, Billerica, MA, USA) or autoclaved for 20 min at 121 °C. The inorganic solution was prepared and inoculated similarly. The inoculated solutions were cultured in the dark at 25 °C on a shaker (170 rpm). From each replicate, solution samples were collected 14 days after the experiment started and incubated at 25 °C for 3 days on Komada's selective medium supplemented with hygromycin B at 100 μg mL^{-1}.

To access the effect of the concentration of the MPM solution, the solution was diluted to 50, 20, 10, 5, and 2% of the original concentration by adding sterile distilled water. To assess the effect of the volume of microorganisms from the MPM solution, the diluted solutions were centrifuged and the resulting microbial pellets were resuspended with 5 mL of the inorganic hydroponics solution. Strain H111-dsRed was added to a test tube filled with 5 mL of diluted MPM solution or inorganic hydroponics solution obtaining microbial pellets at a final density of 1×10^2 cells mL^{-1}. A heat-treatment experiment was also performed in which the MPM solution was pasteurized at 40 to 80 °C for 30 min. The inoculated solutions were then cultured in the dark at 25 °C on a shaker (170 rpm). From each replicate, solution samples were collected 7 days after the experiment started and were incubated on Komada's selective medium. All the *in vitro* experiments used three replicates.

Morphological observation of *F. oxysporum*

F. oxysporum strain H111-dsRed was added to a test tube filled with 5 mL of MPM solution or inorganic hydroponics solution at a final density of 1×10^4 cells mL^{-1}. The inoculated solutions were cultured in the dark at 25 °C for 7 days on a shaker (170 rpm), with three replicates. At 7 days after the inoculation, fungal cells were observed using a fluorescence microscope (BX50) equipped with a U-MWIG filter. To assess *F. oxysporum* growth, more than 200 cells were observed from each replicate. The total number of microconidia, germinated microconidia (< 20 μm), elongated hyphae (> 20 μm), macroconidia, and chlamydospores were counted.

Growth inhibition of *F. oxysporum* by bacterial isolates

For bacterial isolates, bacterial collection was generated from the rhizosphere biofilms of MPM solution. The MPM solution was spread plated in R2A medium (0.5g peptone, 0.5g casamino acids, 0.5g yeast extract, 0.5g dextrose, 0.5g soluble starch, 0.3g dipotassium phosphate, 0.05g magnesium sulfate $7H_2O$, 0.3g sodium pyruvate, and 15g agar), and plates were incubated at 25 °C for 5 days. Bacterial colonies from each plate were picked and transferred into new plates and incubated again. This repeated twice, resulting in a collection of 42 isolates. DNA sequences of the isolates were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3130x1 Genetic Analyzer System (Applied Biosystems) using the standard methods (Sambrook and Russell 2001). The sequences were compared with the NCBI DNA database sequences using the BLAST software (Altschul et al. 1997). The closest bacterial species identified through the BLAST was considered to be the potential bacteria of

interest.

Next, to select bacterial isolates early growing in liquid culture condition, 42 isolates were cultured in R2A and 1/10 NA liquid medium (0.3g beef extract, 0.5g peptone, and 15g agar) for 2 days and ended up with 38 and 13 isolates, respectively. Those were separately transferred into a new liquid medium and incubated again. The cultured solution of each isolates were adjusted to approximately 1×10^9 cfu mL^{-1}, and the resulting bacterial solution was used as the stock bacterial solution for use as inoculum.

To determine suppressive effects of the fast growing bacteria on *F. oxysporum*, the bacterial solution was prepared adjusting to approximately 1×10^7cfu mL^{-1} for each bacterial isolate by filling up to 5 mL with R2A or 1/10 NB liquid medium. *F. oxysporum* strain H111-dsRed was added to a test tube filled with 5 mL of the prepared bacterial solution at a final density of 1×10^2 cells mL^{-1}. The inoculated solutions were then cultured in the dark at 25 °C on a shaker (170 rpm). From each replicate, solution samples were collected 7 days after the experiment started and were incubated on Komada's selective medium. All the *in vitro* experiments used three replicates.

Results

The rhizosphere microbial community

We observed rhizosphere biofilms developing on the roots under a microscope, and we observed the most interesting sites under a scanning electron microscope. Biofilms formed in the MPM system but not in the inorganic system (Figure 1A, B). Microbial colonization of the plant roots was obvious (Figure 1C; Figure 2). The microbial colonization was

observed both on root hairs and in zones where mature main and lateral roots were present. Several microbes with different morphologies were detected on the root surface, and some microbes adhered tightly to the root surface and to other microbial cells (Figure 1D). In short, rhizosphere microorganisms indigenous to the MPM solution colonized the roots and were associated with biofilm formation on the rhizoplane.

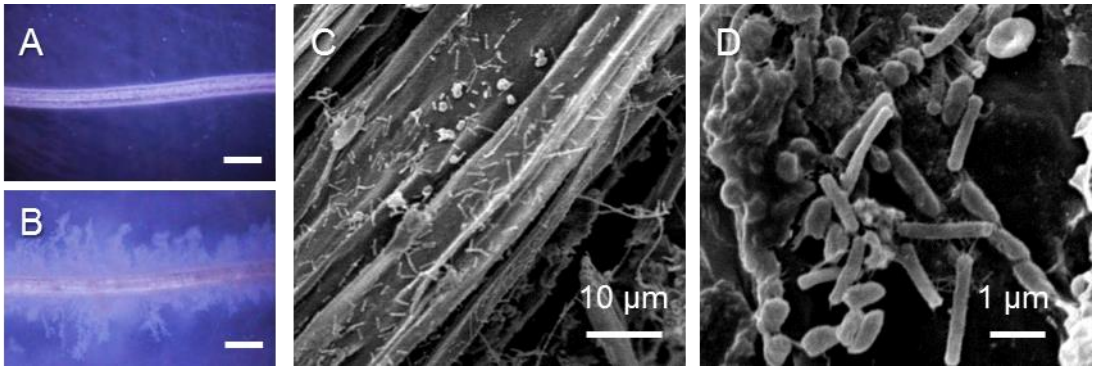

Figure 1. Biofilms adhered to the root surface in the MPM system but not in the inorganic hydroponics system. Tomato roots were grown in (A) conventional inorganic system and (B) in the MPM system for 4 days after transplanting and were observed by a light microscope. Bars for A and B represent 1mm. (C, D) Rhizosphere microbes inhabiting the root surface in the MPM system under a scanning electron microscope.

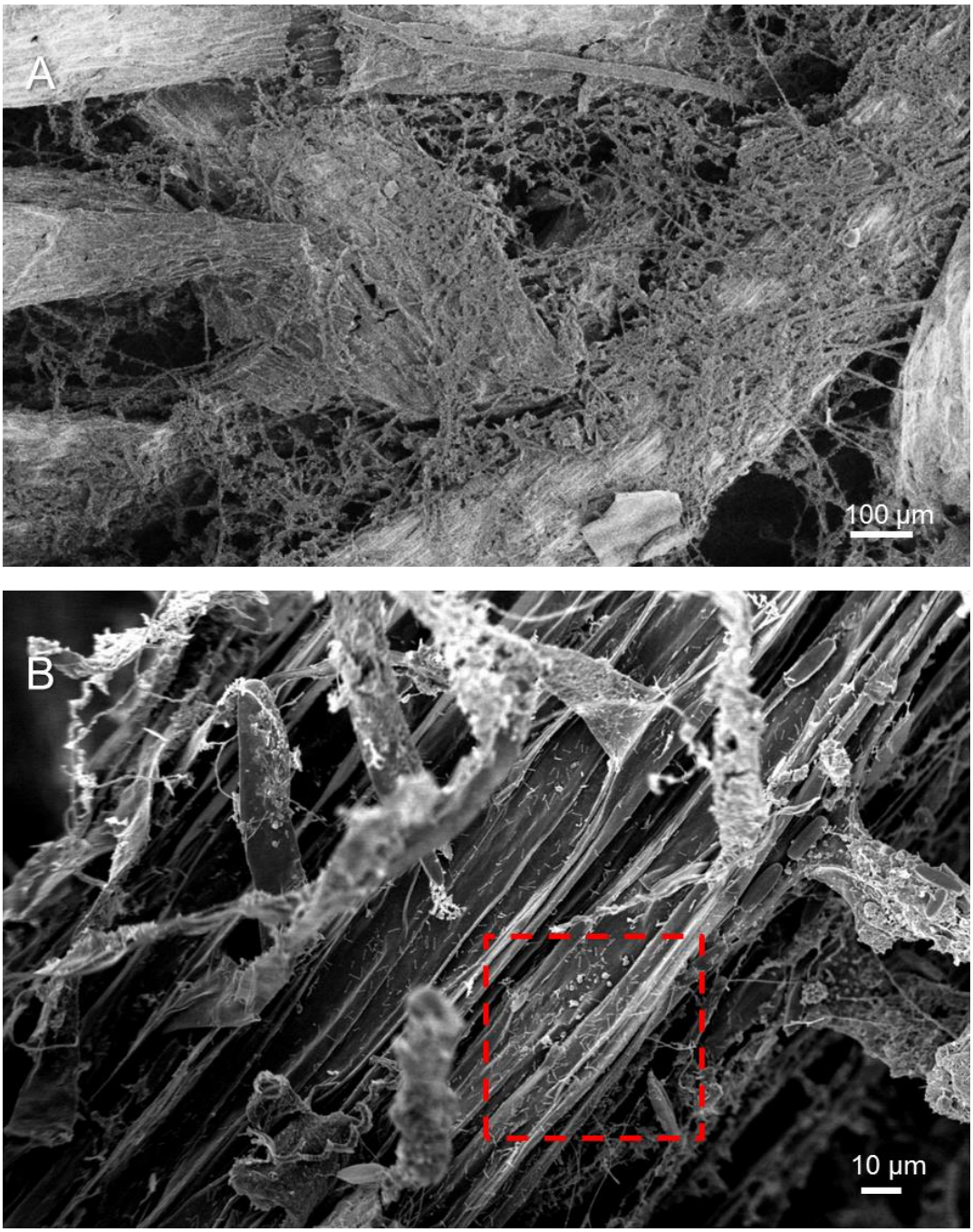

Figure 2. Microscopic observation of biofilms adhering to the root surface in the MPM system. (A, B) Rhizosphere microbes inhabiting the root surface. (A) was taken by a digital microscope and (B) by a scanning electron microscope. The regions bounded by dotted lines are magnified for Figure 1C.

