Screening Soils for Suppressiveness to *Rhizoctonia solani* and *Pythium splendens**

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Wen-hsiung Ko**・Wang-ching Ho** : *Rhizoctonia solani* および
Pythium splendens に対する土壌の抑止作用の検索*

Abstract

Growth of mycelia and germination of sporangia amended with root extract on soil surface were used to detect soils suppressive to *Rhizoctonia solani* and *Pythium splendens*, respectively. In tests with 30 soil samples collected from various locations in Hawaii, 5 inhibited growth of *R. solani* more than 50 % and 8 inhibited sporangial germination of *P. splendens* more than 50 % as compared with conducive soil. Three of the soils were suppressive to both *R. solani* and *P. splendens*. Steaming the soil for 10-15min at 97-98 C eliminated suppressiveness as evidenced by no reduction of either mycelial growth of *R. solani* or sporangial germination of *P. splendens*. Steam treatment reduced populations of fungi, actinomycetes, and bacteria in suppressive soils to approximately 0.04 %, 1 % and 5 %, respectively, of the original numbers. Propylene oxide treatment and autoclaving increased inhibition on some soils. Similar effects were not found on steam-treated soils. The results indicate that suppressive soils may exist in small islands in a field and that the degree of suppressiveness varies from location to location.

(Received May 10, 1982)

Key Words : Suppressive soil, *Rhizoctonia solani*, *Pythium splendens*.

Introduction

Suppressive soils occurring naturally in fields are usually recognized by consistent observation of lower incidence of disease in a certain area in comparison with that in the nearby area^{1,3,7)}. The suppressive fields involved are relatively large and the number of such suppressive soils which have been reported so far is very limited. Since both microorganisms and nonbiological components in soils are very diverse and their distribution is not uniform^{2,5)}, it is considered possible that suppressive soil may exist as islands, small in area, in proportion to the total area of a conducive field. Two different methods were, therefore, used to detect suppressiveness in such small areas. A brief account of this work has been published¹⁴⁾.

* This research was supported in part by a grant under the U.S.D.A. Section 406 Program. Journal Series Paper No. 2652 of the Hawaii Institute of Tropical Agriculture and Human Resources.

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Materials and Methods

Soils. Soil samples collected from forests, pastures and vegetable farms on the island of Hawaii were taken from a depth of 0 to 10 cm after the surface litter was cleared. Each sample about 2 to 3 kg was a composite of five subsamples taken within a circle of approximately 2 m diam. Unless otherwise stated one sample was collected from one location which represented a different vegetation, soil type or elevation. Soils were sifted, moistened to about 65 % field capacity and stored at 24C in loosely closed glass jars for at least 1 mo to let microorganisms exhaust nutrients which may become available due to soil disturbance¹⁷⁾. For measurement of the growth of *Rhizoctonia solani* Kühn on the surface of soil, approximately 50 g of soil adjusted to about 75 % field capacity was placed in a Petri dish (150 mm diam, 25 mm high), compressed to a block measuring about 130×40×8 mm, and the surface was smoothened. For measurement of germination of *Pythium splendens* Braun sporangia on soil surface, approximately 8g of soil was similarly prepared on a glass slide to give a block of soil measuring about 50×25×3 mm. Silt loam (pH 5.4) collected from Lalamilo Experimental Farm was used as a conducive control soil because it did not restrict mycelial growth of *R. solani* nor did it inhibit germination of *P. splendens* sporangia amended with root extract.

Growth of *R. solani* mycelia on soil. *R. solani* (R-434) obtained from M. Aragaki was maintained on 10 % V-8 agar (10 % V-8 juice, 0.02 % CaCO₃, 2 % Bacto agar). A 5mm plug of inoculum was placed on the center of a V-8 agar plate and 20 to 30 sterilized discs (6 mm diam) of polycarbonate membrane (CPR, 0.2 μm; Nuclepore Corporation, Pleasanton, California) were placed around the inoculum. After incubation at 24C for 3 days, a membrane disc containing mycelia was detached from the plate with a surgical blade and placed at one end of a soil block in the Petri plate. All plates were then placed in a large polyethylene bag and incubated at 24C. Growth of *R. solani* was observed directly on soil surfaces every two days for 6 days using a Bausch & Lomb stereomicroscope at the magnification of 7.5¹²⁾.

