J Gen Plant Pathol (2011) 77:331–335
DOI 10.1007/s10327-011-0336-8

DISEASE CONTROL

Fe^{2+} and Mn^{2+}, potential agents to induce suppression of *Fusarium oxysporum* for biological soil disinfestation

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Received: 8 June 2011 / Accepted: 5 September 2011 / Published online: 30 September 2011

Abstract Here *Fusarium oxysporum* was killed in exudates obtained from soil biologically disinfested with ethanol, indicating that physical interaction with soil microorganisms was not essential. Because acetic acid was confirmed to accumulated during the treatment, we evaluated the effect of acetic acid amendment against the pathogen in plastic containers. A drop in the soil redox potential seemed to be correlated with the fungicidal efficacy of acetic acid. Under reductive soil conditions, metal ions such as Mn^{2+} and Fe^{2+} formed, and the pathogen was effectively suppressed in Mn^{2+} and Fe^{2+} solution. Therefore, Fe^{2+} and Mn^{2+} may be the agents that induce suppression of the pathogen during biological soil disinfestation.

Keywords *Fusarium oxysporum* f. sp. *lycopersici* · Biological soil disinfestation · Ethanol · Fe^{2+} · Mn^{2+}

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Introduction

Organic soil amendments such as wheat bran, rice bran, molasses and ethanol have been developed for biological soil disinfestation (BSD) as an alternative to chemical soil fumigation in Japan (Kobara et al. 2007b; Shinmura 2000). After amendment, the soil is irrigated and covered with a conventional agricultural plastic film. Many soilborne pathogens such as *Fusarium oxysporum* f. sp. *lycopersici*, *Ralstonia solanacearum*, and *Meloidogyne incognita* can be suppressed by BSD (Kobara et al. 2007a; Momma 2008; Momma et al. 2005, 2010; Shinmura 2004; Uematsu et al. 2007). Effective BSD treatments are accompanied by changes in the soil environment, for example, a drop in the redox potential, accumulation of acetate and butyrate, and emanation of a peculiar odor from the soil (Kobara et al. 2007a; Momma et al. 2005, 2006). In a BSD study using wheat bran, Momma et al. (2006) focused on organic acids as the potential inducer of BSD effect but was unable to identify the principal agents that killed *F. oxysporum* f. sp. *lycopersici*. Okazaki and Nose (1986) concluded that acetic acid and *n*-butyric acid produced in glucose-amended, flooded soil was fungicidal, but did not confirm any fungicidal activity in soil. Similarly, Katase et al. (2009) demonstrated nematicidal activities of acetic acid and *n*-butyric acid and postulated that a low pH in the soil microsites that surround wheat bran particles could be responsible for nematicidal activity even though the overall soil pH did not decrease to incite nematode suppression.

In the present study, we treated soil with ethanol, then identified and quantitated the organic acids and the concomitant survival of *F. oxysporum* in soil extracts. We also added acetic acid to infested soils and evaluated the subsequent viability of *F. oxysporum*. Ferrous ion (Fe^{2+}) and

other metal ions present in the extracts were also tested as possible fungicidal agents.

Preparation of inoculum

Fusarium oxysporum f. sp. *lycopersici* CU1 (race 1) was grown in potato dextrose broth for 5 days at 25°C with shaking. Fungal cultures were passed through cheesecloth and centrifuged at 3000×*g* for 5 min to precipitate bud cells, which were then resuspended in sterile distilled water. This step was repeated twice.

A 4-mL suspension of bud cells was mixed with 16 g of perlite powder to prepare inoculum. Twenty grams of this inoculum was then packed in a nylon mesh bag (ca. 10 × 7 cm) to prevent mixing of the inoculum and the soil. Inoculum bags were used to easily evaluate the effect of each treatment on the population density of the pathogen in soil. The number of initial viable propagules was measured by dilution on Fo-G1 agar, a medium selective for *F. oxysporum* (Nishimura 2007).