PCR-DGGE analysis of the rhizosphere bacteria

Classification of the rhizosphere bacteria in the MPM system was carried out. To analyze the composition of the rhizosphere biofilm, PCR-DGGE analysis was performed and 174 bands were detected from a series of biofilms that developed before and after plant cultivation (Figure 3). We successfully identified 11 bands for the organisms extracted from the MPM solution before plant cultivation, 45 bands from rice, 51 bands from komatsuna, and 67 bands from tomato by comparative analysis of their 16S rDNA genes (Table 2). Rhizosphere bacteria present in the DGGE profiles belonged to bacterial phylogenetic groups known to exist in the soil and some uncultivated representatives. The bacteria were affiliated with 17 phylogenetic groups (Table 2). The DGGE profiles were assigned to phylogenetic groups of four bacterial phyla (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria) on the basis of reproducible phylogenetic affiliations (Figure 4). The 174 bands were assumed to be OTUs and assigned to bacterial phyla. The overall distribution of the predominant bacterial phyla ranged from approximately 20% for Actinobacteria, Bacteroidetes, and Proteobacteria, respectively, to approximately 40% for Firmicutes.

The diversity of the bacterial community structure based on the OTUs compositions in each bacterial phylum was estimated using a UPGMA dendrogram to identify the biofilm samples with the highest similarity in their rhizosphere microbial community structures (Figure 5). The OTUs compositions showed that microhabitats before plant growth (non-planted) were clustered apart from the microhabitats of the plants whereas no significant differences were found in OTUs compositions (P>0.05). The bacterial community associated with the early growth stages (within 7 days after transplanting) and later growth stages (14 days after transplanting) were clustered separately ($F_{(2,13)}$ = 5.25,

$P<0.005$, $R^2 = 0.45$), with one exception; the community obtained from tomato after 14 days was clustered with the earlier stages ($P>0.05$).

In terms of the abundance of several bacterial taxa (Figure 3; Table 2), three band profiles, which represented *Bacillus* (o, p, and q) were the dominant band patterns both before and after plant cultivation. In addition, five band profiles, which represented Bacillaceae (u, in komatsuna and tomato), *Gordonia* (C, in all three species), Sphingobacteriales (i, in tomato), *Bacillus* (q, in all three species), and *Rhizobium* (s, in all three species), remained constant during plant cultivation (Figure 3). In contrast, bacteria in the Chitinophagaceae (f), Sphingobacteriaceae (l), and *Bacillus* (n and r) were observed only before plant cultivation (Figure 3). The following bacteria from the rhizosphere of the MPM system (Table 2) are among the bacterial taxa in disease-suppressive soils that are known to protect plants from pathogens and confer other beneficial properties (Borneman and Becker 2007; Benitez et al. 2009; Kyselkova et al. 2009; Schreiner et al. 2010): *Bacillus* (n, o, p, q, r, and u), Actinomycetales (A), Burkholderiales (b), Deltaproteobacteria (d, e, and k), Sphingobacteriaceae (l), and Comamonadaceae (j and m). Of these, *Bacillus* (o, p, q, and u) from all but one (q in komatsuna 14) of the plant rhizospheres and Deltaproteobacteria (k) from komatsuna and tomato were detected in the DGGE band profiles, throughout all or most of plant cultivation. Altogether, the population-based approach using DGGE analysis indicates that the structure of the bacterial community inhabiting the MPM system may involve a core bacterial group that includes several dominant bacteria related to disease suppression in soil microbiota (Lundberg et al. 2012).

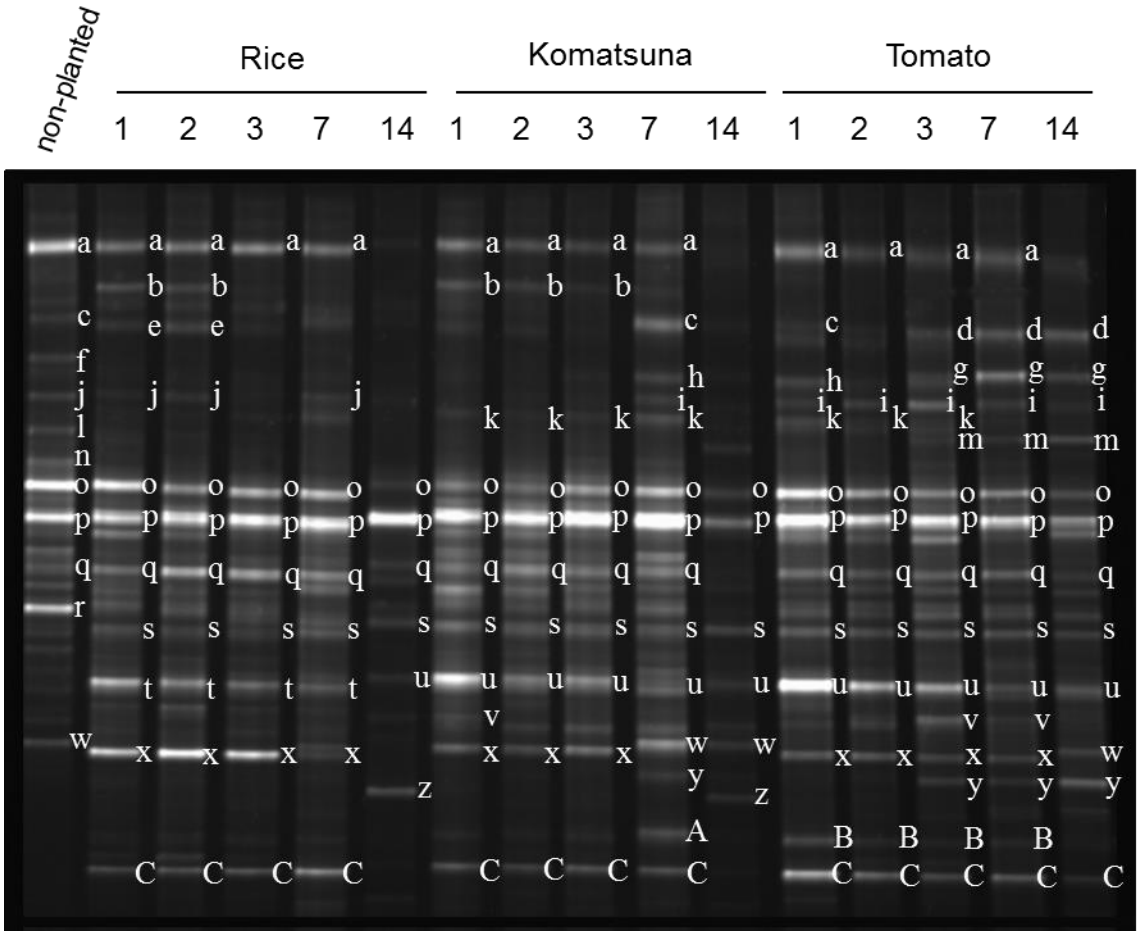

Figure 3. DGGE banding profiles for the bacterial 16S rDNA genes extracted from the rhizosphere biofilms that developed in the MPM system. Lane numbers (1, 2, 3, 7, and 14) correspond to the number of days after transplanting. A control sample ("non-planted") represents a biofilm that developed in the MPM solution before the cultivation of the plants. Bands with the same mobility in the DGGE gel were marked with the same letter (a to z and A to C) and excised for sequencing.

Table 2. Phylogenetic affiliations of the bacterial 16S rDNA gene sequences corresponding to the prominent DGGE bands retrieved from the rhizosphere biofilms.

DGGE band [a]	Microorganisms	Phylogenetic affiliation	Accession number	Identity (%)[b]	Alignment
a	*Lysinibacillus* sp.	Bacillaceae	AB605770	100	194/194
b	*Variovorax paradoxus*	Burkholderiales	AB608026	99.5	193/194
c	Uncultured Sphingobacteria bacterium	Sphingobacteriales	AB610409	96.8	183/189
d	Uncultured deltaproteobacterium	Deltaproteobacteria	AB608046	97.9	190/194
e	Uncultured bacterium	Deltaproteobacteria	AB608031	96.9	188/194
f	*Niastella* sp.	Chitinophagaceae	AB607235	98.4	186/189
g	Uncultured Bacteroidetes bacterium	Chitinophagaceae	AB608045	98.4	186/189
h	Uncultured Bacteroidetes bacterium	Bacteroidetes	AB608040	98.4	186/189
i	Uncultured Sphingobacteriales bacterium	Sphingobacteriales	AB608044	100	189/189
j	*Comamonas* sp.	Comamonadaceae	AB607236	99.0	192/194
k	Uncultured deltaproteobacterium	Deltaproteobacteria	AB608041	94.3	183/194
l	*Olivibacter terrae*	Sphingobacteriaceae	AB605771	97.4	184/194
m	Uncultured *Comamonas* sp.	Comamonadaceae	AB608047	100	194/194
n	*Bacillus niabensis*	*Bacillus*	AB607230	99.5	193/194
o	*Bacillus* sp.	*Bacillus*	AB607231	100	195/195
p	*Bacillus* sp.	*Bacillus*	AB607232	100	194/194
q	*Bacillus horneckiae*	*Bacillus*	AB608027	100	194/194
r	*Bacillus* sp.	*Bacillus*	AB607233	100	194/194
s	*Rhizobium* sp.	*Rhizobium*	AB608042	100	169/169
t	*Dyadobacter* sp.	Dyadobacter	AB608028	98.9	187/189
u	*Bacillus* sp.	Bacillaceae	AB608035	97.4	190/194
v	Uncultured Bacteroidetes bacterium	Bacteroidetes	AB608043	95.2	180/189
w	Uncultured Cytophagales bacterium	Cytophagales	AB607234	99.5	185/186
x	Uncultured *Microbacterium* sp.	Microbacteriaceae	AB608029	100	174/174

y	Uncultured bacterium	Bacteroidetes	AB608048	99.5	188/189
z	*Chelativorans* sp.	Rhizobiales	AB608033	100	169/169
A	Uncultured actinobacterium	Actinomycetales	AB608039	94.8	165/174
B	Uncultured actinobacterium	Thermomonosporaceae	AB610410	99.4	167/168
C	*Gordonia* sp.	*Gordonia*	AB608030	100	174/174

[a] DGGE bands used for the sequences were excised from the DGGE banding profiles (Figure 3)

[b] Percent similarity of the partial 16S rDNA coding sequences to the sequences of their closest bacterial relatives available based on a BLAST search in the NCBI nucleotide sequence database.