Germination of *P. splendens* sporangia on soil. Sporangia of *P. splendens* were suspended in root extract of cucumber to overcome the general fungistasis of soil¹⁷⁾. Cucumber was selected because it is very susceptible to the test organisms and easy to obtain large quantities of roots. For production of sporangia, *P. splendens* (106F) isolated from cucumber was grown in 25 ml of 10 % V-8 broth (10% V-8 juice, 0.02 % CaCO₃) in a 250 ml flask for 7 days at 24C. Mycelia were washed two times with distilled water, and triturated in 25 ml distilled water with an Omni mixer at 5,000 rpm for 2 min. The resulting suspension was passed through a 53 μm screen. Sporangia in the filtrate were concentrated by centrifugation at 5,000 rpm for 10 min and adjusted to 2×10⁵ sporangia per ml with a Pipetman digital microliter pipet (P-20D; West Coast Scientific, Inc., Oakland, California)¹³⁾.

About 100 cucumber seeds in a 250 ml beaker were washed overnight with water dripping from a faucet, and spread in a 1000 ml beaker covered with wet cheesecloth kept moist by twice daily atomization. The beaker with seeds was placed horizontally in darkness at 24C. After 3 to 4 days, seedling roots were excised and ground in water at the ratio of 1:9 (w/v) with a mortar and pestle. Extracts from these roots

were obtained by filtering the ground material through two layers of cheesecloth. The conidial suspension was mixed with an equal volume of root extract and one drop (ca. 0.05 ml) of the resulting suspension was added to the surface of soil block on the glass slide which was then placed on moistened paper towels on a laboratory tray enclosed in a polyethylene bag and incubated at 24 C. After incubation for 6 hr, conidia were stained with rose bengal (1 % rose bengal, 5 % phenol, and 0.01 % CaCl_2) and germination was counted directly on the surface of the soil using a Zeiss Universal Microscope equipped with a Model II C vertical illuminator at the magnification of 200¹²⁾.

For measurement of both mycelial growth and sporangial germination two replicates were used for each treatment and all the experiments were repeated at least once.

Soil treatments. Soil blocks on slides or petri plates were steamed in a steamer for 15 min. The air temperature in the steamer was maintained at 97–98 C. Mycelia of *R. solani* and sporangia of *P. splendens* were placed on the soil surface after the soils were cooled. To determine the effect of steam treatment of soil on microbial populations, soil suspensions were prepared by suspending 10 g of steamed soil with 90 ml of sterile distilled water in an Omni mixer at 4,500 rpm for 1 min. Dilutions of 10^{-1} , 10^{-2} and 10^{-3} were prepared for fungi; and 10^{-2} , 10^{-3} and 10^{-4} for bacteria and actinomycetes before plating on agar media. Suspensions of non-steamed soil were diluted to 10^{-2} , 10^{-3} and 10^{-4} for fungi; 10^{-4} , 10^{-5} and 10^{-6} for actinomycetes; and 10^{-5} , 10^{-6} and 10^{-7} for bacteria. Surfactant-PDA¹⁹⁾, alkaline water agar¹⁰⁾ and PCNB-soil extract agar⁶⁾ were used for determining the populations of fungi, actinomycetes and bacteria, respectively. Plates were incubated at 24 C and colony numbers were determined after 7 days for bacteria and fungi, and 14 days for actinomycetes. Three plates were used for each dilution and the experiments were repeated once. For gas treatment, soil blocks on slide or petri plates were placed in a desiccator (160 mm diam, 255 mm high) and into it 3 ml of propylene oxide was added⁹⁾. After exposure to the gas overnight, soils were allowed to aerate for 24 hr to let the gas disperse before use.

Results

Linear growth of *R. solani* on 30 soil samples tested ranged from 10 to 130 mm in 6 days (Table 1). During the same period, the fungus grew 98 mm on conducive control soil. Soil samples No. 9, 13, 16, 20 and 24 were considered suppressive because they restricted linear growth to less than 50 % of that found on the conducive control. Hyphae of *R. solani* ceased to grow within 4 days on these soils. In general, fewer hyphae grew from the inoculum discs on suppressive soil than on conducive soil. Only 1 or 2 hyphae of *R. solani* were observed after 6 days on 3 suppressive soils, while 5 to 10 hyphae were observed on 12 conducive soils. Germination of *P. splendens* sporangia amended with root extract ranged from 4 to 98 % on the same 30 soil samples tested (Table 1). On conducive control soil the fungus germinated 95 % with root extract and 6 % without root extract. Soil samples No. 1, 7, 13, 15, 16, 21, 24 and 27 were considered suppressive because germination on these soils were less than 50 % of that on conducive control soil. Two of the 10 suppressive soils were suppressive

