Acetic Acid and n-Butyric Acid as Causal Agents of Fungicidal Activity of Glucose-amended Flooded Soil

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Abstract

Volatile fungicidal substances, which affect the survival of *Fusarium oxysporum* f. sp. *raphani* in soil flooded with 3% glucose solution in vitro, were analysed by using a cryogenic trap, by bioassay, gas chromatography and gas chromatography-mass spectrometry. The antifungal volatile substances collected consisted of two components: acetic acid and n-butyric acid. Their concentrations in the centrifuged supernatant of the soil were 3,407 and 2,944 ppm, respectively, when the fungicidal activity in the soil reached maximum values on the 6th day after flooding. The concentrations were high enough to kill the chlamydospores of the fungus. Other volatile substances including hydrogen sulfide were not related to the fungicidal activity of the soil. These results suggest that the fungicidal activity of glucose-amended flooded soil was associated with the production of acetic acid and n-butyric acid in the soil.

(Received October 29, 1985)

Key words: soil, glucose-amendment, *Fusarium*, acetic acid, n-butyric acid, fungicidal activity.

Introduction

Flooding of soil is one of the cultural methods for controlling soil-borne diseases caused by *Fusarium* spp.. Its effectiveness is enhanced by amendment with several organic materials^{18,26}. The beneficial effect of organic amendments has been attributed to either the increase in the number of competitive microorganisms¹⁹ or the production of a volatile inhibitor¹². In the previous paper, it was suggested that the death of *Fusarium oxysporum* f. sp. *raphani* in glucose-amended flooded soil was due to the presence of volatile fungicidal substance(s) evolved from the soil¹⁵. As an extension of the previous work, in the present paper the identification of the volatile substances responsible for the death of *F. oxysporum* f. sp. *raphani* in the soil flooded with a glucose solution is reported. A preliminary account of this work has already been published¹⁶.

Materials and Methods

Fungus. An isolate of *Fusarium oxysporum* f. sp. *raphani* was used. Hereafter this fungus will be referred to as *F. oxysporum*.

Soil. Soil was collected in the cultivated upland field of the National Institute of

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Soil treatment. Ten g of soil was put in 200-ml Erlenmyer flasks, flooded with 10 ml of 3% glucose solution and incubated at 28 C. Soil flooded without glucose was also incubated as a control. The soil suspension was centrifuged at $1,000\times g$ for 5 min to obtain the supernatant.

Collection of volatile substances. Volatile substances in the soil were collected by using the apparatus illustrated in Fig. 1. A silicone rubber stopper with inlet and outlet tubes was attached to the 200-ml Erlenmyer flask containing the soil. The flask

Fig. 1. Apparatus for cryogenic collection of volatile substances. The numbers correspond to the following items: 1: a 200-ml Erlenmyer flask containing 10 g of soil sample, 2: Teflon tube (2 mm i.d.), 3: Teflon tube connection, 4: U-shaped Teflon tube (4 mm i.d.), 5: cooling bath with freezing mixture of ice and salt (-20°C), 6: U-shaped Teflon tube with 500 mg of Flusin T, 7: glass-wool plug, 8: Flusin T, 9: ethanol cooling bath (-20 to -60°C).

was placed in a water bath maintained at 38 C. Nitrogen gas was bubbled via the inlet tube into the soil suspension at a flow rate of 700 ml/min for 10 min. The exhaust gas from the outlet tube was successively passed through traps 1 and 2 which consist of a 4 mm i.d. $\times$ 60 cm U-shaped Teflon tube. The latter was packed with 500 mg of Flusin T (30–60 mesh) as adsorbent. Trap 1 was maintained at -20°C to remove the moisture. Trap 2 was immersed in an ethanol bath at a temperature ranging from -20°C to -60°C .