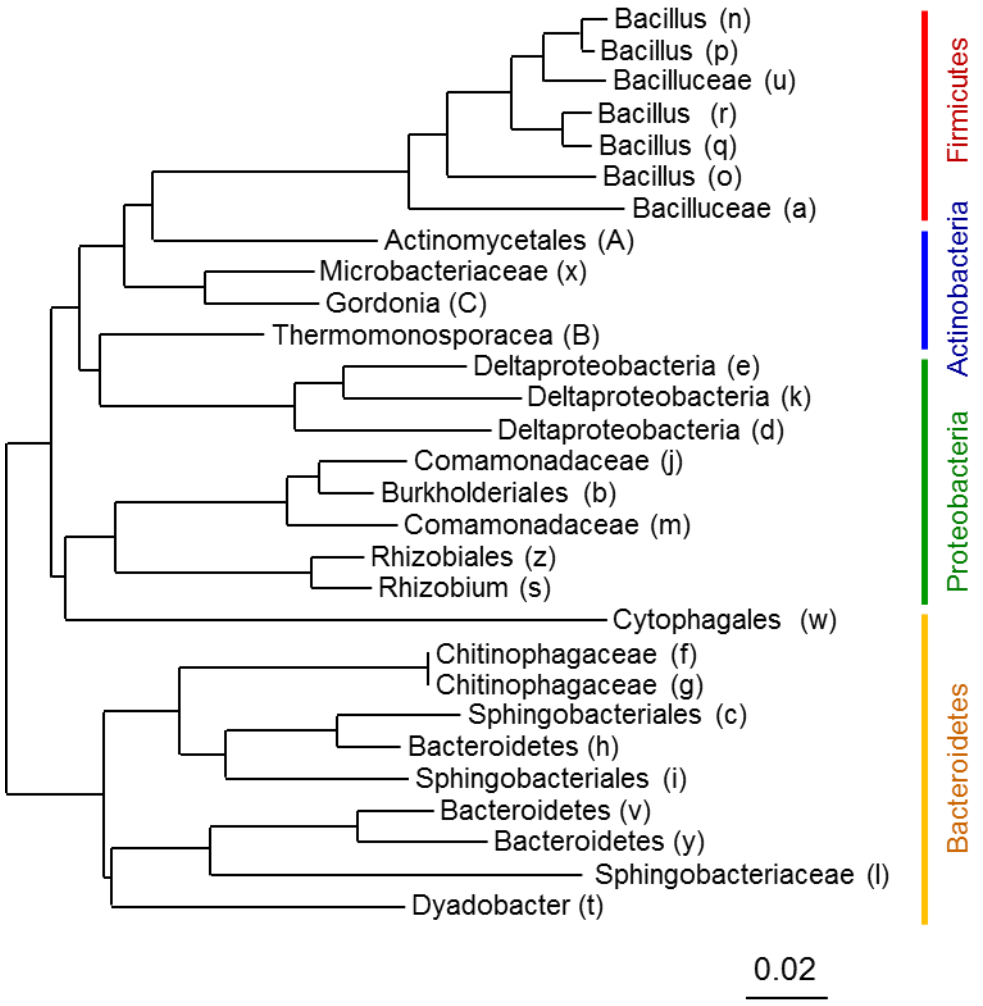

Figure 4. Phylogenetic tree of the rhizosphere microorganisms detected in the biofilms from the MPM system. Letters in parentheses from each phylogenetic taxon represent the band labels in the DGGE banding profiles for the bacterial 16S rDNA genes obtained from the rhizosphere biofilms (Figure 3). The stability of the groups was assessed using 1000 bootstrap replications of the data sets.

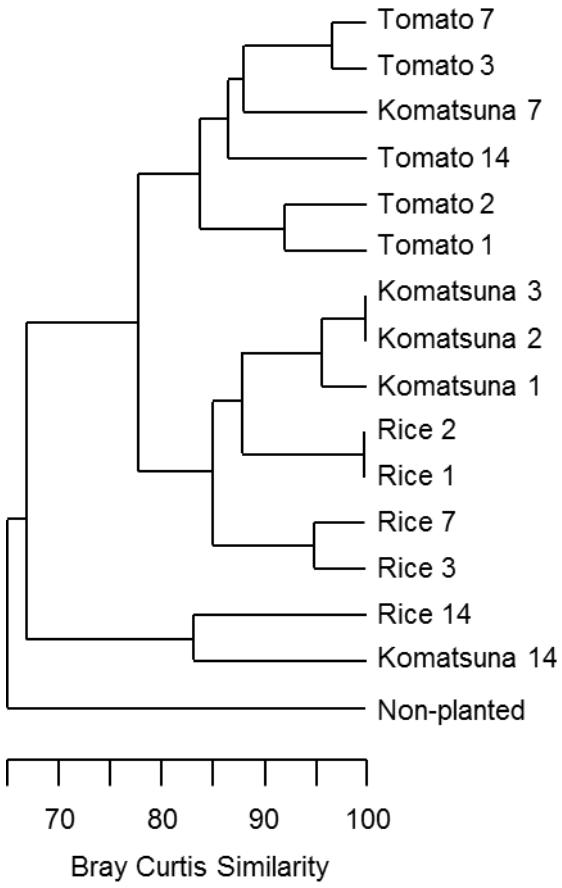

Figure 5. UPGMA dendrogram of the rhizosphere microbial community in a pre- and post-plant cultivation using the Bray–Curtis index. The scale shows the Bray–Curtis similarity value. Sample names correspond to the band lanes shown in Figure 3.

Suppression of bacterial wilt disease in the MPM system

Typical symptoms of *R. solanacearum* wilt were observed on plants in the conventional inorganic hydroponics, but not in the MPM system (Figure 6A). In detail, tomato seedlings were visibly wilted in the inorganic hydroponics by 5 days after inoculation (Figure 6B), and all of the wilted plants died shortly after the wilting symptoms had appeared. A milky-white, sticky exudate, which indicates the presence of bacterial cells, oozed from stem cross sections of infected plants. *R. solanacearum* was abundant in the inorganic hydroponic solution as well as the infected plant roots (Figure 6C). In contrast, tomato plants grown in the MPM system showed no disease symptoms and remained healthy (0% mortality) at 12 days after inoculation (Figure 6A, B). The suppression of bacterial wilt disease was sustained throughout the experiment, and *R. solanacearum* was not detected from either the MPM solution (detection limit $\geq$ 10 CFU/mL) or a homogenate of the seedlings (detection limit $\geq 10^2$ CFU/g fresh mass) after dilution plating (Figure 6C). However, when the tomato seedlings in the MPM system were inoculated with *R. solanacearum* earlier than 4 days after transplanting (data not shown), they wilted. In short, these results showed that the MPM system strongly suppressed the development of bacterial wilt disease if the seedlings had at least 3 days to become established in the system before the introduction of the bacterial pathogen.

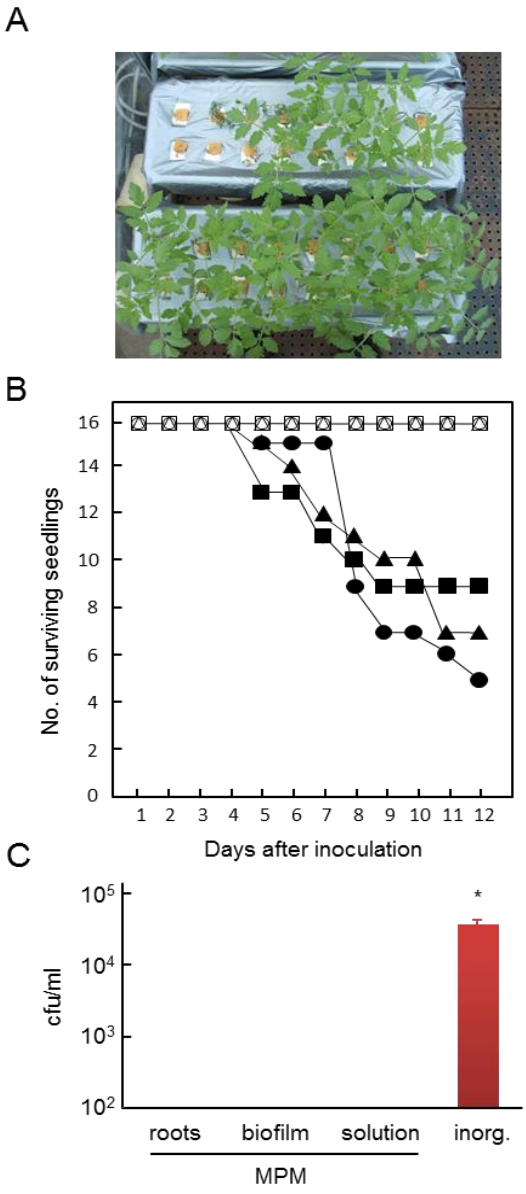

Figure 6. Development of suppression of Bacterial wilt disease in the MPM system. (A) Tomato plants were inoculated with *R. solanacearum* strain MAFF301487 (Approximately 1×10^5 cfu mL^{-1}) 4 days after transplanting. (B) Time course of survivorship of seedlings after inoculation with *R. solanacearum* in the MPM system (open circles, triangles, and squares for the three replicates) and inorganic hydroponics system (closed circles, triangles, and squares for the three replicates). Seedlings in the inorganic hydroponics without pathogen (the control) were not wilted at the similar levels to that in organic hydroponics. (C) The density of *R. solanacearum* in the MPM system and inorganic hydroponics system (inorg.) 12 days after inoculated was examined. Columns with a star were significantly different at the significance level of $P < 0.01$ according to a Tukey-Kramer multiple comparison test.

