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Inhibitory Effects of Super Reductive Water on Plant Pathogenic Fungi

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The antifungal activity of super reductive water (SRW) against plant pathogenic fungi was examined to extend its application to integrated pest management (IPM) for plant diseases. Diluted solutions (×1/10, ×1/25, and ×1/50) of SRW inhibited fungal growth of kiwifruit soft rot pathogen, *Diaporthe actinidiae*, in a concentration dependent manner. When kiwifruits were inoculated on wounds with mycelium blocks, stock and diluted solutions successfully inhibited the disease development. In addition to the high pH of the SRW, fungistatic activity was also considered as the cause of the antifungal effect against the pathogen. Whereas conidial germination of *Magnaporthe grisea* was not affected by the diluted SRW solutions, appressorium formation was significantly inhibited in a concentration dependent manner. With little harmfulness to human health and environment, SRW could be used to control plant pathogenic fungi, particularly appressorium-forming fungal pathogens.

Keywords : appressorium, antifungal activity, *Diaporthe actinidiae*, *Magnaporthe grisea*, super reductive water.

Super reductive water (SRW) and acid electrolytic water (AEW) can be produced by electrolysis-ionizing reaction in which two alloyed electronodes of anode and cathode are located across the ion exchange membrane in the water bath (Shiramizu et al., 1996). Since the water is usually produced with the use of salts such as NaCl and KCl, AEW contains high levels of chlorine (ClO_x^-) and active oxygen, which exert a powerful sterilizing effect on several microorganisms (Sasai-Takedatsu et al., 1997). Chlorine has a strong cytotoxicity and can cause serious problems by reacting with organic matter to form carcinogenic substances in water environment (Dychdala, 1983; Rutala, 1995).

Super reductive water (SRW) has unique characteristics, such as a high negative oxidation-reduction potential (ORP) of -800 mV and strong alkalinity (pH of more than 11.5), which far exceed those of the living environment of microorganisms. The water exerts a sterilizing effect on several bacteria by hydroxyl radical (Katsuragi et al., 2001). Furthermore, SRW is very safe to human health and environment because it has no harmful chemicals in the water.

Recently, a new SRW was manufactured with improved stability of more than 6 months in a special buffer system. The SRW has been used as a safe cleansing and sterilizing agent in medicine (Katsuragi et al., 2001). However, the mechanisms and modes of sterilizing functions are not fully elucidated. Little attempt has been made to evaluate the antagonistic activity of the SRW against plant pathogenic fungi. This study examined whether the new SRW has an antifungal activity against plant pathogenic fungi causing kiwifruit soft rot and rice blast disease, and thus to extend its application to integrated pest management (IPM) for plant diseases.

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Materials and Methods

Preparation of SRW solutions. The SRW used in the study has a stability of more than 6 months in a special buffer system and was supplied by the Bumhwoo Co. (GC100X®, Ansan, Korea). The product is commercially manufactured for cleansing semiconductors and for sterilizing foods and kitchenware. It was previously reported to have excellent bactericidal effects on gram-negative bacteria with little cytotoxicity (Katsuragi et al., 2001). The SRW stock solution has pH 11.6, OPR -800 mV, HClO 0.3 ppm, and EC 2.63 μS/cm. The stock was diluted to gradient concentrations of 1/10, 1/25, and 1/50 with deionized water before the tests.

Determination of organic matter detachment by the SRW from the surface of plant tissue. Bean sprouts were cultivated for 5 days with the circulated irrigation system previously described (Hur and Koh, 2001). The surface of the bean sprouts was heavily contaminated with mucous organic matters. Freshly harvested bean sprouts (200 g in fresh weight) were dipped into 2 L each of diluted SRW solution (×1/20) and distilled water, respectively, and gently agitated for 5 minutes. Total organic carbon (TOC) contents in the washed solutions were determined

with TOC analyzer (Model TOC-VCPN, Shimadzu, Japan).