Organic acids produced during ethanol BSD

Soil (Hydric Hapludand) was collected from an experiment field of the National Institute for Agro-Environmental Sciences (Tsukuba, Japan), passed through a 5 mm sieve, then 4 kg of fresh soil (2.7 kg dry mass) was packed in a plastic box (ca. 17 × 17 × 15 cm) equipped with sealing clamps on the lid. The inoculum bag was buried in the soil at a depth of ca. 7 cm. Then the soil was treated with 1000 mL of distilled water or ethanol solution (2.0%, v/v) and incubated at 30°C. Soil samples (ca. 40 g fresh mass) were collected 1, 2, 3, 6, 9, and 12 days after treatment and centrifuged at 3000×*g* for 5 min. The samples were taken separately from a new individual box each time, and the residual soil was discarded. The supernatant was passed through a 0.2 μm filter (Dismic-25cs, Advantec, Tokyo, Japan). The inoculum bags were retrieved 12 days after treatment, and viable propagules of the pathogen were counted as described earlier. The organic acids in the soil were identified and measured using a high-performance liquid chromatograph (HPLC) equipped with a conductometric detector (SCL-10A, CDD-6A Shimadzu, Kyoto) and a Shim-pack SPR-H reverse-phased column (250 mm × 7.8 mm, 8 μm particle size; Shimadzu GLC Ltd., Tokyo) in conjunction with a security guard column (50 mm × 7.9 mm, 8 μm particle size). The column oven temperature was kept at 50°C, and 10 μL of the sample was injected. The mobile phase was 4 mM *p*-toluenesulfonic acid, and the flow rate was 0.4 mL/min. The composition of reaction solution consisted of 4 mmol/L *p*-toluenesulfonic acid, 100 μmol/L EDTA and 16 mmol/L Bis–Tris, and the flow rate was 0.8 mL/min. In this analysis, succinic acid, lactic acid, formic acid, acetic acid, propionic acid, and butyric acid were determined quantitatively. Three replications were made for each treatment.

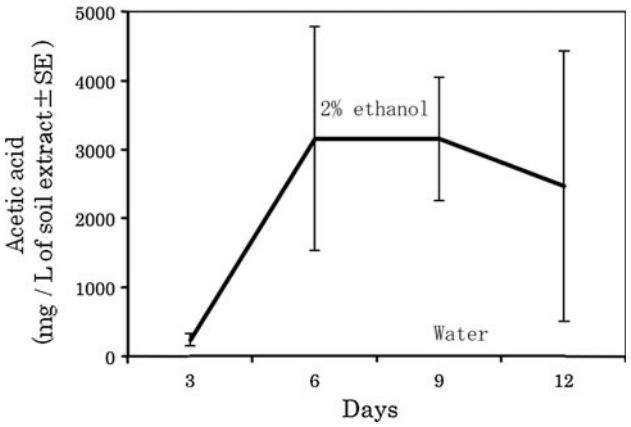

Fig. 1 Acetic acid levels in ethanol-treated soil. Soil extracts were obtained 3, 6, 9, and 12 days after treatment. Concentrations were measured using HPLC

Acetic acid was detected after the ethanol treatment (Fig. 1), but the concentration of the other organic acids was negligible (data not shown). No organic acids were detected in the water treatment. The pathogen was not detected from inoculum bags after the ethanol treatment, but 5.02 ± 0.03 log CFU/g dry perlite (±SE) were viable in the water-treated treatment. Thus, the fungicidal effect of the ethanol-BSD coincided with the production of acetic acid.

Survival of *F. oxysporum* f. sp. *lycopersici* in soil exudates

To check whether a physical interaction between the pathogen and soil microorganisms contributed to the fungicidal effect of BSD, we incubated the pathogen in filtered soil exudates. An inoculum bag and a porous cup (Mizutoru, Daiki Rika Kogyo Co., Ltd., Saitama, Japan) were prepared and buried in soil in each plastic box as already described. Soil exudates were obtained 6 and 12 days after incubation from the porous cups and passed through a 0.2 μm filter (Dismic-25cs, Advantec). Bud cells, collected from potato dextrose broth cultures, were then resuspended in 15 mL of soil extract (ca. 5.0 log cell/mL) and incubated for 5 days at 30°C. Then, at 12 days after treatment, the surviving propagules in these soil extracts and in the inoculum bags in the BSD-treated soil were counted as already described. Three replications were done for both experiments.

Table 1 Survival of *Fusarium oxysporum* f. sp. *lycopersici* in soil extracts 6 and 12 days after treatment with ethanol or water

Treatment	Viable propagules (log CFU/mL ± SE)[a]		Survival in soil[b] (log CFU/g dry perlite)
	6 days	12 days	
2.0% ethanol	4.21 ± 0.08	ND	<1.4
Water	5.06 ± 0.07	5.37 ± 0.02	4.57

ND not detected (minimum detectable density = 0.70 log CFU/mL)

[a] Soil exudates were obtained using a porous cup 6 and 12 days after treatment. Bud cells added to the exudates (ca. 10^5 cells/mL) were incubated at 30°C for 5 days; viable propagules were grown and counted on Fo-G1 agar

[b] Inoculum bags buried in soil were retrieved 12 days after treatment, then viable propagules were counted

A reductive soil condition was confirmed in terms of Fe^{2+} formation, using a phenanthroline indicator solution (1.0 g 1,10-phenanthroline monohydrate in 0.5 L 10% acetic acid): 0.1 mL of the indicator was added to 0.9 mL of the soil extract. Although Childs (1981) originally sprayed α,α′-bipyridyl solution on soil profiles to detect Fe^{2+}, we used phenanthroline, which is likely to be more suitable for testing our soil–water extracts. And we confirmed that phenanthroline test using soil water always gave a positive reaction (red coloration), when the original soil gave a positive reaction in the phenanthroline spray test.