Suppression of Fusarium wilt disease in the MPM system

A fungal pathogen *F. oxysporum* f. sp. *lactucae* causes root rot and wilting of commercial lettuce plants in Japan (Fujinaga et al. 2003, 2005). The virulence of the fungal diseases makes it a widespread problem in hydroponics systems based on an inorganic nutrient solution. We tested disease suppression against Fusarium wilt of lettuce in MPM system. When Boston lettuce seedlings were inoculated with *F. oxysporum* f. sp. *lactucae* strain H111 under greenhouse conditions, the MPM system provided remarkable suppression of *F. oxysporum*, whereas conventional inorganic hydroponics showed a high disease incidence and severe damage (Figure 7A). When strain H111-dsRed was inoculated into the hydroponics solutions of the lettuce under growth chamber conditions, typical symptoms were observed on plants grown in the conventional inorganic hydroponics system, but not in the MPM system 4 days after transplanting (Figure 8A). Infected seedlings began to appear in the inorganic hydroponics system 6 days after inoculation and wilted shortly after disease symptoms had appeared in the leaves (Table 3). In contrast, no disease symptoms were observed in the MPM system (Figure 8A). The suppressive effect was sustained until harvest. The suppression of *F. oxysporum* f. sp. *radicis-lycopersici* was also observed in tomato plants. Strain LS89-1-1 (1×10^4 cells mL^{-1}) was inoculated on the tomato plants 4 days after transplanting, and all plants died or became severely stunted in only the inorganic hydroponics system within 10 days after inoculation (Figure 7B and Table 3). These results demonstrate that the MPM system strongly suppresses the Fusarium wilt disease even under greenhouse conditions.

In the rhizosphere, *F. oxysporum* survived in the rhizosphere microbial community

under the disease suppression. We detected abundant *F. oxysporum* in both infected plant root surfaces and the hydroponics solution of the inorganic system (Figure 8B). However, surprisingly, we also detected the fungal pathogen (though at lower levels) in the rhizosphere biofilms that formed on the root surface and in the hydroponics solution in the MPM system, although seedlings in the MPM system showed no disease symptoms (Figure 8B). We reisolated the strains of *F. oxysporum* from the MPM solution and confirmed strong pathogenicity to lettuce plants (data not shown). These results showed that the development of Fusarium wilt disease was strongly suppressed in the MPM system even though the pathogen was detected in the rhizosphere. They indicate that the suppression of Fusarium wilt disease results from controlling fungal activity rather than from destroying the pathogen.

To clarify the effects of transplanting on disease control, lettuce plants were inoculated with strain H111 from 0 to 14 days after transplanting into the MPM system. The disease suppression toward Fusarium wilt disease developed only 4 days after transplanting (Figure 8C) in addition to disease suppression against Bacterial wilt disease of tomato. These results showed that the MPM system suppressed the development of root diseases caused by *F. oxysporum* if transplanted seedlings had at least 4 days to become established in the system before pathogen introduction. Altogether, these results showed that the rhizosphere microbial community in an MPM system was capable of suppressing both a bacterial pathogen of *R. solanacearum* and a fungal pathogen of *F. oxysporum*, and the suppressive effects on both bacterial and fungal pathogens was generated 4 days after transplanting.

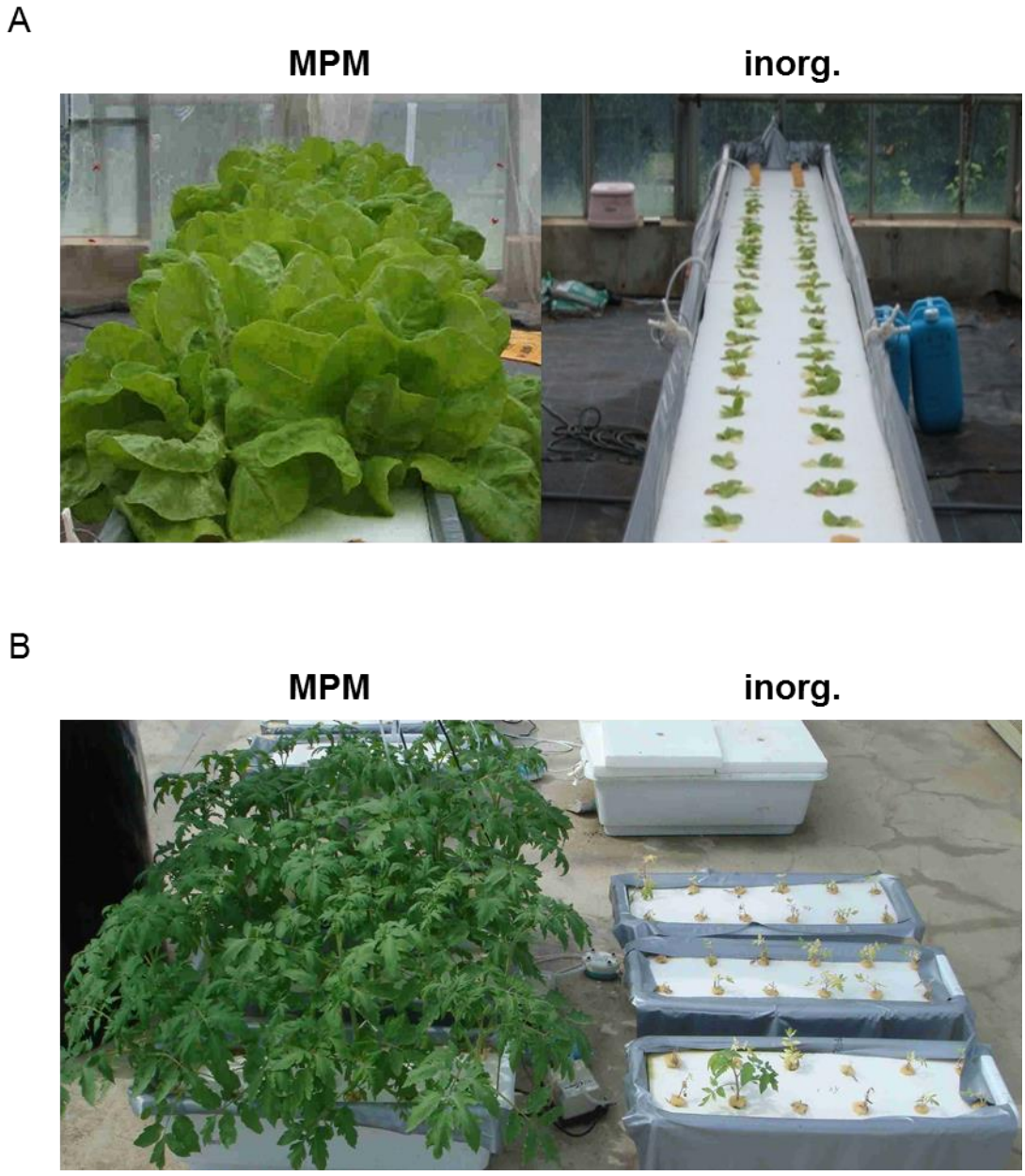

Figure 7. The MPM system suppressed the Fusarium wilt disease. (A) Boston lettuce (*Lactuca sativa* L. cv. Saradana) was grown in the MPM system and inorganic hydroponics system in a greenhouse. Plants were inoculated with *F. oxysporum* f. sp. *lactucae* strain H111 (1×10^4 cells mL^{-1}) 4 days after transplanting and grown for 1 month. The image shows the results 30 days after inoculation. (B) Tomato plants were inoculated with *F. oxysporum* f. sp. *radicis-lycopersici* strain LS89-1-1 (1×10^4 cells mL^{-1}) 4 days after transplanting. The image shows the results 10 days after inoculation. Disease severity in tomato plants is shown in supplemental Table 3.

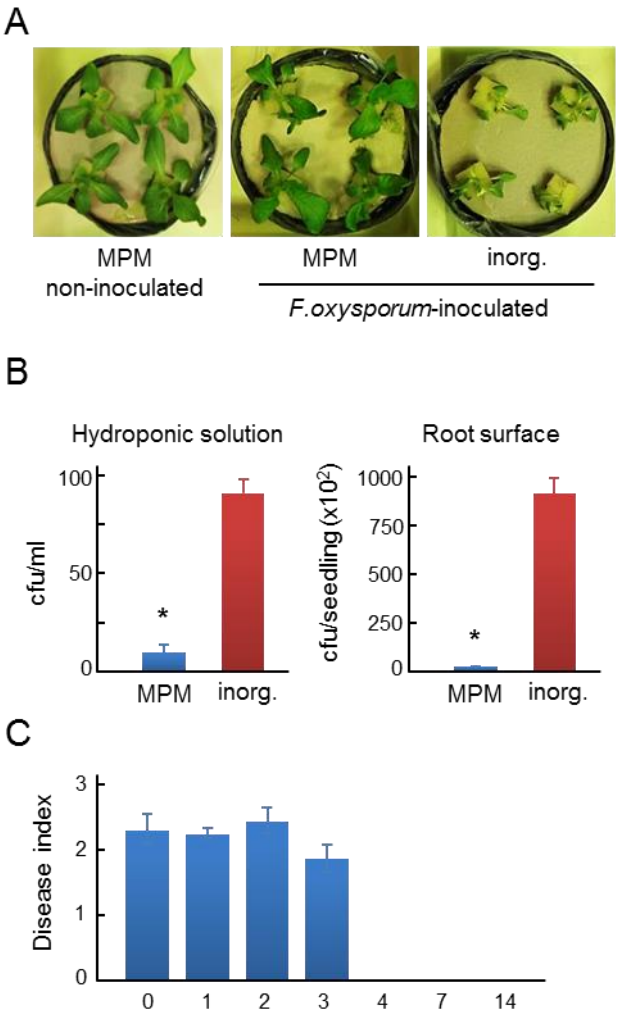

Figure 8. Development of suppression of Fusarium wilt disease in the MPM system. (A) Boston lettuce plants were inoculated with *F. oxysporum* f. sp. *lactucae* strain H111-dsRed (1×10^4 cells mL^{-1}) 4 days after transplanting. (B) The density of *F. oxysporum* f. sp. *lactucae* strain H111-dsRed from the inorganic (inorg.) and MPM solution and root surfaces were examined. Columns with a star were significantly different at the significance level of $P < 0.01$ according to a Student's t-test. (C) Boston lettuce plants were inoculated with the strain H111-dsRed (1×10^4 cells mL^{-1}) on 0 to 14 days after transplanting and then the disease was monitored after the inoculation. The disease severity was determined on a scale of 0 to 3, where 0 indicates no disease, 1 indicates wilting leaves, 2 indicates a wilting plant, and 3 indicates a dead plant, and symptoms were recorded until 10 days after inoculation. The disease rating represents the mean score from 12 seedlings in each treatment replicate.