Antifungal activity of the SRW on kiwifruit soft rot pathogen, *Diaporthe actinidiae*. Kiwifruit soft rot pathogen, *D. actinidiae* (*Phomopsis mali*), was grown on potato dextrose agar (PDA). Freshly transferred isolates were incubated at 25°C for about 10 days and then used for the tests. Antifungal activity of the SRW on mycelial growth of the isolates was estimated on 100 ml potato dextrose broth (PDB) media formulated with the diluted SRW stock solutions at 1/10, 1/25, and 1/50. Three agar plugs (8 mm in diameter) cut from the margin of actively growing cultures were inoculated into the media. The cultures were grown in a shaking incubator at 25°C and 120 rpm for 7 days. To discriminate the sterilizing effect of the SRW solution on the mycelium growth from the inhibitory effect of the high pH level, the fungus was also cultivated in a PDB media adjusted with 0.5 N KOH to pH 7.5, 9.0, and 10.0. All the media were sterilized with microfilter (0.22 μm, Millipore, USA) before mycelium inoculation. After a 7-day incubation, mycelia mats were filtered, harvested, and dried at 70°C for 24 h. Inhibition of the fungal growth was examined by measuring the dry weight of the mycelia mats.

To investigate the antifungal activity of the SRW on spore germination, conidia of the fungal pathogen were harvested by flooding the plates with 10 ml distilled water. The conidia were collected by centrifugation of the spore suspension at 5,000 rpm for 10 minutes, and were re-suspended in the SRW solutions. Several droplets (100 μl) of the conidia suspensions (approximately 10^6 conidia ml^{-1}) were placed on cavity slide glass (Fujiwara glass & Co., Japan). The glasses were sealed in a moistened box, and incubated at 25°C for 24 h. The percentages of germinated and germinating conidia were determined by the direct microscopic examination of 1,000 conidia.

Kiwifruits were wound-inoculated with agar plug of *D. actinidiae*, as prepared above. After surface disinfection with methanol, wounds were induced on the skin of kiwifruits by sterilized pin-punch. Several droplets (200 μl) of the SRW solutions were placed on the wounds and then left until the droplets were completely absorbed into the skin of the kiwifruits. Agar plugs were smeared on the wound with a sterilized razor blade and sealed with parafilm. After incubation of the inoculated kiwifruits at 20°C for 2 weeks, occurrence of lesions and their size were determined.

Inhibitory activity of the SRW on conidial germination and appressorium formation of *Magnaporthe grisea*. Rice blast pathogen, *M. grisea* (race KJ 201), was routinely maintained on PDA and kept in a 25°C incubator under white light (16 h photoperiod). For the preparation of the conidia suspensions, the cultures were grown on oatmeal agar (50 g of oatmeal per liter). After a 7-day incubation, aerial mycelia of the plates were removed with a sterilized razor blade and then the scraped culture plates were exposed to fluorescence light to promote condition (24 h photoperiod) for 3 days. Conidial suspensions were prepared as above. Several droplets (200 μl) of the conidia suspension (approximately 5×10^4 conidia ml^{-1}) were placed on a hydrophobic and transparency film (CG3300, 3 M, Italy), sealed in a moistened box, and incubated at 25°C for 24 h. The percentages of germinated and germinating conidia induced to form appressoria were determined by the direct microscopic examination of 1,000 conidia on three films.

Results and Discussion

Characterization of the SRW solutions. The diluted SRW solutions showed pH levels of 9.93, 8.97, and 7.51 for 1/10, 1/25, and 1/50 diluted stock solutions of SRW, respectively. The deionized water of a control showed pH 6.19. The SRW exhibited strong activity of organic matter detachment from the surface of the bean sprouts. Whereas TOC content in the washed solution with distilled water was 37.3 mg l^{-1}, the content in the washed solution with the SRW was 151.9 mg l^{-1}, which was four times higher than the control. The result demonstrated that the SRW successfully detached organic contaminants from the surface of living plant tissues. This is the first report quantitatively evaluating the efficacy of SRW in organic matter detachment from living plant tissues.