The phenanthroline reaction in ethanol-treated soil was faint 6 days after incubation but was strong after 12 days, indicating Fe^{2+} accumulated over time (data not shown). Bud cells were completely killed only in soil exudates at 12 days after incubation (Table 1). The pathogen buried in soil had also decreased substantially in ethanol-treated soil. Such suppression was never achieved in any of the water-treated controls. These results suggested that water-soluble compounds such as organic acids contributed to the fungicidal effect and that direct physical contact between the pathogen and soil microorganisms was not essential for the fungicidal activity.

Effect of acetic acid

After the inoculum bag and porous cup were buried in the box of soil as described earlier, we poured either 1300 mL of distilled water or 0.66% acetic acid solution (v/v) onto the soil. This application rate simulated the dose corresponding to the maximum acetic acid concentration (ca. 4 g/L) in the soil treated with 2.0% ethanol solution. Soil extract, collected 7 and 14 days after treatment, was then tested with phenanthroline. Survival of the pathogen was assayed 14 days after treatment. The experiments were done with three replications and repeated twice.

In the first trial, the pathogen was not detected after acetic acid treatments but was detected at 4.17 ± 0.07 log CFU/g dry perlite (±SE) after the water treatment. The phenanthroline reaction for the acetic acid treatment was faint 7 days after incubation but became strong 14 days after incubation (Fig. 2). In the second trial, however, the phenanthroline reaction was still faint 14 days after incubation for the acetic acid treatment, and the pathogen was present at 3.75 ± 0.03 log CFU/g dry mass (±SE) in the acetic acid treatment and at 4.82 ± 0.05 log CFU/g dry mass (±SE) in the water control. These results indicated that the intensity of the phenanthroline reaction was positively correlated with fungicidal activity in acetic-acid-amended soil, coincident with data from field trials; spraying phenanthroline solution or bipyridyl solution (1.0 g α,α′-bipyridyl in 0.5 L 10% acetic acid, Childs 1981) turned the soil surface pink to red when BSD was effectively conducted.

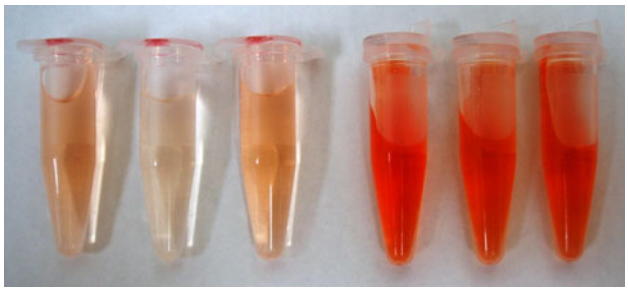

Fig. 2 Phenanthroline test to determine Fe^{2+} concentration in soil extract collected from acetic-acid-amended soil 7 days (three on *left*) and 14 days (three on *right*) after treatment. The stronger the *red* color, the higher is the concentration of Fe^{2+}. Each tube represents a replication

Interestingly, a positive reaction in the phenanthroline reaction was not observed, and the pathogen population was not suppressed after acetic acid was added to sterilized soil (data not shown). Fungicidal activity and a drop in redox potential were evident only after wheat bran or ethanol was added to nonsterilized soil, suggesting that the pathogen was killed in acetic-acid-amended soil as a result of the same mechanism operating in BSD when using wheat bran or ethanol. Because Takai and Kamura (1966) reported that Fe^{2+} and Mn^{2+} were produced in flooded paddy soils under reduced conditions, we then tested the fungicidal activity of Fe^{2+} and Mn^{2+}.

Fe^{2+} and Mn^{2+}: possible compounds involved in fungicidal activity of BSD

Bud cells (5.4 log cell) were incubated in 15 mL aqueous solutions containing $FeSO_4$, $Fe_2(SO_4)_3$, $MnSO_4$, or $MgSO_4$ at 0, 1.0, 0.1, 0.01, 0.001% (w/w). In a preliminary experiment using IRON CHECK (Advantec), we found that Fe^{2+} was present at >50 mg/L soil extracts of ethanol-treated soil. For more precise determinations of metal ions including Fe^{2+}, we needed to use an inductively coupled plasma (ICP) instrument. When $FeSO_4$ and $MnSO_4$ solutions are assumed to be completely ionized, 0.01% solutions contain ca. 20 mg of Fe^{2+} and Mn^{2+} per liter. Viability of the pathogen was measured 1, 4 and 7 days after incubation at 30°C. Three replications were made for each treatment.