Table 3. Timing and severity of symptom development in Boston lettuce and tomato plants inoculated with *F. oxysporum* ff. spp. *lactucae* and *radicis-lycopersici* in the MPM system[a].

Treatment [b]	Disease rating at 5 to 10 days after inoculation					
	5	6	7	8	9	10
MPM	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
MPM inoculated with *F. o. l.*	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
inorg. inoculated with *F. o. l.*	0.0 (± 0.0)	1.4 (± 0.3)	1.8 (± 0.2)	2.2 (± 0.3)	2.3 (± 0.2)	2.4 (± 0.3)
MPM inoculated with *F. o. r.*	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)
inorg. inoculated with *F. o. r.*	0.0 (± 0.0)	0.7 (± 0.2)	1.2 (± 0.2)	1.4 (± 0.3)	1.75 (± 0.3)	2.3(± 0.4)

[a] Disease rating: For tomato, 0, no symptoms; 1, yellowing; 2, wilting; 3, death. For lettuce, 0, no symptoms; 1, wilting leaves; 2, a wilting plant; 3, death. Values are the means (±S.D.) of the disease ratings from 12 lettuce (*F. o. l.*) and 36 tomato plants (*F. o. r.*) in each treatment replicate. The symptoms of Fusarium wilt of tomato 10 days after inoculation are shown in Figure 8B.

[b] Plants cultivated in the MPM and inorganic (inorg.) hydroponics systems for 4 days after transplanting were inoculated with 1×10^4 cells mL^{-1} of *F. oxysporum* f. sp. *lactucae* (*F. o. l.*) or f. sp. *radicis-lycopersici* (*F. o. r.*).

The microbiological factor influencing the disease suppression in MPM solution

We explored the biotic and abiotic forces that contributed to the observed suppression of both bacterial and fungal root diseases in the MPM system. We treated samples of the MPM solution by filtration and autoclaving to sterilize the solution and inoculated the resulting samples with bacterial strain MAFF301487 and fungal strain H111-dsRed. In the dynamics of bacterial and fungal pathogens in the MPM solution, densities of *R. solanacearum* and *F. oxysporum* increased greatly in both the sterilized samples and the inorganic hydroponics solution (Figure 9A, B). On the other hand, consistent with the results of an inoculation test, the density of *R. solanacearum* decreased, and the density of *F. oxysporum* did not increase in the untreated MPM solution (Figure 9A, B). These results demonstrated that microbes with certain exudates in microenvironment rather than the copious amount of at least water-soluble and heat-stable substances secreted by these microbes were likely to be the main factor that controls both *R. solanacearum* and *F. oxysporum* activities based on different suppressive mechanisms.

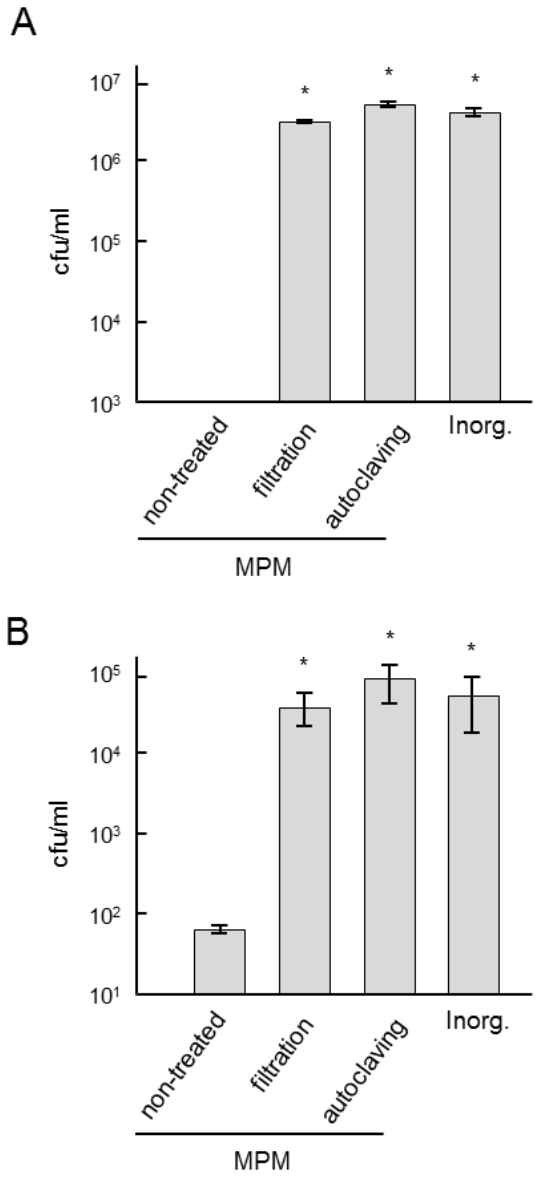

Figure 9. The suppression of root-borne pathogens by the MPM solution in 14 days after inoculation. The factors suppressing (A) *R. solanacearum* and (B) *F. oxysporum* growth in the MPM system were evaluated by sterilization of the solution by filtration, autoclaving. Columns with a star were significantly different at the significance level of $P < 0.05$ according to a Tukey-Kramer multiple comparison test.

Microbial density required to maintain fungal disease suppression

To investigate the mechanisms of root-bone disease suppression in the MPM system, we preliminarily focused on evaluating disease suppressiveness of the MPM solution toward *F. oxysporum* based on research strategies to elucidate the microbiological basis of disease suppression (Weller et al. 2002). Firstly, we examined the growth of *F. oxysporum* in the MPM solution by several heat treatments (40 to 80 °C). The suppression of pathogen growth was retained by the MPM solution after heat treatment at 50 °C. However, treatment at temperatures greater than or equal to 60 °C completely eliminated the solution's suppressive ability (Figure 10A) and resulted in an increase in the pathogen's growth rate to a level similar to that in the untreated inorganic hydroponics, in which substantial disease incidence occurred (Figure 9B). These results showed that the microbiota that suppressed the growth of the fungal pathogen was temperature-sensitive and that the microbial community structure changed as a result of the heat shock.

Next, we examined the growth of *F. oxysporum* in the MPM solution at several dilutions (2 to 50% w/w) after mixing with sterile distilled water (Figure 10B). At dilutions to 2, 5, and 10% of the original concentration, growth suppression of strain H111-dsRed was fully lost, resulting in increases in the fungal cell density to approximately 1×10^4 CFU mL^{-1}, whereas at 20% or more of the original concentration, suppression of fungal growth was comparable to that in the undiluted solution.

We then investigated the effect of the volume of biofilm to detect its influence on suppression of the fungal pathogen. We prepared a microbial pellet from a stock biofilm solution by centrifugation and collected approximately 1.7 (± 0.2) mg mL^{-1} of microbes. We detected approximately 3.8×10^9 CFU g^{-1} or 1.8×10^{13} CFU g^{-1} of culturable bacteria

from the pellet on CSL medium or 1/10 NA medium, respectively. We resuspended the resulting microbial pellets in the inorganic hydroponics solution at the same dilutions (between 2 and 50% of the original concentration) and then tested their ability to suppress the growth of the strain H111-dsRed (Figure 10C). The growth of this strain was suppressed to the same level as in the undiluted solution when at least 20% of the original amount of microbes was present, but was not inhibited at 5% or less of the original concentration, and there was a noticeable but clearly lesser suppression at 10% of the original concentration. The impact on *F. oxysporum* growth was minimized when the microbial population was reduced by the dilution. These findings allowed us to indicate the importance of the presence of microbiota for control of the fungal pathogen growth.

We also performed an inoculation test on lettuce plants using strain H111-dsRed in the inorganic hydroponics solution with the diluted microbial pellets (Figure 10D). Disease suppression decreased as the dilution rate of the solution increased, with nearly complete suppression at a concentration of 50% of the original, but a large reduction at 20% and little or no reduction at 10% or less. These results showed that the disease suppression provided by the MPM solution was microbial density dependent.