Inhibitory activity of the SRW on kiwifruit soft rot pathogen, *Diaporthe actinidiae*. Mycelial growth of *D. acinidiae* was definitely inhibited by the SRW treatment (Fig. 1). Dry weight of mycelial mats grown on the PDB

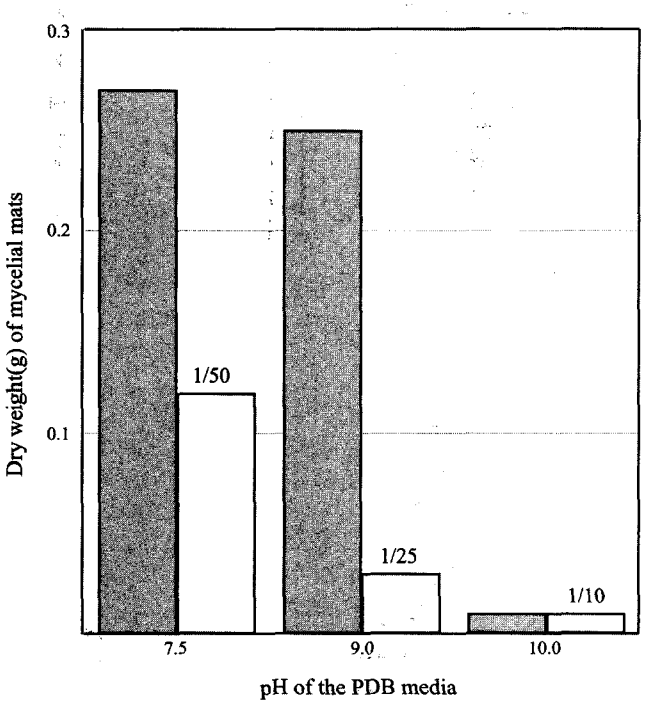

Fig. 1. Inhibitory effects of SRW on mycelial growth of *Diaporthe actinidiae*, kiwifruit soft rot fungus. The whte stack (□) indicates dry weight of mycelial mats grown on the PDB media formulated with diluted SRW stock solutions of 1/10, 1/25 and 1/50; the shadow stack (■) indicates those grown in the PDB media adjusted with 0.5 N KOH to pH levels similar to that of the SRW treated media. The data are means of six replicates.

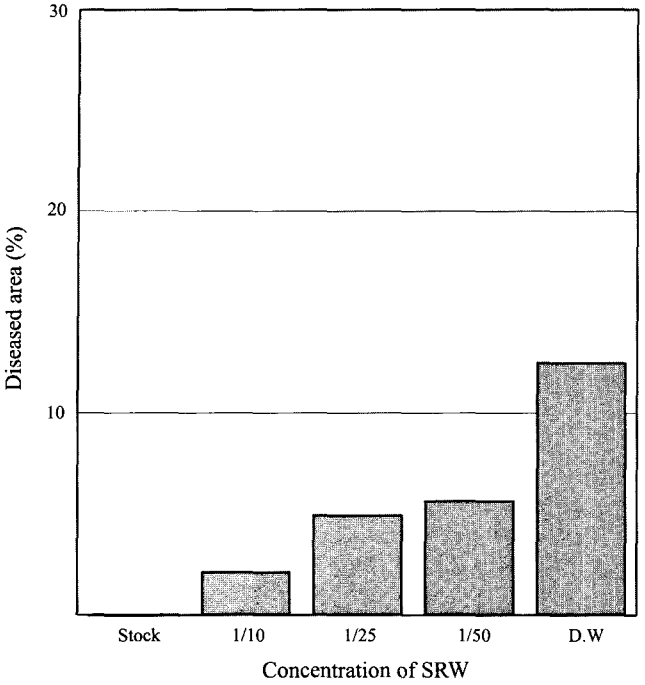

Fig. 2. Inhibitory effects of SRW on disease development of *Diaporthe actinidiae* artificially wound-inoculated on kiwifruits. D.W indicates distilled water treatment as a control. The data are means of 10 replicates.

media formulated with the diluted SRW solutions was significantly reduced in a concentration dependent manner. Compared with the PDB cultures adjusted to pH levels similar with that of the SRW solutions, fungal growth in the SRW treated cultures was significantly inhibited. The result suggests that the inhibitory effect of the SRW on fungal growth could be caused not only by the fungistatic activity of the SRW solutions, but also by the high pH.

The SRW treatments also inhibited the disease development of *D. actinidiae* on kiwifruits (Fig. 2). Soft rot was clearly induced on the kiwifruits artificially inoculated with mycelium blocks in the control treatment. However, complete inhibition of lesion development was found in stock SRW treatment, which was likely to be concentration dependent. However, spore germination of *D. actinidiae* was not affected by the SRW solutions (data not shown).