Analysis of volatile substances. Qualitative and quantitative determinations of hydrogen sulfide (H_2S), carbonyl sulfide (COS), phosphine, ethylene and ethylene oxide in the soil flooded with a glucose solution were carried out by using the combined cryogenic collection-gas chromatographic system described by Farwell *et al.*⁶⁾ with a slight modification. The gas chromatograph used was a Hitachi model 163 instrument equip-

ed with a flame photometric detector (FPD) and a flame ionization detector (FID). The columns and their temperatures were as follows: a Teflon tube (3 mm i.d. $\times$ 2 m) packed with chromosorb 101 (80-100 mesh) at 80 C for the detection of sulfur compounds and phosphine, and a glass column (3 mm i.d. $\times$ 1 m) packed with Porapak Q (50-80 mesh) at 40 C for the determination of ethylene and ethylene oxide. The flow rate of the carrier gas (He) was 50 ml/min.

Quantitative determination of acetic acid and n-butyric acid in the soil solution was performed by using a gas chromatograph equipped with FID. A portion (1-5 μ l) of the soil solutions, whose pH was adjusted to 2, was directly injected onto a gas chromatographic column. Operating conditions were as follows: column; glass column (3 mm i.d. $\times$ 1 m) packed with Chromosorb 105 (60-80 mesh), column temperature; 180 C, flow rate of carrier gas (He) 50 ml/min.

Mass spectra of the volatile substances collected cryogenically were obtained with a JEOL JMS-D 300 gas chromatograph-mass spectrometer (GC-MS) at 70 eV. Gas chromatographic separation was carried out using a glass column (2 mm i.d. $\times$ 1.2 m) packed with Chromosorb 105 (60-80 mesh) at 180 C. The flow rate of the carrier gas (He) was 30 ml/min.

Formic acid and lactic acid concentrations in the soil solutions were determined by a high performance liquid chromatograph equipped with a Shodex Ionpak S-811 column and a UV absorbance detector (210 nm). The acids were eluted with 0.01 N H_2SO_4 at a flow rate of 1.0 ml/min.

Bioassay. Chlamydo spores of *F. oxysporum* were obtained by the method described previously¹⁴⁾. In the germination test, the spore suspension was diluted to 1×10^4 spores/ml with the supernatant fraction (BMS) of a mineral salt solution¹⁴⁾ and with BMS amended with glucose at 100 mM (BMSG). The suspension was diluted with sterile water for the fungicidal test.

Effect of H_2S on chlamydo spore germination was assayed as follows. Chlamydo spore suspension was mounted on a microscopic glass-slide coated with a collodion film in three places. Two sheets of the slide were placed in the head-space of a 9,000 ml closed chamber containing 2,000 ml of H_2S aqueous solutions. The initial concentrations of these solutions were 0.2, 0.48 and 0.93 ppm, respectively. The concentration was determined by applying the methylene blue method⁸⁾.

Activity of the volatile substances collected by the cryogenic-trap was assayed by the germination test. The adsorbent in the trap was transferred into a small Petri dish (4.0 cm i.d. $\times$ 15 cm height) and the chlamydo spore suspension mounted on a microscopic cover-glass coated with a collodion film was placed in the head-space of the petri dish. The top of the Petri dish was exchanged with the bottom of another Petri dish and was sealed with a Teflon tape.

Inhibitory activity of acetic acid and n-butyric acid on chlamydo spore germination was tested by exposing the chlamydo spore suspension mounted on microscopic glass-slides to the head-space gas above 20 ml of aqueous solutions of acetic acid or n-butyric acid placed in a 15-cm Petri dish. Germination was examined after 15 hr of incubation at 28 C in all the experiments.

Fungicidal activity of the soil flooded with the glucose solution was assayed as follows. An aliquot (1.0 ml) of the chlamydospore suspension (5×10^6 spores/ml) was seeded to each of the flooded soils amended with and without glucose in 200-ml Erlenmeyer flasks. After 24 hr of incubation at 28 C, the number of fungi was counted by the dilution plate method described previously¹⁵⁾. The fungicidal activity was expressed as the percentage of non-colonizing spores in the treated soil to the colonizing spores in the control soil.

