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EVALUATION OF ROLES OF INHIBITORS AND NUTRIENT-SINK IN SOIL MYCOSTASIS

BY
M. BHATTACHARYA and K. R. SAMADDAR
Department of Botany, Kalyani University, Kalyani, West Bengal

Nutrient-independent propagules of *H. oryzae*, *C. lunata*, *P. oryzae* and *M. phaseolina* failed to germinate when incubated on nutrient-free cellophane discs kept in contact with soil. Cellophane discs pre-incubated on soil were not inhibitory to test propagules. No fungistatic substance (s) could be extracted from cellophane discs incubated on soil. Nutrient-free agar discs exposed to the atmosphere of alkaline natural soils were not inhibitory to the test propagules. The data indicated that volatile fungistatic factor(s) were not emanated from the soils, Aqueous, acid, alkali and organic solvent extracts from the fallow soil were not inhibitory to the test propagules. All test propagules failed to germinate when exposed to continuous leaching with dripping water, but did so when leached with 0.1% glucose solution or propagules leachates obtained previously. Exposure to leaching decreased the germination potential of the test propagules. Loss of endogenous metabolites from the spores to the soil was demonstrated by rapid diffusion of ^{32}P and ^{14}C metabolites from spores preloaded with these labelled isotopes and incubated on soil. There was little or no loss of radioactive metabolites from control spores incubated on slides. The data lend support to nutrient sink hypothesis for the causation of soil mycostasis.

INTRODUCTION

Occurrence of fungistasis in soils of India including the district of Nadia of West Bengal has been reported (Bhattacharya and Sammaddar, 1975). The fungistasis of these soils could be annulled by heat sterilization or by amendment with nutrients and therefore is of "Biotic ' type.

In general, the biotic fungistasis is explained either by the presence of inhibitors in the soil (Dobbs and Hinson, 1953 ; Watson and Ford, 1972, Balis and Kouyeas, 1979) or by the nutrient-sink imposed by soil microorganisms (Ko and Lockwood, 1967, Lockwood, 1977 ; Filonow and Lockwood, 1983).

The objective of the investigation was to test the 'inhibitor' and "nutrient-sink" hypotheses with respect to two soils of Kalyani (Nadia) using a few Nutrient dependent and independent propagules. Attempts were made to detect or extract the diffusible and/or volatile fungistatic factor (s) from the test soils. Attempt was also made to determine the loss of metabolites from the spore to the soil as a cause of mycostasis.

MATERIALS AND METHODS

A cultivated sandy loam (pH, 7.0; ED_{50} 8) and a fallow loam (pH, 7.4; ED_{50}

very high) were used. Soils after collection were stored as described previously (Bhattacharya and Samaddar, 1977). This soil is referred as natural soil. Soil was sterilized when required by autoclaving at 15 psi for 40 min. on two successive days.

Nutrient-dependent conidia of *Penicillium chrysogenum* and Nutrient-independent conidia of *Curvularia lunata, Helminthosporium oryzae, Pyricularia oryzae* and sclerotia of *Macrophomina phaseolina* were used. The maintenance of the fungi and preparation of propagule suspensions were done following the method of Setua and Samaddar (1983).

The double cellophone (DC) method of Weltzien (1963) was used for detection of diffusible fungistatic principle. For assay of volatile fungistatic factor, the soil emanation (SEA) method of Hora and Baker (1970) was used.

For soil extracts, 200 g of air - dried, natural or of sterilixed soil was extracted with 100 ml of water, HCI (0 1 N and N), NaOH (0.1 N and N), ethanol, methanol, ether, pet ether, and chlorofrom ethanol (2 : 1) mixture. Soil-water suspensions were vigorously shaken in a rotary shaker for 1 hr, the slurry was centrifuged (3000 g for 10 min) and the supernatant was filter sterilized (Bact. filter No. 5, Corning) before assay. Soil suspensions in acid or alkali were shaken overnight, centrifuged, the pH adjusted to 6.8 with acid or alkali and filter sterilized before assay. Soil suspensions in organic solvents were shaken for 1 hr and centrifuged. The supernatant was evaporated to dryness in a flash evaporator at 30°C. The residue was dissolved in 50 ml of distilled water, centrifuged and filter sterilized before assay.