Inhibitory activity of the SRW on conidial germination and appressorium formation of *Magnaporthe grisea*. Whereas conidial germination of *M. grisea* was not affected by the diluted SRW solutions, appressorium formation was significantly inhibited in a concentration dependent manner (Fig. 3). Conidial germination and appressorium formation were not observed in SRW stock solution. Appressorium, the specialized infection structure of many plant pathogenic fungi, is induced by environmental stimuli including thigmotropic and chemical signals (Podila et al., 1993).

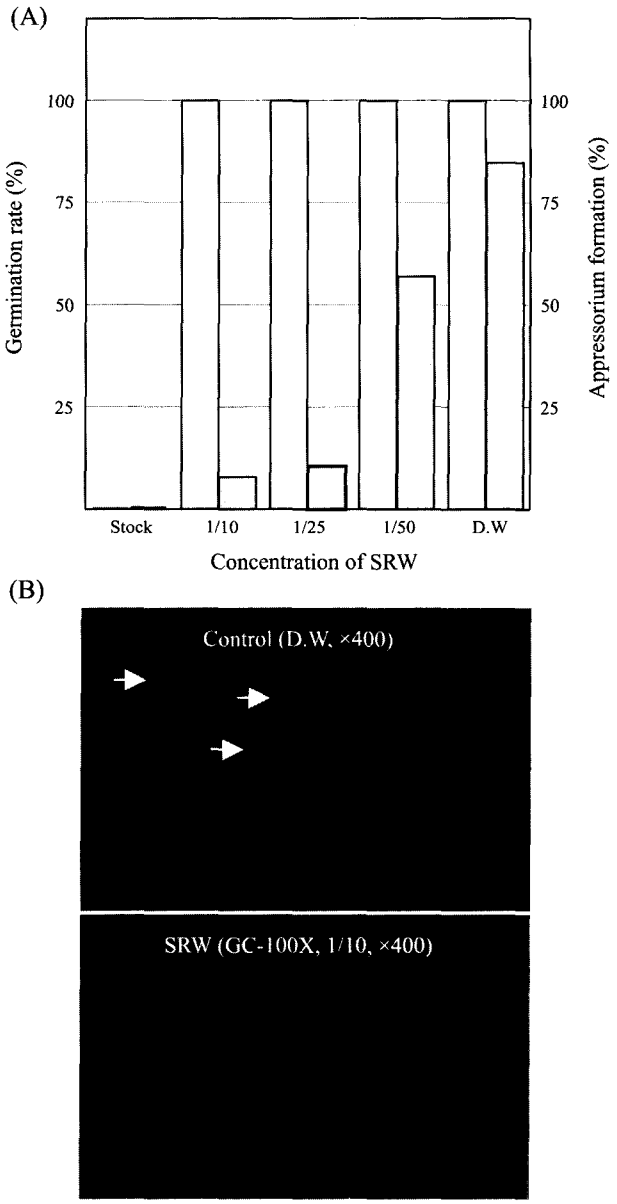

Fig. 3. (**A**) Inhibitory effects of SRW on conidial germination and appressorium formation of *Magnaporthe grisea*, rice blast fungus. D.W indicates distilled water treatment as a control. (□)=spore germination; (■)=appressorium formation. (**B**) Suppression of appressorium formation in diluted SRW solution (lower), compared with control (upper). Arrows indicate appressoria.

Environmental cues inducing appressoirum formation in *M. grisea* include the hydrophobicity and hardness of the contact surface and chemicals from the plant surface (Gilbert et al., 1996; Lee and Dean, 1993; Xiao et al., 1994). Since the SRW already showed an excellent function of detaching organic matter from the bean sprout surface, SRW-induced inhibition of appressorium formation was considered to be due to conidial detachment from the hydrophobic film surface. SRW-mediated conidial detach-

ment could interfere with conidial recognition of the surface and prevent induction process of appressorium formation thereafter. This implies that the SRW can control the plant pathogenic fungi that form appressoirum before successful infection and further disease development.

With little harmfulness to human health and environment (GCL Co. 2002; Katsuragi et al., 2001), the SRW can be applied to plant diseases caused by pathogenic fungi, particularly appressorium-forming fungal pathogens.

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