Fungicidal activity of H_2S , COS, formic acid, acetic acid and n-butyric acid was assayed as follows. Aqueous solutions of these substances were prepared to reach twice the final concentration, and each 10 ml of the solutions was put in a 100-ml Erlenmeyer flask containing 1 ml of spore suspension and 9 ml of distilled water. The flasks were plugged tightly with a rubber stopper covered with a polyethylene film. The solution was substituted for 10 ml of distilled water for the control. Microconidia of *F. oxysporum* were used as an inoculum (1×10^5 spores/ml) in assays of H_2S and COS, and the number of colonizing and non-colonizing spores was counted by the dilution plate method after 3 days of incubation at 28 C. Chlamydospores of *F. oxysporum* (1×10^6 spores/ml) were used in assays of formic acid, acetic acid and n-butyric acid, and the colonizing spores were counted after 1 day of incubation at 28 C.

Results

Time-course of fungicidal activity in the soil flooded with a glucose solution

Fig. 2 shows the changes in the fungicidal activity of the soil flooded with the glucose solution. The activity appeared during the first 3 days after flooding and reached the maximum values on the 6th day. About 90% of the chlamydospores were killed in the soil at that time after incubation for 24 hr. Evidently, glucose-amendment induces fungicidal capacity in the soil, reconfirming the findings reported in the previous paper¹⁵⁾. About 15% of the chlamydospores were killed in the soil even on the 14th day.

*Determination of hydrogen sulfide and carbonyl sulfide concentrations in the soil flooded with a glucose solution and their effect of *F. oxysporum**

Hydrogen sulfide and COS concentrations were measured to determine whether these substances were associated with the fungicidal activity of the soil flooded with the glucose solution. The soil solution contained $1.3\text{--}2.4 \times 10^{-3}$ ppm of H_2S on the 6th day, and 0.03×10^{-3} ppm of COS on the 7th day. Other sulfur compounds were hardly detected among the active volatile substances collected from the soil flooded with the glucose solution by the cryogenic trap 2 cooled at -35 C, as described later. Chlamydospore germination reached 64 and 0.5% in the head-space gas above the H_2S solutions at the concentrations of 0.2 and 0.48 ppm, respectively, when BMS was used as the medium, and germination was about 90% in the case of BMSG. About 90% of the microconidia were killed in 0.4 ppm of H_2S solution during the 3 day incubation period. None of the spores were killed at a concentration of 0.18 ppm. No effect was observed when the microconidia were placed in 2×10^{-3} ppm of COS solution during 3 days. These results indicate that the volatile sulfur compounds are not associated with the fungicidal activity of the soil. Beside the volatile sulfur compounds, phosphine, ethylene

Fig. 2. Time-course of fungicidal activity in glucose-amended flooded soil. The activity was represented as the rate of the death of chlamydo spores of *F. oxysporum* in the soil after 24 hr of incubation at 28 C. The rate was calculated by the following equation:

$$\left(1 - \frac{\text{number of survivors in glucose-amended flooded soil}}{\text{number of survivors in unamended flooded soil}} \right) \times 100$$

Vertical line at each point represents 95 % confidence limit.

and ethylene oxide were also analyzed, but they could not be detected.

Isolation and identification of volatile fungicidal substances from the soil flooded with a glucose solution

The volatile substances inhibiting chlamydo spore germination were caught by the cryogenic trap when the trap temperature was kept below -35°C . However, the substances remained in the soil at pH 5.5 or above during the expulsion as shown in Table 1. This observation suggests that the substances are acids. The active components were collected from the soil at pH 3.4 after removal of the inactive volatile substances by bubbling N_2 gas into the soil at pH 5.5, and then subjected to GC and GC-MS analysis. Three peaks with Rt. 0.7, 2.4 and 12.0 were observed on the gas chromatogram. Comparing the Rt. on the gas chromatogram and the mass spectra of authentic substances, the peaks with Rt. 2.4 and 12.0 were identified as acetic acid and n-butyric acid, respectively (data not shown). The Rt. of 0.7 coincided with the Rt. of authentic ethanol. These results suggest that acetic acid and n-butyric acid are the main components of active volatile materials in the soil flooded with a glucose solution.