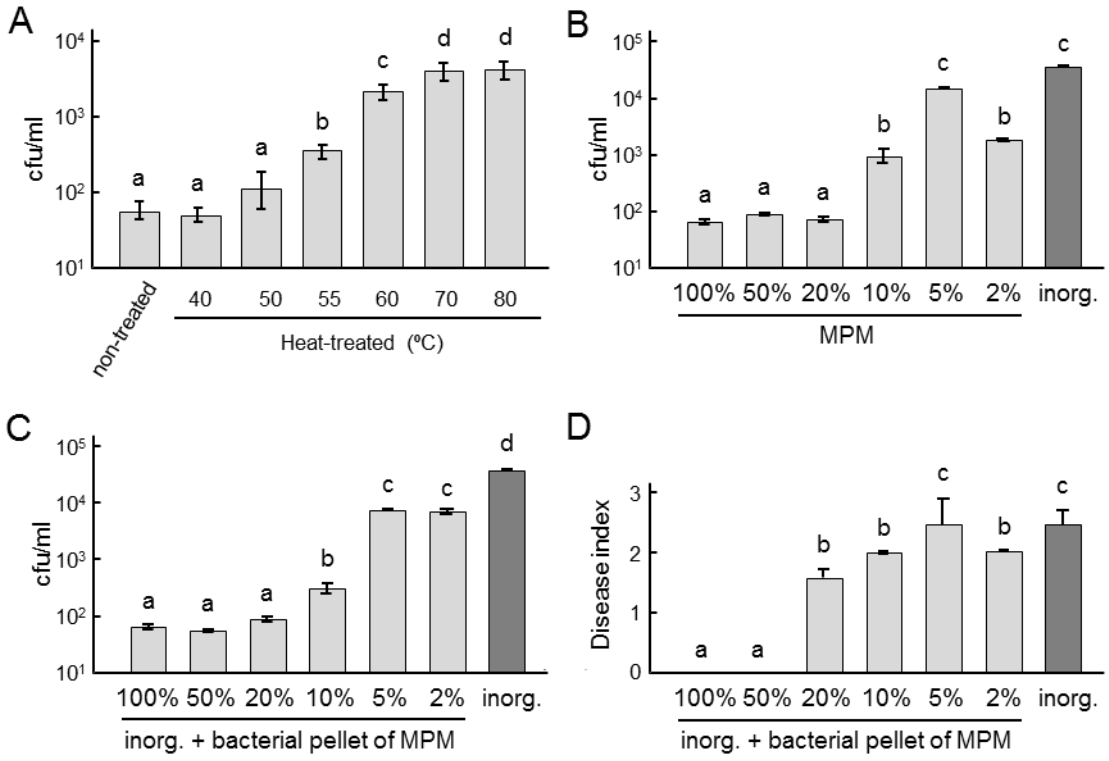

Figure 10. Microbial density required to maintain disease suppressiveness. Microbial factors affecting suppressiveness to Fusarium wilt in the MPM system were evaluated by pasteurization or a suppressiveness transfer test in which MPM solution was diluted. *F. oxysporum* f. sp. *lactucae* strain H111-dsRed was added after (A) pasteurization of the MPM solution at 40 to 80 ºC for 30 min or (B) dilution of the MPM solution or (C) dilution of the microbial pellet collected by centrifugation of the MPM solutions. The inoculated solutions were cultured at 25 ºC for 7 days, and were examined using Komada's selective medium supplemented with hygromycin. (D) Suppression of *Fusarium* wilt was maintained using the diluted microbial pellet. The disease severity was determined on a scale of 0 to 3, where 0 indicates no disease, 1 indicates wilting leaves, 2 indicates a wilting plant, and 3 indicates a dead plant, and symptoms were recorded until 10 days after inoculation. The disease rating value represents the mean (±S.D.) for 12 seedlings from each treatment replicate and was recorded until 10 days after inoculation. Columns with different letters were significantly different at the significance level of $P < 0.05$ according to a Tukey-Kramer multiple comparison test.

Morphological characterization of *F. oxysporum* in rhizosphere microbial community

Fusarium oxysporum is unique in its asexual reproduction that includes three kinds of asexual spores (conidia): microconidia, macroconidia, and chlamydospores (Nelson 1981). In nature, these conidia germinate, elongate, and develop into hyphae under a suitable environment. Microconidia and macroconidia are produced from phialides, which are specialized hyphae, whereas globose chlamydospores are endurance organs in the soil, where they act as the primary inocula (Couteaudier and Alabouvette 1990; Katan et al. 1997) (Figure 11A).

We compared the formation of these conidia and the status of the inoculated microconidia of the strain H111-dsRed between the MPM and inorganic hydroponics solutions for 7 days after incubation. The fungal cells in the MPM solution exhibited dsRed fluorescence, indicating that the *F. oxysporum* inhabiting the MPM solution was alive (Figure 11B). *F. oxysporum* cells from the MPM solution formed germinated microconidia and chlamydospores but those from the inorganic hydroponics solution were likely to produce microconidia and macroconidia (Figure 11C). In the status of inoculated *F. Oxysporum* cells, the fungal cell growth in the production of microconidia and macroconidia resulted in an increase to approximately 1×10^5 CFU mL^{-1} in the inorganic hydroponics solution. In contrast, fungal cell density in the MPM solution remained stable at approximately 1×10^4 CFU mL^{-1} (Figure 11C). Macroconidia production was not identified for *F. oxysporum* morphogenesis in the MPM solution (Figure 11B). Microconidial germination occurred in the MPM solution, accounting for approximately 30% of *F. oxysporum* cells (Figure 11C). The germinated microconidia present in the MPM solution showed dramatic changes in morphology, and we identified chlamydospores with a

preserved lipid body and thick walls that let the resting spore survive unfavorable conditions. The level of chlamydospore production varied depending on the microbial volume in the MPM solution. At a dilution to 10% or less of the original concentration, chlamydospore formation decreased dramatically (data not shown). These results showed that *F. oxysporum* in the MPM solution retained viable cells in an inactive state under the unfavorable conditions in the solution. These findings indicate that microbial consortia in the MPM solution prevent *F. oxysporum* from elongation of its hyphae and from conidia production.

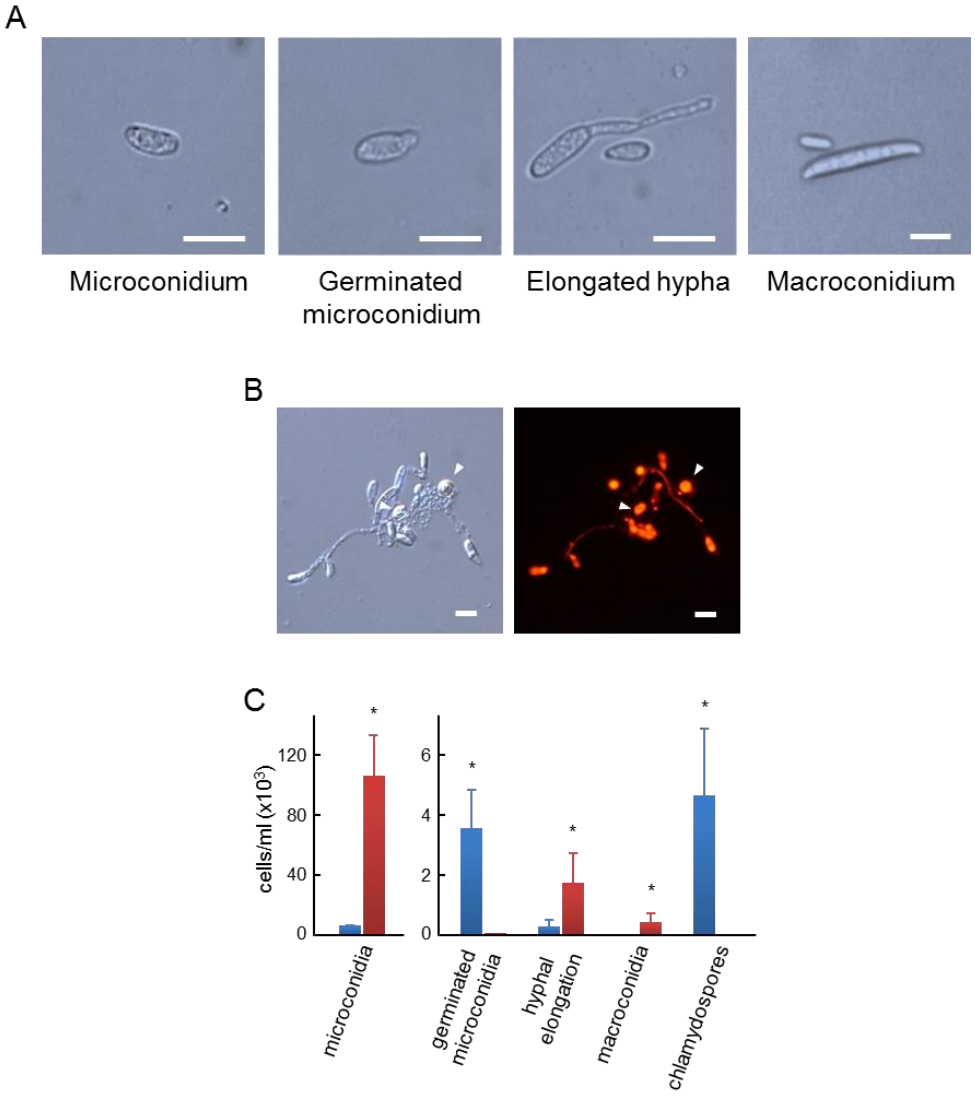

Figure 11. Control of *F. oxysporum* morphology in the MPM solution. *F. oxysporum* f. sp. *lactucae* strain H111-dsRed was added to the MPM and inorganic hydroponics solutions, and cultured at 25 ºC for 7 days. (A) Morphological characteristics of *F. oxysporum* f. sp. *lactucae* strain H111-dsRed (microconidia, germinated microconidia, elongation hypha and macroconidia). Bars represent 10 μm. (B) Survival of cells of the transformants in the MPM solution was observed using a light microscope. Strain H111-dsRed exhibited red fluorescence in the MPM solution. Chlamydospores (arrow heads) were formed on the bacterial cells. Bars represent 10 mm. (C) Cell formations of *F. oxysporum* f. sp. *lactucae* strain H111-dsRed was assessed using the numbers of microconidia, germinated microconidia (< 20 μm), macroconidia, and chlamydospores and by the number of microconidia that exhibited hyphal elongation > 20 μm. Blue bars, MPM system; red bars, an inorganic hydroponics system. Columns with a star were significantly different at the significance level of $P < 0.05$ according to a Student's t-test.

Growth inhibition of *F. oxysporum* by the rhizosphere bacterial isolates

We explored suppressive effects of bacterial isolates from MPM solution against *F. oxysporum*. Initially, we used culture-based approaches to identify antagonists involved in the disease suppression. We assayed the ability of bacterial isolates from the rhizosphere biofilm to reduce *F. oxysporum* growth *in vitro*. To do so, we collected 42 isolates from the MPM solution, but none of the isolates suppressed *R. solanacearum* strain MAFF301487 and *F. oxysporum* strains H111-dsRed and LS89-1-1 on several media (data not shown).