Survival of the pathogen was significantly reduced in $FeSO_4$, $Fe_2(SO_4)_3$, and $MnSO_4$ solutions (Table 2); $Fe_2(SO_4)_3$ was the least effective. Thus, Fe^{2+}, Fe^{3+} and Mn^{2+} did reduce the population of *F. oxysporum*. Fe^{2+} is known to be toxic to rice plants and several bacterial species (Fakih et al. 2008; Foy et al. 1978; Murata et al. 2008). Iron and manganese exist as Fe^{2+} and Mn^{2+} in soil solution, and they increase in reduced conditions (Takai and Kamura 1966). In addition, Fe^{3+} is strongly adsorbed to soil particles. $MgSO_4$ was also tested to determine whether SO_4^{2-} was critical but failed to induce fungicidal activity. SO_4^{2-} is one of the predominant, naturally occurring substances in the soil. Furthermore, because it is one of the least toxic anions, SO_4^{2-} is not monitored under the Drinking Water Quality Standards (DWQS) of the Ministry of Health, Labour and Welfare of Japan.

Table 2 Time-course of survival of *Fusarium oxysporum* f. sp. *lycopersici* after treatment with metal ions or water

Treatment	Viable propagules (log CFU/mL ± SE)		
	1 day	4 days	7 days
Water	4.8 ± 0.1	4.8 ± 0	4.8 ± 0
Fe^{2+} (%)			
1	2.6 ± 0	ND	ND
0.10	1.9 ± 0.1	ND	ND
0.01	3.5 ± 0	ND	ND
0.001	4.2 ± 0	2.1 ± 0.1	ND
Fe^{3+} (%)			
1	2.6 ± 0.1	ND	ND
0.10	3.6 ± 0	1.6 ± 0.1	ND
0.01	4.0 ± 0	3.8 ± 0	3.8 ± 0
0.001	4.2 ± 0	4.3 ± 0	4.3 ± 0
Mg^{2+} (%)			
1	4.7 ± 0	4.7 ± 0	4.8 ± 0
0.10	4.8 ± 0	4.9 ± 0	4.8 ± 0
0.01	4.8 ± 0	4.8 ± 0	4.9 ± 0
0.001	4.8 ± 0	4.8 ± 0	4.9 ± 0
Mn^{2+} (%)			
1	2.4 ± 0	ND	ND
0.10	2.5 ± 0	ND	ND
0.01	2.6 ± 0	ND	ND
0.001	3.5 ± 0	2.6 ± 0	1.9 ± 0.1

Bud cells were incubated in $FeSO_4$, $Fe_2(SO_4)_3$, $MgSO_4$, or $MnSO_4$ solution, and viable propagules were grown and counted on Fo-G1 agar after 1, 4, and 7 days of incubation at 30°C

ND not detected (minimum detectable density = 0.70 log CFU/mL)

Although we did not investigate the mode of action of Fe^{2+} and Mn^{2+} in suppressing the fungus in the present study, we presume that reactive oxygen species might be derived from a chemical reaction such as Fenton's reaction. Murata et al. (2008) suggested that reactive oxygen species caused the cell death of *Escherichia coli* in Fe^{2+} solution.

On the basis of these observations, we hypothesize that Mn^{2+} and Fe^{2+}, released under anaerobic conditions, work synergistically with acetic acid to show suppress the fungus in BSD-treated soil. The present study gives us new insight into the well-known but poorly explained suppression of pathogenic fungi under reduced conditions such as flooded paddy soils and BSD-treated soils. However, we cannot rule out direct fungicidal activity by acetic acid on the basis of our experiments. Katase et al. (2009) considered that organic acids produced in microsites around organic materials locally reduced the pH and induced fungicidal activity.

Whether the application of metal ions can reproduce the fungicidal activity of BSD treatments needs to be confirmed with further study. Additive effects of Mn^{2+}, Fe^{2+}, and other metal ions with organic acids should also be investigated to help elucidate the mechanism of disease suppression by BSD.

Acknowledgments We thank Dr. N. Matsumoto for helpful discussion and polishing this paper. This research work was supported in part by the research and development projects for promoting new policy of agriculture, forestry and fisheries, funded by the Ministry of Agriculture, Forestry and Fisheries of Japan.

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