Spores or sclerotia of test fungi were leached by dripping glass distilled water, 0.01 M phosphate buffer, nutrient solution or leachates of propagules obtained previously following the method of Ko and Lockwood (1967).

Test fungi were grown on PDA supplemented with ^{14}C-glucose (50μc/ml) or^{32}p -orthophosphate (100μc/ml). ^{32}p-orthophosphate was diluted with phosphate buffer (pH 6.0) and added to the medium. After 2 weeks growth propagule samples were collected and washed 3 times by centrifugation. The stored soil was remoistened to 60% water-holding capacity, packed in a small Petri dish (2.5 cm. diam) and surface smoothed. A large cellophane (4 cm diam) disc was placed over the soil surface and on top of this was placed a smaller disc (2 cm. diam) of dialysis casing. Cellophane and dialysis casing discs were boiled, washed and sterilized (15 psi for 15 min.) before use. Samples of spore suspension (0.05 ml) were placed on the small disc and the Petri dishes were placed inside sealed chambers containing $C0_2$ traps (100μl of 1M NaOA and 0.2 M $Ba(OH)_2$ in a planchet). Spore-bearing discs placed on sterile glass plates and placed inside sealed chambers with $C0_2$ traps served as controls. The spore-bearing discs were periodically

removed and transferred to planchets, glued, dried quickly under a lamp and radioactivity measured. The $C0_2$ traps containing $^{14}C0_2$ due to respiration of the propagules and that respired by soil organisms were also counted. The $C0_2$ traps in control chambers gave the amount respired by spores only. The loss of radioactivity from the spore to the soil was calculated by subtracting the count due to spore respiration in $C0_2$ - traps. The results presented are average of three replicate spore-bearing disc.

Measurrent of ^{32}P-activity was carried out in a lead shielded end window counter attached to a scaler (Atomic Energy Establishment, Trombay) using stainless steel planchets. A window less gas-flow counter (Atomic Energy Establishment, Trombay) was used for counting ^{14}C-activity.

Phosphate buffer was prepard following the method of Gomori (1955). Carbohydrate determination was done by the anthrone method of Morris (1948). Amino acids and related compounds were estimated by the ninhydrin method of Moore and Stein (1954).

Table 1. *Germination of propagules of nutrient-independent fungi on cellophane discs incubated on soil*

Organisms	Slide[b] control	Mean (%) germination					
		1		7		21	
		Cult	Fal	Cult	Fal	Cult	Fal
C. lunata	93	25	17	30	11	16	6
H. oryzae	90	16	10	6	5	12	8
P. oryzae	87	0	0	14	8	19	10
M. phaseolina	88	16	12	20	12	18	7

a Soil samples were removed on indicated days after remoistening, packed in Petridishes, propagules placed and assayed. Percentage germination was determined after 24 hours of incubation.

b Cellophane discs with propagules were incubated on sterile glass slides in moist chambers.

c The cultivated soil (cult.) had a cropping history of rice and jute in rotation. The fallow soil (Fal) had no crop for last 20 years.

RESULTS

Test for diffusible fungistatic factor

To test the diffusible nature of the fungistatic factor present in soils, propagules were separated from the soil by a cellophane membrane and their germination studied. Results indicated significant inhibition of germination of the propagules on the cellophane disc kept in contact with both cultivated and fallow soils. The fallow soil appeared to be slightly more fungistatic than the cultivated soil. The observed inhibition of germination could be due to diffusion of fungistatic substance

through cellophane or due to loss of endogenous nutrients from propagules through cellophane.

To test the latter possibility cellophane disc were incubated in contact with soil for 24 hrs, removed, seeded with propagules and incubated on glass slides for another 20 hrs. Germination of propagules on dics pre-incubated on soil was comparable to controls. In another experiment cellophane discs were incubated on soil at 1° and 28°C for 24 hrs and then extracted with sterile water at 0°C and the extracts assayed for fungistatic activity by slide germination bioassay. Germination of propagules of test fungi was not inhibited in any of the extracts.

Test for volatile fungistatic factors

Sterile water agar discs were placed on glass slides kept in the atmosphere above the soil. Agar discs exposed above distilled water were used as controls. Fungistatic activity of these agar discs was tested. Results indicated (Table 2) that there was no inhibition of germination of the propagules on the agar discs preincubated in the atmosphare of the soils irrespective of whether the discs were removed from the soil atmosphare after 24 hrs or left there.