Table 1. Growth of *Rhizoctonia solani* and germination of *Pythium splendens* sporangia coated with root extract on soils collected from various locations on the island of Hawaii

Soil sample designation	<i>R. solani</i>		<i>P. splendens</i> (% germination)
	Linear growth (mm/6 days)	Mycelium density	
1	120 ^{a)}	+ ^{b)}	33 ^{a)}
2	58	+	90
3	90	++	76
4	130	+	88
5	75	++	78
6	120	++	88
7	55	±	44
8	69	±	92
9	34	±	80
10	75	+	89
11	75	++	84
12	120	++	90
13	10	+	4
14	100	++	90
15	60	+	42
16	30	±	4
17	50	+	98
18	85	++	88
19	120	++	98
20	25	+	88
21	55	+	32
22	78	+	90
23	100	+	98
24	12	±	8
25	86	+	94
26	70	+	84
27	55	+	32
28	65	++	84
29	75	++	82
30	98	++	56
Control	98	++	95

a) Two replicates were used and the experiments were repeated once.

b) Density rating scale: ±, only 1 or 2 hyphae from the inoculum and showing lysis on 4th day. +, with 3 or 4 hyphae; ++, with 5 to 10 hyphae barely visible with naked eye.

only to *R. solani* and five were suppressive only to *P. splendens*; whereas, the other three were suppressive to both pathogens.

Five soil samples were collected from a 3-acre area of uniform soil texture at Lalamilo Experimental Farm. It is a silt loam and the area has been used for growing Chinese cabbage. One sample was found to be suppressive to *R. solani* (21 mm/6 days) and the other four were conducive (83–123 mm/6 days). Germination of *P.*

splendens sporangia on one of the same five soil samples was only 40 %, while 82 to 97 % germination on the other four. In other experiments 12 soil samples were randomly collected at least 5 m apart from within one-acre area of pasture at South Kohala. The soil is a very fine sandy loam. Nine out of 12 samples were conducive to *R. solani* supporting 65–130 mm mycelial growth in 6 days, whereas the other three were suppressive with growth limited to only 2–33 mm in 6 days. However, all 12 samples were to a certain degree suppressive to *P. splendens* resulting in only 12–54 % sporangial germination as compared to 97 % on conducive control soil.

Suppressive soils No.9 and 21 were steamed for 5, 10, 15, 30 and 60 min, cooled, and tested for effect on growth of *R. solani* and germination of *P. splendens* sporangia, respectively, as described previously. Maximum growth of *R. solani* and germination of *P. splendens* sporangia on suppressive soil occurred on soil steamed for 10 and 15 min, respectively (Fig. 1). Steam treatment for 15 min was, therefore, selected for further study. The population study revealed that steam treatment for 15 min eliminated about 93–99 % of the total microbial populations in soils (Table 2). In all three soils tested fungi were most sensitive to steam treatment, followed by actinomycetes and bacteria. The populations of fungi, actinomycetes, and bacteria were reduced to about 0.04 %, 1 % and 5 %, respectively, of the original numbers.

Increased linear growth of *R. solani* was observed on all five suppressive soils after they were steamed for 15 min. The same treatment did not result in an effect on linear growth on the conducive control soil (Table 3). Density of *R. solani* mycelium on suppressive and conducive soils increased after steam treatment. Increased germination of *P. splendens* sporangia was also observed on 6 of 8 steamed suppressive soils (Table 4). However, germination on soil samples No.13 and 24, and the conducive

Fig. 1. Linear growth of *Rhizoctonia solani* and germination of *Pythium splendens* sporangia amended with root extract on suppressive soils steamed at 97–98°C for various time intervals. Suppressive soils used were No.9 for *R. solani* and No.21 for *P. splendens*.

Table 2. Numbers of microorganisms in suppressive soils before and after steaming at 97-98C for 15 min

Soil sample	Fungi		Actinomycetes		Bacteria	
	Natural	Steamed	Natural	Steamed	Natural	Steamed
13	3×10^5 ^{a)}	240	131×10^5	2.0×10^5	123×10^5	17×10^5
16	5×10^5	75	78×10^5	0.9×10^5	126×10^5	4×10^5
24	2×10^5	84	212×10^5	0.2×10^5	177×10^5	1×10^5

a) No. propagules/g dry soil. Three dilutions of soil suspension were used for each group of microorganisms for each soil sample, and three plates were used for each dilution. Data were from one of two experiments with similar results.