Determination of acetic acid and n-butyric acid concentrations in the soil flooded with a glucose solution and the effect of these substances on *F. oxysporum*

Table 1. Effect of soil pH on volatility of antifungal substances in glucose-amended flooded soil

Treatment ^{a)}	pH of soil	Germination (%)
No treatment	4.4	0
2N HCl (1.0 ml)	2.9	0
2N NaOH (1.0 ml)	9.7	88
1N NaHCO ₃ (1.0 ml)	5.5	89
1N NaHCO ₃ (1.0 ml) 2N HCl (1.0 ml) ^{b)}	3.4	0

- a) Volatile substances were collected from glucose-amended flooded soil after addition of 2N HCl, 2N NaOH or 1N NaHCO₃. Figures in parenthesis indicate the amount of the acid or bases added to the soil. Percentage germination of the spores was 89% in a control. The medium used was BMSG.
- b) Nitrogen gas was bubbled through the soil to which 1.0 ml of N NaHCO₃ had been added and then the volatile substances were collected after addition of 1.0 ml of 2N HCl.

Table 2. Concentrations of acetic acid and n-butyric acid in the centrifuged-supernatants from the soils flooded with and without glucose

Flooded period (days)	Soil condition	Concentration(ppm)	
		Acetic acid	n-Butyric acid
3	Flooded	120±33	N. D. ^{a)}
	Flooded+Glucose	534±74	564±49
6	Flooded	80±20	N. D.
	Flooded+Glucose	3407±171	2944±259
12	Flooded	N. D.	N. D.
	Flooded+Glucose	428±162	587±247

a) Not detected (less than 30 ppm).

To determine whether acetic acid and n-butyric acid play a role in the fungicidal activity of the soil flooded with a glucose solution, their concentrations in the soil and fungicidal activity were determined. Table 2 shows the concentrations of acetic acid and n-butyric acid in the soil solutions from the soils incubated for 3, 6 and 12 days after flooding with and without glucose. Acetic acid and n-butyric acid were present at the concentrations of 3,407 and 2,944 ppm, respectively, in the soil solution on the 6th day, when the fungicidal activity reached maximum values. In contrast to the soil flooded with a glucose solution, no significant amounts of acetic acid and n-butyric acid were detected in the absence of glucose. As shown in Table 3, n-butyric acid almost completely inhibited chlamydospore germination at a concentration of 500 ppm, and acetic acid at 3,000 ppm. The inhibitory activity of the acids slightly decreased in a medium with glucose. Fig. 3 shows the concentration-response curves of acetic acid, n-butyric acid and a mixture containing equal amounts of these substances for killing the chlamydospores. Each of them almost completely killed chlamydospores at a concentration of 3,000 ppm or higher during the 24 hr period of incubation. Concentrations of acetic acid and n-butyric acid lethal for 50% of the spores (LC 50) were 1,506 and 1,522 ppm, respectively. LC 50 value of the mixed-solution was 1,252 ppm. These re-

Table 3. Inhibitory activity of acetic acid and n-butyric acid on germination of chlamydospores of *F. oxysporum* f. sp. *raphani*^{a)}