Table 2. *Germination of popagules of nutrient-independent fungi on agar discs exposed to atmosphere above the remoistened soil*

Organism	Control [b]	Mean (%) germination											
		Days after remoistening the soil [a]											
		Cultivated						Fallow					
		1		7		21		1		7		21	
		PL[c]	PR[d]	PL	PR	PL	PR	PL	PR	PL	PR	PL	PR
C. Innate	89	89	88	94	94	89	85	90	96	92	91	85	80
H. oryzae	96	95	95	96	93	95	94	98	98	98	95	93	94
P. oryzae	81	81	80	91	90	81	80	84	75	85	83	90	87
M. phaseolina	85	82	80	80	85	80	81	82	83	85	80	81	84

a Air-dried soils were remoistened to 60% WHC and stored in stoppered containers. Samples were removed on indicated days after remoistening, taken in Petri dishes and assayed for "volatile factor" using SEA method. The agar discs after exposure to soil atmosphere were handled as PL and PR as discribed below.

b Agar discs exposed over distilled water for 24 hrs were seeded with propagules and percentage germination de ermined after 24 hrs.

c PL=Pre-incubated and left ; agar discs exposed to soil atmosphere for 24 hrs, seeded with propagules and left in the soil atmosphere for another 24 hrs, after which percentage germination was determined.

d PR=Pre-incubated and removed; agar discs exposed to soil atmosphere for 24 hrs, removed, seeded with propagules and incubated on slides in moist chambers for another 24 hrs after which percentage germination was determined.

Effect of soil extracts

The effects of soil sxtracts on the germination of propagules of the test fungi are shown in Table 3. It is evident that the soil extracts were not inhibitory to the germination of the test organisms (Table 3).

Table 3. *Germination of fungal propagules in extracts of a fallow soil*

Organisms	Control[c]	Mean (%) germination[a] in soil extracts[b] with									
		water	HCl N/10	HCl N	NaOH N/10	NaOH N	ethanol	methanol	Ether	Pet ether	Chloroform: ether (2:1)
C. lunata	91	88	85	75	95	95	98	94	92	85	91
H. oryzae	92	90	75	80	98	95	90	85	91	91	94
P. oryzae	84	82	80	78	85	82	75	80	81	92	79
M. phaseolina	88	85	82	80	86	84	82	85	83	85	80

a Average of 400 propagules

b Soil extracts were mixed with washed propagule suspensions

c Germination in distilled water.

Effect of leaching on propagules germination

Propogules of test fungi were exposed to continuous leaching. Results indicated (Table 4) significant inhibition of germination of all test fungi. When the propagules were leached with 0.1% glucose solution, little or no inhibition of germination was observed. Germination potential of *C. lunata* decreased following leaching for 24 hrs with distilled water. Germination of pre-leached spores was reduced to 10% of the controls of *C. lunata*. Leaching with water for 24 hrs had no effect on the germination of *H. oryzae*, but after 72 hrs of leaching germination

Table 4. *Germination of fungal propagules exposed to natural soil and during leaching*

Organisms [a]	Mean (%) germination			
	Slide[b]	Nat. Soil[c]	Leached with water	Leached with 0.1% Glucose
C. lunata	90	10	8	85
H oryzae	87	16	3	80
M. phaseoli	85	8	5	80
P. chrysogenum	0(82)[d]	0	0	75

a Except *P. chrysogenum* all test fungi were nutrient-independent.

b Washed cellophane discs placed on slides kept in moist chambers served as controls.

c Washed cellophane discs placed on natural soil using DC method.

d Percentage germination in presence of 0.1% glucose solution.

potential was decreased 30-40% of controls. The linear growth rate of germ tubes of conidia of *H. oryzae* pre-leached for 24 hrs was significantly reduced (40-60%) as compared to non-leached controls.

Propagules leached with 0.1% glucose or leachates obtained by leaching a separate batch of propagules, germinated readily. Leachates of *H. oryzae*, *C. lunata* and *M. phaseolina* contained authrone and ninhydrin positive materials. The stimulation of germination by propagule washings in the leaching system was found to be non-specific since spore washings of *H. oryvae* could stimulate the germination of *C. lunata* or *M. phaseolina* and *Vice-Versa*.