Table 3. Effect of steam treatment on suppressiveness of soils as measured by growth of *Rhizoctonia solani*

Soil sample	Soil treatment			
	Steaming	None	Steaming	None
9	73 A ^{a)}	32 B	+++ ^{b)}	+
13	30 A	10 B	++	+
16	43 A	24 B	++	+
20	44 A	25 B	++	+
24	35 A	22 B	++	+
Conducive soil	63 A	67 A	+++	++

a) Linear growth (mm/4 days). Values followed by the same letter for each soil sample were not significantly different at $P=0.05$.

b) Density of mycelial growth. Rating scale: ±, only 1 or 2 hyphae from the inoculum and showing lysis on 4th day; +, with 3 or 4 hyphae; ++, with 5 to 10 hyphae barely visible with naked eye; +++, with more than 10 hyphae visible with naked eye.

Table 4. Effect of steam treatment on suppressiveness of soils as measured by germination of *Pythium splendens* sporangia amended with root extract

Soil sample	Soil treatment	
	Steaming	None
1	76 A ^{a)}	33 B
7	82 A	41 B
13	3 A	4 A
15	83 A	46 B
16	96 A	8 B
21	92 A	25 B
24	13 A	4 A
27	81 A	31 B
Conducive soil	96 A	95 A

a) Germination, %. Values followed by the same letter for each soil sample were not significantly different at $P=0.05$.

control soil was not significantly increased as a result of steam treatment.

Suppressive soils were steamed for 15 min, exposed to propylene oxide vapor overnight or autoclaved for 15 min to partially or completely eliminate soil microorganisms. Because the amount of some suppressive soils left was not enough for these tests, different suppressive soils obtained during the latter screenings were also used. Similar to steam treatment, autoclaving also resulted in increased linear growth of *R. solani* on all the suppressive soils tested (Table 5). However, propylene oxide treatment not only failed to increase growth of *R. solani* on two of the suppressive soils, but decreased the growth on conducive

Table 5. Effect of steaming, autoclaving and propylene oxide treatment on growth of *Rhizoctonia solani* on suppressive soils

Soil sample	Soil treatment			
	Steaming	Autoclaving	Propylene oxide	None
13	20.5 A ^{a)}	24.3 A	12.5 B	5.5 C
20	46.8 A	47.8 A	18.5 B	20.0 B
79	47.3 A	30.8 B	19.0 C	15.5 C
90	50.0 B	62.3 A	35.0 C	12.0 D
94	31.0 B	31.5 B	44.5 A	20.5 C
Conductive soil	50.8 B	80.0 A	18.0 C	51.0 B

a) Linear growth (mm/4 days). Values followed by the same letter for each soil sample were not significantly different at $P=0.05$ according to Duncan's new multiple range test.

Table 6. Effect of steaming, autoclaving and propylene oxide treatment on suppressiveness of soils as measured by germination of *Pythium splendens* sporangia amended with root extract

Soil sample	Soil treatment			
	Steaming	Autoclaving	Propylene oxide	None
13	8 A ^{a)}	9 A	5 A	7 A
78	79 A	53 B	80 A	21 C
79	82 A	28 C	82 A	54 B
90	82 A	83 A	82 A	7 B
94	80 A	75 A	71 A	21 B
Conductive soil	90 A	94 A	45 B	94 A

a) Germination, %. Values followed by the same letter for each soil sample were not significantly different at $P=0.05$ according to Duncan's new multiple range test.

control soil. Germination of *P. splendens* sporangia increased following autoclaving of three suppressive soils, was not affected on soil sample No. 13, and was decreased on soil sample No. 79 (Table 6). Propylene oxide treatment of 4 of 5 suppressive soils resulted in increased sporangial germination, but not on soil sample No. 13. Germination on conductive control soil was decreased by propylene oxide treatment.

Discussion

The results indicate that suppressive soil may exist in small islands in a field and that the degree of suppressiveness varies from location to location. About 17 % of soil samples collected from various locations in Hawaii were suppressive to *R. solani*, and 27 % were suppressive to *P. splendens*. The soil in a field at Lalamilo Experimental Farm was generally conducive to *R. solani* and *P. splendens*. However, one of the five soil samples collected from that area was found to be suppressive to *R. solani* or *P. splendens*. The area at South Kohala selected for this study was generally suppressive to *P. splendens*, but conducive to *R. solani*. However, 3 of the 12 soil samples collected from this area were suppressive to *R. solani*, and the soil samples inhibited sporangial germination of *P. splendens* from 44 % to 88 %.