Next, we examined the growth inhibition of *F. oxysporum* with the fast-growing bacteria under liquid culture condition. A mixture of 38 bacterial isolates and of 13 bacterial isolates selected with R2A and 1/10 NB liquid medium, respectively were inoculated with *F. oxysporum* strain H111-dsRed. The 38 bacterial isolates suppressed the growth of *F. oxysporum* (data not shown), and 13 bacterial isolates resulted in significant suppression toward strain H111-dsRed (Figure 12A), which was similar to that in the MPM system. Due to experimental facility, we focused on the 13 bacterial isolates to evaluate the core bacterial activity underlying disease suppression (Table 4). Of 13 isolates, we separated them into two groups (six and seven bacterial isolates) based on growth rate and evaluated their abilities to inhibit the growth of *F. oxysporum* strain H111-dsRed. A mixture of the seven bacterial isolates (relatively slow growth) suppressed *F. oxysporum* while mixture of six bacterial isolates (relatively rapid growth) did not show significant suppression (Figure 12A). We then investigated suppressive effects of an individual bacterium among the seven isolates. In the bacterial group composed of the seven bacterial isolates which showed significant suppression against a fungal pathogen, none of them showed suppressive effects on *F. oxysporum* individually (Figure 12B). However, some combinations among the

following 6 bacteria: *Kaistia soli*, *Bosea minatitlanensis*, *Cupriavidus laharis*, *Bosea minatitlanensis*, *Brevibacillus centrosporus*, and *Ancylobacter polymorphus* obtained a significant suppression toward *F. oxysporum* (Figure 12B). These results showed that the disease suppression in the MPM system was reproducible by bacterial isolates from the MPM system and generated by complex interactions within the rhizosphere bacterial community rather than originated simply from a single bacterium or antagonist.

Table 4. Phylogenetic groups of the bacterial 16S rDNA gene sequences of bacterial isolates from MPM solution.

Microorganisms	Phylogenetic groups[a]	Phylogenetic affiliation corresponding to DGGE[b]
Kaistia sp.	PB	Rhizobiales
Sphingopyxis sp.	PB	–
Bosea sp.	PB	Rhizobiales
Ancylobacter sp.	PB	Rhizobiales
Cupriavidus sp.	PB	Burkholderiales
Brevibacillus sp.	FC	–
Sphingopyxis sp.	PB	–
Paenibacillus sp.	FC	–
Microbacterium sp.	AC	Microbacteriaceae
Agrobacterium sp.	PB	Rhizobiales
Brevibacillus sp.	FC	–
Arthrobacter sp.	AC	Actinomycetales
Wautersia sp.	PB	Burkholderiales

[a] Phylogenetic groups of bacterial phyla: AC, Actinobacteria; FC, Firmicutes; PB, Proteobacteria.
[b] Phylogenetic affiliation of bacterial isolates were correspondent with those of the DGGE banding profiles (Table 2). –: not detected.

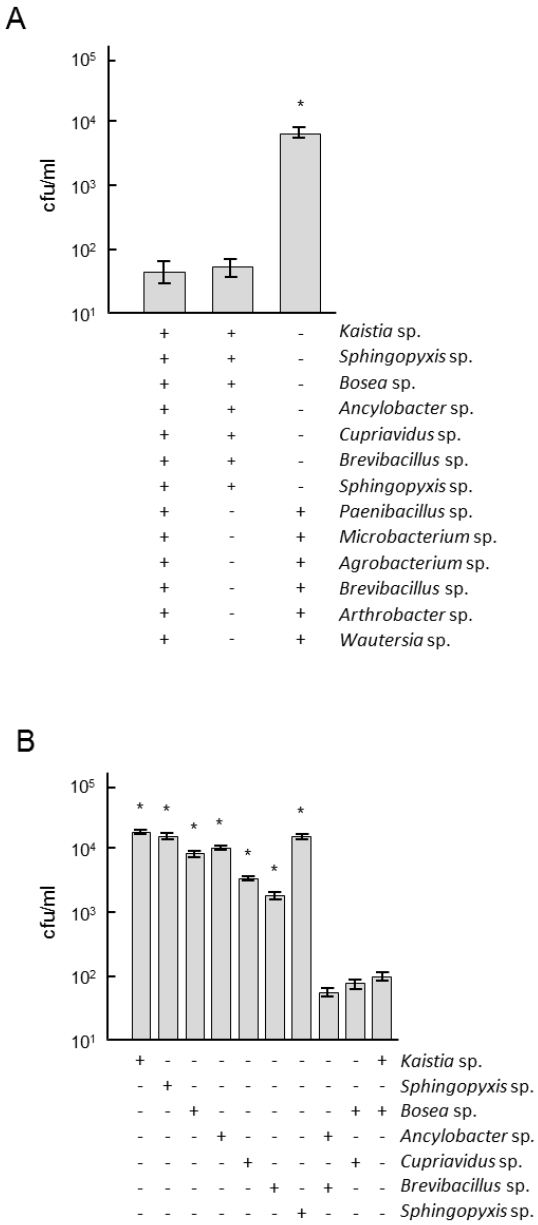

Figure 12. Growth inhibition of *F. oxysporum* by the rhizosphere bacterial isolates was explored. (A) Bacterial combinations of 13 isolates from MPM system, 6 isolates (a day to increase), and 7 isolates (two days to increase) of the 13 isolates as well as (B) the single and two combinations of the 6 bacterial isolates were separately inoculated with *F. oxysporum* strains strain H111-dsRed using 1/10 NB liquid medium. Columns with a star were significantly different at the significance level of $P < 0.05$ according to a Tukey-Kramer multiple comparison test. The presence or absence of bacteria is indicated by + or －, respectively.

Discussion

A major objective of the present study was to evaluate the disease suppression in MPM system. Our results demonstrated that the rhizosphere microbiota constructed in the MPM system can suppress both *R. solanacearum* and *F. oxysporum* based on distinctive mechanisms. To elucidate the suppressive mechanisms, we specifically focused on disease suppression against Fusarium wilt disease in MPM system. Our studies allowed us to assess pathogen-suppressing activities expressed by biofilm bacterial community and reveal possible bacterial communities influencing the suppression of *F. oxysporum*.

The microbiological characteristics under the disease suppression in the MPM system were similar in nature to that in naturally occurring disease-suppressive soils (Weller et al. 2002). *F. oxysporum* growth suppression in the MPM system was eliminated when the solution was pasteurized or transferred to sterilize water. These treatments decreased the suppression of Fusarium wilt disease in lettuce and tomato cultivation. The microbiological features have been reported in various suppressive soils including soils capable of suppressing Fusarium wilt (Scher and Baker 1980; Alabouvette 1986; Haas and Défago 2005). In our population-based approach to explain microbial community abundance, we detected bacteria or antagonists that were previously reported in disease-suppressive soil, including *Bacillus*, Actinomycetales, Burkholderiales, Deltaproteobacteria, Sphingobacteriaceae, and Comamonadaceae, in the rhizosphere bacterial community of the MPM solution. They are specific bacteria within the microbiota underlying the suppression of soil-borne disease and belonged to the following four bacterial phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Sivan and Chet 1989; Weller et al. 2002;

Benitez and Gardener 2009). These bacteria are assumed to be bacterial indicators of disease suppression from suppressive soils (Kyselkova et al. 2009; Mendes et al. 2011). We reported here about suppression of root-borne disease by microbiological activities in hydroponic agriculture.

Microbiota including antagonistic bacteria is present in the rhizosphere of the MPM system. Bacterial strains belonging to the Bacillus genus are known as potential biocontrol agents for *F. oxysporum* (Weller et al. 2002). The Bacillus strains produce a wide range of diversity in the type and the number of antibiotics (Raaijmakers et al. 2002). Our results showed that biofilms including the above-mentioned taxa and individual isolates from the biofilm did not show any antibiotic effects on *F. oxysporum* on the media that we used. The growth suppression of *F. oxysporum* was eliminated when the MPM solution was filtered or autoclaved to sterilize the solution. The suppression ability was also eliminated by diluting the solution or reducing the microbial biomass. Moreover, antagonistic bacteria were unlikely to contribute to the disease suppression during 4 days after transplanting although the antagonists inhabited in the MPM solution. These results suggest that the disease suppression by the MPM system is governed by microbial consortia rather than by potential bacterial antagonists and that antimicrobial substances which are at least diffusible, degradable, or sensitive to heat and concentrations were unlikely. Possible antimicrobial agents such as organic volatile compounds (Minerdi et al. 2009) are under investigation.

Fusarium oxysporum can form chlamydospores under unfavorable environmental conditions, such as low temperatures, and these resting bodies act as primary inocula in soil-borne infections, whereas the microconidia and macroconidia formed by *F. oxysporum* are important in a secondary infection (Nelson 1981; Couteaudier and Alabouvette 1990).

Controlling morphogenesis of *F. oxysporum* by which interactions between microbiota and *F. oxysporum* stimulate chlamydospore formation in the rhizosphere is part of the mechanism for the inhibition of this pathogen in the MPM system. The disease suppression that we observed was associated with reproduction silencing in *F. oxysporum* caused by the interactions. The present results provide evidence that the suppression of *F. oxysporum* in the MPM system resulted from interactions with the rhizosphere microbiota that affected the pathogen's morphogenesis. *F. oxysporum* survived in the microbiota from the MPM system but did not cause disease symptoms. The fungal pathogen retained its pathogenicity when it was reisolated from the solution. However, *F. oxysporum* formed chlamydospores when exposed to the rhizosphere microbiota, even though those in MPM solution did not form them 3 days after the inoculation (Figure 13A). The fungal cells in the MPM solution had a higher germination rate of microconidia but the lower hyphal elongation rate than those in the inorganic hydroponics solution. The hyphal elongation is necessary for the production of microconidia and macroconidia (Nelson 1981). The insufficient hyphal elongation in MPM solution may lead to no increase macroconidia and microconidia, resulting in *F. oxysporum* cell density remaining unchanged. These findings revealed that the rhizosphere microbial consortia established in the MPM system is not suitable for the growth of *F. oxysporum* and suggested that bacterial–pathogen interactions negatively affect the pathogenic ability of *F. oxysporum* within the rhizosphere microbial community.