Loss of endogenous metabolites from spore to soil

Spores of *P. chrysogenum* and *H. oryzae* labelled with radioactive metabolites (^{32}P-or ^{14}C-) were incubated on soil and the amount of radio-activity lost as a function of time was determined. Loss of radioactivity from spore-bearing discs kept on slides in moist chambers served as controls. Results indicated (Fig. 1) rapid loss of ^{32}P- and ^{14}C- metabolites from spores of both the test fungi when incubated in contact with natural soil. The rate of diffusion of labelled metabolites from spores to the soil was initially very high and about 30-50% of the endogenous metabolites were lost within 24-41 hrs. of exposure to soil. Thereafter, the rate

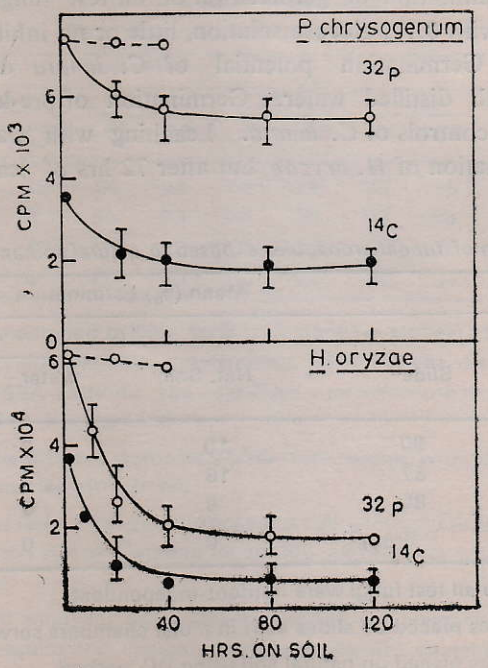

Fig. 1

declined and reached a steady state which persisted during the period of observation.

DISCUSSION

The observation that the propagules separated from the soil by cellophane membrane were significantly inhibited can be interpreted as an evidence of existence of diffusible fungistatic substance in soil. The results, however, can be explained by the nutrient sink hypothesis. Diffusion of endogenous nutrients from propagules to the soil through the membrane may be responsible for decreased germination of propagules in such assay systems (Lingappa and Lockwood, 1964 ; Ko and Lockwood, 1967). This possibility has been supported by the fact that cellophane discs previously kept in contact with soil were not inhibitory to germination and no fungistatic substance (s) could be extracted from such discs. The results are in confirmation of previous works (Ko and Lockwood, 1967 ; Lockwood, 1977).

Volatitle fungistatic substance (s) as measured by SEA method (Hora and Baker, 1970) were not released from air-dried and remoistened soils used in this study. This is in confirmation of the earlier observation that the volatile inhibitors are restricted to certain soils and are not responsible for the wide-spread occurrence of fungistasis in most natural soils (Ko and Hora, 1972).

No fungistatic substance (s) could be isolated from soils tested using a variety of solvents and extraction procedures. Falure to isolate the fungistatic substances from the soil may be interpreted as due to absence of such substances in natural soils or inactivation or removal of such substances during isolation (Griffin, 1962; Weltzien, 1963; Dix, 1967; Schuepp and Green, 1968).

Germination of spores and sclerotia of nutrient-independent organisms was significantly inhibited when these propagules were subjected to continuous leaching with water. Failure of germination in such leaching system has been thought to be due to depletion of soluble endogenous nutrient reserves by dripping water (Ko and Lockwood, 1967; Lockwood, 1975). Decrease in germination potential of such leached spores, presence of carbohydrate and amino acid and related materials in the leachates and good germination of propagules when leached with glucose solutions or previously obtained spore-washings support the contention of loss of endogenous nutrients during leaching. The leaching induced depletion of nutrients, however, was much greater than that of natural soil. Spores of *H. oryzae* and *C. lunata* germinated well in distilled water after incubation on soil for 3 days, whereas significant decrease in germination potential of these spores occurred even after 24 hrs exposure to dripping water.