Concentration (ppm)	Medium ^{b)}	Germination (%)	
		Acetic acid	n-Butyric acid
0	BMS	93.8±0.6	93.8±0.6
	BMSG	94.3±0.8	94.3±0.8
50	BMS	94.0±0.7	94.6±1.0
	BMRG	95.9±1.1	97.8±0.5
100	BMS	95.7±0.6	90.3±1.4
	BMSG	95.5±1.4	97.2±0.5
250	BMS	90.9±0.7	70.6±3.2
	BMSG	94.5±0.6	97.1±0.7
500	BMS	87.7±2.6	1.1±0.4
	BMSG	96.5±0.4	4.6±1.9
1,000	BMS	65.4±1.5	0
	BMSG	96.2±1.0	0.2±0.2
2,000	BMS	15.7±2.4	—
	BMSG	71.7±4.7	—
3,000	BMS	0	—
	BMSG	0.7±0.4	—

a) The germination test was conducted by exposing chlamydospore suspensions to the head-space gas above 20 ml of aqueous solutions of acetic acid or n-butyric acid placed in a 15-cm petri dish.

b) BMS: supernatant fraction of a mineral salt solution.
BMSG: BMS+glucose.

sults indicate that the levels of acetic acid and n-butyric acid exceeded the lethal concentration for chlamydospores in the soil on the 6th day. In a separate experiment, formic acid concentration was below 30 ppm in the soil solution on the 6th day and formic acid did not kill the chlamydospores at a concentration of below 50 ppm. Lactic acid was not detected.

Discussion

Hydrogen sulfide which is one of the sulfur compounds commonly formed in flooded soils²⁰⁾ is toxic to animals²⁵⁾, plants¹¹⁾ and microorganisms¹⁰⁾. Beside H₂S, a number of volatile sulfur compounds are formed in flooded soil^{2,13)}. In the present studies, however, the concentration of each of H₂S and COS in the soil flooded with a glucose solution was much lower than that required for killing the spores. Other sulfur compounds were hardly detected in the cryogenic-collection from the soil. Furthermore, although it has been reported that phosphate is reduced to phosphine by anaerobic bacteria or during the decomposition of organic materials under waterlogged conditions³⁾, such a compound could not be detected in this study. These compounds were, therefore, not associated with the fungicidal activity of the soil. In contrast to these compounds, significant amounts of acetic acid and n-butyric acid were present as components of antifungal volatile substances from the soil. Their concentrations in the soil solution

Fig. 3. Concentration-response curves of acetic acid, n-butyric acid and their mixture in relation to the death of chlamydospores of *F. oxysporum*. A: curve for acetic acid, B: curve for n-butyric acid, C: curve for a mixture of equal amounts of these acids. The response represents the rate of death of chlamydospores of *F. oxysporum* in the solutions after 24 hr of incubation at 28 C. The rate was calculated by the following equation:

$$\left(1 - \frac{\text{number of survivors in the solutions}}{\text{number of survivors in water}}\right) \times 100$$

Vertical line at each point represents 95% confidence limit.

were high enough to kill the chlamydospores (Table 2, Fig. 3). Changes of the concentration were positively correlated to the fungicidal activity of the soil (Fig. 2, Table 2). These data indicate that both acids play an important role in the fungicidal activity of the soil. Butyric acid has already been shown to be one of the antifungal substances in the soil flooded with a sucrose solution⁵. Acetic acid has also been considered to be one of the causal agents for the inactivation of radish-yellow fusarium in radish-residue fermentation under anaerobic conditions⁹. Production of organic acids in flooded soils amended with and without organic materials has been extensively studied with reference to their harmful effects on the growth of rice plants in paddy fields^{1,22,23}. The acids mainly consist of acetic acid and n-butyric acid. It was shown that acetic acid and n-butyric acid are produced at concentrations of 1,100–1,860 ppm and 150–860

ppm, respectively, when four paddy field soils amended with 0.3-0.4% of milk vetch powder were incubated under flooded conditions *in vitro*²²⁾. About 1,500 ppm of each of both acids was similarly produced in flooded sand amended with 5% of rice straw powder in an *in vitro* experiment⁷⁾. It is, therefore, probable that a sufficient amount of the acids for killing the fungus would be produced in the soil amended with a large amount of readily decomposable organic materials or in a micro-site of soil around the materials under flooded conditions. Fungicidal value of the soil flooded with a glucose solution, however, was always lower than anticipated by the concentrations of acetic acid and n-butyric acid detected in the soil. This discrepancy may be ascribed to the difference of 1-1.5 pH unit between the soil and the solutions of the acids used for measuring the concentration-response curves, since the toxicity of fatty acids increases with the lowering of the pH⁴⁾. Fungicidal activity of acetic acid and n-butyric acid may also be weakened in the soil solution because it has been shown that the toxicity of fatty acids to roots of rice plants was weakened in a soil solution²¹⁾. Although the activity may be reduced to some extent by the rise of the pH and the presence of mineral salts, the concentrations of these acids seemed to be high enough to kill the fungus.