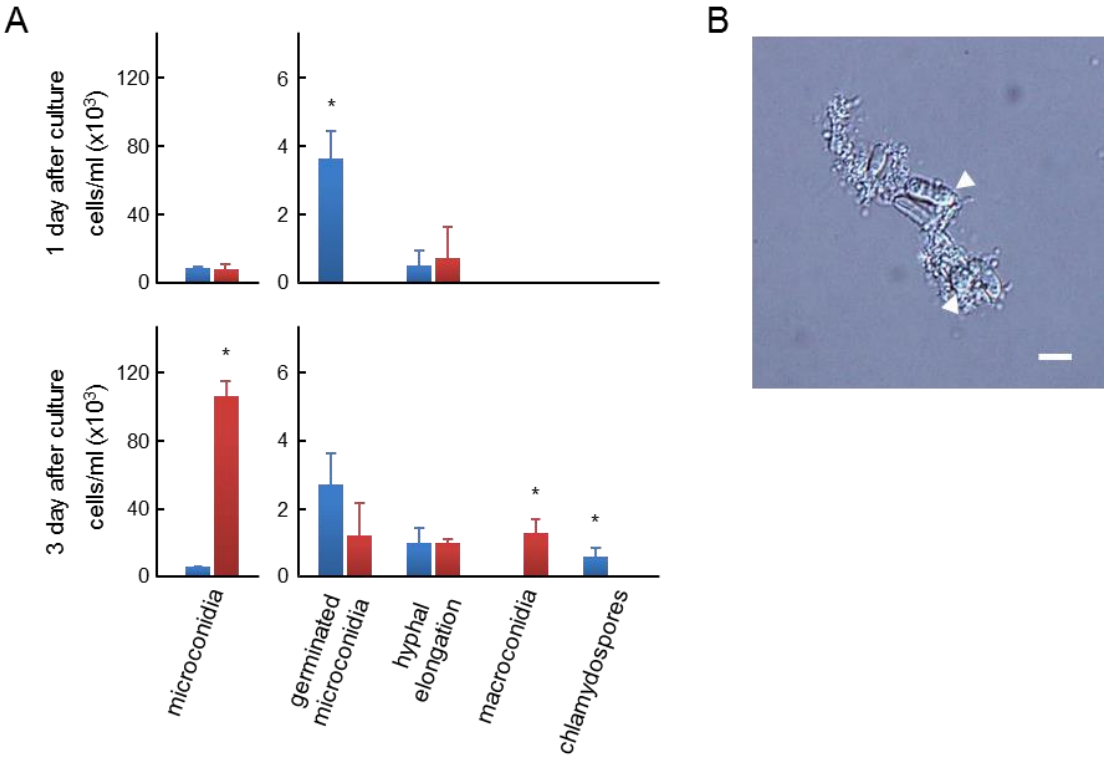

Figure 13. Observations of *F. oxysporum* morphology in the MPM solution. (A) Cell formations of *F. oxysporum* f. sp. *lactucae* strain H111-dsRed was assessed 1 and 3 days after inoculation by the numbers of microconidia, germinated microconidia, macroconidia, and chlamydospores and by the number of hyphae with elongation (> 20 μm). Blue bars, MPM system; red bars, an inorganic hydroponics system. Columns with a star were significantly different at the significance level of $P < 0.05$ according to a Tukey-Kramer multiple comparison test. (B) Bacterial adhesion on chlamydospore of *F. oxysporum* f. sp. *lactucae* strain H111-dsRed. Chlamydospores (arrow heads) were formed on bacterial cells. Bars represent 10 mm.

The microbiological factors influencing the chlamydospore formation of *F. oxysporum* under the disease suppression in the MPM system could be ascribed to direct interactions between rhizobacteria and *F. oxysporum* cells in microenvironment. Some bacteria that are known to be biocontrol agents adhere to its hyphae (Elvers et al. 2001) and alter its hyphal morphology (Bolwerk et al. 2003). Ford et al. (1970) demonstrated that inclusion of soil microbes in the water induced chlamydospore formation in *Fusarium* spp.. Minerdi et al. (2008) reported that microbial symbionts lead to virulence silencing of *F. oxysporum*, and that changes of cell morphogenesis of *F. oxysporum* underlie the suppression. In soils that suppress Fusarium wilt disease, natural suppression is associated with morphological changes in *F. oxysporum* that reduce saprophytic growth and inhibit chlamydospore germination (Sneh et al. 1987; Lemanceau and Alabouvette 1993; Weller et al. 2002). In the present study, chlamydospore formation was detected in fungal cells surrounded by rhizosphere microbiota (Figure 13B), and hyphal elongation was suppressed in the MPM solution. Although antagonists in biofilms lacked activities against *F. oxysporum* when tested in a culture-based method, their antibiotic effects could synergistically control the fungal pathogen in the microenvironment. It is suggested that the complex phenomenon of disease suppression in the MPM system can be established by both physical and functional attributes of microbiota influencing morphological changes in *F. oxysporum*.

Strong suppressive effects on *F. oxysporum* in MPM solution was attributed to assembly of multiple suppressive effects induced by interplays between the rhizosphere bacteria. We obtained 13 bacterial isolates to successfully reproduce suppressive effects on *F. oxysporum*. Consistent with phylogenetic groups in the rhizosphere bacterial population revealed by DGGE analysis, the majority of the bacterial isolates presented in the rhizosphere biofilms

where they contribute to disease suppression toward Fusarium wilt disease. The most of bacterial isolates were not able to contribute to *F. oxysporum* growth suppression by their own although *Microbacterium* sp., *Paenibacilllus* sp., and *Brevibacillus* sp. have been known as the potential biological control agent against Fusarium spp. including *F. oxysporum* (Cavaglieri et al. 2005: Chandel et al. 2010; Dijksterhuis et al. 1999). Some combinations of the single bacteria which did not suppress the fungal pathogen obtained suppressive effects on *F. oxysporum*. As for the development of the growth suppression, bacterial cell-cell communication may cooperate closely in various ways to develop functional abilities (Bodman et al. 2008). A core bacterial network within complex microbiota was likely to influence soil-borne disease suppression (Mendes et al. 2011). We suggest that the suppressive effects in the rhizosphere microbiota of MPM system are derived from bacterial consortium rather than a single bacterial antagonist.

Bacterial interactions with plant roots can lead to plant resistance to *F. oxysporum*. Induced systemic resistance (ISR) against this pathogen can be elicited by some strains in the genera *Bacillus*, *Pseudomonas*, *Serratia*, and *Achromobacter* (Van Peer et al. 1991; Someya et al. 2000; Lugtenberg and Kamilova 2009). Our results demonstrated that one or more potential biocontrol agents in genus *Bacillus* exist in the MPM system. Although ISR may be one of the causes of the suppression of root-borne disease by the MPM system, there is conflicting evidence as to whether ISR plays a major role in disease suppression. Our results indicated that tomato plants grown in the MPM system were still susceptible to diseases of the aerial parts of the plant, including airborne pathogens such as powdery mildew (*Oidium neolycopersici*), leaf mold (*Cladosporium fulvum*), or gray mold (*Botryotinia fuckeliana*) during cultivation in the greenhouse (M. Shinohara et al.,

unpublished data). Because *F. oxysporum* exhibited morphological changes that allowed it to survive in the rhizosphere microbial community, but with reduced infectious ability, the disease suppression we observed in the MPM system is likely to result from microbiological interactions rather than ISR.

Our confirmation of the suppression of Fusarium wilt disease in the MPM system provides substantial insights into the development of biocontrol actions in the rhizosphere. The important characteristic in biocontrol actions is to construct microbial ecosystem where microbes can be functionally performed against root-borne pathogens. We demonstrated that the rhizosphere microbiota in the MPM system suppress Fusarium wilt disease by affecting the morphological characteristics of *F. oxysporum* under the disease suppression. The disease suppression had fully developed by 4 days after transplanting whereas the microbiota potentially including antagonistic bacteria was not capable of contributing to the disease suppression before the 4 days. The changes of rhizosphere structures involving the roots and biofilm formation begin to occur during the 4 days (Figure 14).

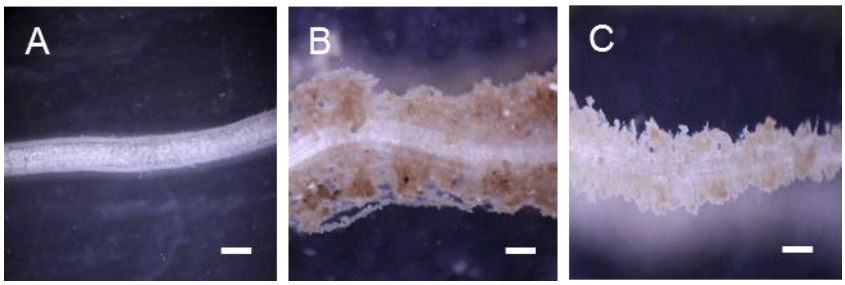

Figure 14. Rhizosphere of tomato seedlings grown (A) in the inorganic hydroponics and (B, C) MPM system. (B) A thick biofilm formed within half a day of transplanting into the MPM system. (C) Thinner biofilm that formed on new root by 4 days after transplanting. Bars represent 0.5 mm.

For the significance of plant-microbial interactions in the rhizosphere, it had been reported that the biocontrol actions are associated with interplays between plant and several microbes (Weller et al. 2002; Lugtenberg and Kamilova 2009), and the plant–microbial interactions can select a root microbiota to physiologically and functionally construct the plant-preferable rhizosphere (Bulgarelli et al. 2012; Lundberg et al. 2012). Bolwerk et al. (2003) proposed that bacterial biocontrol agents need a few days to initiate the mechanical actions that are capable of influencing *F. oxysporum* cell growth. These findings suggest that the development of microbiological abilities for biocontrol actions needs initial establishment periods. The present study can provide a new perspective on the way we visualize plant–microbial interactions in the rhizosphere and studies using the MPM system could be the effective approach to elucidate plant-microbial dynamics in the rhizosphere.

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