The experiments in which radio-labelled spores were exposed to soil provide further evidence that soil fungistasis may be to due to loss of endogenous metabolites from

spores to the soil. About 30-50% of the initial radioactivity of spores were lost very rapidly within a short time of exposure to soil Evidently spores in contact with soil lose endogenous nutrients. The results are in confirmation of earlier works (Bristow and Lockwood, 1975a, b; Sneh and Lockwood, 1976; Filonow and Lockwood, 1983). Why loss of endogenous nutrients prevents germination of nutrient-independent spores, however, is not clear and warrants investigation.

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REFERENCES

Balis, C., and Kouyeas, V. (1979). Contribution of chemical inhibitors to soil mycostasis. pp. 97-106. *In* : B. Schippers and W. Gams. (eds.). *Soil-Borne Plant pathogen*. Academic Press, New York. 686 p.

Bhattacharya, M., and Samaddar. K. R. (1975). Widespread occurrence of fungistasis in Indian Soils. *Indian phytopath.* **28** : 376-378.

—— and —— (1977). Fungistasis, lysis and survival of *Pyricularia oryzae* and *Helminthosoporium oryzae* in soil *Phytopath. Z.* **89**, 60-67.

Bristow, P. R., and Lockwood, J. L. (1975a) Soil fungistasis ; Role of spore exudates in the inhibition of nutrient-independent propagules. *J. Gen. Mycrobiol.* **90**, 140-146.

Bistow, P. R., and Lockwood, J. L. (1975b). Role of the micrabial nutrient sink and of fungistatic substances in two soils. *J. Gen. Microbiol.* **90**, 147-156.

Dix, N. J, (1967). Mycostasis and root exudation : factors influencing the colonization of bean roots by fungi. *Trans, Brit. mycol. Soc.* **50**(1), 23-31.

Dobbs, C.G., and Hinson, W.H. (1983). A widespread fungistasis is soils *Nature*, 172 : 197-199.

Filonow, A. B., and Lockwood, J. L. (1983). A Mycostasis in relation to the microbiol sinks of five soils. *Soil, Biol. Biochem.* **15**, 557-565.

Gomori, G. (1955). Preparation of buffers for use in Enzyme studies pp. 138-146. *In* : S P. Colowick aud N. P. Kaplan (eds.) *Methods in Enzymlogy,* Vol. I. Academic Press, New York.

Griffin, G. J., (1962) Production of a fungistatic effect by soil microflora in autoclaved soil. *Phytopathology* **52** : 190-191.

Hora, T. S., and Baker, R. (1972a). Extraction of a volatile factor from soil-inducing fungistasis *Phytopathology* **62** : 1475-1476.

Ko, W. H., and Hora, F. K (1972). The nature of a volatile inhibitor from certain alkaline soils. *Phytopathology* **62** : 573-575.

—— and Lockwood, J. L. (1967). Soil fungistatis : relation to fungal spore nutrition. *Phytopathology* **57** : 894-901.

Lingappa, B. T., and Lockwood, J. L. (1964). Activation of soil microflora by fungus spores in soil fungistasis. *J. Gen. Microbiol.* **35**, 215-227.

Lockwood, J. L. (1975). Quantitative evaluation of leaching model system for soil fungistasis *Phytopathology* **65**, 460-464.

—— (1977). Fungistasis in soils. *Biological Reviews.* **52**(1) : 1-43.

Moore, S., and Stein, W. H. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211** : 907-913.

Morris, D. L. (1948). Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science **107** : 254-255.

Schuepp, H., and Green, R. J. (1968). Indirect assay methods to investigate soil fungistasis with special consideration of soil pH. *Phytopath. Z.* **61** : 1-26.

Setua, G. C. and Samaddar, K. R. (1980). Evaluation of the role of volatile ammonia in fungistasis of soils. *Phytopath. Z.* **98** : 310-319.

Sneh, B., and Lockwood, J. L. (1976). Quantitative evaluation of the microbial nutrient sink in soil in relation to a model system for soil fungistasis. *Soil. Biol. Biochem.* **8** : 65-69,

Waston, A. G., and Ford, E. J. (1972). Soil fungistasis — a reapprasial.

Weltzien, H. C., (1963) Studies on the causes of inhibition of fungus spore germination in soil *Zbl. Bakt. Abt.* **2116** (2) : 131-170.