In the previous paper¹⁵⁾ it was shown that chlamydospore germination was strongly inhibited in the head-space gas above the soil flooded with a glucose solution. The spore germination was also strongly inhibited in the head-space gas above the n-butyric acid and acetic acid solutions. In the case of n-butyric acid, the inhibitory concentration was about one-sixth compared to the concentration found in the soil solution on the 6th day (Table 2, Table 3). This finding indicates that the acid is the causal agent of the inhibition in the head-space gas above the soil. In the previous paper¹⁵⁾ it was also observed that the germination of the chlamydospores was suppressed by one-half in the head-space gas above the unamended flooded soil compared with that of the control water. The soil, however, did not contain both acids at inhibitory levels (Table 2). Smith¹⁷⁾ suggested that ethylene may act as a soil fungistatic and its production in flooded soil has been reported²⁴⁾. Ethylene was not detected under the current conditions. However, the possibility that other volatile inhibitors are produced in unamended flooded soil can not be ruled out.

In summary, volatile fungicidal substances evolved from the soil flooded with a glucose solution *in vitro* were found to consist of acetic acid and n-butyric acid, providing a possible clue for the mechanism of fungicidal activity observed in flooded soil amended with readily decomposable organic materials.

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和 文 摘 要

岡崎 博・能勢和夫：グルコース添加湛水土壤中に発現する殺菌作用の原因物質について

グルコース添加湛水土壤の殺菌作用は土壤中に産生する揮発性抗菌物質と密接な関係がある。この揮発性抗菌物質を明らかにするために畑土壤 (10 g) に 3% グルコース液 (10 ml) を加えて保温静置した土壤の揮発性成分の分析および検出された成分の *Fusarium oxysporum* f. sp. *raphani* に対する抗菌性の検定を行った。揮発性成分の分析は土壤の殺菌力が最高になる湛水後 6 日目の土壤について行った。硫化水素, 硫化カルボニルを含めて土壤中の揮発性硫黄化合物の濃度はきわめて低く本菌の死滅に関係するとは考えられなかった。エチレン, エチレンオキシド, ホスフィンは検出されなかった。抗菌性を有する揮発性成分は土壤に窒素ガスを通じ, それを -35°C に冷却したトラップに導くことによって捕集された。土壤の pH が 5.5 以上では抗菌性成分は駆出されず有機酸であることが示唆された。トラップで捕集した抗菌性成分をガスクロマトグラフィーおよびガスクロマトグラフィー質量分析法によって分析した結果, 捕集成分は酢酸, n-酪酸および微量のエタノールであった。このことから抗菌性は酢酸, n-酪酸によるものと推定された。一方, 酢酸および n-酪酸の本菌厚膜胞子に対する 24 時間後の LC₅₀ はそれぞれ 1,506 ppm および 1,522 ppm であった。また, 6 日目の土壤溶液中には酢酸および n-酪酸がそれぞれ 3,407 ppm および 2,944 ppm 存在し, それは厚膜胞子を死滅させるに十分な濃度であった。さらに, グルコース添加湛水土壤の本菌厚膜胞子に対する殺菌力と土壤溶液中の酢酸および n-酪酸の濃度との間には正の相関が認められた。以上の結果からグルコース添加湛水土壤における *Fusarium oxysporum* f. sp. *raphani* の死滅は土壤中に産生する酢酸および n-酪酸によるものであると結論した。