Growth of mycelia of most fungi is restricted in natural soil¹⁷⁾. *R. solani* has been reported to be among the very few species which are not subjected to such restriction^{4,8)}. Present results show that the distance *R. solani* travelled was greatly affected by the type of soils used. On 30 soil samples collected from various locations the range of linear growth by this fungus extended from 10 to 130 mm in 6 days. The fungus ceased to grow on suppressive soils within 4 days. Sporangia of *P. splendens* germinated poorly on natural soil without addition of nutrients due to the presence of general soil fungistasis¹⁷⁾. Amending *P. splendens* sporangia with root extract before addition to the 30 soil samples mentioned above resulted in germination ranging from 4 % (no annulment of fungistasis) to 98 % (complete annulment). This suggests that the degree of annulment of soil fungistasis by nutrients may be affected by soil type. Germination of *Calonectria crotalariae* conidia on soils was reported to be 80–89 % by Hwang and Ko¹¹⁾, and 28 % by Roth and Griffin¹⁸⁾. In addition to the clonal differences as suggested by Roth and Griffin¹⁸⁾, according to this study soil type differences may also account for the variation of the results.

Steam treatment reduced the total microbial population to 0.3–7.4 % of the originals. Therefore, the reduced inhibition in suppressive soils is probably mainly due to the destruction of soil microorganisms. However, the extent to which the increase in growth and germination was due to nutrients released from killed microorganisms is still unknown. Although microorganisms appear to be an important factor responsible for inhibiting the growth of *R. solani* on suppressive soils tested, nonbiological factors also may be involved because on some steamed suppressive soils growth of *R. solani* was not as good as that on a conducive soil (Table 3). Inhibition of *P. splendens* germination by soil samples No. 13 and 24 appears to be caused mainly by nonbiological factors, whereas inhibition by the other soil samples seems to be of biological origin (Table 4).

Steaming appears to be a better treatment of soil than gas treatment and autoclaving for studying the relative importance of biological and nonbiological factors in suppressing test organisms. Propylene oxide treatment reduced both linear growth of *R. solani* and germination of *P. splendens* sporangia on the conducive control soil. It is considered possible that in certain types of soils inhibitory substances may appear due to propylene oxide treatment, or relatively high concentration of residual propylene oxide may be retained. Germination of *P. splendens* sporangia was lower on autoclaved than on natural soil sample No. 79 (Table 6). Similar results were reported by Ko and Hora¹⁵⁾ who showed that the inhibitory effect of certain acid soils against germination of *Neurospora tetrasperma* ascospores was enhanced by autoclaving. Enhanced inhibition by steam treatment was not observed in any of the soils tested.

It is considered possible that the principles involved in the inhibition of mycelial growth of *R. solani* and sporangial germination of *P. splendens* in these soils may be applicable to controlling diseases caused by these two pathogens. Control of damping-off of beets caused by *R. solani* by means of PCNB has been shown to be due to the suppression of growth of this pathogen in soil¹⁶⁾. When one of the suppressive soils obtained in this study was infested with *P. splendens* sporangia and planted with cucumber seeds, more than 90 % of the seedlings survived after two weeks. However, during the same period, more than 90 % of the seedlings were killed in conducive

control soil inoculated with the same amount of *P. splendens* sporangia (C. W. Kao and W. H. Ko, *unpublished*).

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和文摘要

Phizoctonia solani および *Pythium splendens* に対する土壌の抑止作用の検索

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Rhizoctonia solani および *Pythium splendens* に対する土壌の抑止作用を探索するために、キュウリの根の搾汁を添加した土壌試料表面における *R. solani* の菌糸の生長および *P. splendens* の胞子のうの発芽を観察した。ハワイ島の各地で採集した30コの土壌試料を用いて実験を行ったが、そのうち5コは *R. solani* の菌糸の生育を50%抑制し、8コは *P. splendens* の胞子のうの発芽を50%抑制した。また3コは両菌に対して抑制的に作用した。土壌を97~98℃の蒸気で10~15分間処理すると、抑止作用は消失し、*R. solani* の菌糸生育の抑制および *P. splendens* の発芽抑制がみられなくなる。蒸気処理により、菌類、放線菌および細菌数はそれぞれ約0.04%、1%および5%に減少する。土壌試料によっては、ポリブレンオキサイドでくん蒸あるいは高圧蒸気殺菌をすると抑制が増加する例があった。しかしこの様な効果は上記の蒸気処理によっては認められなかった。抑止土壌は圃場のなかに点在し、抑止作用の程度は場所によって異なる。